DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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This thesis has been submitted with our approval as university supervisors

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DEDICATION

This work is dedicated to the Catholic Academic Exchange Service (KAAD), office in Bonn, for granting me the scholarship to enable me undertake my Master of Science degree in medical virology, my Parents Mr. and Mrs. Odari, my uncle Dismas Wangoye, my siblings; Nancy, Gordon, Emily, Victor and Brenda.

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LIST OF ABREVIATIONS AND ACRONYMS

Aa	Amino acids
Ab	Antibody
Ag	Antigen
ALT	Alanine aminotransferase
anti-HCV	antibody to hepatitis C virus
cDNA	Complementary DNA
DNA	Deoxyribonucleic Acid
EIA	Enzyme immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
HCV	Hepatitis C virus
IDUs	Intravenous drug users
MvPI	Max von Pettenkofer Institute
NAT	Nucleic acid Amplification Technology
NS	Non-structural protein
Nt	Nucleotides
NTR	Non-translated Region
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RIBA	Recombinant immunoblot assay
RNA	Ribonucleic Acid
RT-PCR	Reverse transcriptase polymerase chain reaction

UTR Untranslated Region

ABSTRACT

Many developing countries are reluctant to intensively screen blood bank samples and employ genotype specific treatment strategy for Hepatitis C virus (HCV). This is mainly due to high costs, time and high technical skill requirements associated with Nucleic Acid Amplification Technology (NAT)-based tests. The study aimed to evaluate *Monolisa® HCV Antigen-Antibody Ultra* (Bio-Rad Laboratories Limited, Marnes La Coquette, France), a new combination ELISA assay designed to detect in parallel antigens for and antibodies to HCV, and further determine the genotyping performance of Restriction Fragment Length Polymorphism (RFLP) assay on HCV genotypes 1 to 4 samples. The study involved retrospective and prospective analysis of samples stored at the Max von Pettenkofer Institute (MvPI) and samples obtained from patients attending HCV treatment at the two main Ludwig Maximillian University hospitals in Germany. Sensitivity, Specificity and Predictive values of the new ELISA kit was evaluated and compared with the AXSYM HCV version 3.0 (Abbot Diagnostics, Germany), an antibody based ELISA kit. Seventy four samples were tested on the two ELISA assays while fifty PCR positive samples were genotyped by Restriction Fragment Length Polymorphism assay. The study further measured the viral loads of twelve samples using random primers and compared the results with the measurements obtained by 5'UTR specific primers. The two ELISA assays realized comparable results both recorded a similar sensitivity of 91% with positive predictive values of 100% and 98% for the two assays respectively. Specificity of *Monolisa® HCV Ag-Ab Ultra*

was recorded as 100% with a negative predictive value of 87% against a specificity of 93% with a negative predictive value of 86% recorded for AxSYM. Two samples with high viral loads of 780.000 and 8.900.000 IU/mL were not detected by the *Monolisa® HCV Ag-Ab Ultra* assay. Genotyping of these two samples revealed genotype 1b, a HCV-subtype. Restriction Fragment Length Polymorphism assay genotyped and showed clear results on forty two (84%) samples, unclear results on six (12%) samples and conflicting results on two (4%) of the fifty samples genotyped. Although RFLP realized difficulty with genotype 2 samples, all other genotypes were easily genotyped. Finally the study showed similarity in the viral load measurements between random and specific primers. The study concludes that although *Monolisa® HCV Antigen-Antibody Ultra* assay depicts high sensitivity and specificity in detecting antibodies to HCV, it does not add further benefit to detect HCV infections by enhanced sensitivity due to the potential contingency to trace viral capsid antigens, a fact that needs further evaluation. On the other hand, RFLP is an effective genotyping tool among HCV genotypes 1 to 4. The study also reveals the importance of random primers and asserts the primers as a point of focus in the future projection in Hepatitis C genotyping. It recommends further evaluations of these assay platforms using Kenyan samples, as their introduction would be instrumental in HCV diagnosis and management in Kenya. This work provides a baseline for further studies on evaluation of antigen sensitivity of *Monolisa® HCV Antigen-Antibody Ultra*, restriction performance of various enzymes used in RFLP genotyping and performance of random primers in HCV diagnosis.

CHAPTER ONE

INTRODUCTION

1.1 Introduction

Hepatitis C virus remains a major health care burden to the world. Incidence rates across the world fluctuate and are difficult to calculate given the asymptomatic, often latent nature of the disease prior to clinical presentation. Further, prevalence rates across the world have changed due to the fact that more countries are now aware of transfusion-related hepatitis C infections. Several research studies have also shown more and more evidence supporting intravenous drug use (IDU) as the leading risk factor for the spread of the virus. It is also clear that most studies on HCV prevalence use blood donors to report the frequency of HCV usually by anti-HCV antibodies and do not report follow-up HCV testing. It is important to note, however, that using blood donors as a prevalence source may underestimate the real prevalence of the virus because donors are generally a healthier population stratum.

1.2 Background information

Hepatitis C virus (HCV) is a prevalent pathogenic bloodborne infection leading to an estimated 170 million people with chronic infections worldwide (Lauer and Walker. 2001). The highest prevalence rates have been suggested to be found in Africa, Eastern Mediterranean, South East Asia and the Western Pacific (Sy and Jamal 2006).

Muasya *et al.*(2008) estimates a current prevalence rate of 0.2% to 0.9% among the general population in Kenya. This estimate seems to be supported by findings of

Ilako *et al.*(1995) who recorded a prevalence of 0.9% among volunteer blood donors. This however may not reflect the true picture since no major survey on disease burden of HCV has been carried out in Kenya in recent times. The findings by Muasya and his research group of a 22.2% prevalence rate among a cohort of drug users in Kenya depicts a possible higher prevalence than the estimates in the general population. Secondly, Kenya currently concentrates more on the screening for Hepatitis B, HIV and bacterial related sexually transmitted diseases, with little attention to Hepatitis C virus in donated blood. Furthermore, HCV infection can proceed with no apparent clinical signs during the early phase of infection and is often diagnosed only by chance in asymptomatic populations. Success of HCV treatment among those infected mainly depends on the infecting HCV genotype. Due to high costs involved in this type of treatment, many developing countries are yet to employ this treatment strategy, thus it is not assured whether all those who are diagnosed for HCV fully recover. Finally, Kenya borders Tanzania without any major cross-border movement health related restrictions. Tanzania has been shown to be among those countries with the highest HCV prevalence rate in the world (WHO, 1997). These factors highlighted seem to suggest a higher prevalence rate of HCV in Kenya than the current rates, as assumed so far.

Just like Kenya, many developing countries are yet to introduce Hepatitis C screening for donated blood and at the same time employ the strategy of genotype dependent treatment of HCV infection. This is due to the fact that as much as HCV diagnostic kits and genotyping assays are commercially available, they are still very expensive for introduction into the regular healthcare system by most of these

governments. This calls for the development of more affordable, effective and reliable assays for clinical diagnosis of HCV, as this will accelerate the introduction of these strategies, just as seen in Europe, U.S.A and other developed countries.

This study evaluating *Monolisa® HCV Ag-Ab Ultra*, a "combination" ELISA assay and the genotyping performance of Restriction Fragment Length Polymorphism (RFLP) assay, a technique for HCV genotyping was undertaken at the Max von Pettenkofer Institute (MvPI) Diagnostic Virology Department in Munich, Germany. This was mainly due to the Department's role in the development and evaluation of diagnostic techniques for human pathogenic viruses, apart from the routine diagnostic work and other activities.

The samples used were mainly obtained from patients who had been diagnosed with new infections or those who were undergoing treatment for HCV, mainly from Germany and other parts of Europe, with a few from different regions of the globe as Munich is a cosmopolitan city. The choice and use of these samples, as assumed during the study, provided a high probability of detecting mutant strains. This was due to the fact that this country already employs genotype specific treatment for HCV infection hence the possibility of mutation-related resistance to the available regiments used in the treatment of HCV.

Mutated strains are of utmost importance to Africa and other developing countries due to the economic pressure they put in the healthcare systems of these countries which often have unstable economies. Although no published data to support this

assertion was found, Kenya specifically stands at a high risk of importing these mutant strains, not just from Europe but from other regions of the world as well, due to the growing trend of sex tourism mainly at the Kenyan coast. There is also currently lack of established blood donor population, and limited contact tracing mechanisms for foreign blood donors.

The results obtained from this study are important in the HCV diagnosis and management as a basis of making an informed opinion in the choice and selection of diagnostic assays that are affordable. Further the worldwide significant genetic variations in HCV are evidence of its frequent rates of mutation and rapid evolution. This calls for constant research in the development of new and better tools in HCV diagnosis. Therefore, findings of this study may also form a baseline for future research in the development of more effective and affordable diagnostic assays for Hepatitis C virus.

1.3 Statement of the problem

Hepatitis C virus (HCV) is a major cause of liver disease worldwide and a potential cause of substantial morbidity and mortality in the future. The complexity and uncertainty related to the geographic distribution of HCV infection and chronic hepatitis C, determination of its associated risk factors, and evaluation of cofactors that accelerate its progression, underscore the difficulties in global prevention and control of HCV. Currently, in most industrialized countries, HCV antibody screening and mini-pool nucleic acid amplification testing (NAT) is carried out for virtually all collected blood hence the reduction of HCV transmission through blood

transfusion. However in many developing countries, HCV transmission through blood transfusion remains a critical health problem. The fact that HCV does not show clear clinical signs during the long window period further complicates the risk of transmission through other routes in the general population. Even though success of treatment of HCV is genotype specific indicating the importance of genotyping for HCV prior to treatment, these developing countries including Kenya have not introduced genotype specific strategy in HCV management. Therefore, the fact that these countries do not have cheaper alternative methods for early diagnosis of HCV infection and lack of genotype specific strategy in treatment of HCV seems to have aggravated the incidence of HCV infections through transfusion related transmission. These factors seem therefore to have hampered the control and management of HCV transmission in these countries.

1.4 Justification of the study

Currently there is no vaccine and no post-exposure prophylaxis for HCV. There is thus a need for prevention of HCV infection mainly through safer blood transfusion. The long window period before detection of the first antibodies to HCV infection calls for the development of a more reliable assay that could help reduce the window period, as opposed to the available expensive nucleic acid amplification techniques. The fact that "combination" assays able to detect both antigens and antibodies could offer a promising alternative to NAT in early HCV detection, justifies the need to evaluate their efficacy among positive samples, hence evaluation of *Monolisa*® *HCV Ag-Ab ULTRA.*

Success of treatment for HCV has been shown to be dependent on the specific infecting genotype. Thus, together with viral load measurement and determination of different hosts-related markers, HCV genotyping is used to predict the response to antiviral therapy and to optimize the duration of treatment. It is also an essential tool in epidemiological studies and for tracing the source of infection. For clinical management of the infection, however, determination of a specific genetic group is sufficient. Commercially available genotyping assays are too expensive especially for the developing countries, which are yet to fully embrace genotyping in clinical management of HCV hence the development of assay platforms that are considered cheaper compared to currently available assay platforms. The fact that these new assay platforms are developed as a matter of urgency justifies the need to evaluate the efficacy and reliability of these assay platforms if HCV control and management is to be fully realized.

The 5' untranslated region (5'UTR) is the genome section of HCV often used when genotyping this virus, mainly for epidemiological reasons and for purposes of tracing source of infection. This section has however been shown to be inappropriate in discriminating the virus strains especially at the subtype level (Cantaloube *et al.,* 2000; Chen and Weck 2002; Germer *et al.,* 1999). This calls for analysis of other sections in the genome. Genotyping specific sections require use of specific primers thus limiting the study of other regions of the genome. This fact justifies the need to evaluate the performance of random based primers that can be used in genotyping all other regions of the genome.

This study, therefore, was aimed at evaluating the diagnostic performance of *MONOLISA® HCV Ag-Ab Ultra* (Bio-Rad) ELISA kit based on its simultaneous detection of Anti-HCV Antibodies and HCV Capsid antigens among known HCV positive samples and determining the ability of RFLP to correctly genotype samples of known HCV 1 to 4 genotypes. It further looked at the reverse transcribing ability of random primers in comparison to 5'UTR specific primers in genotyping HCV samples.

1.5 Hypotheses

I. *Monolisa® HCV Ag-Ab Ultra* (Bio-Rad) ELISA kit is able to detect all antigens and antibodies to HCV from positive blood samples.
II. RFLP genotypes with ease all HCV genotypes 1 to 4 positive samples.
III. There is no difference between random and 5'UTR specific primers in HCV amplification and genotyping.

1.6 Objectives of the study

1.6.1 Broad Objectives

To evaluate the diagnostic performance of MONOLISA® *HCV Ag-Ab Ultra* (Bio-Rad) ELISA kit and to determine the ability of RFLP to correctly genotype samples of known HCV 1 to 4 genotypes.

1.6.2 Specific objectives

1. To assess the antibody sensitivity, antigen sensitivity, specificity and the predictive values of *Monolisa® HCV Ag-Ab Ultra* (Bio-Rad) ELISA kit.

2. To evaluate RFLP by genotyping HCV- PCR positive amplicons of known genotypes and comparing the results with the blasted sequence results of the same amplicons.
3. To compare the performance of random primers versus HCV 5'end specific primers in reverse transcribing purified HCV RNA extracts.
4. To compare the genotype variations of HCV obtained by sequencing both the 5'UTR "conserved" region and Non-structural "hyper-variable" regions of the HCV genome for selected PCR positive samples.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 HCV morphology and genome characteristics

into three genera: flavivirus, pestivirus, and hepacivirus. Flaviviruses include yellow Hepatitis C virus infection is a major cause of acute and chronic hepatitis and may eventually lead to cirrhosis and hepato-cellular carcinoma (Alter *et al.*, 1989; Kiyosawa *et al.*, 1990). It is an enveloped, single-stranded RNA virus that has been classified in the *Flaviviridae* family. The virus is spherical in shape; the size of the viral particles is between 36 and 62 nm and has an estimated density of 1.08 g/ml (Kiyosawa *et al.*, 1990).

The *Flaviviridae* family is divided fever virus, dengue fever virus, Japanese encephalitis virus, and Tick-borne encephalitis virus. Pestiviruses include bovine viral diarrhea virus, classical swine fever virus and Border disease virus. HCV, with its 6 known genotypes (1-6) and numerous subtypes, belongs to the hepacivirus genus, which also include tamarin virus and GB virus B -GBV-B (Lindenbach and Rice, 2001).

Like all other members of *Flaviviridae* family, HCV is enveloped in a lipid bilayer in which two envelope proteins (E) are anchored. The envelope surrounds the nucleocapsid, which is composed of multiple copies of a small basic protein (core or C), and contains the RNA genome. The genome is a positive-strand RNA molecule, with an open reading frame (ORF) encoding a polyprotein of about 3000 amino acids (aa) or slightly more depending on the genotype. This ORF contains 9024 to

9111 nucleotides depending on the genotype and encodes at least 11 proteins including; 3 structural proteins (C or core, E1 and E2), a small protein (p7) whose function has not yet been definitively defined, 6 nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B), and the so-called "F" protein which results from a frameshift in the core coding region, as illustrated in Figure 2.1.

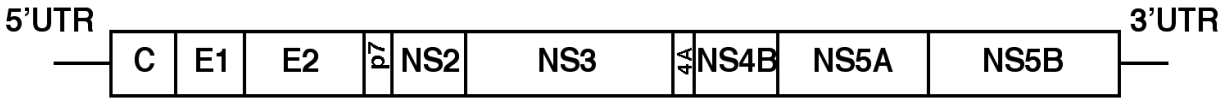


Figure 2.1: Organization of Hepatitis C genome (Thurner *et al.,* 2004)

Briefly, 5'UTR and 3'UTR are the un-translated regions. The HCV 5'UTR contains 341nt located upstream of the ORF translation initiation codon and is the most conserved region of the genome. The 3'UTR consists of stable stem-loop structures and an internal poly (U)-poly (U/C) tract. It contains approximately 225nt and interacts with the NS5B RdRp and with two of the four stable stem-loop structures located at the 3' end of the NS5B-coding sequence (Cheng *et al.,* 1999; Lee *et al.,* 2004). The polyprotein processing and the location of the 10 HCV proteins relative to the ER membrane are schematically represented in Figure 2.2. Scissors indicate ER signal peptidase cleavage sites; cyclic arrow, autocatalytic cleavage of the NS2-NS3 junction; black arrows, NS3-NS4A protease complex cleavage sites; intra-membranous arrow, cleavage by the signal peptide peptidase. The trans-membrane domains of E1 and E2 are shown after signal-peptidase cleavage and reorientation of the respective C-terminus hydrophobic stretches (dotted rectangles). Spots denote glycosylation sites of the E1 and E2 envelope proteins.

Structural proteins include; **C** which is the HCV core protein and is a highly basic, RNA-binding protein, which is presumed to form the viral capsid. **E1** and **E2** are envelope glycoproteins and are essential components of the HCV virion envelope, necessary for viral entry and fusion (Bartosch *et al.*, 2003; Nielsen *et al.*, 2004).

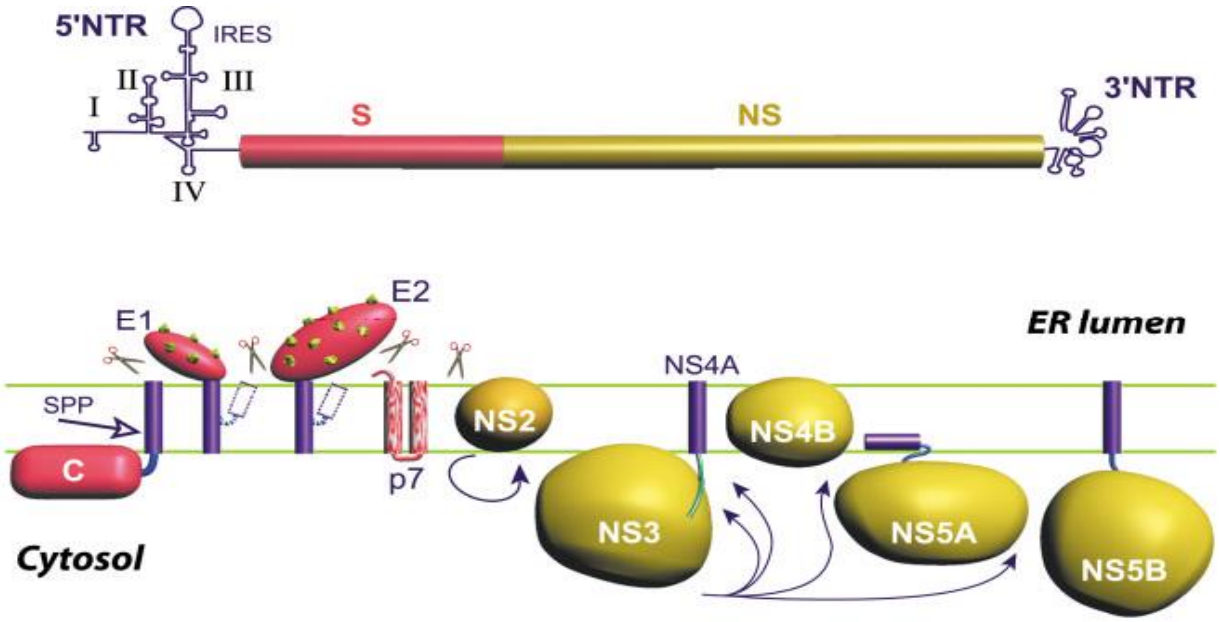


Figure 2.2. Polyprotein processing and the location of the 10 HCV proteins relative to the ER membrane (Penin *et al.*, 2004).

Nonstructural proteins include; P7 which is a small, 63 aa polypeptide, that has been shown to be an integral membrane protein (Carrere-Kremer et al., 2002). Other Nonstructural proteins are designated NS and include; NS2 which is a non-glycosylated trans-membrane protein. It is short-lived and loses its protease activity after self-cleavage from NS3 and is degraded by the proteasome in a phosphorylation-dependent manner by means of protein kinase - casein kinase 2 (Franck *et al.*, 2005). **NS3** is a multi-functional viral protein containing a serine protease domain in its N-terminal third and a helicase/NTPase domain in its C-

terminal two-thirds. **NS4A** is a cofactor of NS3 protease activity. NS3-4A also bears other properties through its interaction with host cell pathways and proteins hence may be important in the lifecycle and pathogenesis of infection, for this reason it has been one of the most popular viral targets for anti-HCV therapeutics (Pawlotsky and McHutchison, 2004; Pawlotsky, 2006). **NS4B** is an integral membrane protein of 261 aa with an ER or ER-derived membrane localization. One of the functions of NS4B is to serve as a membrane anchor for the replication complex (Gretton *et al.,* 2005). **NS5A** is a 56-58 kDa phosphorylated zinc-metalloprotein that probably plays an important role in virus replication and regulation of cellular pathways. **NS5B** is an RNA-dependent RNA polymerase. It has been 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 Epidemiology of Hepatitis infections

2.2.1 Genotype distribution

Hepatitis C virus is classified into eleven major genotypes (designated 1-11), many subtypes (designated by alphabetical letters), and about 100 different strains (numbered 1, 2, 3, etc.) based on the sequence heterogeneity (Simmonds. 1999). Of these, genotypes 1- 6 show clear genotype distinction between genotypes.

Genotypes 1-3 have a worldwide distribution. Subtypes 1a and 1b are the most common, accounting for about 60% of global infections. They predominate in Northern Europe and North America, and in Southern and Eastern Europe and

Japan, respectively. Type 2 is less frequently represented than type 1. Type 3 is found mainly in south-east Asia and is variably distributed in different countries. Genotype 4 is principally found in the Middle East, Egypt, and central Africa. Type 5 is almost exclusively found in South Africa, and genotypes 6-11 are distributed in Asia (WHO, 2008).

In a study done in Kenya by Muasya *et al.* (2008) among 333 drug users, 73% of the 38 HCV RNA positive samples sequenced belonged to genotype 1a with the remaining 27% being of genotype 4. This study did not find genotypes 2, 3, 5 and 6 among these drug users. However, owing to the sample size of the study, other genotypes cannot be ruled out in this country.

2.2.2 Global distribution of HCV infections

Hepatitis C virus is prevalent worldwide, however due to the fact that most infections are asymptomatic and since many studies are mainly conducted in population sub-groups, limited epidemiological data are currently available describing the true picture of the global distribution.

A review of 263 journal articles reporting HCV seroprevalence rates in different countries (WHO, 1997) estimated that 160 million people were infected by HCV globally. This data excluded Botswana, Peru and Zambia. Countries where high prevalence rates were found included Egypt, Bolivia, Burundi, Cameroon, Guinea, Mongolia, Rwanda, and Tanzania. In this report WHO reported high prevalence mainly in Africa, the Eastern Mediterranean, South East Asia and the Western

Pacific, with lower prevalence rates in North America and Europe. In this review, a seroprevalence of 0.9% among blood donors had been reported in Kenya by Ilako *et al.* (1995).

These figures were updated in 1999 using data from 131 countries (WHO, 1999). Currently, about 3% of the world‘s population (an estimated 170 million people) are now thought to be chronic carriers (WHO, 2002). The prevalence of HCV in Kenya is still estimated at between 0.2 and 0.9 (Muasya *et al*., 2008). This however may not reflect the true picture as no major survey has been conducted in the country. Further the fact that Kenya borders Tanzania which is one of those countries with high prevalence rates of 13.8% (WHO, 1997; Talatela, 2007) the prevalence rates could be different. The global distribution of HCV is shown in Figure 2.3.

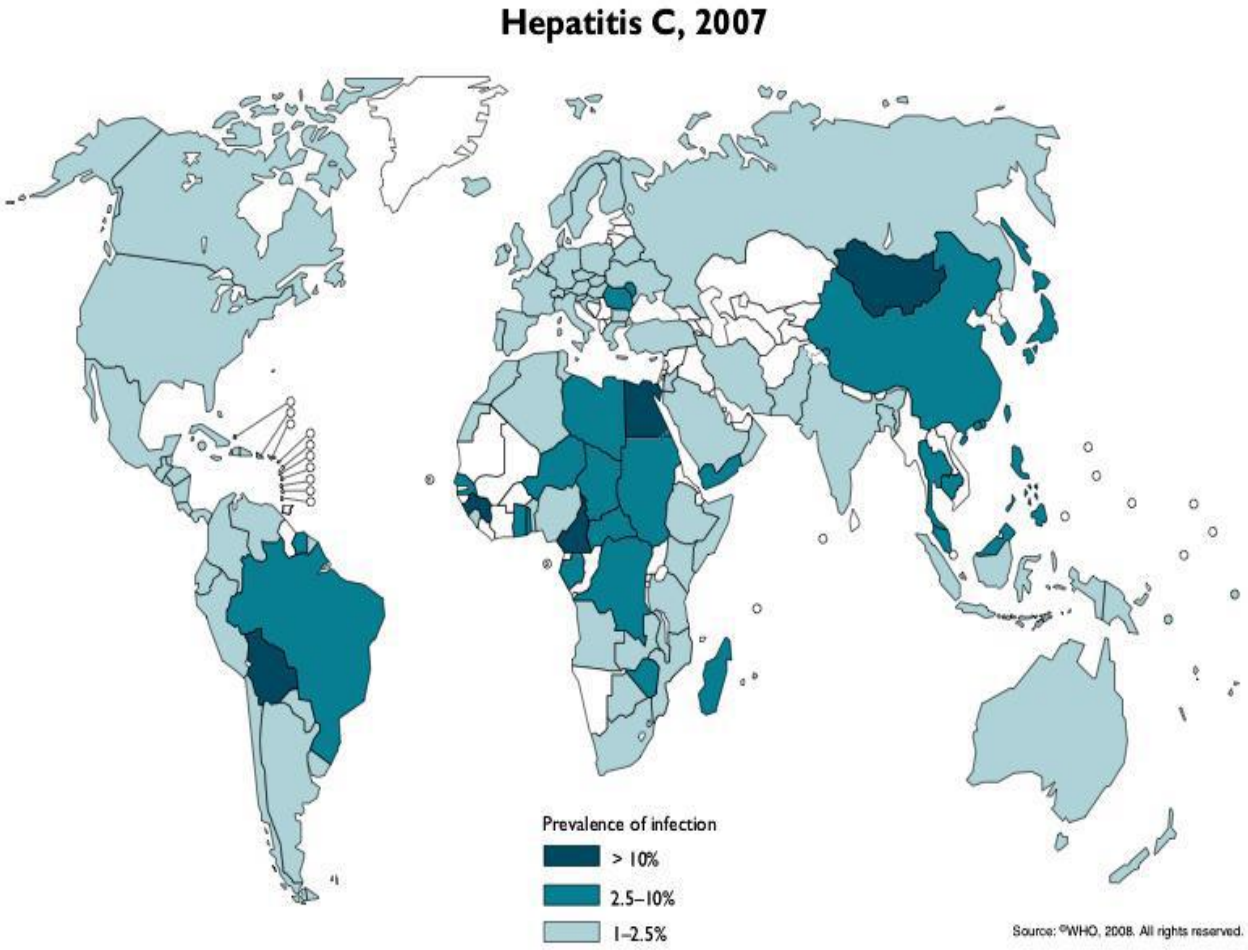


Figure 2.3: Global distribution of HCV infections (W.H.O, 2008)

2.3 Risk factors for HCV transmission

2.3.1 Blood Transfusion

Transfusion of blood or blood products has been a leading cause of transmission of HCV. However, due to improved screening, transmission through blood transfusions has decreased in most developed countries. However, incidences of transfusion related hepatitis C transmission are still higher in other areas of the world mainly in the developing countries.

A study by Ilako *et al.*(1995) - to assess the prevalence of hepatitis C virus antibodies in renal patients, blood donors and patients with chronic liver disease- found the prevalence of HCV infection in Kenya to be similar to that found mainly in England and Western Europe, all recording 0.9%. This finding of 1995 however may not reflect the situation today. This is mainly attributed to the fact that blood donations by 1995 were mainly from high school students and prison inmates. It is noted here that better screening for selecting blood donors mainly seen in the developed countries is yet to be realized in many developing countries. To enhance speedy realization, therefore, there is an urgent need for affordable, effective and reliable HCV laboratory diagnostic assays for HCV screening of blood in these developing countries.

2.3.2 Intravenous drug use

Transmission of Hepatitis C virus has been strongly associated with intravenous and percutaneous drug and needle use. Studies have provided varied data concerning HCV prevalence among these drug users. A study carried out in Antwerp and Limburg in Belgium showed that 71% and 46% respectively of the 310 drug users sampled had anti-HCV antibodies (Mathei *et al., 2005*).

The Hepatitis C European Network for C-operative Research (HENCORE) group reported a prevalence of hepatitis C of 80% among intravenous drug users (IVDU). The latest study by Aceijas and Rhodes (2007), found varying regional estimates, ranging from 10% to 96% in Eastern Europe and Central Asia, from 10% to 100% in South and South-East Asia, from 34% to 93% in East-Asia and the Pacific, from 5%

to 60% in North Africa and the Middle-East, from 2% to 100% in Latin America, from 8% to 90% in North America, from 25% to 88% in Australia and New Zealand, and from 2% to 93% in Western Europe. Only in Colombia and Lebanon were all HCV prevalence estimates below 20%. A similar study in Kenya realized a prevalence of 42.2% (66/146) of HCV versus 36.30% (53/145) of HIV among IDUs with only 3.24% (6/185) of HCV versus 13.51 (25/185) of HIV among non injectors (Odek-Ogunde *et al.,* 2004).

2.3.3 Hemodialysis

It has been well documented that dialysis patients have a higher risk of acquiring HCV infection. In the 1990's many regions of the world reported anti-HCV prevalence rates of 10% -50% among hemodialysis patients with lower rates of about 1.7% in such places as Ireland (Conlon *et al.,* 1993). However, there has been a decreasing tendency in the sero-prevalence of HCV among the hemodialysis patients reported from many hemodialysis facilities worldwide (Taziki and Espahbodi, 2008; Almroth *et al.,* 2002; Yang *et al.,* 2003; Silva *et al.,* 2006). Little is known about the prevalence among this group in Africa. Data compiled by Rahnavardi *et al.* (2008) indicate that the rate of infection among this group of patients is high in Senegal which recorded 12/15 (80%) of HCV positive cases from one hemodialysis center in the year 2000 (Diouf *et al.,* 2000), with the least (5%) of similar cases recorded in Kenya in 2003 (Otedo *et al.,* 2003).

2.3.4 Sexual contact

The role of sexual contact in the transmission of HCV remains unclear. One hypothesis is that many of the hepatitis C patients may have injecting sexual

partners. This was elucidated in a study where 15% of non IVDU women with injecting male partners had HCV infection (Goldberg *et al.*, 2001).

Vandelli *et al.* (2004) in a 10-year prospective follow-up study (8060 person-years) showed no evidence of sexual transmission among monogamous couples in Italy. This finding was, however, contradicted by the results from a similar study among spouses in Egypt which estimated that wife to husband transmission was 34% and 10% among women with and without detectable HCV RNA respectively. This study further estimated husband to wife transmission at 3%. Overall, 6% of the cases were estimated to have contracted HCV from their spouses (Magder *et al.*, 2005). However, when evaluating the findings from Magder´s group, it is important to note that the prevalence of HCV is much higher in Egypt and this study did not emphasize monogamous sexual relationships, though transmission between spouses can only be assumed to be sexual in nature.

Further, Alary *et al.* (2005) found no evidence for sexual transmission of HCV among gay men in a prospective Omega Cohort Study in the US (2653 person-years of follow-up). It suffices therefore to note that the studies highlighted above, support the notion that sexual transmission of HCV is still rare but for some reason, is higher among those with high-risk sexual contact. Other routes of transmission suggested have included vertical transmission, poor sterilization of invasive equipments in hospitals, among many other invasive procedures.

2.4 Treatment and Prevention of Hepatitis C infection

2.4.1 Mode of treatment

The National Institutes of Health (NIH) recommends treatment for HCV under the following conditions; a positive test result indicating hepatitis C virus circulation in the bloodstream; a biopsy that indicates significant liver damage and elevated levels of the liver enzyme, alanine aminotransferase (ALT) in the blood.The standard of care for hepatitis C treatment is a weekly injection with pegylated interferon alfa combined with twice-daily oral doses of ribavirin (Rebetol) which is a broad-spectrum antiviral agent. Two pegylated interferon medications are available, peginterferon alfa-2b (Peg-Intron) and peginterferon alfa-2a (Pegasys). The goal of HCV treatment is to clear the virus from the bloodstream. Combined pegylated interferon and ribavirin clear HCV infection in 40% to 80% of those treated.

Successful treatment of HCV, however, has been found to be dependent on the specific infecting genotype. The existing literature, although limited, suggests that patients with chronic hepatitis C genotypes 4–6 may exhibit different clinical courses and treatment outcomes. Ethnicity-related factors may contribute to the presence of more advanced disease in patients with genotype 4, who also tend to have a poor response to interferon-based therapy. Hepatitis C genotype 5 virus appears to be an easy-to-treat virus with response rates similar to those of genotypes 2 and 3 after a 48-week course of therapy. Response to treatment in patients with HCV genotype 6 may be at an intermediate level between that seen with genotype 1 and genotype 2 or 3 (Nguyen and Keeffe, 2005).

2.4.2 Side effects of medication

Interferon side effects include severe flu-like symptoms, irritability, depression, concentration and memory problems, skin irritation, fatigue and insomnia. Ribavirin can cause a low red blood cell count (anaemia), itchiness, nasal congestion, skin irritation, fatigue and birth defects. Combination therapy including pegylated interferon and ribavirin may cause psychosis or suicidal behavior in a limited number of people (Nguyen and Keeffe, 2005). For this reason, treatment with interferon is not recommended for patients with a history of uncontrolled major depression. Likewise, patients who are pregnant or have untreated thyroid disease, low blood cell counts or autoimmune disease, or addicted alcohol or drug users are not candidates for this therapy.

Side effects from combined pegylated interferon and ribavirin are generally most severe during the first few weeks of treatment, and may be improved with pain relief medications and anti-depressants. However, some people taking interferon need their dosage reduced because of severe side effects, whereas in others, treatment must be stopped due to these side effects. In worst cases of HCV liver damage, a patient may need to undergo liver transplantation (Nguyen and Keeffe, 2005).

2.4.3 Prevention of HCV infection

Currently, there is no vaccine for the prevention of HCV. However, vaccination against hepatitis A and B viruses may be recommended to avoid any liver damage and or complication of treatment of hepatitis C which may be caused by these viruses (Nguyen and Keeffe, 2005). Other preventive measures should target the

suspected routes of transmission.

2.5 Diagnosis of HCV infection

2.5.1 HCV Diagnosis (Moyer *et al.*, 1999)

The diagnosis of HCV infection can be made by detecting either anti-HCV (antibodies against HCV) or HCV RNA circulating in the blood. Detection of anti-HCV is recommended for routine testing of asymptomatic persons and should include use of both enzyme immunoassay (EIA) and supplemental or confirmatory testing with an additional, more specific assay (Figure 2.4). Use of supplemental antibody testing, such as recombinant immunoblot assay (RIBA) tests, for all positive anti-HCV results of EIA is preferred, particularly in settings where clinical services are not provided directly.

Supplemental anti-HCV testing confirms the presence of anti-HCV, hence eliminating false-positive antibody results, which indicates past or current infection. This testing can be performed on the same serum sample collected for the Enzyme Immunoassay (EIA) in routine serology. Confirmation or exclusion of HCV infection in a person with indeterminate anti-HCV supplemental test results should be made on the basis of further laboratory testing, which might include repeating the anti-HCV test in two or more months or testing for HCV RNA and determining the ALT level.

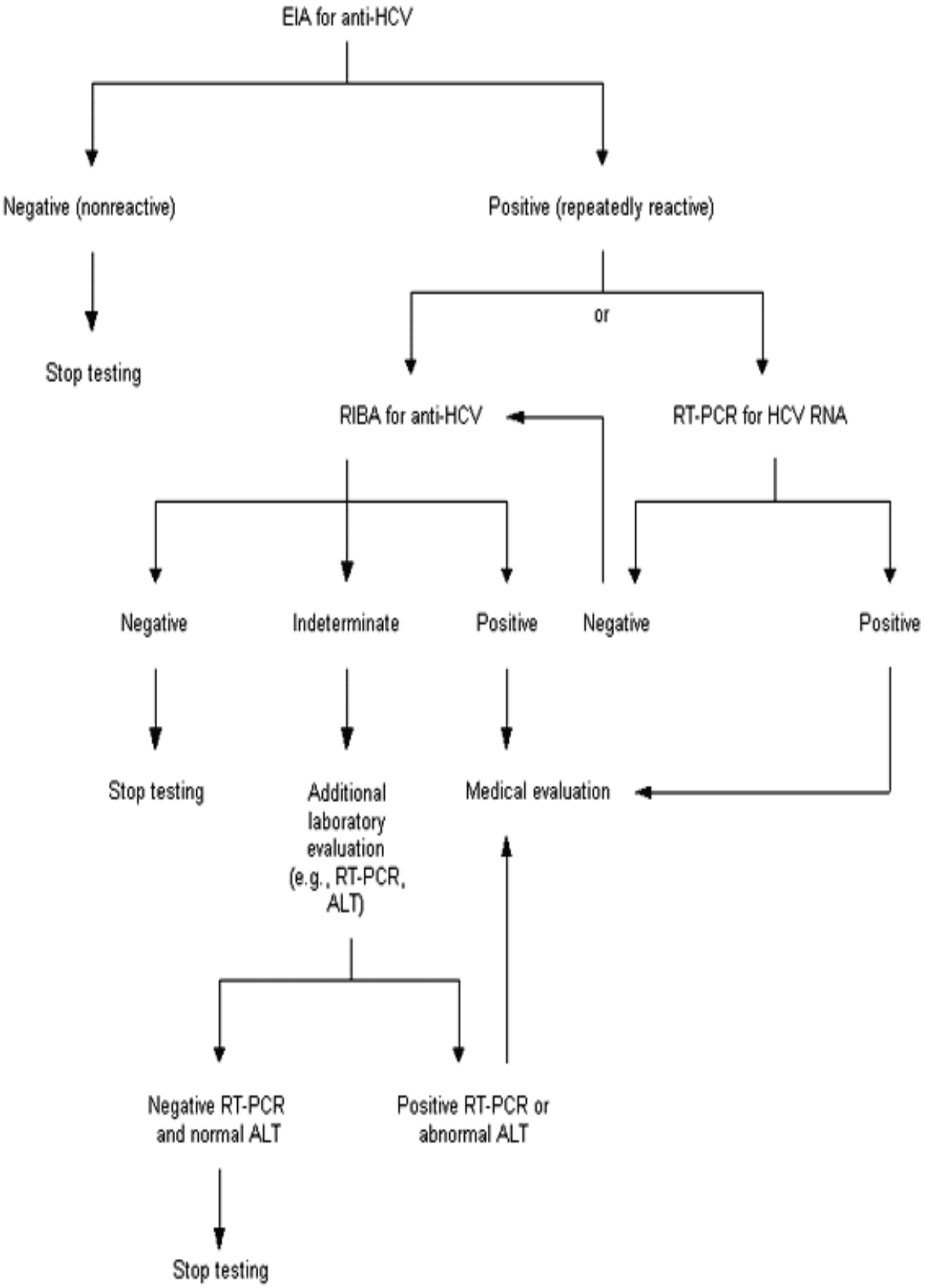


Figure 2.4. Algorithm for Hepatitis C virus infection testing in asymptomatic patients (Moyer *et al.*, 1999)

The diagnosis of HCV infection can also be made through detection of HCV RNA using reverse transcriptase polymerase chain reaction (RT-PCR) techniques (Figure 2.4). Hepatitis C virus RNA can be detected within one to two weeks after exposure to the virus, weeks before the onset of ALT elevations or the appearance of anti-HCV by PCR diagnosis. In some patients, the detection of HCV RNA may be the only evidence of HCV infection. Quantitative assays for measuring the titer of HCV RNA have been developed, including a branched chain DNA assay and a quantitative PCR. Several different nucleic acid detection methods also have been developed to group isolates of HCV based on genotypes.

2.6 Chronological sequence in the development of HCV detection techniques

The Hepatitis C virus was identified by Houghton and colleagues in 1988 (Choo *et al.,* 1989), making it the first time in the history of virology that a virus was characterized by genetic cloning and not by isolation. In early 1990s, antibody testing was widely used in detecting exposure to hepatitis C virus and became important worldwide in identifying HCV carriers in blood donor populations and in diagnosis of HCV infection (Chokephaibulkit *et al.,* 1992; Kleinmann *et al.,* 1992). By the late 1990s, nucleic acid tests (NAT) that directly detected HCV RNA in the blood became available, both for blood screening and diagnostic testing (Moyer *et al.,* 1999).

In 1995, a third type of assay that detected HCV circulating core antigen in serum or plasma in chronically infected individuals, was developed (Tanaka *et al.,* 1995). This was estimated to have an overall sensitivity of about 10^4–10^5 HCV RNA

copies/ml, and several studies have since then indicated that HCV core antigen is detected in 71% – 98.7% of the HCV NAT positive samples obtained during the pre-seroconversion period (Ayogi *et al.,* 1999; Courouce *et al.,* 2000; Muerhoff *et al.,* 2002; Nubling *et al.,* 2002; Leary *et al.,* 2006).

Recent studies on affordable and reliable diagnostic assay platforms have led to the development of ‘‘combination’’ immunoassays which are able to detect HCV antibodies and HCV antigens within a single kit. This approach is aimed at enabling for the detection of samples within the pre-seroconversion window period (Shah *et al.,* 2003; Laperche *et al.,* 2005; Ansaldi *et al.,* 2006). They include Chemiluminescence Immunoassay; a PRISM-based assay developed at Abbott Laboratories and *Monolisa® HCV Ag-Ab ULTRA* assay from BioRad laboratories. A research version of the HCV combination assay, developed on the ARCHITECT instrument has also been developed for research purposes by the research group at Abbott Laboratories.Even with all these improvements, pre-seroconversion window period has remained a major challenge to many of these tests especially in early detection of the virus.

2.6.1 Pre-seroconversion window period

Pre-seroconversion period is summarized in figure 5. Following infection with HCV, there is a pre-seroconversion window period of 6–10 weeks during which time antibodies to HCV cannot be detected using currently available antibody based assays (Barrera *et al.,* 1995). Immediately after infection, there is a brief period referred to as the ‘‘eclipse phase’’ during which HCV viral products cannot be

detected in serum or plasma. This period is followed by a preramp-up phase, characterized by low-level viremia (less than 10^4 HCV RNA copies/ml), or by periods of intermittent viremia. During this time, which can range from several days to weeks, the viral titers remain low or increase slowly (Glynn *et al.*, 2005).

The ramp-up phase ensues, with an exponential growth phase, characterized by viral doubling times ranging from 10.8 to 17 hrs (Busch, 2001; Nubling *et al.*, 2002). This ramp-up phase is estimated to persist for an average of about nine days.

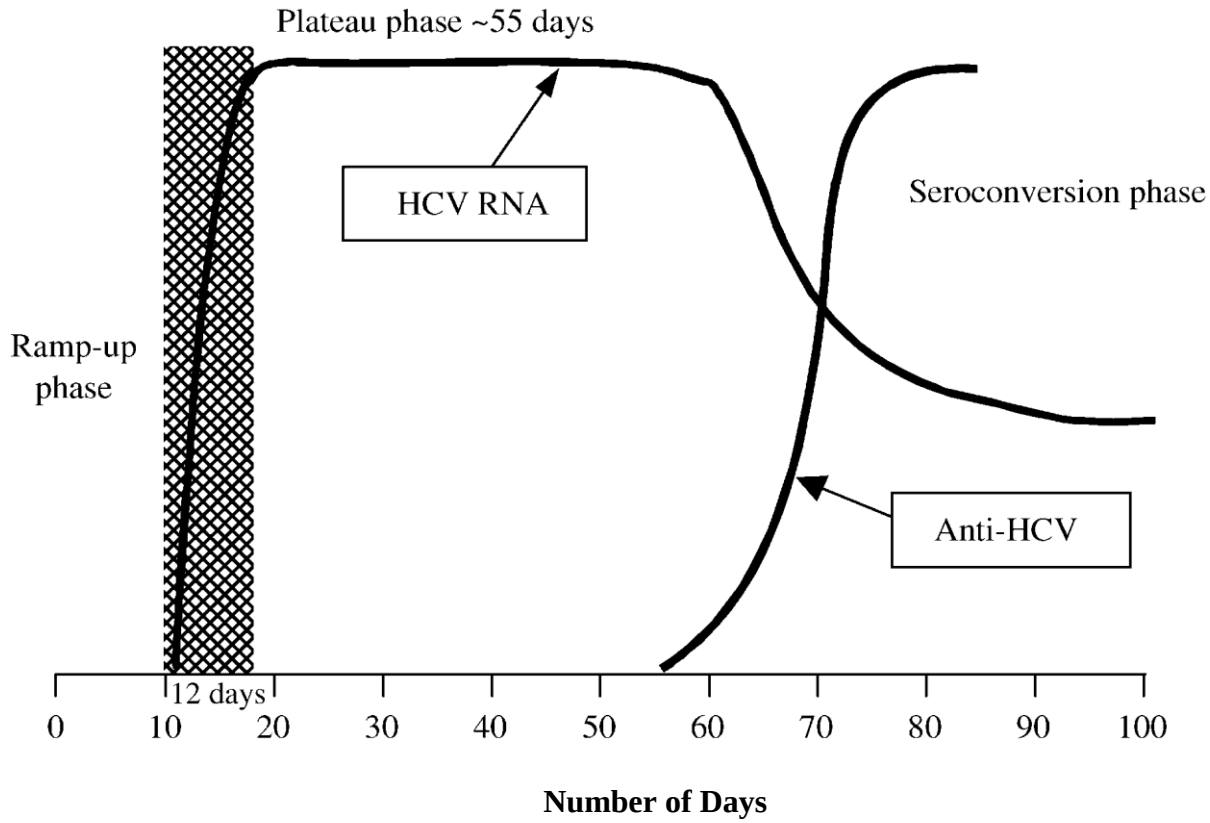


Figure 2.5. Early events in hepatitis C infection (Tobler *et al.*, 2005)

This is followed by a plateau phase, during which time the viral load range is $4.1x10^4$ to $7.2x10^7$ HCV RNA copies/mL with a mean value of $3.8x10^6$ HCV RNA

copies/mL (Glynn *et al.*, 2005). The plateau phase is believed to last for 50 to 60 days prior to seroconversion (Busch 2001; Glynn *et al.*, 2005), and occupies a major proportion of the pre-seroconversion window period.

Following seroconversion, there is typically a transient reduction in viral load even among those individuals who become chronically infected. Within 60 days in most cases, HCV RNA levels develop a relatively stable state and may persist for many years (Cox *et al.*, 2005). While about 15% of HCV infected individuals are able to eliminate the virus, 85% of infected individuals develop chronic infection characterized by the continual ability to detect both antibodies to HCV and HCV RNA for many years (Hoofnagle, 1997).

In summary, it is noted that HCV is one of the blood borne infections that poses major health challenges. These challenges need proper preventive measures put in place to reduce infections in the populations, which can only be achieved with proper and timely diagnosis of the virus in the infected patient or detection of the virus in materials that pose possible threat of infection to populations such as blood or blood products intended for transfusion. Many of the commercially available ELISA assays are antibody based, thus conclusive determination of HCV infection involves use of other assays mainly Nucleic Acid based techniques or core antigen detection assay, thus increasing the cost. A “combination” assay that is able to detect both antigens and antibodies within a single kit is therefore seen to reduce the cost, besides reducing the window period. Successful treatment of infection is also dependent specifically on the infecting genotype. This coupled with the various side

effects of antiviral drugs currently available are a clear indicator of genotype determination in infected patients before commencement of treatment. Though available, commercial genotyping assays are still very expensive. For example, during this study it was realized that to genotype fourty samples using a line probe assay (LiPA) specifically Versant™ HCV Genotype assay 2.0 (Innogenetics®, Belgium), the cost involved was approximately EUR 6,500 translating to EUR 162.5 (U.S $255.36) for each sample against about EUR 30 (U.S $ 47.14) for the same sample by RFLP when all the restriction enzymes are used. This cost by RFLP could reduce when only enzymes targeting specific genotypes suspected within a specific geographical region are used to confirm infection. Due to these high costs involved, many developing countries are, therefore, yet to adopt the strategy of intense blood screening for HCV in regular blood transfusion set ups and genotype specific treatment for HCV in regular HCV management. This fact is therefore an indication that constant research is needed in the development and evaluation of reliable, effective and affordable assays. This study therefore set out to evaluate *MONOLISA® HCV Ag-Ab Ultra* (Bio-Rad), an ELISA assay that could be instrumental in early serological detection of HCV infection and Restriction Fragment Length Polymorphism (RFLP), a genotyping assay still viewed as reliable and cost effective. These two could provide an alternative to currently available commercial assays and Nucleic acid based techniques, regarded as expensive mainly by the developing countries. This could accelerate implementation of strategies that integrate screening for HCV in regular blood transfusion settings and genotype specific treatment of HCV infected patients.

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Study site

The study took place at the Diagnostic Virology Laboratory of Max von Pettenkofer Institute in Munich. Max von Pettenkofer is a research center and medical school of the Ludwig Maximillan University in Munich Germany. The laboratory mainly receives samples from the University hospital and the main hospital (*Klinikum Grosshadern*) in Munich. Munich is one of the major cities in Germany and the capital of the Bayern federal state of Germany. Patients whose samples are tested here mainly come from Munich city as well as other cities in Germany. In certain cases, patients are referred to the University Hospital (*Klinikum der Universitat*) from the neighboring countries such as Austria and France. Since Munich is a cosmopolitan city, other patients who attend this hospital may belong to different nationalities.

3.2 Description of assays under evaluation

I. *Monolisa® HCV Ag-Ab Ultra* (Bio-Rad) ELISA kit

The kit is composed of **a microplate**, coated with; monoclonal antibodies against the capsid protein of HCV, two recombinant proteins produced by *E. coli* from the NS3 region, one recombinant antigen from the nonstructural NS4 region, and a peptide from the capsid area of the viral genome. **Conjugate 1,** containing mouse biotinilated monoclonal antibodies against HCV capsid, which does not react with the capsid-mutated peptide with which the microplate is coated and **conjugate 2,** containing mouse peroxidase antibodies to human IgG and peroxidase-labeled

streptavidin. **Washing solution** containing 10× concentrate Tris NaCl buffer, 1% Tween-20. Chromogen solution containing tetramethylbenzidine (TMB). **1 N sulfuric acid stop solution**. Negative (Tris HCl buffer: 10mM Tris–HCl, 0.5mM MgCl2, and 150mM NaCl, pH 7.4), positive (human serum containing antibodies to HCV diluted in Tris HCl buffer, photochemically inactivated), and antigen-positive controls obtained from lyophilized capsid synthetic peptide.

II. Restriction Fragment Length Polymorphism assay

This is a molecular biological technique where restriction enzymes (endonucleases) are used to cleave the DNA at specific locations. The strands generated are then separated according to their lengths by gel electrophoresis.

3.3 Study design

The study involved both retrospective and prospective analysis of samples. It used stored samples as well as samples from patients undergoing treatment for HCV as well as new patients. “Difficult” or “sticky” sera from bone marrow transplant patients and HCV negative patients were also used in evaluating *Monolisa® HCV Ag-Ab Ultra* (Bio-Rad) ELISA kit. Samples determined as PCR positive samples belonging to genotypes 1 to 4 were used in the evaluation of the genotyping performance of RFLP. Six of these samples used in RFLP evaluation were further sequenced on both conserved and “hyper” variable regions of the HCV genome.

3.4 Sample population

The study used stored samples, samples from patients undergoing treatment for HCV as well as new patients diagnosed of HCV infection. “Difficult” or “sticky”

sera (sera that could not necessarily generate antibodies to HCV) from bone marrow transplant patients and samples from HCV negative patients were also used in evaluating *Monolisa® HCV Ag-Ab Ultra* (Bio-Rad) ELISA kit. Only PCR positive samples belonging to genotypes 1 to 4 were used in evaluating the genotyping performance of RFLP. These samples were readily available at Max von Pettenkofer Institute virology laboratory. They were categorized as shown in the table based on PCR results and results obtained by Ortho HCV 3.0 ELISA test system with an enhanced SAVe, (Ortho Clinical diagnostics, Johnson and Johnson, United Kingdom) an antibody based ELISA kit.

Table 3.1: Samples for evaluation of *Monolisa® HCV Ag-Ab Ultra* ELISA kit.

Test samples	
Sample type	**Total number used**
PCR positive samples	32
Antibody positive- PCR Negative	8
HCV Negative samples	27
Bone marrow transplant patient	7
Total	**74**

Table 3.2: Samples for evaluation of RFLP

Genotype		Number
	Subtype	
1	1a	4
	1b	10
2	2a	8
	2b	1
3	3a	10
4		10
Mixed	Mixed	2
Unknown		5
Total		**50**

3.5 Ethical Considerations

The study used archived samples or samples forwarded by the Diagnostic Virology Department of the Max von Pettenkofer institute Germany. Therefore, there was no direct contact with the patients, hence no direct risk to the patient was involved. Further, no names of the patients were published anywhere during or even after study as samples were identified by personal identification numbers. The permission to use these samples was obtained from the Max von Pettenkofer institute in Germany which is authorized to conduct research as well as perform routine diagnosis.

3.6 Data management and analysis

i. Data management

Data generated was entered in excel worksheets for management. Samples were identified with specific codes assigned to them upon reception. All patients details were password protected and no information was released to anyone except for the investigators who were pertinent to the study.

ii. Data analysis

Sensitivity, specificity, confidence limits (CL) and predictive values of anti-HCV or antigen tests

Sensitivity, specificity and predictive values were calculated as represented diagrammatically represented in Table 3.3.

Table 3.3. Criteria for sensitivity, specificity and predictive values

		Reference / Gold standard test		
		+	**-**	
Test under Evaluation	**+**	a True positives	b False positives	**a + b**
	-	c False negatives	d True negatives	**c+d**
		a+c	**b+d**	

Sensitivity = a/ (a+c) x 100 Positive predictive value = a/ (a+b) x100

Specificity = d/ (b+d) x 100 Negative predictive value = d/ (c+d) x 100

Sensitivity: Is the ability of the assay under evaluation to identify correctly specimens that contain antibodies to HCV and or antigens of HCV based on a reference assay a gold standard test (WHO, 2001). Thus, sensitivity is the number of true positive specimens recognized by a test under evaluation as positive **(a)**, divided by the number of specimens identified by the reference assays as positive **(a+c)**, expressed as a percentage. Thus it is a probability of true positives by the new test system based on a gold standard test.

Specificity: Is the ability of the assay under evaluation to identify correctly specimens that do not contain antibody to HCV and or antigens of HCV based on a reference assay also known as a gold standard test (WHO, 2001). Thus specificity is the number of true negative specimens recognized by an evaluated test as negative **(d),** divided by the number of specimens identified by the reference assays as negative **(b+d),** expressed as a percentage. Thus it is the probability of detecting true negative samples by the assay under evaluation based on a gold standard assay.

Confidence limits (CL): The 95% confidence limits are a means of determining whether observed differences in sensitivity or specificity between assays are significant or not (WHO, 2001). Exact 95% confidence limits for Binomial proportions were calculated from the F-distribution. This was calculated as described by Armitage and Berry (1987).

Predictive Values:

The **positive predictive value (PPV)** is the probability that when the test is reactive, the specimen does contain antibody to HCV or antigen of HCV. This was calculated using the simple formula **a/ (a+b)** which gave approximate values for the tests.

The **negative predictive value (NPV)** is the probability that when the test is negative, a specimen does not have antibody to HCV or antigen of HCV. This was also calculated using the simple formula **d/(c+d) x 100** which again gave approximate values.

3.7 Laboratory procedures

3.7.1 Serological diagnosis for evaluation of Monolisa® HCV Ag-Ab ULTRA assay

Serological evaluation of *Monolisa Ag-Ab HCV Ultra* assay was performed as described by the manufacturer. Step by step procedure was done as shown in Appendix 1. Briefly, samples were added into the wells and incubated with conjugate 1 (R6) at 37°C for one hour before washing was done. Another incubation followed with conjugate 2 (R7) at 37°C for thirty minutes. Once more washing was done and the plate finally incubated in the dark for thirty minutes with a freshly prepared enzymatic development solution before the reaction was stopped and optical readings taken.

The presence or absence of antibodies to HCV or/ and HCV capsid antigen was thus determined by comparing for each sample the recorded absorbance with that of the calculated cut- off value. The cut- off value was calculated by dividing the mean of optical density readings for the three positive controls by 4. Samples with optical

density values below the cut off value were considered to be non-reactive; samples however just below the cut off value, i.e. by less than 10% were interpreted with a lot of caution, and thus retested. Samples above the cut- off values were considered initially reactive and retested in duplicate before the final interpretation (Appendix 2 and 3).

The assay was compared with two other antibody based ELISA kits, Ortho HCV 3.0 ELISA test system with an enhanced SAVe, (Ortho Clinical diagnostics, Johnson and Johnson, United Kingdom) and AxSYM system HCV version 3.0 (Abbott, USA) to evaluate different parameters.

Any samples that were found to present conflicting results with the above ELISA assays were subjected to a confirmatory test by western blot using INNO-LIA HCV score (Innogenetics® N.V, Belgium) and PCR for confirmation. INNO-LIA HCV score is describes in Figure 3.1.

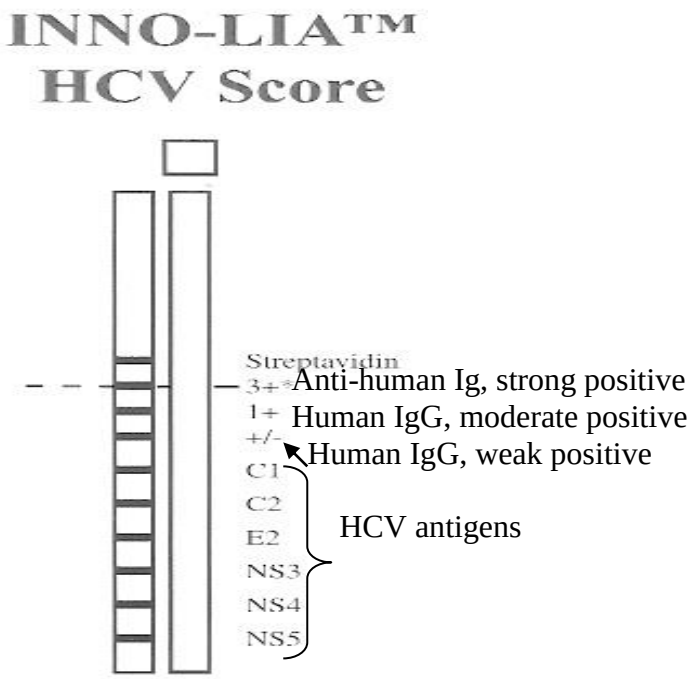


Figure 3.1. INNO-LIA^{TM} HCV score test strip (Innogenetics® N.V, Belgium)

3.7.2 Genotyping assay

3.7.2.1 Restriction Fragment Length Polymorphism (RFLP)

During RFLP for HCV, RNA was first extracted from samples using High Pure Viral Nucleic Acid kit (Roche applied science; Cat no. 11858874001), according to the manufacturer's instructions. Steps for extraction were followed as shown in Appendix 9. The target RNA was then reverse transcribed into cDNA amplification in a nested PCR system to produce millions of copies of strands of DNA identical to the original. The amplified DNA copies were then combined with a set of restriction enzymes namely Drd-I, BsmA-I, Bsr-I, Sau3A and Hinf-I. The sample mixture was then incubated at a specific temperature and time, depending on the type of enzyme used (Appendix 12). Other restriction enzymes used included, Fok-I and Nci-I. Any sample that did not give any restriction pattern was cleaved by Sma I to confirm

whether it was positive for HCV. Each of these enzymes used are known to have restriction sites as follows;

Drd-I: 5’ …..GACNNNN NNGTC….3’
3’ …..CTGNN NNNNCAG….5’

Bsma-I: 5’…..GTCTC$(N)_1$ …3’
3’…..CAGAG$(N)_5$ ...5

Bsr-I: 5’ ….ACTGGN ...3’
3’ ….TGAC CN….5’

Sau 3A-I: 5’..GATC.....3’
3’.... CTAG ..5’

Hinf- I: 5’....GANTC...3’
3’...CTNA G....5’

Fok 1: 5’....GGATG$(N)_9$ ...3’
3’....CCTAC$(N)_{13}$ ...5’

NCI-I: 5’...CCSGG....3’
3’....GGS CC....5’

S= C or G

Restriction patterns by the enzymes were then viewed as bands by gel electrophoresis under the UV light. An In-house RFLP interpretation criteria from Max von pettenkofer institute was used, as shown in Figure 3.7.

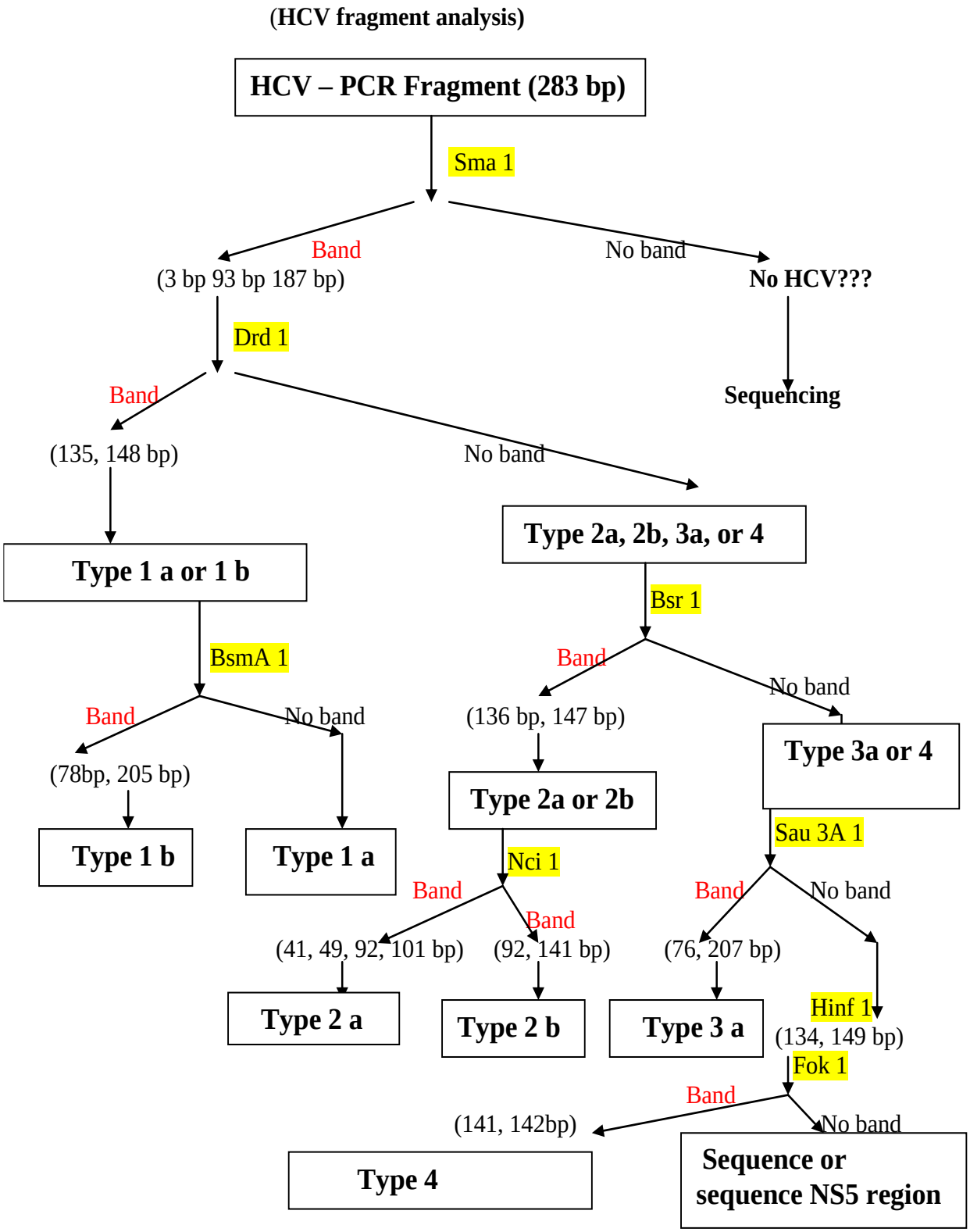


Figure 3.2. Schematic representation of the HCV genotyping process using enzymes Sma-1, Drd-1, BsmA-1, Bsr-1, Nci-1, Sau3A-1, Hinf -1 and Fok-1 (Inhouse protocol, MvPI)

Any samples giving conflicting genotypes by RFLP was subjected to sequencing using the assay platforms designed by and guidelines from Agencourt Bioscience Corporation. Sequencing was done using CEQ 8800 Beckman for HCV sequencing, and sequence results edited and blasted using the Hepatitis C database on gene sequencing to establish the genotypes for the analyzed sequences.

Performance of random primers (5'- NNN NNN – 3') was based on its ability to reverse transcribe sample known to be PCR positive and the results compared with those obtained by 5'UTR specific primers, (HCVN 02: 5' – gTg CAC ggT CTA CgA gAC C – 3' and HCVN 08: 5' –TAC TCA CCg gTT CCg CAg A – 3').

Upon reverse transcription, the cDNA was then subjected to amplification by PCR in a nested system using primers designated as; Outer primers for first PCR reaction, HCVN 01: 5' – ggC gAC ACT CCA CCA TRR A – 3' (forward primer) and HCVN 02: 5' – gTg CAC ggT CTA CgA gAC C – 3' (reverse primer). Inner primers for the second PCR reaction; HCVN 03: 5' – CAC TCC CCT gTg Agg AAC T – 3' (forward primer) and HCVN 04: 5' - CCC ggg gCA CTC gCA AgC A – 3' (reverse primer). Cycles were as shown;

PCR 1		PCR 2	
94°C ..... 4 minutes		94°C..... 4 minutes	
94°C..... 30 seconds		94°C..... 30 seconds	
50°C..... 1 minute	X **35 cycles**	50°C..... 1 minute	X **30 cycles**
72°C..... 1 minute		72°C..... 1 minute	
72°C.....8 minutes		72°C..... 8 minutes	
4°C .....∞		4°C..... ∞	

Generated PCR products were then run on 2% gels to asses the products. Upon generation of bands, indicating amplification of the nucleic acids viral load measurements were done for the two sets of primers and the results compared.

CHAPTER FOUR

4 RESULTS

4.1 Evaluation of *Monolisa® HCV Ag-Ab ULTRA*

4.1.1 Overall performance of *Monolisa® HCV Ag-Ab ULTRA assay* kit (Sensitivity, Specificity and Predictive Values).

A total of 74 samples were tested by AxSYM and the new *Monolisa® HCV Ag-Ab ULTRA assay* detection ELISA kits. As shown in Table 4.1, both kits were able to detect 31 out of the 32 PCR reactive samples. Both kits further detected all samples categorized as PCR negative - antibody positive. On the contrary though, *Monolisa® HCV Ag-Ab ULTRA assay* kit detected as negative all the negative samples whereas AxSYM detected as reactive two sample from this group.

Table 4.1. Results for all samples tested on AxSYM and *Monolisa Ag/Ab* Elisa kits (n=74)

Test samples		**AxSYM**		***Monolisa® HCV Ag-Ab***	
Sample type	Total number	Reactive	Non-Reactive	Reactive	Non- Reactive
PCR positive samples	32	31	1	31	1
Antibody positive- PCR negative	8	8	0	8	0
HCV negative samples	27	2	25	0	27
Bone marrow transplant patient	7	4	3	4	3
Total	**74**	**45**	**29**	**43**	**31**

One of the thirty two PCR positive samples not detected by the two ELISA kits in Table 4.1 was realized to belong to genotype 1b that is known to be universally distributed, and had a viral load of 8,900,000 IU/Ml above the suggested 260,000

IU/Ml theoretical threshold limit of detection (Turke *et al.*, 2008). This had previously been obtained from a bone marrow transplant patient, and formed part of the seven samples whose optical signals were measured and the trend of optical signals assessed and compared, as shown in Figure 4.1, between the two ELISA kits. Results depicted a similar trend in the optical signal generation between the two assay kits. Two of the three samples detected negative at cut off points of 1 (S/CO) and 0.5 for Axsym and *Monolisa® HCV Ag-Ab ULTRA assay* respectively, had viral loads of 8,900,000 and 780,000 IU/Ml, above the theoretical threshold limit of detection for *Monolisa® HCV Ag-Ab ULTRA assay*.

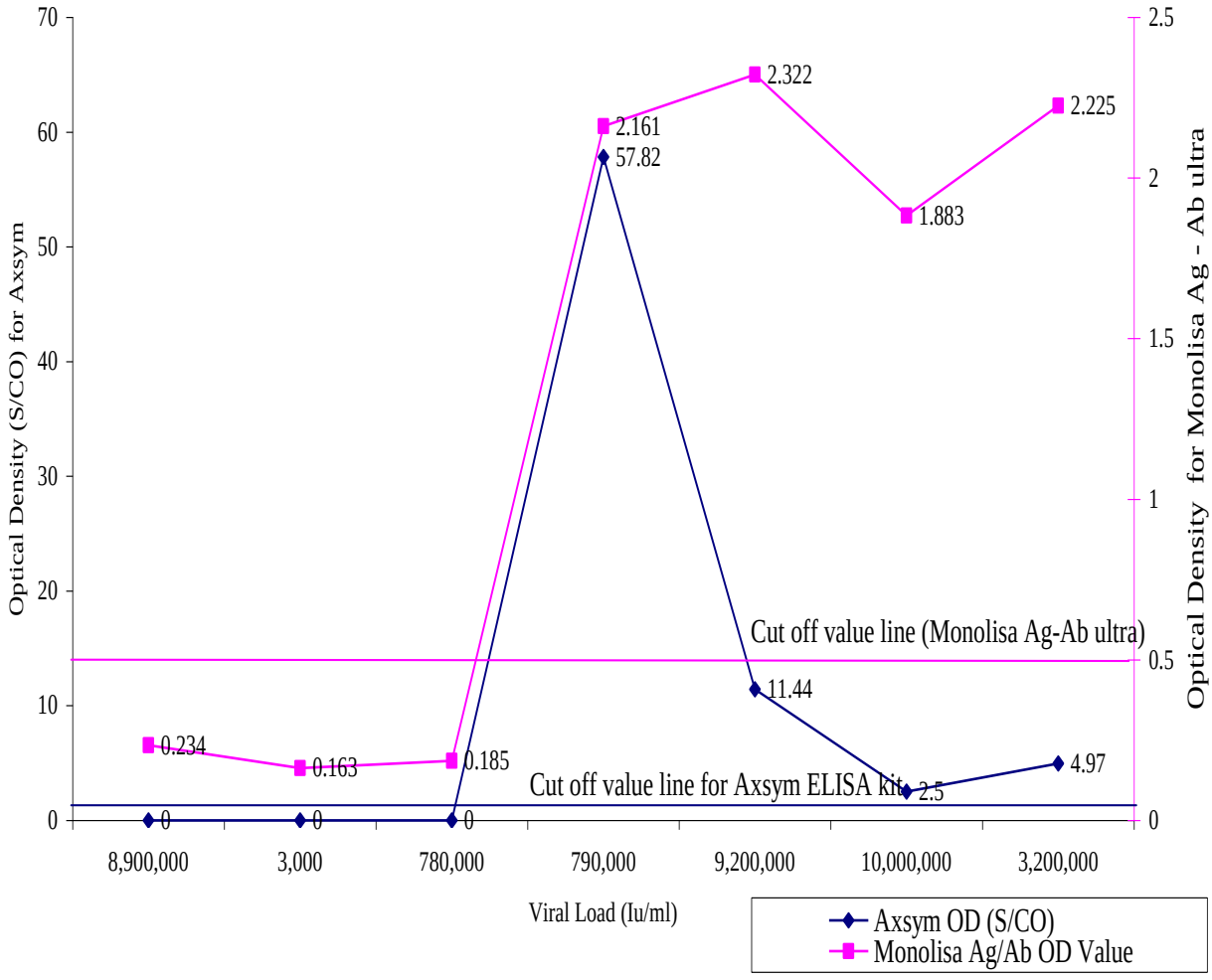


Figure 4.1: Optical density results for samples from bone marrow transplant patient (n=7)

The two negative samples (V0901987 and V0904153) detected as positive by AxSYM antibody ELISA kit were further subjected to another antibody test, Ortho HCV version 3.0, and further subjected to a confirmatory test by Western Blot using INNO-LIA^{TM} HCV score test strip (Innogenetics® N.V, Belgium) and results obtained as shown in Table 4.2. The rationale behind subjecting the samples to another Ortho HCV version 3.0, was based on the fact that the latter antibody assay the most commonly used for routine diagnosis of HCV in many laboratories across the world, and therefore it was of interest comparing the results for the two samples in the two antibody tests.

Table 4.2. Western Blot Results for samples V0904153 and V0901987 (n=2)

Sample number	**Results**			
	Ortho HCV 3.0	AxSYM	*Monolisa® HCV Ag-Ab ULTRA*	Western Blot
V0904153	Negative (0.048)	Positive (1.46)	Negative (0.048)	Negative
V0901987	Indeterminate (0.317*)	Positive (20.3)	Negative (0.071)	Negative

Evaluation of *Monolisa® HCV Ag-Ab ULTRA assay* and AXSYM ELISA kits were further characterized based on their performance among samples with different viral loads. Samples with different viral loads including those with viral loads below the suggested theoretical threshold of 260,000 IU/mL - corresponding to approximately 13.7 pg of total HCV core antigen(Krajden *et al.*, 2004; Fabrizi *et al.*, 2005) for *Monolisa® HCV Ag-Ab ULTRA assay* kit (Turke *et al.*, 2008) were tested on the two assay platforms. The results showed no direct correlation between viral load and the optical density (Figures 4.2a and 4.2b) with some samples having very low viral

loads but giving high optical density signals whereas some with very high viral loads above the threshold limit giving lower optical signals compared to those with viral loads lower than the threshold limit of detection.

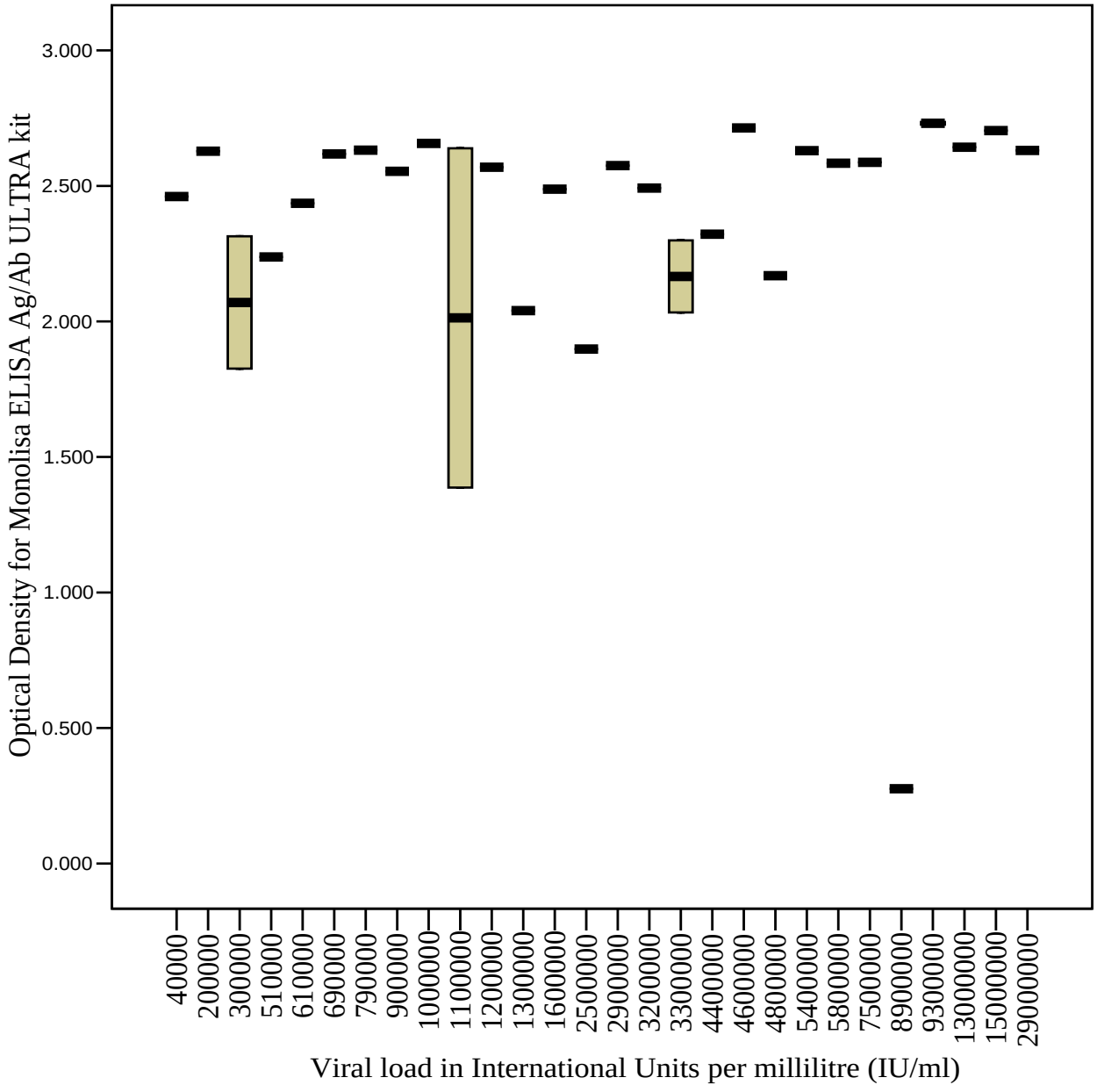


Figure 4.2a Optical Density versus viral load for *Monolisa ELISA Ag/Ab ULTRA*

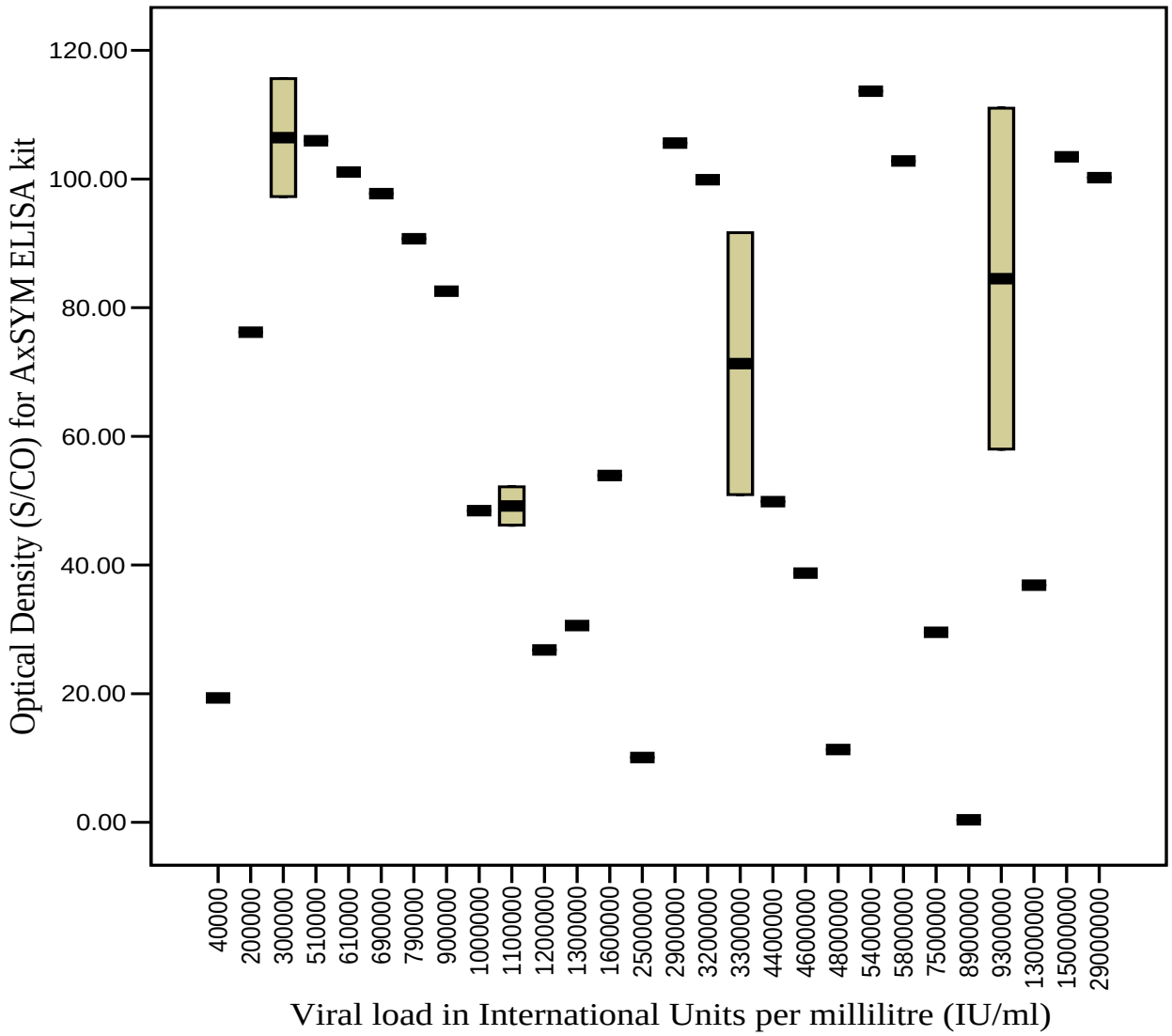


Figure 4.2b: Optical Density versus viral load for AxSYM ELISA kit

Samples categorized as PCR negative- antibody positive recorded similar trends for optical density signals for the three ELISA kits compared. However, AxSYM recorded high optical signals for sample number V0901987, depicting it as positive, *Monolisa® HCV Ag-Ab ULTRA assay* and Ortho HCV 3.0 recorded lower optical signals below the detection limit, thus depicting the same sample as negative (Figure 4.3a and 4.3b).

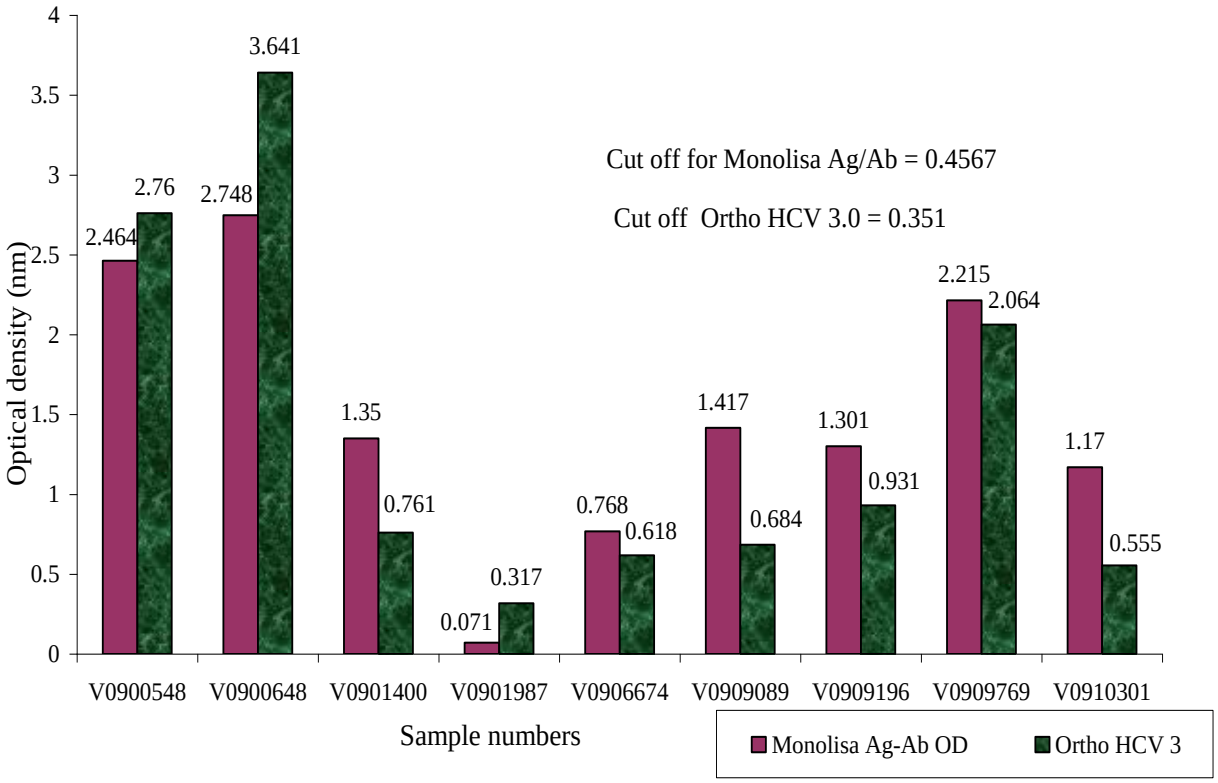


Figure 4.3a: Optical density signals for PCR negative - Antibody positive samples by Monolisa HCV Ag-Ab ultra and Ortho HCV 3.0 ELISA Kits

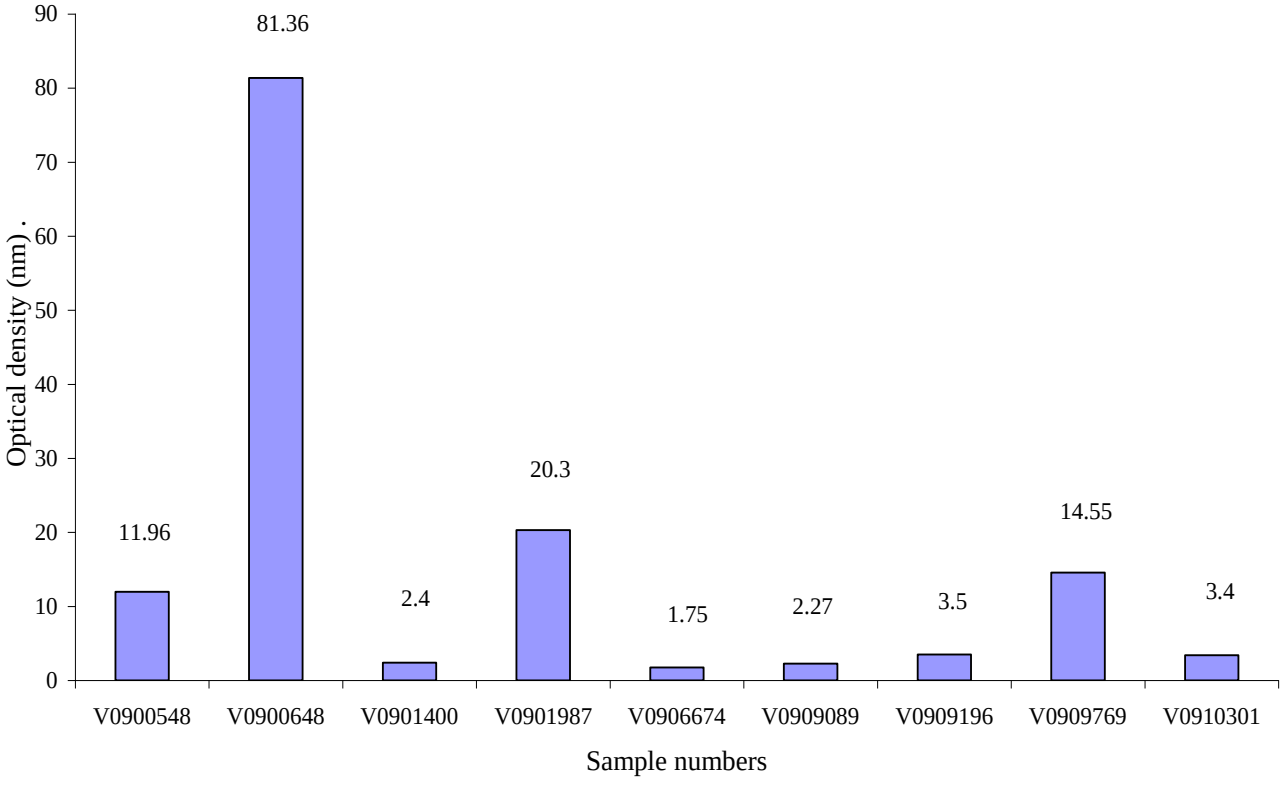


Figure 4.3b: AxSYM ELISA optical density results for PCR negative - Antibody positive samples (n=9).

Overall sensitivity, specificity and predictive values for *Monolisa Ag-Ab HCV Ultra* and AxSYM kits were therefore determined based on PCR and Western blot as the reference (gold standard assays) as shown in Table 4.3a and 4.3b. The rationale behind using the results based on the two (PCR and Western blot) was due to the fact that *Monolisa Ag-Ab HCV Ultra* is designed to detect both antigens for and antibodies to HCV.

Table 4.3a. Calculation of sensitivity, specificity and predictive values for *Monolisa HCV Ag-Ab* ULTRA assay kit

		Results by PCR/ Western blot		
		+	-	
Results by *Monolisa*	+	43	0	**43**
	-	4	27	**31**
	Total	**47**	**27**	**74**

Table 4.3b. Calculation of sensitivity, specificity and predictive values for AxSYM Antibody ELISA

		Results by PCR/ Western blot		
		+	-	
Results by AxSYM	+	43	2	**45**
	-	4	25	**29**
	Total	**47**	**27**	**74**

An overall sensitivity of 91.5% at a confidence limit of 95% was therefore realized for *Monolisa Ag-Ab HCV Ultra* and AxSYM ELISA kits with varying specificity and predictive values as shown in Table 4.4a.

Table 4.4a. Overall sensitivity, specificity and predictive value results for *Monolisa HCV Ag-Ab Ultra* and AxSYM ELISA kits

ELISA kit	Assay parameters evaluated			
	Sensitivity % (95 CL)*	Specificity % (95 CL)*	PPV %	NPV %
Monolisa Ag-Ab Ultra	91.5 (86.6 - 95.1)	100 (98.1 - 100)	100	87.1
AxSYM ELISA kit	91.5 (86.6 - 95.1)	93 (89.1 – 96.6)	96	86

The study further evaluated sensitivity and specificity at a confidence limit of 95% for various categories of samples. This was important in depicting the performance of the assay within different categories of patients to whom tests for HCV diagnosis have always given varied results (WHO. 2001). The results were obtained as shown in Table 4.4b.

Table 4.4b : Sensitivity, Specificity at 95% CL and predictive values of Monolisa HCV Ag-Ab ULTRA ELISA kit versus AxSYM ELISA kit among different samples (n=74)

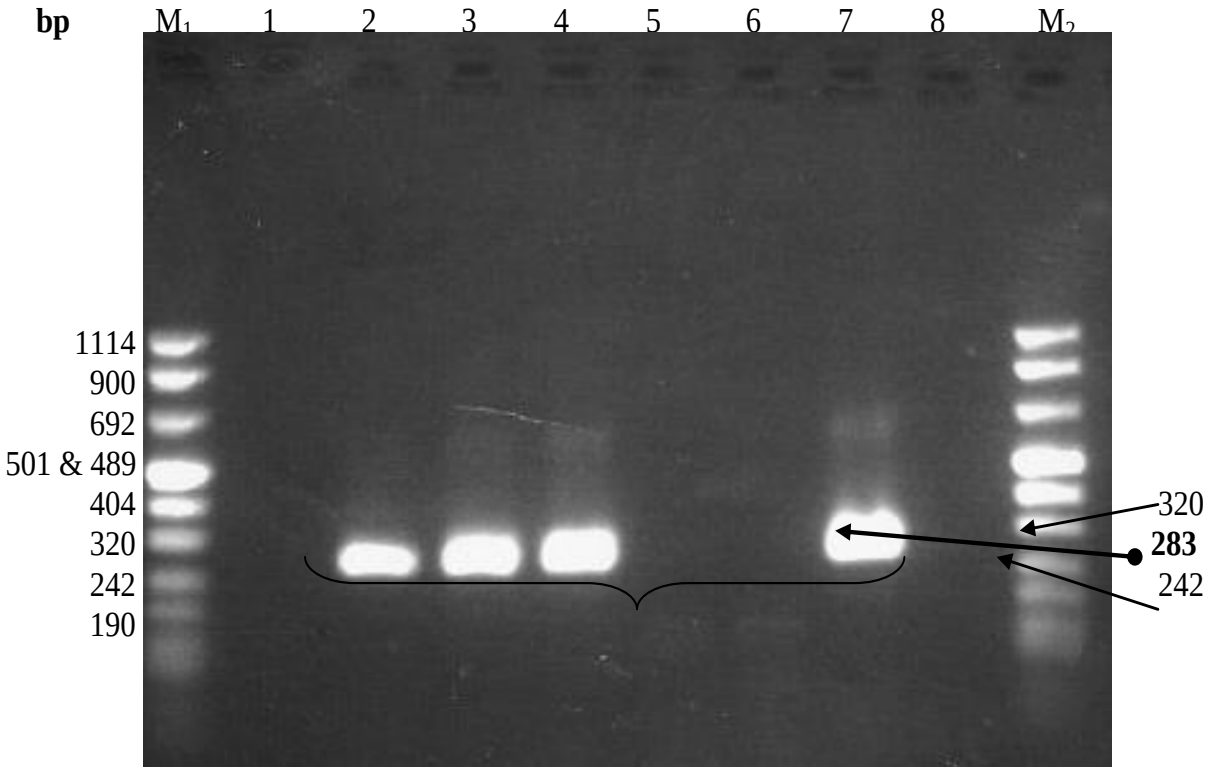
Evaluation	Type of sample	Total number	Axsym ELISA kit		Monolisa HCV Ag-Ab ULTRA	
			Evaluation (%)	PV(%)	Evaluation (%)	PV (%)
Sensitivity and Positive PV	PCR positive samples	32	31/32 (96.875)	31/31 (100)	31/32 (96.875)	31/31 (100)
	Antibody positive - PCR negative	9**	8/8* (100)	8/9 (88.89)	8/8* (100)	8/8 (100)
	"Difficult" or "sticky" sera	7	4/7 (57.143)	4/4 (100)	4/7 (57.143)	4/4 (100)
Specificity and Negative PV	HCV negative samples	26	25/26 (96.154)	25/25 (100)	26/26 (100)	26/26 (100)

****** One sample confirmed negative by western blot. ***** total number of samples confirmed PCR negative – antibody positive

PV= Predictive Value

4.2 Genotyping performance of RFLP

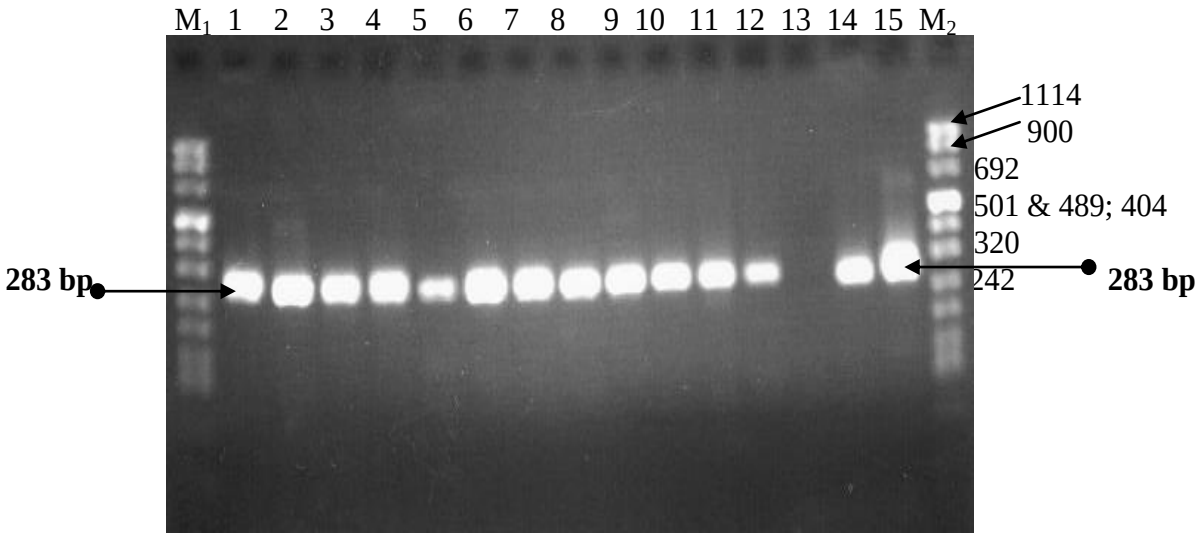
All the 50 samples for analysis were reverse transcribed by both random primer (5'-NNN NNN- 3') and 5' specific primers. This was shown by band formation at position 283bp (Plate 4.1) designed for hepatitis C detection by gel electrophoresis. A quality control had been performed (Plate 4.1) prior to gel electrophoresis to detect the positive samples.



Key: M_1 and M_2 - DNA molecular weight marker VIII (0.019 – 1.11 kbp). Wells 2, 3, 4 and 7 - bands at 283 base pairs (bp) (positive controls). Wells 1, 5, 6 and 8 - no band formation (negative controls).

Plate 4.1 Quality control gel

The study also realized that 18 of the 50 samples could not be reverse-transcribed by random primers, which was shown by lack of band formation on the gels (Plate 4.2).



Key: M_1 and M_2 -DNA molecular weight markers (0.019 – 1.11 kbp), wells 1 to 12, 14 and 15 - bands at 283bp for positive samples. Well 13 no band indicating a PCR negative result from a known PCR negative – antibody positive sample.

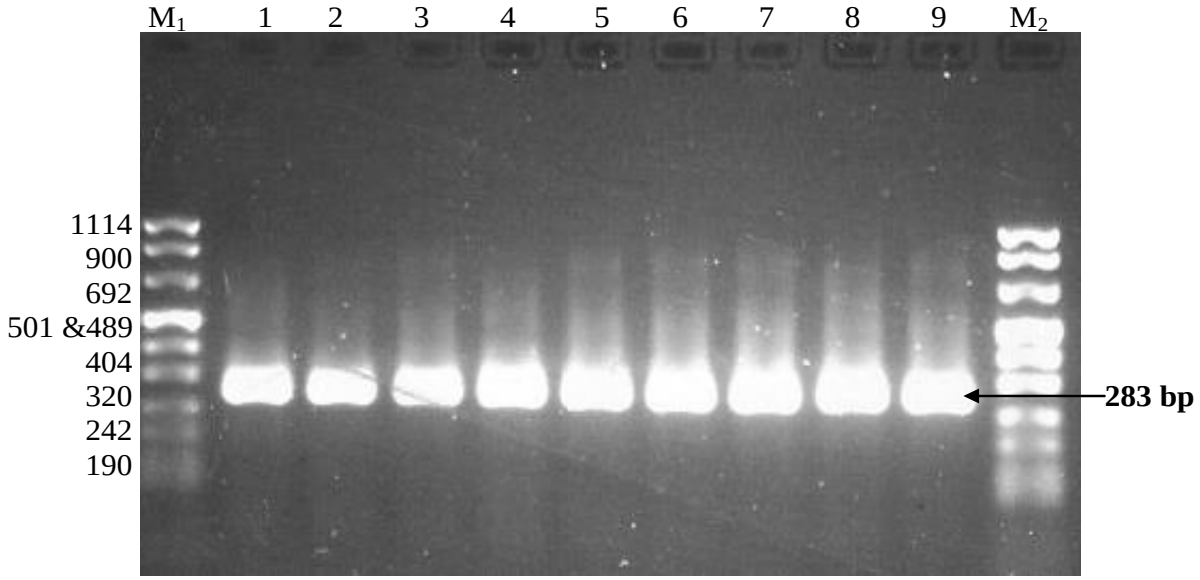
Plate 4.2 A sample of results generated by gel electrophoresis

The study noted that most of the samples that were not reverse-transcribed by random primers had under the manual operation had viral load measurements above 1 million IU/mL (Table 4.2.1). The samples also included one sample known to be of a "mixed" HCV genotypes infection, indicating that low viral loads were not the reason for failure of reverse transcription by random primers. The table also shows one sample with viral load below 100,000IU/mL reverse transcribed.

Table 4.2.1: Results from PCR products reverse transcribed using random primer (n=50)

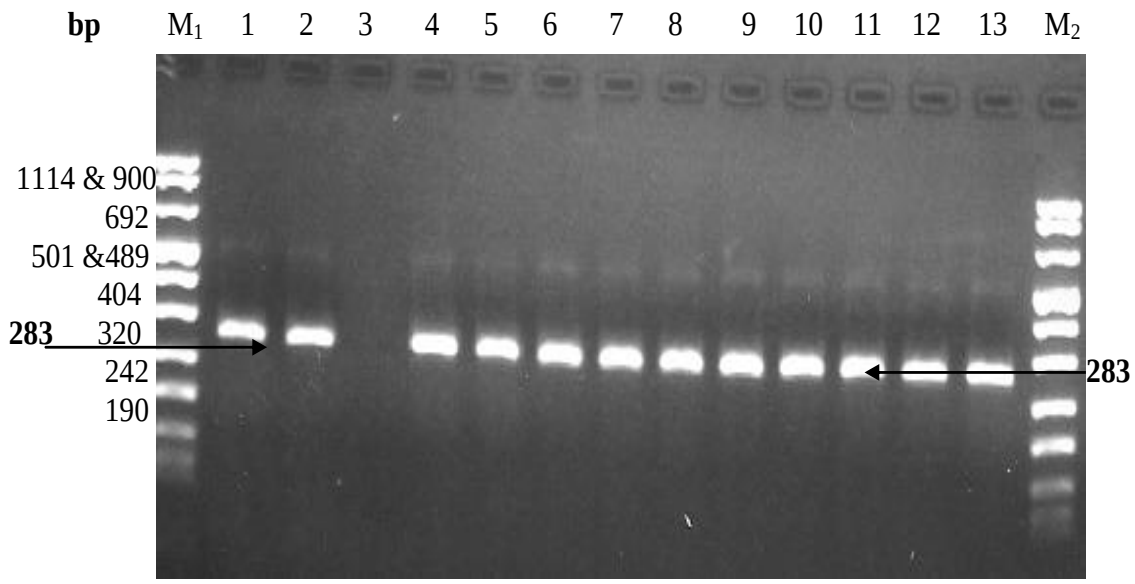
Viral loads (Iu/ml)	total number	Results	
		Positive	Negative
≥ 10,000,000	4	3	1
1,000,000 - 9,999,999	21	15	6
100,000 - 999,999	13	7	6
≤100,000	6	5	1
Not known	6	2	4
TOTAL	**50**	**32**	**18**

These 18 samples were later reverse transcribed by both random primers and primers specific for 5'UTR, HCVN 02: 5' – gTg CAC ggT CTA CgA gAC C – 3' and HCVN 08: 5' –TAC TCA CCg gTT CCg CAg A – 3' in an automated system and results compared. In both cases, it was realized that very clear bands were generated by both primers (Plates 4.3a and 4.3b).



M_1 and M_2 - DNA molecular weight marker (0.019 – 1.11 kbp), wells 1 to 9 - bands formed at 283 bp indicating positive results.

Plate 4.3a A sample of results from samples that generated results by 5'UTR specific primers in an automated system



Key; M_1 and M_2 are DNA molecular weight markers (0.019 – 1.11 kbp). Band formations at 283bp for all wells except well 3. No sample was loaded into well 3.

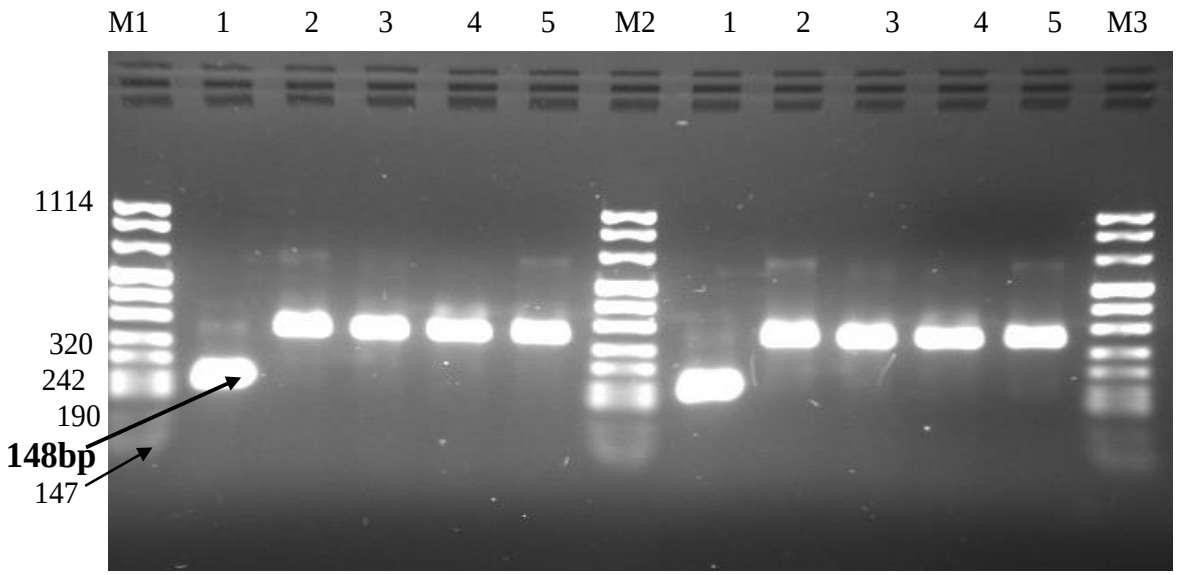
Plate 4.3b PCR products from some samples reverse transcribed by random primers in an automated system.

Twelve samples shown in plates 4.3a and 4.3b, obtained by random primers and specific primers, were then used in viral load measurements and the results compared (Appendix 11 and 14). Results showed that viral loads from samples reverse transcribed by specific primers were higher compared to those reverse transcribed by random primers. Random primers however, recorded high viral load measurements compared to specific primers for sample V0908908 and V0910995 (Table 4.2.2). This was despite the sharp bands that had been generated by products of the specific primers when plates 4.3a and 4.3b were visually compared.

Table 4.2.2. Viral load differences between specific and random primers in HCV extraction

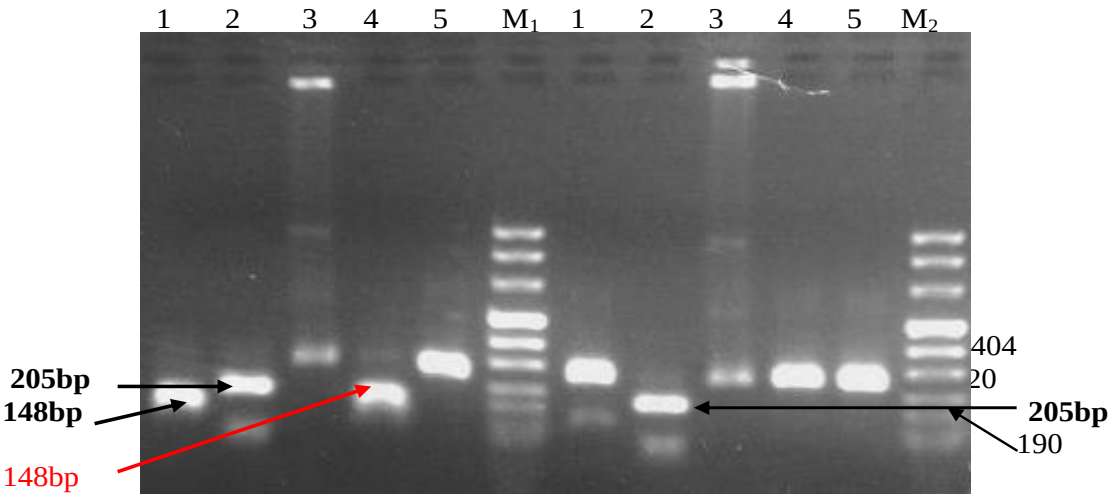
Sample Number	**Viral Load**		
	5' Specific Primer	Random primer	Difference in Viral Load (%)
V0836795	$2.1x\ 10^5$	$9.0x\ 10^4$	57
V0836012	$6.0x\ 10^4$	$1.2x\ 10^4$	80
V0832282	$2.0x\ 10^6$	$8.9x\ 10^5$	56
V0831394	$7.1x\ 10^5$	$7.0x\ 10^5$	0.1
V0908908	$1.9x\ 10^6$	$2.0x\ 10^6$	-5
V0838927	$9.8x\ 10^4$	$9.0x\ 10^3$	9
V0743586	$2.8x\ 10^6$	$3.6x\ 10^5$	87
V0910995	$1.5x\ 10^5$	$5.9x\ 10^5$	75
V0821001	$4.4x\ 10^5$	$5.1x\ 10^4$	88
V0910475	$2.9x\ 10^5$	$1.2x\ 10^5$	59
V0909125	$5.6x\ 10^5$	$5.7x\ 10^4$	90
V0804272	$1.4x\ 10^5$	$4.7x\ 10^4$	66

All the PCR products from the 50 samples were then used in evaluating the genotyping performance of RFLP among HCV genotypes 1 to 4. Genotype 1a samples were identified by a specific restriction by Drd-I enzyme only (Plate 4.4), while genotype 1b underwent a restriction by either Bsma-I only, which was considered unusual or Drd-I and Bsma-I as shown in Plate 4.5.



Key; M1, M2 and M3 are DNA molecular weight markers (0.019 – 1.11 kbp), wells; 1 - Drd-I; 2 - BsmA-I; 3 - Bsr-I; 4 - Sau3A; 5 - Hinf-I.

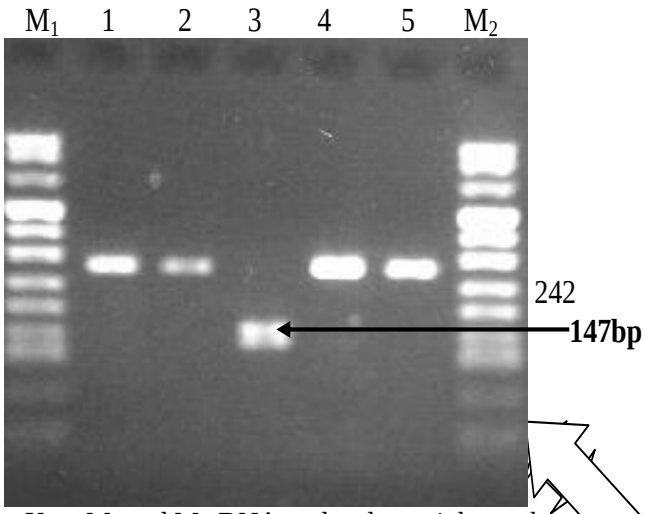
Plate 4.4: A gel film showing genotype 1a samples restricted by Drd-I



Key; M_1 and M_2 - DNA molecular weight markers (0.019 – 1.11 kbp), Well 2 (right) restriction by BsmA-I only at position 205bp, Wells 1 and 2 (left) restriction by Drd-I and BsmA-I at positions 205bp and 148bp. Well 4 (indicated in red) an unspecific restriction by Sau 3A at position 148bp instead of the usual position 207bp or 76bp.All wells; 1- Drd-I, 2- BsmA-I, 3- Bsr-I, 4- Sau 3A and 5- Hinf-I.

Plate 4.5 Genotype 1b shown by restriction by BsmA-I and Drd - I

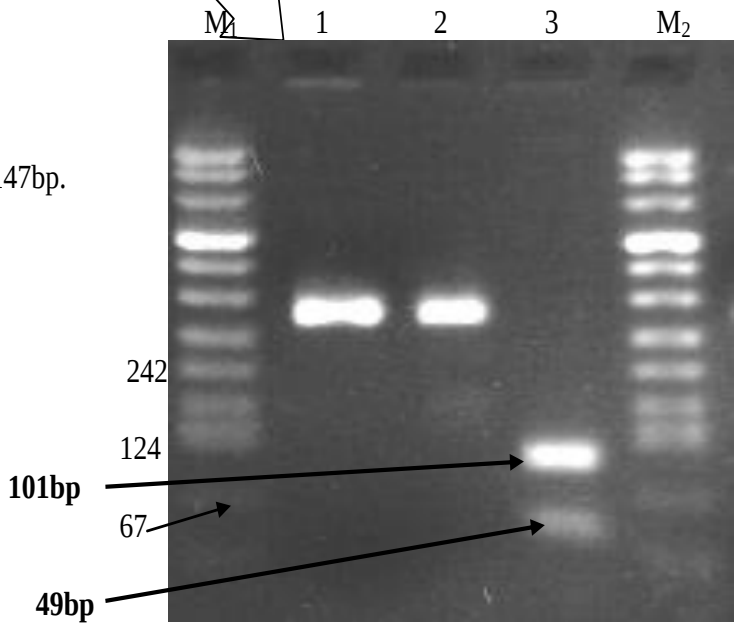
Genotype 2 fragments were restricted by Bsr-I enzyme. However for confirmation of genotype 2, and differentiation of 2a and 2b, the samples were further subjected to restriction by Nci-I and the bands positively identified appeared as shown in Plates 4.6a and 4.6b.



Key; M_1 and M_2 DNA molecular weight markers.

Wells 1-Drd-I, 2- BsmA-I, 3- Bsr-I, 4- Sau 3A, 5- Hinf- I

Plate 4.6a Restriction by Bsr- I at 147bp.



Key; M_1 and M_2. DNA molecular weight markers (0.019 – 1.11 kbp),

Wells 1-HCV sample, 2- BsmA-I (for quality control), 3- NCi - I

Plate 4.6b Restriction by Nci- I at positions 101bp and 49bp

Restriction patterns for Genotypes 3a and 4 samples were also corresponded to their specific restriction enzymes, restricting at the Sau 3a and the Hinf-I sites respectively. However, for genotype 4, a confirmation was done with Fok-I enzyme, partly due to unspecific restriction of some genotypes by recorded for Hinf-I enzyme during the study.

Of the 50 samples genotyped and analyzed, clear results by specific enzyme restrictions were realized on 42 (84%) samples (Table 4.2.3), unclear results on 6 (12%), with 2 (4%) of the samples genotyped giving results that were different from the known genotypes.

Table 4.2.3. Samples giving clear RFLP results similar to results obtained by the diagnostic section of MvPI

		Results obtained during the study	
Sample number	Known Genotype	Restriction enzymes	Genotypes by RFLP
V0907444	1b	Drd-I and Bsma-I	1b
V0910242	1a	Drd-I	1a
V0910475	-	Drd-I and Bsma-I	1b
V0911307	-	Drd-I	1a
V0909818	1b	Drd, Bsma and Sau 3a	1b
V0908613	1b	Bsma-I	1b
V0909726	1a	Drd-I	1a
V0911178	1a	Drd-I	1a
V0910747	2a	Bsr-I and Nci-I	2a
V0842389	2a	Bsr-I and Nci-I	2a
V0907069	3a	Sau 3a	3a
V0907465	3a	Sau 3a	3a
V0910196	-	Drd-I	1a
V0908159	1b	Drd-I and Bsma-I	1b
V0908953	1b	Drd-I and Bsma-I and Sau 3a	1b
V0908913	1b	Bsma- I	1b
V0910434	1a	Drd- I	1a
V0829687	2a	Bsr-I and Nci-I	2a
V0808075	2a	Bsr-I and Nci-I	2a
V0904088	3a	Sau 3a and Hinf-I ***	3a
V0901670	3a	Sau 3a and Hinf-I ***	3a
V0915977	4	Hinf-I and Fok-I	4
V0842545	4	Hinf-I and Fok-I	4
V0911205	1b	Drd-I and Bsma-I	1b
V0829371	4	Hinf-I and Fok-I	4
V0827550	4	Hinf-I and Fok-I	4
V0740685	4	Hinf-I and Fok-I	4
V0840185	3a	Sau 3a and Hinf-I ***	3a
V0836796	3a	Sau 3a and Hinf-I ***	3a
V0818483	1b	Drd-I and Bsma-I	1b
V0813056	1b	Drd-I and Bsma-I	1b
V0724864	4	Hinf-I and Fok-I	4
V0924227	4	Hinf-I and Fok-I	4
V0835157	3a	Sau 3a and Hinf-I ****	3a
V0833733	3a	Sau 3a and Hinf-I ****	3a
V0908001	3a	Sau 3a and Hinf-I ****	3a
V0909868	-	Sau 3a and Hinf-I*****	3a
V0908908	-	Hinf-I and Fok-I	4
V0909252	1b	Drd-I and Bsma-I	1b
V0836716	2a	Bsr and Nci-I	2a
V0728602	2b	Bsma-I, Bsr-I and Nci-I	2b
V0717734	4	Bsma-I, Hinf-I and Fok-I	4

**** Indicate unspecific restriction by Hinf – I enzyme. Samples in red also underwent unspecific restrictions by either Sau 3A or Bsma – I enzymes.

Samples that were considered to have unspecific restrictions are those whose genotypes could not be resolved unless subjected to sequencing. They either showed no restriction at all or had undergone restrictions by enzymes known to confirm different specific genotypes (Table 4.2.4) and therefore further genotyping of these samples either required sequencing or use of other more sensitive and specific assays.

Table 4.2.4. Samples that were considered to have unclear genotypes by RFLP

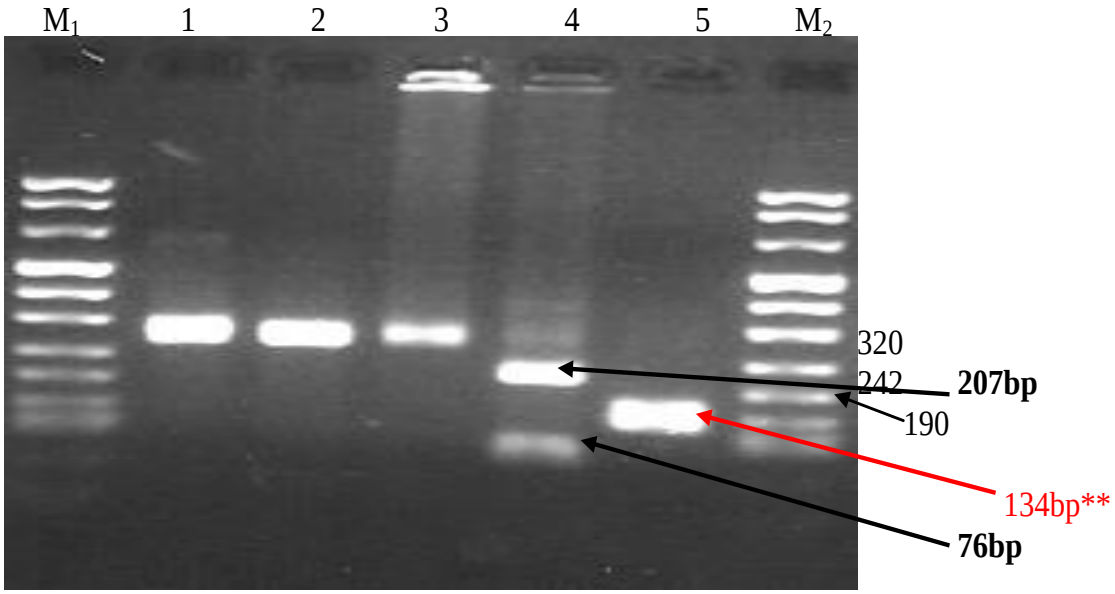
		Results obtained during the study	
Sample number	Known Genotype	Restriction enzymes	Genotypes
V0908873	4	Drd-I, Bsma-I, Hinf-I and Fok-I	Unclear
V0832720	2a	No restriction by any enzyme	Unclear
V0832719	4	Bsma-I, Sau 3a and Hinf-I	Unclear
Mixed 1	1b and 4	Drd-I, Bsma-I, Sau 3a and Hinf-I	Unclear
V0908229	2a	No restriction by any enzyme	Unclear
Mixed 2	1b, 3a and 4	Drd-I, Hinf-I and Fok-I	Unclear

Genotype results obtained by RFLP for the final group of 2 samples in Table 4.2.5 completely varied from the known genotypes of the respective samples. Sequencing results for the two samples tallied with the known genotypes for the two samples.

Table 4.2.5. Samples giving conflicting results from the known genotypes

		Results obtained during the study		
Sample number	Known Genotype	Restriction enzymes	Genotype	Sequencing Results
V0838927	2a	Drd-I and Bsma-I	1b	2a
V0908324	3a	Drd-I and Bsma-I	1b	3a,3b

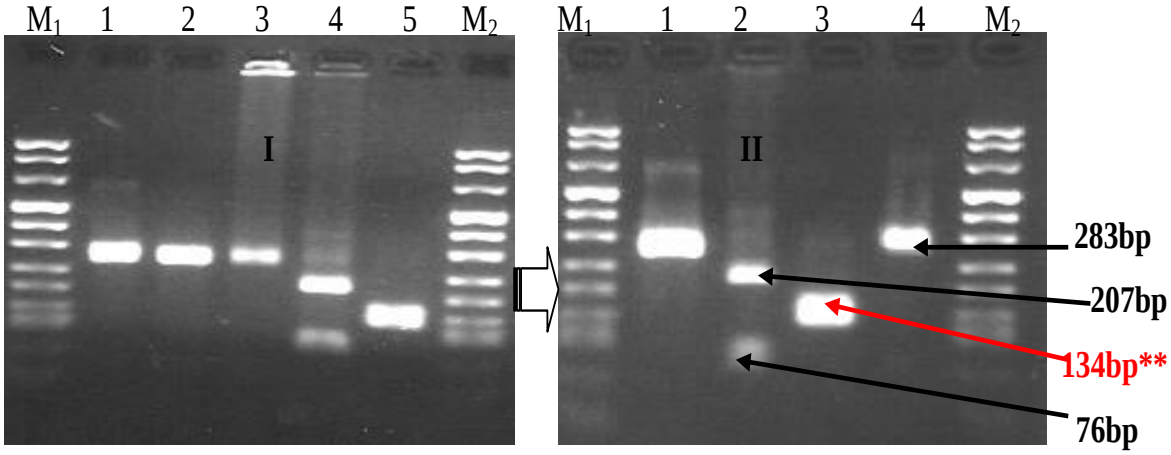
As already seen in plate 4.5 and among samples highlighted in red in Table 4.2.4, some genotype 1b samples showed unspecific fragment restriction with Sau 3a. Further all genotype 3a samples also marked with red stars in Table 4.2.3, showed unspecific restriction with Hinf-I enzyme known to have a restriction site in genotype 4 samples (Plate 4.7a).



Key; M_1 and M_2 - DNA molecular weight markers (0.019 – 1.11 kbp); Well 4 – restriction by Sau 3A at positions 207bp and 76bp. Well 5 – restriction by Hinf – I enzyme at position 134bp** (shown by red arrow). Wells 1- Drd – I, 2- BsmA- I, 3- Bsr- I, 4- Sau 3A, 5- Hinf- I enzymes. (Sample number V0835157).

Plate 4.7a Unspecific restriction of suspected genotype 3a restricted by Hinf – I enzyme

Suspected genotype 1b samples with unspecific Sau 3a restriction were later chosen for sequencing, and the results obtained confirmed the genotypes of the samples as 1b. Suspected genotype 3a samples which showed unspecific Hinf-I restriction were further subjected to restriction by Fok-I enzyme. Non-restriction by Fok-I enzyme, were used as a basis to rule out genotype 4 and conclude infection by genotype 3a, based on the positive restriction by Sau 3a as seen in the example in Plate 4.7b.

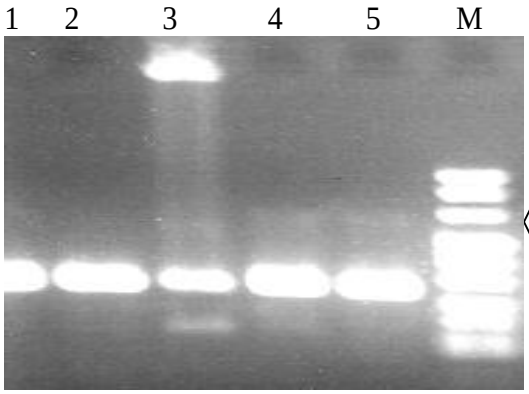


Key; M_1 and M_2 are DNA molecular weight markers (0.019 – 1.11 kbp), (**I**) wells 4 and 5 – restriction by Sau 3A and Hinf – I; (**II**) restriction by Sau 3A at positions 207bp and 76bp (well 2), continued restriction by Hinf – I enzyme at position 134bp** (shown by red arrow) and failed restriction by Fok – I (well 4). (**I**) Wells 1- Drd – I, 2- BsmA- I, 3- Bsr- I, 4- Sau 3A, 5- Hinf- I enzymes. (**II**) wells 1- HCV sample, 2- Sau 3A, 3- Hinf-I, 4- Fok-I (Sample number V0835157).

Plate 4.7b Confirmed genotype 3a

Among those samples which showed unclear results by fragment restriction using the 5 primary restriction enzymes (Drd-I, Bsma-I, Bsr-I, Sau 3A and Hinf-I) two samples of sample number V0832720 and sample number V0908229 shown in Plate 4.8a and 4.8b, were of particular interest. This was due to the fact that they did not

undergo any restriction despite repeated testing. These two samples were therefore subjected to restriction by Sma-I, which positively restricted the Sma-I fragment, positively confirming HCV infection of the two samples.

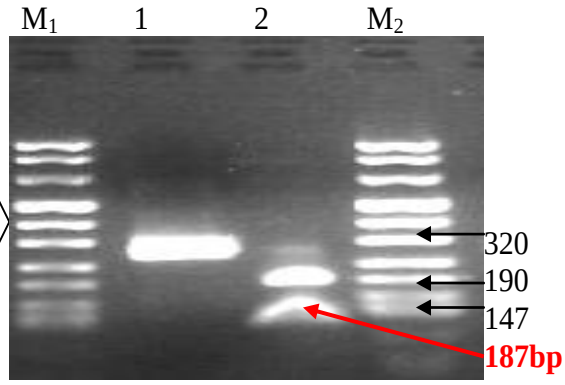


Key; M– molecular weight marker

Wells 1- Drd-I, 2-BsmA-I

3-Bsr-I, 4-Sau 3A and 5- Hinf-I enzymes

Plate 4.8a (i): Sample V0832720 showing no restriction

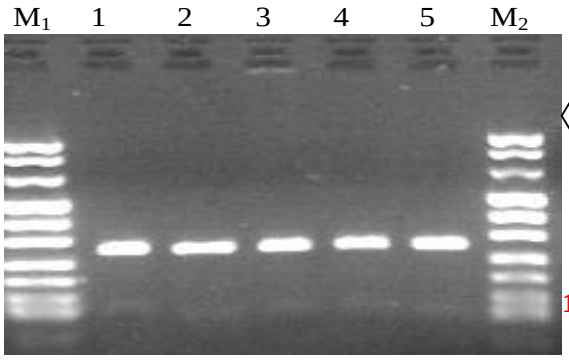


Key; M1 and M2 molecular weight markers

well 2- restriction by Sma- I at 187bp (red).

Wells 1- HCV sample, 2- Sma- I enzyme

Plate 4.8a (ii): Sample V0832720 showing restriction by Sma -I

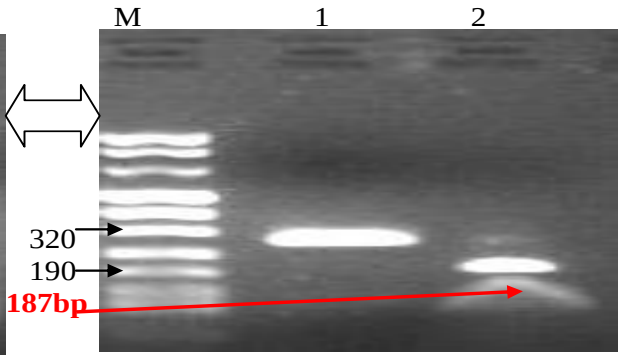


Key; M– molecular weight marker

Wells 1- Drd-I, 2-BsmA-I

3-Bsr-I, 4-Sau 3A and 5- Hinf-I enzymes

Plate 4.8b (i): Sample V0908229 showing no restriction



Key; M1 and M2 molecular weight markers

well 2- restriction by Sma- I at 187bp (red).

Wells 1- HCV sample, 2- Sma- I enzyme

Plate 4.8b (ii): Sample V0908229 showing restriction by Sma -I

These two samples which could not undergo fragment restriction by any of the 5 standard enzymes for genotyping HCV 1 to 4 genotypes belonged to known genotype 2a samples. This lack of restriction probably is a pointer towards the difficulty that may be experienced with genotyping genotype 2 samples in an RFLP genotyping system. This was seen in the fact that the ease of genotyping with RFLP varied across genotypes (Figure 4.2.1).

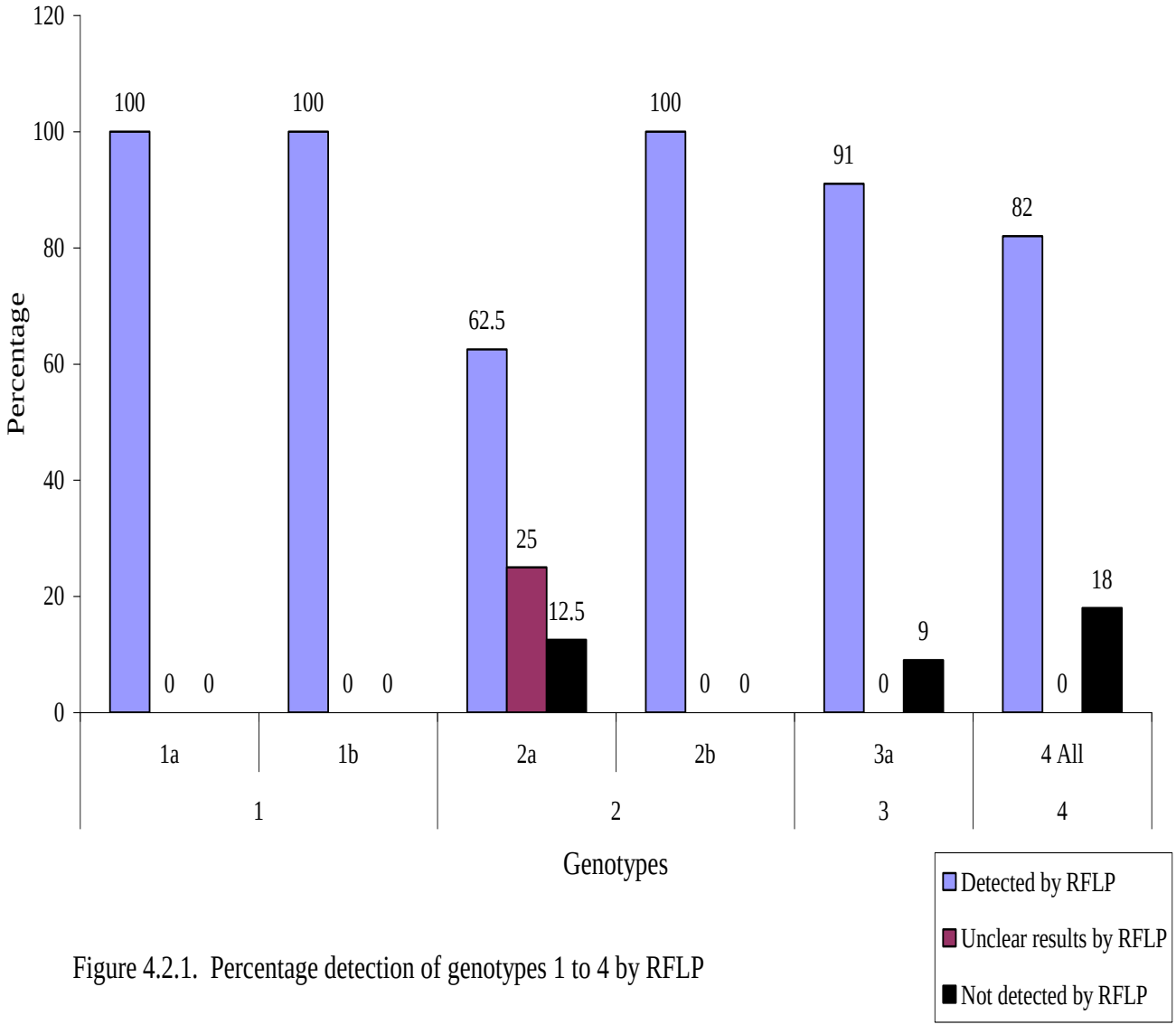


Figure 4.2.1. Percentage detection of genotypes 1 to 4 by RFLP

All samples with unclear restriction and those that showed different genotyping results from known genotypes were then chosen for sequence analysis. Samples with

positive RFLP genotyping results tallied across board with the sequencing results obtained after blasting the sequences in the HCV data base (Table 4.2.6). Other samples sequenced in this group included those samples whose genotypes were unknown before RFLP analysis. They were thus sequenced to assess whether there were any similarities in the results obtained between RFLP analysis and sequencing. Their results also tallied except for sample V0908908 whose sequence was similar to 3 different genotypes and one mixed (1a/2a) genotype. Further analysis of sequence alignment for this sample (V0908908) identified Hinf-I, Fok-I and Drd-I restriction fragment sites, however Fok-I and Drd-I restriction fragments were found to be overlapping as seen in Figure 4.2.2

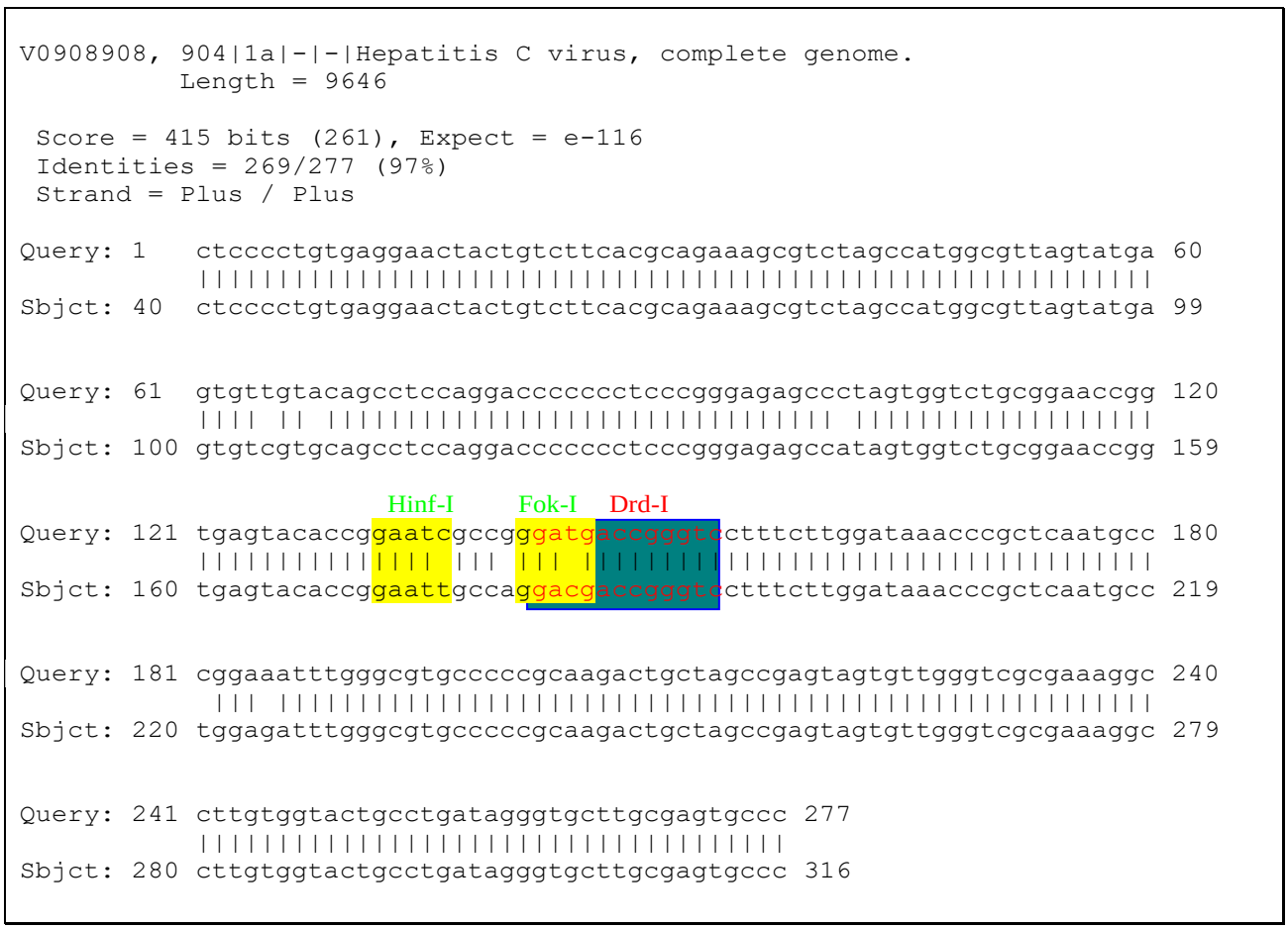


Figure 4.2.2 Restriction fragment sites for Hinf-I and Fok-I (yellow area) with fragment site for Drd-I (blue area) overlapping with Fok-I restriction site.

Table 4.2.6. Samples with conforming genotypes both by RFLP and sequence results

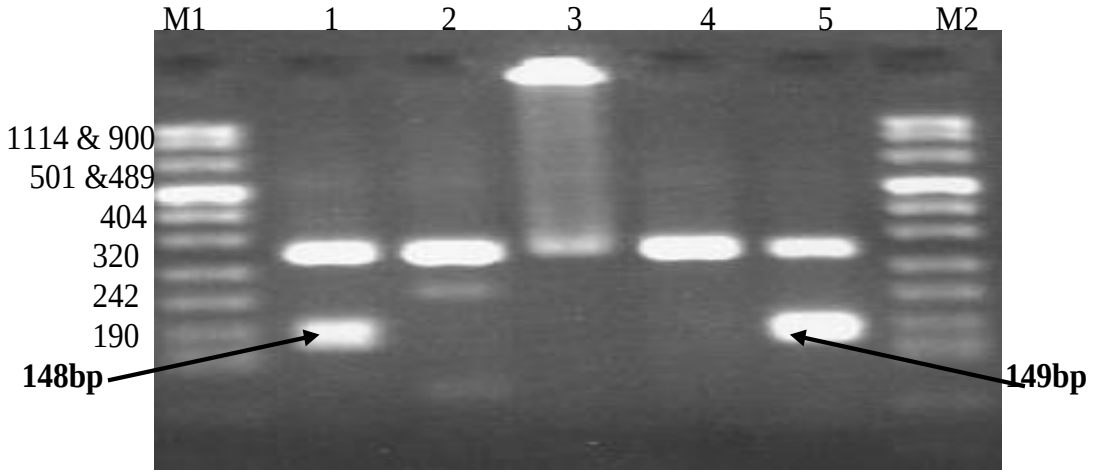
Sample number	Known Genotype	Results obtained during the study		
		Restriction enzymes	Genotypes by RFLP	Genotypes by Sequencing
V0910475	-	Drd-I and Bsma-I	1b	1b
V0909818	1b	Drd, Bsma and Sau 3a	-	1b
V0909726	1a	Drd-I	1a	1a
V0907069	3a	Sau 3a	3a	3a
V0908159	1b	Drd-I and Bsma-I	1b	1b
V0908953	1b	Drd-I and Bsma-I	1b	1b
V0829371	4	Hinf-I and Fok-I	4	4
V0827550	4	Hinf-I and Fok-I	4	4
V0836796	3a	Sau 3a and Hinf-I	3a	3a
V0908001	3a	Sau 3a and Hinf-I	3a	3a
V0909868	-	Sau 3a and Hinf-I	3a	3a
V0908908	-	Hinf-I and Fok-I	4	4,5a,1a,1a/2a
V0909252	1b	Drd-I and Bsma-I	1b	1b
V0728602	2b	Bsma-I, Bsr-I and Nci-I	2b	2b

All samples sequenced including samples V0832720 and V0908229 which were not genotyped by RFLP realized clear results, similar to the sample genotypes that were known for the samples (Table 4.2.7). It was also noted that samples which had intentionally been mixed at the start of evaluation did not show all the genotypes that had been mixed (Table 4.2.7).

Table 4.2.7: Samples that were considered to have unclear genotypes by RFLP

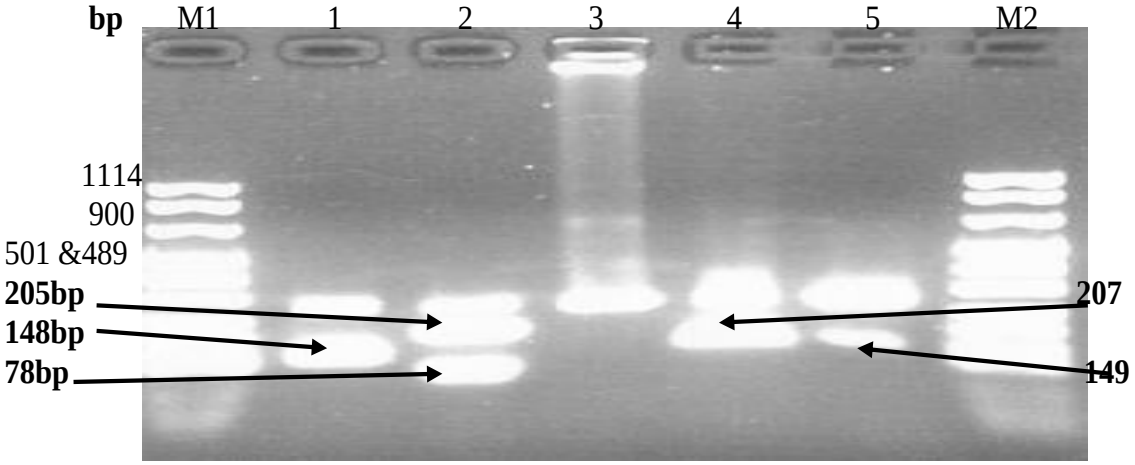
Sample number	Known Genotype	Results obtained during the study		
		Restriction enzymes	Genotypes by RFLP	Genotypes by Sequencing
V0908873	4	Drd-I, Bsma-I, Hinf-I and Fok-I	Unclear	4
V0832720	2a	No restriction by any enzyme	Unclear	2a
V0832719	4	Bsma-I, Sau 3a and Hinf-I	Unclear	4
Mixed 1	1b and 4	Drd-I, Bsma-I, Sau 3a and Hinf-I	Unclear	1b
V0908229	2a	No restriction by any enzyme	Unclear	2a
Mixed 2	1b, 3a and 4	Drd-I, Hinf-I and Fok-I	Unclear	4, 1b

Sample labeled "Mixed 1", which was a mixture of samples of known genotype 1b and 4, only gave a sequence result of genotype 1b leaving out genotype 4, whereas "Mixed 2" of genotypes 1b, 3a and 4 samples, did not produce any sequence results of genotype 3a. This was contrary to the earlier RFLP results which had previously indicated suspicion of mixed genotypes for each sample as seen in Plates 4.9a and 4.9b.



Key; M1 and M2 - DNA molecular weight markers (0.019 – 1.11 kbp), well 1 – restriction by Drd – I at at 148 bp, well 5 restriction by Hinf- I at 149bp. Wells 1-Drd-I, 2- BsmA- I, 3- Bsr-I, 4- Sau 3A and 5-Hinf-I enzymes.

Plate 4.9a: Sample "Mixed 1" showing restriction bands for Drd-I and Hinf - I



Key; M1 and M2 are DNA molecular weight markers (0.019 – 1.11 kbp). Well 1 restriction for Drd-I (148bp), 2 - BsmA- I (205, 78bps), 4- Sau 3A (207bp) and 5 - Hinf-I (149bp). Wells 1- Drd-I, 2- BsmA- I, 3- Bsr-I, 4- Sau 3A and 5-Hinf-I enzymes.

Plate 4.9b: Sample "Mixed 2"showing restriction bands

Further analysis of the sequence alignment obtained for the two samples was done in other regions of the viral genome. The results obtained were compared to the 5'UTR which is frequently used in developing diagnostic assays and in sequencing. These results were based on the similarities in percentage identities and the e- values for each particular database sequence in comparison to the sample sequence blasted in the database. Results obtained varied across the samples as summarized in Table 4.2.8.

Table 4.2.8 Sequence analysis of different regions of HCV genome for non- restricted and mixed samples

Number	**5' UTR**			**Other regions**				**Complete genome**		
	Genotype	Length	Identity	Region	Genotype	Length	Identity	Genotype	Length	Identity
V0832720	2a	321	210/212	Core	2	395	210/212	2a	9711	210/212
				Core/E1	2c	1195	210/212			
				polyprotein (p7)	1b/2k	3620	210/212			
				E2/NS1	2f	1585	210/212			
V0908229	2a	340	271/277	core, env &E2/NS1	2e	1585	272/277	2a	9711	210/212
				polyprotein (p7)	2/5	9246	272/277			
Mixed 1				Core, E1, NS1/E2, NS2, NS3, NS4a, NS4b, and NS5	1b	9408	275/276			
Mixed 2				Core	4, 1a	395	276/280	1a	9646	274/282
				Core, env & E2/NS1	5a	1848	275/282			

5'UTR: The untranslated region section commonly used in HCV molecular diagnosis and sequencing; Other regions: are the "hyper"-variable regions including Core, Envelope, polyprotein and Non-structural proteins, that are known for frequent mutations; Complete genome: Is rarely sequenced in routine HCV diagnosis.

Table 4.2.8 further reveals genotype similarity in sequencing 5'UTR and the whole genome for samples V0832720 and V0908229. This however is not the case especially when the polyprotein p7 is selected for sequencing as this region reveals mixed genotypes for the two samples.

The finding that the polyprotein region revealed mixed genotypes 1b/2k and 2/5 for samples V0832720 and V0908229 respectively and the fact that the samples did not show any restriction patterns was of a special interest in the study. Further analysis of the sequences for the two samples however located the restriction sites for Bsr-I and Nci-I, known for identification of genotype 2a in RFLP (Figures 4.2.3 and 4.2.4).

```
V0832720|seid 37|2a|-|-|Hepatitis C virus genotype 2, complete
          genome
         Length = 9711
 Score = 321 bits (202), Expect = 1e-87
 Identities = 210/212 (99%), Gaps = 1/212 (0%)
 Strand = Plus / Plus


Query: 1   gtacagcctccaggcccccccctcccgggagagcc-tagtggtctgcggaaccggtgagt 59
           |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct: 104 gtacagcctccaggcccccccctcccgggagagccatagtggtctgcggaaccggtgagt 163

                       Nci-I      Bsr-I
Query: 60  acaccggaattgccgggaagactgggtcctttcttggataaacccactctatgcccggcc 119
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 164 acaccggaattgccgggaagactgggtcctttcttggataaacccactctatgcccggcc 223


Query: 120 atttgggcgtgcccccgcaagactgctagccgagtagcgttgggttgcgaaaggccttgt 179
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 224 atttgggcgtgcccccgcaagactgctagccgagtagcgttgggttgcgaaaggccttgt 283


Query: 180 ggtactgcctgatagggtgcttgcgactgccc 211
           |||||||||||||||||||||||||| |||||
Sbjct: 284 ggtactgcctgatagggtgcttgcgagtgccc 315
```

Figure 4.2.3: Sequence pattern for V0832720 showing the restriction sites for Bsr-I and Nci-I

Even though the restriction site for Bsr-I and Nci-I are very clear in Figure 14 above, the restriction site for Bsr-I appear to be located at between point 80 and 84nt instead of either 136 nt or 147nt, as designed in the evaluation criteria. It is thus not clear whether this could have contributed to lack of recognition by the restriction enzyme Bsr-I. It is worth noting that Nci-I used to confirm genotype 2a upon restriction by Bsr-I was not used due to the negative results obtained by Bsr-I restriction. This however was not the case with sample V0908229 where the restriction sites appear to be located at the regular points as seen in Figure 4.2.4.

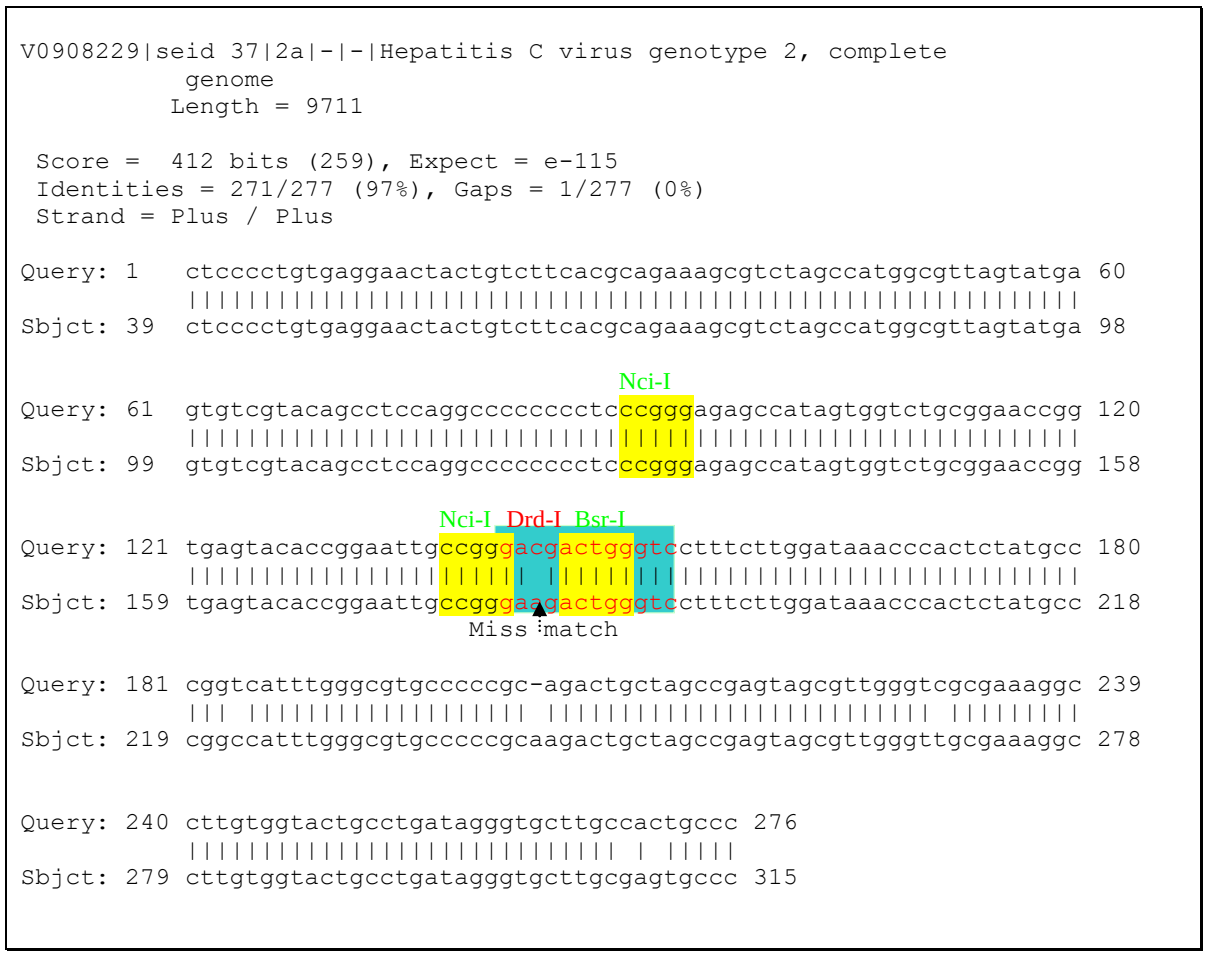


Figure 4.2.4. Sequence pattern for V0908229 showing the restriction sites for Bsr-I and Nci-I

Although the restriction sites in the Figure 4.2.4 show restriction fragments at the

regular sites by the evaluation criteria for Bsr-I and Nci-I restrictions, the fragments can be seen to be sandwiched within the restriction site for Drd-I that restricts for HCV genotype 1a. Further, a miss-match can also be seen at position 144nt. This probably could have contributed to lack of restriction by Drd-I enzyme, but it may not be clear whether the miss-match coupled with a sandwich by Drd-I could have contributed to lack of Bsr-I restriction, factors that require further evaluation.

CHAPTER FIVE

5 DISCUSSION

5.1 Evaluation of *Monolisa*® HCV Ag-Ab ULTRA assay

This study realized a sensitivity of 91.5% for both *Monolisa® HCV Ag-Ab ULTRA assay* and AxSYM ELISA kits, with differing positive predictive values (PPV) of 100% and 97.7% for *Monolisa® HCV Ag-Ab ULTRA assay* and AxSYM respectively. Difference in the positive predictive value is important as it depicts *Monolisa® HCV Ag-Ab ULTRA assay* as superior compared to AxSYM due to a reduced number of false positive results realized for this ELISA kit. A similar sensitivity as the overall sensitivity was also realized among PCR positive samples with the same positive predictive values for the two ELISA kits, however among PCR negative – antibody positive samples, sensitivity of the two assay kits varied with AxSYM recording false positive a false positive result a finding which depicts AxSYM to be more sensitive compared to *Monolisa® HCV Ag-Ab ULTRA assay.* This realization depicts AxSYM as a suitable screening test mainly in populations with low HCV prevalence. On the other hand *Monolisa® HCV Ag-Ab ULTRA assay* is depicted by these results as confirmatory on detection of circulating antibodies.

The superiority of *Monolisa® HCV Ag-Ab ULTRA assay* is also supported by a specificity of 100% with a negative predictive value (NPV) of 100% for *Monolisa® HCV Ag-Ab ULTRA assay* kit against a specificity of 93% recorded for AxSYM ELISA kit, with both kits further recording a difference in the negative predictive values. The finding on specificity also depicts *Monolisa® HCV Ag-Ab ULTRA assay*

as an ideal confirmatory test as it was able to detect all negative samples including two samples that were identified as positive by AxSYM and showed varying results on Ortho HCV 3.0, both antibody ELISA kits.

A similar sensitivity for *Monolisa® HCV Ag-Ab ULTRA assay* as realized in this study has been realized by other studies done on the same assay platform. Ansaldi *et al.* (2006) in their findings reported that this assay presented optimal sensitivity as concerns antibody detection, a result supported by the realization that this assay kit detected all PCR negative – antibody positive samples with ease, further detecting the negative sample that showed varying results on the two antibody ELISA kits. Also in their work, even though based on antigen detection alone, this research group realized a sensitivity of 91.4% which is similar to the overall sensitivity of *Monolisa® HCV Ag-Ab ULTRA assay* kit realized in this study. The results realized in this study also support the observation by Shah *et al.,* (2003) and Laperche *et al.,* (2005) that "combination" assays detect all the seropositive samples detected in the "antibody only" assays and also detect 70.5% to 91.4% of HCV RNA positive samples obtained during the preseroconversion window period. It should however be noted that this work did not determine the sensitivity of this kit in the preseroconversion window period samples and thus these results cannot justify the claims by Shah or Laperche and their research groups.

The sensitivity of 96.9% among PCR positive samples realized in this study supports works which have reported the presence of detectable HCV antigens using other different assay platforms in between 82 – 98% HCV RNA positive – antibody

negative samples (Courouce *et al.,* 2000; Muerhoff *et al.,* 2002; Nubling *et al.,* 2002; Shah *et al.,* 2003; Laperche *et al.,* 2005; Leary *et al.,* 2006). This study however did not use any PCR positive - .antibody negative samples since such early phase preseroconversion window period samples are only diagnosed by chance.

The mean absorbance value of this assay platform among negative samples of 0.083 (0.033 – 0.219) was less than 30% of its cut off value of 0.327 (CO/OD), a performance comparable to that of ELISA kits routinely used in screening for blood-transmitted infections such as hepatitis B virus (HBV) and Human immunodeficiency virus (HIV) infections (Decker *et al.,* 1993; WHO, 1991). Similar results have also been reported by Ansaldi *et al.* (2006) and Dean *et al.* (2006).

ELISA assays that combine detections of both antibodies and antigens in a single kit have effectively been developed and introduced in the field of HIV detection (Ly *et al.,* 2004). The introduction of a similar assay platform in the detection of acute HCV infection, which has previously relied mainly on classical serological methods (Alter *et al.,* 2003), offers advantage especially over existing antibody assays that have substantial limitations; First, on early HCV detections due to slow development of HCV specific antibodies and second, due to the fact that immunocompromised patients may fail to develop a strong and rapid specific immune response against HCV. Therefore the overall sensitivity and specificity results obtained for assay kit depicts *Monolisa Ag-Ab HCV Ultra* as an ideal ELISA system for early detection of HCV circulating antigens or antibodies. It also depicts the assay system as ideal for

detection of HCV infection in immunocompromised populations.

Despite the expected advantages on this assay highlighted above, this study also reports the failure of *Monolisa® HCV Ag-Ab ULTRA* to detect three PCR positive samples from a bone marrow transplant patient belonging to genotype 1b. Two samples had significant viral loads of 8,900,000 and 780,000 IU/mL. Failure by this assay kit to detect genotype 1b is also important since genotype 1 has a world wide geographical distribution (WHO, 2008). Similarly, Turke *et al.* (2008) reported complete failure of the *Monolisa® HCV Ag-Ab ULTRA assay* to detect genotype 3a sample with a viral load of 7.76 million IU/mL and also failed to detect genotype 1b samples with a viral load of 8,190,000 IU/mL. Schnuriger *et al.* (2006) also reported a failure by *Monolisa® HCV Ag-Ab ULTRA assay* to detect a genotype 4 HCV positive sample despite the fact that the virus had a normal core sequence. The fact that all these findings show undetected samples to be above the theoretical threshold limit of 260,000 Iu/ml, corresponding to approximately 13.7 pg of total HCV core antigen (Krajden *et al.,* 2004; Fabrizi *et al.,* 2005) for *Monolisa® HCV Ag-Ab ULTRA assay* (Turke *et al.,* 2008) has a significant implication for *Monolisa® HCV Ag-Ab ULTRA assay* kit which is designed to detect both antigen and antibody proteins, as the optical signals generated by this assay platform are probably emanating from solely from the antibodies and as such, the assay does not seem to add further benefit to detect HCV infections by enhanced sensitivity due the potential contingency to trace viral capsid antigens.

The results in figure 4.2a show two samples whose viral loads of 200,000 IU/mL

and 40,000 IU/mL fall below the theoretical threshold of 260,000 Iu/ml which correspond to approximately 13.7 pg of total HCV core antigen (Krajden *et al.*, 2004; Fabrizi *et al.*, 2005). These samples however presented significantly high optical signals above 2.5 nm compared to some samples with high viral loads above the theoretical threshold limit of detection. A further evaluation of viral loads versus optical densities realized no direct correlation between the two parameters. Although the stage of disease progression in the patients was not determined, the observation in the two samples suggest that the patients were probably recovering and therefore there was a reduction in the HCV- RNA, thus a reduction in antigen levels with a corresponding increase in the levels of antibodies produced. If this is the case, then this could point to the fact that the source of optical signals generated by *Monolisa HCV Ag-Ab Ultra* from these samples was mainly antibody based as opposed to being antigen based.

A closer analysis of the results obtained by Dean *et al.* (2006) in their evaluation of *Monolisa® HCV Ag-Ab ULTRA assay* kit using different panels of samples also suggest that the optical signals generated by this assay are antibody and not antigen based.

Despite the arguments highlighted above, the interpretation of the source of optical signals basing on viral load alone may not be sufficient enough, and must be done with caution. This is due to the fact that some studies have failed to demonstrate the presence of antigens in RNA positive sera, for example, Ansaldi *et al.* (2006), showed antigen negative sera with viral load values between 100,000 and 850,000

IU/mL, this was despite the fact that this research group had found antigen positive sera with viral load levels below 850, 000 IU/mL. The findings that the analytical sensitivity of antigen assays does not correspond constantly to the HCV- RNA titre in different subjects have been demonstrated by Nubling *et al.* (2002), who reported 14 sera negative to the antigen test with viremia ranging between 10^5 and 10^6 IU/mL and 12 sera positive to antigen test with viremia below 10^5 IU/mL. Similar results were also realized by Maynard *et al.* (2003) who reported 9 sera negative to antigen test with HCV RNA above 1,000,000 IU/mL and 2 sera positive to the antigen test having viremia of below 100, 000 IU/mL. Even though the experimental results on the variable ratio of HCV RNA to viral core antigen in patient sera by Schüttler *et al.* (2004) supported the results as they suspected that the levels of HCV do not strictly correlate to HCV RNA, experimental results by Bouvier-Alias *et al.* (2002) were on the contrary. In their work, Bouvier-Alias and colleagues determined that 1 pg/ml of HCV core antigen corresponded to 8000 IU/ml of HCV RNA; thereby concluding that core antigen quantification can be used to monitor viral loads. Their argument seems to be supported by laboratory In-house results obtained on the same by Max von Pettenkofer Institute virology department on a similar evaluation (unpublished data). In their evaluation, an antigen detection limit of 1.5pg/ml corresponding to about 16,000 IU/mL was realized. Since no similar comparison has been done for capsid antigens, it is just assumed that the arguments presented above, though based on core antigens would well fit as well for capsid antigens.

5.2 Restriction Fragment Length Polymorphism (RFLP)

A summary in figure 4.2.1 and individual results realized in this study depict RFLP

as a reliable and effective genotyping technique, among HCV genotypes 1 to 4, feasible for routine clinical virology laboratories in genotyping HCV for clinical management of patients. This method was able to detect all the genotypes that were selected for analysis. Evaluation of RFLP across the genotypes realized that genotyping with RFLP however, varied across the genotypes tested showing the varying degrees by which different genotypes are able to be detected by various diagnostic techniques.

Among all the genotypes analyzed, RFLP easily distinguished genotype 1 samples. All genotype 1 samples were subtyped as either 1a or 1b based on the restriction enzymes Drd-I or Bsma-I. Other studies where genotype 1 HCV samples were easily detected by RFLP include those done by Buoro *et al.* (1999). The ease by which genotype 1 samples were easily detected could probably be attributed to the fact that subtypes within genotype 1 are assigned according to nucleotide located at position 243 i.e. G or A. It has been documented that approximately 10% of genuine genotype 1a posses a G at position 243 whereas 2% of genotype 1b show an A at the same location (Simmonds *et al.,* 1993), however it is worth noting that other subtypes within this same genotype could also exhibit indistinguishable restriction patterns for example genotype 1c which is identical to genotype 1a (Davidson *et al.,* 1995; Pawlotsky *et al.,* 1995).

In this study RFLP performed poorly in genotyping HCV genotype 2a samples, having an overall performance of 62.5%, giving unclear results for 25% and wrongly genotyping 1 sample (12.5%). Failure to detect genotype 2 samples was

also realized by Buoro and the group in their work among intravenous drug users HCV patients (Buoro *et al.,* 1999). Sequencing results however, were able to correctly genotype these samples that showed unclear results. In-depth analysis into the sequences generated was able to identify the restriction fragments though situated at different positions or sandwiched within Drd-I restriction site. This realization cannot be explained in detail currently as there were no border line conditions on the site specific preferences for enzymes used in RFLP genotyping. Further during the study, some samples belonging to genotype 2a were also restricted by Bsma-I. This was unusual as the two enzymes have not been known to restrict at the same time. Further, it was realized that the genotype 2a that was genotyped as 1b lacked the Bsr-I restriction site, similar result was obtained by the diagnostic department during the regular diagnosis of HCV (data not shown). This calls for a possible revision in the evaluation criteria among genotype 2 samples with regard to RFLP genotyping and interpretation. Or better still, it may call for a possible revision in the enzymes used for determining group 2 genotypes.

Although samples belonging to genotype 3a were easily genotyped, it was realized that most of them had unspecific restriction with Hinf-I, enzyme. However restriction with Fok-I was able to give a clear discrimination on the genotypes. This could mainly be attributed to the fact that Hinf-I is a weak and unstable enzyme. It could also be a pointer to close enzymatic conditions of the two enzymes. RFLP genotyping demonstrated only genotype 3a strains. As is the case with other genotypes, the limitations are also pertinent for genotype 3, since genotypes 3c, 3d, and 3e cannot be distinguished from 3a at the 5'UTR and those classified as 3b show

the same restriction pattern exhibited by 3f (Davidson *et al.,* 1995; Smith *et al.,* 1995). Similar results were obtained for genotype 4 that could not be distinguished into subtypes. However, for genotype 4 samples, clear restrictions with Hinf-I and Fok-I were clearly distinguishable. This could probably be a pointer to non-stability of Hinf-I in comparison to the rest of the enzymes used in this study.

Although the gold standard method for genotyping HCV samples remains to be nucleotide sequencing as proposed by Simmonds in 1995, this technique is still expensive in terms of cost of operation and is not economical for use in large scale. Other alternative methods such as temperature gradient gel electrophoresis (Lu *et al.,* 1995), dideoxy finger printing (Fox *et al.,* 1995) and type specific nested PCR (Ohno *et al.,* 1997) have also proved difficult to adopt in routine laboratories as they require expertise, proper equipments and high level of standardization. Therefore the realization in this study that RFLP method was able to detect all genotypes tested (though with varying frequencies) is a pointer towards effective introduction of this assay platform for genotype specific treatment strategies in developing countries.

Phylogenetic analysis has led to description of HCV into 6 major viral genotypes (Simmonds *et al.,* 1994), each including several subtypes. HCV "isolates" with less than 66-69% nucleotide homology are considered to belong to different genotypes, different subtypes are considered to contain less than 77 – 80% homology, whereas those strains which have 90% or more nucleotide homology are considered variants within the same subtype (Ohba *et al.,* 1995). These differences in genotypes have been known to play an important role in HCV therapy.

Interferon (IFN) - based regimens have been used in the treatment of HCV infections for nearly 2 decades (Di Bisceglie *et al.,* 1989; Davis *et al.,* 1989). Currently though, recommended therapy for chronic hepatitis C infection is a combination of peginterferon and ribavirin given for 24 to 48 weeks depending on the viral genotype (Hoofnagle *et al.,* 2006; NIH, 2002; Strader *et al.,* 2004; Dienstag *et al.,* 2006). This combination therapy is highly effective for patients infected with HCV genotype 2 or 3, where a 24- week course of peginterferon and a reduced dose of ribavirin results in a sustained virological response (SVR) in 75% - 80% of patients (Hadziyannis *et al.,* 2004; Shiffman *et al.,* 2007), whereas genotypes 1 and 4 have been shown to be more resistant than genotypes 2 or 3 (Cantaloube *et al.,* 2001), and require a full 48- week course of treatment with full doses of peginterferon and ribavirin for an undetectable HCV RNA level in only 65% - 70% of patients and a sustained virological response in 45% - 55% (Hadziyannis *et al.,* 2004; Manns *et al.,* 2001; McHutchison *et al.,* 2002). This observation underscores the importance of viral factors in determining the response to interferon- based therapy, thus the prior knowledge of the infecting genotype is of paramount importance in deciding the type of HCV interferon based therapy. Therefore the ability of RFLP to clearly detect and distinguish majority of tested samples could offer an opportunity to most developing countries to introduce genotype specific strategy in treatment of HCV infections.

This study also sought to compare random primers versus HCV 5'end specific primers in reverse transcribing purified HCV RNA extracts and further to compare

the genotype variations of HCV obtained by sequencing both the 5'UTR "conserved" region and Non-structural "hyper-variable" regions of the HCV genome. These two objectives are therefore discussed in tandem as the results seem to depict random primers as a point of focus in the future projection of HCV genotyping.

During the study, some of the samples whose extracts were reverse transcribed by random primers did not generate PCR products when RNA extraction was performed manually despite several repeat tests. The fact that when later extraction was done by automated systems, these samples were able to generate cDNA by random primers confirms the efficiency in the use of automated systems over manual operation and reveals the problems of using manual systems.

Another parameter used in comparing random primers versus 5'UTR specific primers in this study, apart from the ability of the primers to reverse transcribe HCV RNA extracted, was to quantify the viral loads from the cDNA generated by both primers using Real Time (RT) PCR. This parameter was based on the fact that studies have shown a major difficulty of reverse transcription to be mainly the generation of low yields of full-length cDNA transcripts, caused in part by RNase H activity inherent in reverse transcriptases (Sambrook *et al.,* 1989). It was therefore argued in this study that the low yield in viremia values would be expected with random primers due to their hexanucleotide (5'-NNN NNN- 3') thus their ability to give a longer piece of cDNA that can be amplified for sequencing all regions of the HCV genome. This longer piece therefore would majorly be affected by RNase

activity in reverse transcriptases, leading to low yields in viremia values. It was also based on the realization that the specific 19 – oligonucleotide (HCVN 02: 5' – gTg CAC ggT CTA CgA gAC C – 3' and HCVN 08: 5' –TAC TCA CCg gTT CCg CAg A – 3') primers from the 5'UTR, although highly sensitive, they give a very small piece of cDNA from the 5'UTR. Despite these expectations, this study realized comparable viral loads between random primers and 5'UTR specific primers with random primer recording higher measurements in some instances. This fact points to the possibility of using random primers in routine diagnostic testing for HCV infection. Other studies have also shown improved sensitivity in using random primers compared to specific primers for the detection of the HCV RNA by reverse-transcriptase polymerase chain reaction (Radhakrishnan *et al.,* 1999).

It therefore suffices to state that the results realized in this study on the use of random primers confirm the efficiency of using these primers in generating cDNA in HCV reverse transcription and contradicts the notion that these random primers are not very sensitive and therefore associated with low yields in viremia values. cDNA generated by these random primers are not site specific, hence generation of longer templates that are very important when genotyping various regions of HCV genome. This ability to use random primers in generating cDNA used in genotyping various regions of the HCV genome offers an advantage of these primers to their 5'UTR specific counterparts especially in epidemiological studies and in tracing the source of infection where sites of mutations and subtypes respectively are of utmost importance.

For purposes of treatment management, current 5'UTR based genotyping assays are acceptably accurate since they have been shown to have more than 95% concordance with genotypes identified by nucleotide sequencing (Gault *et al.,* 2003; Lau *et al.,* 1995; Simmonds *et al.,* 1993; Tanaka *et al.,* 1994; Zheng *et al.,* 2003). Though the data presented here is not sufficient enough, this assertion could partially be supported by the fact that similar genotypes were obtained among genotype 2a samples between sequence results of the 5'UTR site and the sequence results of the complete genome in table 4.2.8. However, several studies have demonstrated that 5'UTR region may not be appropriate for definitive genotype identification of subtypes (Cantaloube *et al.,* 2006; Laperche *et al.,* 2005; Tamalet *et al.,* 2003). Further, problems have been described with 5'UTR- based genotyping including; misclassification of genotype 1a frequently identified as 1b (Cantaloube *et al.,* 2006; Chan and weck, 2002; Laperche *et al.,* 2005), lack of subtyping of genotypes 2, 3 and 4 related to the diversity within these genetic groups which could not be correctly distinguished from each other when only the 5'UTR is analyzed since these genotypes are not divergent enough in this region (Simmonds *et al.,* 2005) and finally, the misclassification of some genotype 6 samples, classified as genotype 1 (Chinchai *et al.,* 2003; Tamalet *et al.,* 2003), due to the identity of genotype 6 and genotype 1a or 1b at the 5'UTR (Mellor *et al.,* 1996; Simmonds, 2001; Tokita *et al.,* 1995; Tokita *et al.,* 1994).

Although not frequent, but seen to be on the increase due to the hybrid generation in multiple infected , the recombinant forms (Colina *et al.,* 2004; Kalinina *et al.,* 2002; Noppornpanth *et al.,* 2006) limit the accuracy of genotyping assays when only a

section of the genome is sequenced. This assertion is also seen in this work when in some instances RFLP was only able to detect one genotype in samples with mixed infections in this study and when sample coded as “mixed 2” in this study could only be detected as 1a when blast results of the complete genome were realized using products of the 5’UTR primer. All the observations highlighted above, coupled by the fact that the “hypervariable” region of the genome is important in epidemiological studies (Bourliere *et al.,* 2002; Martial *et al.,* 2004; Martinot-Peignoux *et al.,* 1999; Pawlotsky *et al.,* 1995) and for tracing a source of infection (Ackerman *et al.,* 2000; Cantaulobe *et al.,* 2001; Halfon et al., 2001; Izopet *et al.,* 1999) confirm that other sections mainly in the “hypervariable” region of the HCV genome are continually becoming important points in HCV genotyping process. Further, new genotyping assay platforms are being developed based on these other non conserved regions. This can easily and mainly be achieved by use of random primers instead of specific primers. The importance of random primers may also be realized in the future in case of probable resistance when specific antiviral drugs such as protease inhibitors, are introduced in HCV treatment, and therefore PCR products from different regions of the HCV genome must be generated for analysis to counter these resistances developed by the virus.

Analysis of different regions of the genome was a confirmation of different subgroups that we are able to obtain from the “hypervariable” region in comparison to the conserved region. These variations could be a pointer towards the ability of HCV to persist in a host. Different variations observed in the “hypervariable” region may be as a result of mutation, leading to “quasispecies” resulting from mutations

due to high error rates in RNA replication. If this is the case, then these results would support the assertion that the persistence of HCV appears to result from its ability of the virus to mutate rapidly under immune pressure, giving rise to related but immunologically distinct variants (Alter, 1995). Any of these variants could become a predominant strain. This may have a significant meaning for RFLP genotyping as such variants could eventually be missed by this system or improperly genotyped.

Of importance, however, is the fact that the data generated in table 8 revealed the similarity in genotypes, between the 5'UTR and the complete genome, hence confirming the conserved nature of the 5'UTR.

Although RFLP was able to clearly show the possibility of mixed infection in two samples, sequence results for the two samples failed to detect at least one genotype in the sample with mixed genotype infections. This finding could be a pointer towards the possibility that only predominant genomes are detected by sequencing the 5'UTR, leaving out HCV minor populations, which contribute to mixed infections, within each of the directly sequenced isolates. Thus RFLP was found to be very sensitive in determining mixed infections.

Based on the discussion above, it is apparent to note that for subtype assignment, it is mandatory to sequence, simultaneously, genomic regions which exhibit a greater degree of nucleotide heterogeneity within a given genotype, such as E1, Core or NS5.

Analyzing amplicons from the 5'UTR by RFLP is still one of the most widely used methods in HCV genotyping. Significant nucleotide conservation at this location among different strains, which allows the use of universal primers, and therefore maximal sensitivity for detection and subsequent typing explains RFLP's current acceptance. Paradoxically, this study also accepts the fact that such conservation is at the same time a hindrance for conclusive subtyping thus other regions of HCV genome need to be analyzed, hence the need for use of random primers. This study however recognizes that fact that such clear distinctions within the genotypes, where other regions need to be analyzed, are more important in epidemiological studies and tracing sources of infections, as opposed to regular clinical diagnostic work where currently RFLP is ideal.

CHAPTER SIX

6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study concludes the following;

That the sensitivity, specificity and predictive values realized for *Monolisa® HCV Ag-Ab ULTRA assay* kit is comparable to those of antibody based ELISA assays, mainly AxSYM and Ortho HCV 3.0 kits, currently used in routine laboratories for diagnosis of HCV infections.

That although *Monolisa® HCV Ag-Ab ULTRA assay* showed high sensitivity and specificity in detecting antibodies to HCV, it does not seem to add further benefit to detect HCV infections by enhanced sensitivity due the potential contingency to trace viral capsid antigens.

That whereas genomic information is limited, the study showed RFLP as valuable in massive typing and routine diagnostic testing of HCV suspected patients due to costs involved as compared to other commercially available assay platforms as success in HCV treatment is mainly based on the genotype as opposed to the subtype.

That random primers are important in reverse transcribing HCV genome, as the primers reverse transcribed all samples just as did the 5'UTR specific primers. Random primers are useful in genotyping other areas of the HCV genome as opposed to 5'UTR specific primers.

That the “hypervariable” region of HCV genome depicts a variation in HCV genotypes as opposed to the 5’ UTR, and that this region is a point of focus in HCV mutation studies, thus importance of random primers.

6.2 Limitations of the study

Antigen sensitivity of *Monolisa Ag-Ab Ultra* could not be conclusively determined since it was realized that “antigen only” based ELISA kits including *Trak – C* detection ELISA kit, commonly used, were not commercially available. Due to time limitation, it was also not possible to clone antibodies for pre-absorption of antigens in the samples prior to testing of circulating antigens. Further pre-treatment of samples with anti-rheumatoid factors 2 and 3 did not depict any antibody pre-absorption in the samples pre-analyzed.

6.3 Recommendations

Based on the results of this work, the following are recommended:

I. That there is need to assess in detail the antigen sensitivity of *Monolisa® HCV Ag-Ab ULTRA assay* kit in order to conclusively determine the sensitivity of this assay platform.
II. That *Monolisa® HCV Ag-Ab ULTRA assay* kit should be evaluated further using samples from the Kenyan population and if similar results are obtained as in this study then the new kit should be considered for introduction in Kenya for screening donated blood by the medical fraternity and the policy makers, performance of the assay platform should however be compared with other available assays in Kenya before its introduction
III. That there is need to introduce the strategy based on genotype specific

treatment of HCV infection, in the treatment and management of HCV in Kenya. RFLP could provide a reasonable solution in meeting this challenge.

IV. That further evaluation of random primers versus 5'UTR specific primers needs to be done using many samples before a conclusive decision is made on the use of these primers in HCV genotyping. This work provides a baseline for such a study to be carried out on a large scale.

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APPENDICES