

**PREVALENCE OF INTRAVENOUS DRUG USE and
HEPATITIS C VIRUS (HCV) INFECTIONS AMONG
PATIENTS VISITING MALINDI SUB COUNTY
HOSPITAL COMPREHENSIVE CARE CENTRE, KILIFI
COUNTY, KENYA.**

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**Prevalence of intravenous drug use and hepatitis C infections among patients
visiting the Malindi Sub County hospital comprehensive care clinic, Kilifi County,
Kenya.**

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**A thesis submitted in partial fulfillment for the Degree of Master of
Science in Epidemiology in the Jomo Kenyatta University of
Agriculture and Technology
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DECLARATION

This thesis is my original work and has not been presented elsewhere for a degree award

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DEDICATION

To my loving parents and husband who have sacrificed so much for me to reach where I am.

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ABBREVIATIONS and ACRONYMS

- AIDS**- Acquired immunodeficiency syndrome
- CCC**-Comprehensive care clinic
- CRFs**- Circulating recombinant forms
- DNTPs**- Deoxy ribonucleotide tri-phosphates
- DNA**-Deoxyribonucleic acid
- ELISA**-Enzyme linked immune sorbent assays
- HCV**-Hepatitis C virus
- HIV**-Human immunodeficiency virus
- IDUs**-Intravenous drug users
- KEMRI**-Kenya Medical Research Institute
- NTRI**-Nucleoside reverse transcriptase inhibitors
- NNRTI**-Non-nucleoside reverse transcriptase inhibitors
- PBMCs**-Peripheral blood mononuclear cells
- PCR**-Polymerase chain reaction
- PI**- Protease inhibitors
- PLWHA**- People living with HIV and AIDS
- RNA**-Ribonucleic acid
- RT**-Reverse transcriptase
- SPSS**-Statistical Package for Social Sciences

ABSTRACT

Introduction of highly active antiretroviral therapy (HAART) has improved the life of people living with Human Immunodeficiency Virus (HIV). But the persistence of opportunistic infections during HAART still presents a huge burden of disease, which often reverses the direct benefits of anti-retroviral treatment. Hepatitis C virus (HCV) is one of the many known opportunistic infection agents that together or individually, continues to escalate the risk of deaths among HIV/AIDS individuals. This study aimed at determining the prevalence of intravenous drug use and hepatitis C infections among patients attending the comprehensive care clinic (CCC) at the Malindi district hospital. A total of 452 subjects were enrolled into the study from the Malindi sub county Hospital comprehensive care clinic. Socio-demographic information was collected using individual questionnaires. Blood samples were collected in EDTA tubes and processed for downstream application. Approximately 41.2% (186/452) subjects were intravenous drug users (IDUs), of which 152 were aged 40 years or younger while 34 were above 40 years. HCV was solely found among IDUs at a prevalence of 16.4% and not among non-injectors. Due to limited resources, only 25 of the HIV positive samples were sequenced to determine HIV subtype and drug resistance. By HIV subtype, 60% of the infections were due to subtype A (majority of them being A1 at 88.66% while A2 and AE respectively were 6.67%); 12% were subtype C and; 16% were subtype D sixteen percent (4/25) of the virus isolates had mutations that confer resistance to nucleoside reverse transcriptase inhibitor (NRTI) class of antiretroviral drugs, while 24% (6/25) had mutations that confer resistance to non-NRTIs (NNRTIs). For the HCV infections by genotype, 75% were subtype 1a and 25% were subtype 4a. There were no drug resistant mutations among the HCV positive samples. This study has revealed a high prevalence of HCV infections among the IDUs. Public health programs should consider HCV testing alongside HIV testing.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Human Immunodeficiency Virus remains one of the world's leading epidemic today, with approximately 36 million of the global population living with the virus and another 2 million new infections occurring each year (UNAIDS 2016). By the year 2015, there were about 19 million people living with HIV/AIDs in Eastern and Southern Africa alone, though the annual new infections showed demonstrable decline of about 14% between 2010 and 2015 (UNAIDS 2016). According to the Kenya AIDS Indicator Survey, the prevalence rate of HIV in Kenya was 5.6% at the end of 2012, with that of Kilifi county being below the national average at about 3.7% (KAIS 2012). Over the years access to antiretroviral therapy has resulted to longer AIDS-related life expectancy among HIV-1 infected persons. However, HIV infection is accompanied by incidents of opportunistic infections due to waning host immunity, an occurrence that continuously threaten to reverse the benefits of antiretroviral treatment and progression to AIDS (Barreiro , Fernez-Montero , de-Mendoza , Labarga & Soriano 2014).

There are two types of HIV; HIV-1 and HIV-2. HIV-1 is the most common type and globally responsible for AIDS. HIV-1 can be divided into four major groups namely; group M, group O, group N and P. Group M is responsible for majority of global HIV pandemic and is further subdivided into nine genetically distinct subtypes; A, B, C, D, F, G, H, J and K (Powell, Konings, Nanfack , Burda , Urbanski , & Saa 2007). Subtypes A and F are subdivided into sub-subtypes A1, A2 and A3 and into F1 and F2 respectively. In East Africa, subtypes A, D and C are responsible for a majority of the HIV infections, while recombinants these subtypes cause infection in another 30% of the HIV population in the region (Gao , Trask , Hui , Mamaeva , Chen , Theodore & Foley 2001). Two different subtypes of HIV may combine their genetic material in the same cell of infected persons to form a new hybrid virus, a process similar to sexual reproduction, sometimes called "viral sex" (Burke D, 1997). However, many of these strains do not

survive for long, but those that persist and infect more than one person evolve into distinct genotypes often referred to as “circulating recombinant forms” (CRFs). These genotypic variability result in discrete phenotypic and functional properties that the virus exploits to escape immune clearance by the host (such as neutralizing or vaccine antibodies), or therapeutic sensitivity to treatment (drug resistance).

HIV drug resistance has been on the rise since the scale up of antiretroviral treatment. Drug resistant mutants arise due to high rate of viral mutations occurring under drug pressure, or naturally during replication. The prevalence of drug resistance in Sub-Saharan Africa has been on an upward trajectory since the massive roll out of antiretroviral therapy and this is equally evident in Kenya by the high proportions of patients failing treatment (Ochieng, Kitawi, Nzomo, Mwatelah , Kimulwo & Aman 2015, Lwembe, Ochieng, Panikulam, Mongoina, Palakudy & Koizumi, 2007) . A study carried out in a subset of drug naïve Kenyan patients showed the prevalence of reverse transcriptase inhibitors-resistance is around 7.5% (Lihana, Khamadi, Raphael , Kiptoo & Lagat 2009) although this could be higher at population level. This is a clear indication that drug resistant HIV-1 may already be circulating in the population long before exposure to antiretroviral treatment.

Hepatitis C virus occurs as a major opportunistic infection in HIV infected individuals, although mono-infection with HCV in HIV negative persons is equally a public health challenge. HCV is a member of the *flaviviridae* family of viruses, which consists of 4 genera namely *hepacivirus*, *flavivirus*, *pegivirus* and *pestivirus*. Hepatitis C belongs to the genus *hepacivirus*, which only affects humans. HCV consists of a positive (sense) single-stranded RNA genome approximately 10 kb long, containing a single large open reading frame. The virus is grouped into 11 genotypes, 100 strains and multiple subtypes. The HCV subtypes share between 80-85% genetic similarities, with the remaining 8-12% divergence occurring between isolates from independent patients. The most prevalent of HCV strains is subtype 1, although subtypes 1-3 are globally distributed. Subtypes 1a and 1b are responsible for nearly 60% of all global HCV infections, with subtype 3 being restricted mostly to South East Asia and subtype 4 to regions in Central

Africa, North Africa and the Middle East. Subtype 5 is found commonly in South Africa whereas subtype 6 in South East Asia.

Drug use, particularly alcohol consumption, greatly hastens the progression of HCV, in addition to age at time of infection, with subjects at >45 years of age at infection being at a higher risk of developing liver disease. Other predisposing factors to HCV infections include co-infection with viruses like HIV or HBV.

HCV has natural resistance to anti-HCV drugs. and replicates at a very high rate (Transcriptional error) associated with this replication rate often results in mutations that are associated with the generation of drug resistant viral mutations, or virus clones that evade host defense mechanisms. Such events have been evident from studies using infectious molecular clones that contain R155Q, A156T, or D168V mutations in the presence of ciluprevir (drug) exposure, confirming these clones became less susceptible to treatment with the drug compared to the wild-type viruses (Soriano, Eugenia, Pablo, Jose & Pablo 2010).

The genetic variability of HCV, contributes to difference in response to therapy. For instance, over 80% of HCV subtype 2 infected patients are responsive to therapy within six to twelve months compared to about 50% response rate in subtype 1 treatment (Manns, McHutchison , Gordon , Rustgi , Shiffman & Reindollar 2001, Fried M 1999, Strader, Wright, Thomasand Seeff 2004) . Genotype 1b is predominant in subjects infected through nosocomial contamination whereas other subtypes are commonly found in illicit drug users (Ndong, Makuwa , Njouom 2008)

Additionally, disease course in persons co-infected with both HIV and HCV is significantly altered as is individual responses to either anti HIV or anti HCV drugs, a situation that escalates progression to AIDS (Tizzot, Grisbach, Beltrame, & Messias-Reason 2016). Thus, understanding the co-infection status of at-risk persons in addition to the specific infecting virus genotypes of both HIV and HCV is an important step towards epidemiological surveillance and clinical management of patients

1.2 Statement of the problem

Introduction of highly active antiretroviral therapy has resulted into longer survival rates of people living with HIV. However, the incidents of morbidities and mortalities have recently begun to rise, largely due to secondary complications like chronic liver disease that are linked co-morbid infections, HCV is an example. Yet a comprehensive understanding of the magnitude of HIV and HCV co-infection is still lacking as only a few studies have focused assessing these two viruses as co-existing epidemics. Results from another study that assessed the cause of acute hepatitis among patients showed that HCV accounted for 7.1% of the infections. HIV infections were also found to be high among the same patients (Atina, Ogutu, Hardison & Mumo 2004).

Research has shown that HIV increases the progression rate of HCV due to weakened cellular immune response (Irena & George 2002). Conversely, HIV also increases the rate of mutation of HCV by almost an order of magnitude. HIV patients who are also infected with HCV progress faster to end stage liver disease and death when compared to those with just HIV, due to a significantly shortened period within which these patients develop cirrhosis and imbalance in inflammatory cytokine levels (Chung, Andersen and Volberding 2004, Abdallah, Abdel, Hamed & Gamal 2010).

The Coastal region of Malindi is heterogeneously cosmopolitan as a local and international tourist destination. It is apparent that the unique socio-demographic profile in this region in addition to economic factors that limit access to basic health services, is playing an important role in escalation of illicit drug use (NASCO 2012). The study aimed at determining the prevalence of intravenous drug users and hepatitis C infections among patients visiting the Malindi Sub- County hospital in Kilifi County.

1.3 Justification of the study

HCV affects 150-200 million people worldwide, 50% of whom are also HIV positive (Irena *et al* 2002). These two viruses share a common mode of transmission that include risk metrics such as intravenous drug use (IDU). The risk for acquiring both viruses is elevated among IDUs primarily due to the practice of sharing of needles. There is

limited information about the proportion of Kenyans who inject drugs, with the consequence that the circulating risk of both HIV and HCV is often underestimated. Appreciating injection drug use as a health epidemic is a vital first step to alleviating the burden of infection associated with this practice. Malindi is one of the most cosmopolitan regions in Kenya, which, also serves as an international tourist destination. This region therefore also serves as a safe harbor for drugs both in terms of direct use and proliferation into neighboring cities and countries. This study is significant since it sought to understand the most pressing public health infection burdens of HIV and HCV in the unique context of associated variables such as illicit substance use, and in a setting, that presents diverse risk metrics for these epidemics.

Research on HIV and HCV co-infections is scarce especially in developing countries that also bear the brunt of these infectious epidemics yet without appropriate mitigation measures. It is known that co-infection with HIV and HCV produces negative but synergistic effects on disease outcomes (ICAD, 2009). Whereas treatment options exist for HCV, effectiveness of these drugs range from as low as 40% to 80%. These treatments however, are very expensive and out of the reach of most patients in the developing countries. Besides, the efficacies and breadth of the available drugs are limited by the heterogeneity of the infecting virus strains (ICAD, 2009), a phenomenon that further dampens the breadth and scope of effectiveness of available treatment. As a consequence, early identification and prevention of behavior risks and practices that predispose to HCV as well as other viral infection, is likely to be mitigating with respect to reducing infection and disease burden.

In Kenya in particular, there is no treatment or prevention programs that are designed to simultaneously address co-infection with both HCV and HIV. This remains a significant bottleneck to the success of any therapy program that is focused on just a single viral infection in a patient who is otherwise infected with multiple viruses. The consequences of such disparate treatment modules is an apparent loss in overall efficacy of the administered drugs.

Data generated from this study seeks to fill the gaps in knowledge and provide much needed source of guidance on setting up prevention and treatment programs for both HIV and HCV in high-risk populations. The IDUs stand out as one of the risk groups with rapidly increasing infectious epidemic rates, particularly HIV and HCV. Some studies report the co-infection prevalence among IDUs may surpass 90% (Maier & Wu 2002), with equally rising probability of mortality among HIV positive subjects being linked to cirrhosis and liver disease resulting from co-infection with HCV (Muriuki, Gicheru, Wachira, Nyamache & Khamadi2013, Karuru, Lule, Joshi & Anzala 2005, Atina *et al* 2004,). This study in addition, assesses the genetic diversity of infecting HIV and HCV strains, as well as associated mutations that confer resistance to commonly used antiretroviral drugs. Overall, information on co-infection burden and genetic variability of infecting viruses are of immediate benefit to the study population, and when expanded to the entire population as recommended here, will offer useful guidance on comprehensive antiretroviral and infection management programs.

1.4 Research questions

The study was carried out in patients attending the CCC clinic in Malindi sub county hospital. The following research questions were addressed:

- What was the prevalence of intravenous drug users among CCC patients in Malindi sub county hospital?
- What was the prevalence of HCV, its genotypes and anti-HCV drug resistance-associated mutations among CCC patients in Malindi sub county hospital?
- What were the HIV genotypes and anti-HIV and anti-HCV drug resistance-associated mutations among CCC patients in Malindi sub county hospital?

1.5 Objectives

1.5.1 General objective

To determine the prevalence of intravenous drug use and hepatitis C infections among patients attending the comprehensive care clinic (CCC) at the Malindi sub county hospital.

1.5.2 Specific objectives

1. To determine the prevalence of intravenous drug use among the CCC attendees in Malindi.
2. To determine the prevalence of HCV, genetic profile and drug resistance associated mutations among CCC attendees.
3. To assess the different genetic profiles of HIV and drug-specific resistance mutation among CCC attendees.

CHAPTER TWO

LITERATURE REVIEW

2.1 Hepatitis C Virus structure

HCV is a member of the genus *hepacivirus* from the family *flaviviridae*, which consist of three other genera; *flavivirus*, *pegivirus* and *pestivirus*. The structural and virological characteristics of the family *flaviviridae* include enveloped lipid bilayer which anchors two or more envelope proteins. The envelope surrounds the nucleocapsid, made of multiple copies of a small basic protein (core or C) and the RNA genome (Figure 2.1). The *Flaviviridae* is a positive-strand RNA molecule ranging in size from 9.6 to 12.3 thousand nucleotides (nt), and an open reading frame (ORF) encoding a polyprotein around 3000 amino acids (aa). The HCV core (Figure 2.2) is a multifunctional protein but its main role is formation of the viral capsid together with the envelope glycoproteins E1 and E2 to surround and protect the RNA. The core consists of two domains; D1 and D2 distinguished by the differences in their amino acids and their location on the terminal ends of the core. D1 is located at the N terminal and mainly involved in RNA binding and oligomerization. D2 is located at the C terminal and its main function is enhancing the association of the core and the endoplasmic reticulum (Gawlik, Baugh, Chatterji, Lim, Bobardt 2014, Esser-Nobis, Romero-Brey, Ganten, Gouttenoire, Harak, Klein, 2013).

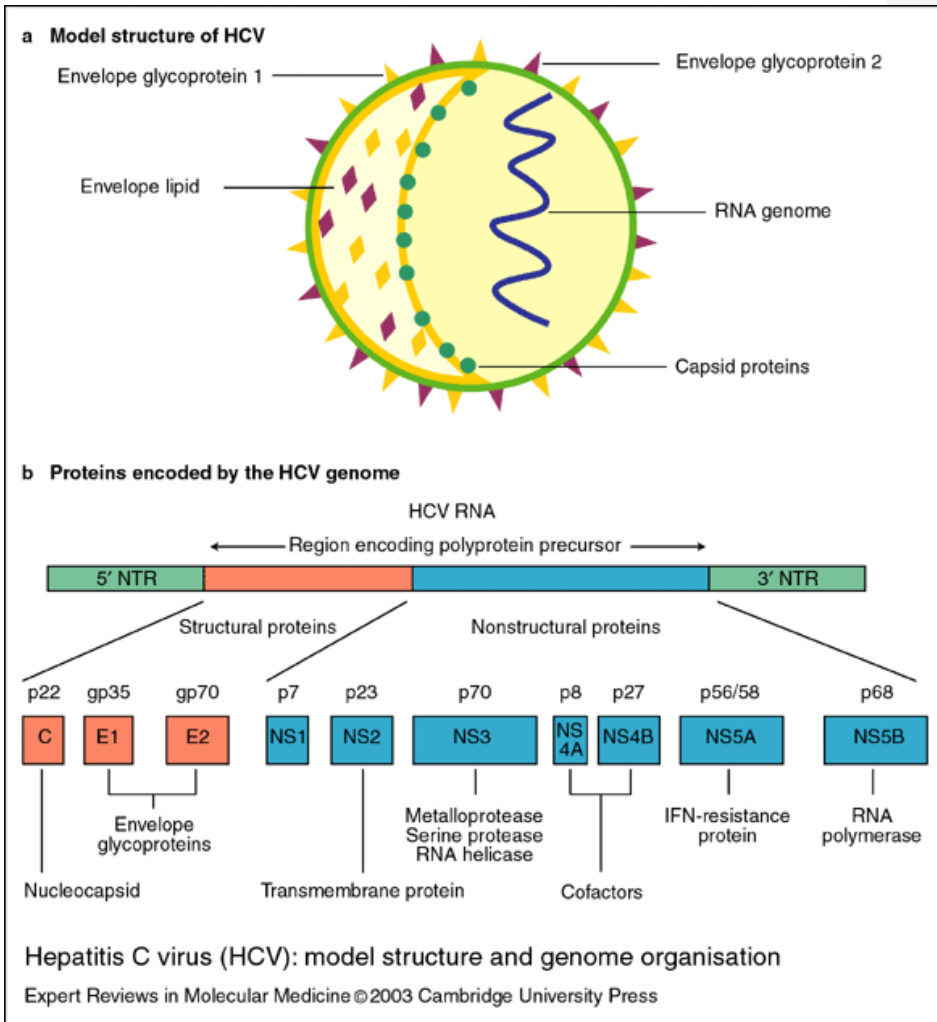


Figure 2.1: HCV structure and genome (Cambridge university press)

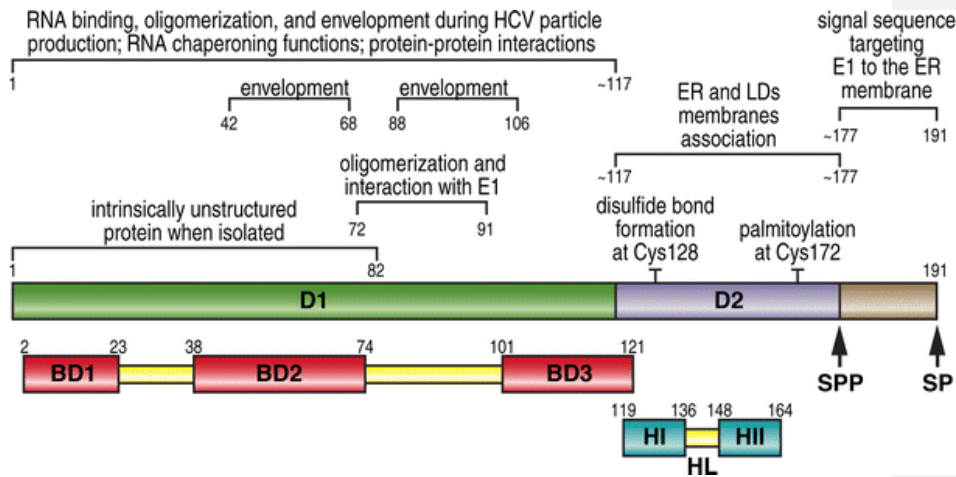


Figure 2.2: Structure of the HCV core protein (Gawlik *et al* 2014)

The non-structural proteins NS3 to NS5B are mainly required for viral replication. The N and C terminal of the NS3 region have different functions. The N terminal contains serine protease domain that cleaves the four downstream non-structural protein junctions, and at least three host targets. The C terminal region is a helicase responsible for replication. NS4A is a co-factor for NS3 region and acts as a regulator during replication. NS4B is responsible for remodeling endoplasmic reticulum. NS5B is the RNA-dependent RNA polymerase (RdRp) with a C-terminal membrane-anchoring segment (Lee, Choi, Ou, & Lai 2014).

2.2 Distribution of HCV genotypes

Recent estimates indicate that there are approximately 8 million people infected with HCV in Central and Eastern Sub-Saharan Africa the infection rate escalates to 15 million people are infected across North Africa and the Middle East (Moh'd, Groeger, Flaxman & Wiersma 2013). There are diverse genotypes circulating in the population and the subsequent effect is seen on the response of specific genotypes to anti HCV

treatment. Genotype 1 has been found to occur worldwide whereby 1a is more prevalent in the Western world while 1b is more prevalent in the Eastern world (Matheï, Robaeys, van-Damme, Buntinx, & Verrando 2005). Genotype 3 is mainly found in Asia, North America and parts of Europe, genotype 4 is common in Europe, Middle East and Central Africa. Genotype 5 is found in parts of Africa and Europe while genotype 6 is found in South East Asia and North America. In China four genotypes have been found: 1, 2, 3 and 6 respectively (Zhao, Yi & Lu 2012). In studies whereby the distribution of HCV was reviewed worldwide showed that the prevalence of genotypes was as follows; - genotype 1, 3, 2 and 4 at 46%, 22% and 13% for the last two respectively (Gower, Estes, Blanch, Ravazi-shearer & Ravazi 2014).

A study carried out among intravenous drug users in Nairobi showed that the most prevalent genotype among this population was 1a at 73% and 4 at 27% respectively (Muasya, Lore, Yano, Yatsuhashi, Owiti, Fukuda 2008). In Iran the most prevalent genotype was 1 followed by 3 respectively (Afzali, Momen-Heravi, & Farokhzad, 2016). A study evaluating HIV/HCV co-infection among HIV patients in Iran showed that 63% of the co-infections were found among IDUs where the most prevalent genotype was 1 followed by 3 respectively.

2.3 HCV drug interaction and resistance.

The main treatment options for HCV include a combination of interferon- α plus ribavirin, this has often acted as a double-edged sword as interferon- α is known to mediate HIV infection. Interferon- α is a cytokine produced by the body in response to inflammation at the mucosal sites, nevertheless its systemic presence directly affects its mucosal availability. It has been strongly associated with increased (rather than decreased) HIV acquisition in a prospective cytokine analysis of a large South African clinical trial (Masson, Passmore, Liebenberg, Werner, Baxter, Arnold, 2015).

HCV drug response is greatly affected by the genotype, effective response of up to 80% is the highest ever observed. Individuals that have been infected by genotype 1 experience lower response to antiretroviral therapy (42-50%) than those having

genotype 2 and 3 (78-80%), (Manns, McHutchison, Gordon, Rustgi, Shiffman, Reindollar 2001, Zeuzem, Hultcrantz, Bourliere, Goeser, Marcellin 2004).

Recent studies that have been focused on sofosbuvir with a combination of other anti HCV drugs have shown increased drug response. A comparison was made between sofosbuvir-ribavirin and pegylated interferon-sofosbuvir, showed that none of the two combinations was found to be superior to the other. There is evidence of increased drug response when sofosbuvir is used compared to the previously used pegylated interferon α -ribavirin combination (Lawitz, Poordad, Brainard, Hyland, Ann, Dvory-Sobol 2013). A combination of sofosbuvir and ledipasvir showed good response was observed especially against genotype 1 (Ahamad, Debeck, Feng, Sakakibara & Kerr 2014). As much as there is sustained viral suppression in response to sofosbuvir, the drug is very expensive thus it is not easily available.

2.3.1 Interaction with interferon- α

Interferon- α is a group of cytokines that belong to the hosts' natural immune response to various stimuli especially during viral infections. They act by binding to specific receptors on target cell resulting in an interaction that leads to up-regulation of interferon stimulated genes (ISGs) and subsequently, the expression of multiple antiviral effector proteins (Wohnsland, Hofmann & Sarrazin 2007). Interferon - α also affects processes that regulate cell growth in HCV and modulate the immune response. In vitro studies that are based on recombinant expression of the whole HCV poly-protein that inhibits signal transducers and activators of transcription 1 (STAT-1) function due to elevated levels of phosphate 2A which results in hypo methylation of STAT-1. This result in increased binding of protein inhibitor of activated STAT called PIAS. This action results in reduced activation of ISGs (Blindenbacher, Duong, Hunziker, Stutvoet, Wang, Terracciano 2003).

2.3.2 Interaction with ribavirin (1- β -D-ribofuranosyl-1, 2, 4-triazole, 3-carboxamide).

Ribavirin is a guanosine analogue that possess a broad spectrum of antiviral activity of RNA and DNA viruses in vitro. Its monotherapy has shown no effect on reducing HCV RNA therefore, an indicator of little or no effects on HCV replication (Pawlotsky, Dahari, Neumann, Hezode, Germanidis, Lonjon 2004). When ribavirin was combined with pegylated interferon- α (PEG-IFN) it resulted in improved sustained virologic response rates.

Modes of action of ribavirin;

- It enhances the host adaptive antiviral immune response.
- Inhibits the hosts' Inosine-5'-monophosphate dehydrogenase .
- Direct inhibition of HCV NS5B RNA dependent, RNA polymerase.
- RNA virus mutagenesis whereby it acts on RNA virus mutagens leading to increased mutational frequency that exceeds the mutational threshold of virus fitness and drive RNA viruses into error catastrophe (Maag, Castro, Hong, and Cameron 2001, Crotty, Maag, Zhong, Hong & Cameron 2000).

In studies involving human liver HUH7 cell lines harboring a HCV replicon system, treatment with increasing concentrations of ribavirin showed resistance to the drug. But this was not conferred by mutations in HCV RNA dependent RNA polymerase. Reduced ribavirin importation into the cells probably was by down regulation of a nucleotide transporter. Mutations were detected within NS5A, G404S and E442G regions that were present only in the ribavirin resistant HCV replicon cells but not in the ribavirin sensitive cell lines (Pfeiffer & Kirkegaard 2005).

2.3.3 Interaction with Sofosbuvir

Sofosbuvir is an NS5B nucleotide polymerase inhibitor which was recently discovered and has undergone different testing phases. Sofosbuvir is a pro-drug of 2'-F-2'-C-methyluridine monophosphate and was licensed in 2013 by the Food and Drug Agency for treatment of genotype 1 and 4 in combination with peglyated interferon α or

ribavirin while it is used in combination with ribavirin only to treat genotypes 3 and 2 (Auda, Fabio, White, Lloyd & Bull 2015). Studies that have assessed the efficiency of sofosbuvir against genotype 1 have shown reduced HCV RNA quantities when it is used in combination with pegylated interferon α or ribavirin (Maribel, Eric, Kris, David, Edwin, John 2013). This combination has also been assessed among treatment-experienced patients who are affected by genotype 2 and 3 and it has shown to have high sustained virological response (Lawitz, Poordad, Brainard, Hyland, Ann, Dvory-Sobol 2015).

2.4 Human immunodeficiency Virus structure

HIV-1 and HIV-2 belong to the genus Lentivirus, family of *Retroviridae* and subfamily *Orthoretrovirinae* (International Agency for Research on Cancer., 1996). Retroviruses transcribe their genome from RNA into DNA using the enzyme reverse transcriptase. The integrase enzyme then integrates the viral DNA into the host's genome, becoming part of the host's cellular DNA. The integrated virus genome subsequently undergoes replication during the natural cellular processes (Nisole and Saib 2004). HIV is a single-stranded, positive-sense, enveloped RNA virus (Nester, Anderson, Roberts, Pearsall, Nester & Hurley 2004).

The viral envelope contains a lipid bilayer and viral spikes called glycoproteins (Carter & Saunders 2007). The surface glycoproteins gp120 are anchored to the virus *via* the transmembrane (TM) glycoprotein gp41. A matrix shell of the matrix-associated protein p17 lines the inner surface of the viral membrane. At the viral core is the capsid protein p24, located at the center of the virus (Figure 2.3). The capsid particle encapsulates two copies of the RNA viral genome. The viral genome is stabilized within the capsid particle by the nucleocapsid (NC) protein p7. The capsid particle also contains three essential virally encoded enzymes: protease (PR), reverse transcriptase (RT) and integrase (IN). The HIV genome consists of 3 structural genes – env, gag and pol (Figure 2.4) that contain information necessary for making structural proteins (Nester *et al.*, 2004). It also has 6 regulatory genes - *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*- that contain

information needed to produce proteins that control the ability of HIV to infect a cell, produce new copies of virus, or cause disease.

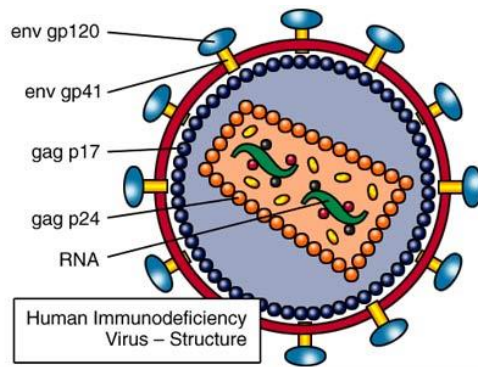


Figure 2.3: Diagram of mature HIV virion
 (Source: <https://www.uhavax.hartford.edu/bugl/hiv.htm>).

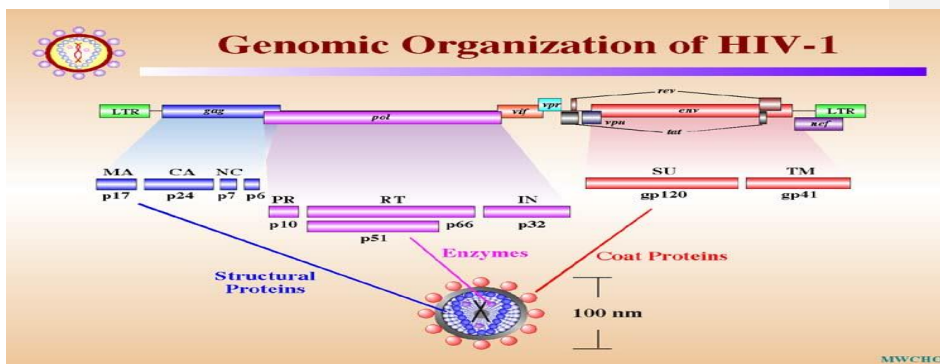


Figure 2.4 : Structure of the HIV genome
 (Source: <http://www.stanford.edu/group/virus/retro/2005gongishmail/HIV-1b.jpg>)

2.5 Distribution of HIV genotypes

Due to improved access to HIV treatment programs there has been a decline in HIV prevalence since 2007 according to the national survey carried out in 2012 (Kimanga *et al.*, 2014). Non-B subtypes are the major cause of infection that occurs in resource limited settings and accounts for 90% of the disease burden while subtype B causes most of the infections in the developed world (Chaplin, Eisen, Idoko, Onwujekwe, Idigbe, Adewole, 2011).

Studies carried out in different regions of Kenya including Western Kenya, North Rift, Kiambu, Malindi among others, subtype A was found to be more prevalent (Cheriro , Kiptoo , Kikui , Mining , Emonyi , and Songok 2015, Nzomo , Kitawi , Mwatelah , Kimulwo, Aman , Masankwa 2015). Although HIV-1 subtype A is dominant in Kenya there is an increasing prevalence of other subtypes and recombinant viruses as well; CRF10 strain was first identified in western Kenya (Songok, Lihana, Kiptoo, Genga, Kibaya , 2015).

Serotyping of C2V3 envelop region of HIV showed that the most prevalent subtype is A (Kitawi, Nzomo , Mwatelah , Aman , Kimulwo , Masankwa 2015). These results prove that subtype A is the dominating HIV subtype circulating among Kenyan patients. This was followed closely by subtype C and D, where a slow increase in the prevalence of subtype C has been shown (Cheriro *et al.*, 2015, Nzomo *et al.*, 2015).

2.6 HIV drug interactions and resistance

There are three main classes of commonly used antiretroviral; Nucleoside inhibitors, Non-nucleoside inhibitors and protease inhibitors. The optimum time for commencing ART in HIV-infected individuals has been a subject of debate for a long time (Siegfried, Davies, Penazzato, Muhe & Egger 2013). However, it is clear that initiation of treatment should be done before irreversible damage has occurred to the immune system. Early initiation of treatment is important as it is easier to control viral replication. This reduces

the risk of resistance with complete viral suppression being achieved in many cases and also decreases the risk of HIV transmission (Rutstein, Sellers, Ananworanich, & Cohen, 2015). Antiretroviral treatment was previously initiated when the CD4 counts of an individual are below 350 cells/mm³ for stage 1 and 2 while those that are classified as stage 3 and 4 are started on ART regardless of their CD4 counts though recent guidelines by the WHO stipulate that an individual is started on ART when their CD4 counts are less than 500/mm³ (NASCOPI 2012, NASCOPI 2014). The HIV treatment algorithm in Kenya includes the following 2 NRTIs (3TC and AZT/ TDF/d4T) and 1 NNRTI (EFV or NVP) for the first line therapy (NASCOPI, 2012).

Table 2.1: First line and second line treatment regimen

FIRST LINE TREATMENT REGIMEN	SECOND LINE TREATMENT REGIMEN
TDF+3TC+EFV or NVP	AZT+3TC+LPV/r or ATV/r*
AZT+3TC+EFV or NVP	TDF+3TC+LPV/r or ATV/r*
D4T+3TC+EFV or NVP	TDF+ 3TC+ LPV/r or ATV/r*

TDF-Tenofovir, AZT-Zidovudine, EFV-Efavirenz, 3TC-lamivudine, LPV-lopinavir, ATV/r- atazanavir/ritonavir. The table gives a summary of the first and second line treatment regimen in Kenya according to NASCOPI guidelines.

2.6.1 HIVs' interaction with nucleoside reverse transcriptase inhibitors (NRTIs)

Nucleoside reverse transcriptase inhibitors (NRTIs) lack the 3' hydroxyl group at the sugar which prevents the formation of the 3'-5' phosphodiester bond between the NRTI and the incoming viral 5'-nucleoside triphosphates resulting in termination (Arts & Hazuda 2012). Resistance mechanisms to NRTIs occur in two main ways; first one is mutations that occur near the drug binding site of the reverse transcriptase gene, examples include: M184V and K65R. The second mode is by mutations that work to undo the actions of the drug such that no matter how much of the drug binds to the virus, its effect is not transferred to the virus.

2.6.2 HIVs' interaction with non-nucleoside inhibitors (NNRTIs)

Non-nucleoside reverse transcriptase inhibitors bind and form hydrophobic pockets proximal to the overlapping site. This results in changes in the spatial conformation of the substrate binding site and reduces polymerase activity. Mechanisms of resistance of the virus against NNRTIs include mutations of the amino acids that form part of the hydrophobic pocket e.g. L100I and Y181C. The mutation KS103 does not occur in the hydrophobic pocket. Nevertheless, it is notorious for resistance against all NNRTIs by creating a hydrogen bond in unliganded reverse transcriptase preventing entry of the NNRTI (Arts *et al.*, 2012).

2.6.3 HIVs' interaction with protease inhibitors

In the structure of HIV-1, protease enzyme is responsible for the cleavage of the viral gag and gag-pol polyprotein precursors during maturation (Park & Marrow 1993, Miller, Hiller & Shaw 2001). This forms the structural units of the virus. Protease inhibitors work by inhibiting the protease enzyme. The virus acquires mutations in the protease gene that confer resistance to the protease inhibitors.

2.7 Human immunodeficiency Virus/Hepatitis C Virus co-infection

Hepatitis C commonly occurs as an opportunistic infection among high-risk individuals, including HIV positive patients, intravenous drug users and sex workers. HIV/HCV co-

infection among high risk groups has been found to be 2-fold higher than the general population in Kenya (Muasya *et al.*, 2008, Karuru *et al.*, 2005). The incidence of HCV is higher among intravenous drug users especially those whose HIV prevalence is quite low; therefore, HCV has been perceived as an indicator of high HIV risk among IDUs (Geane, Adilson, Juliana, Helena, Moyr, Letícia 2016). Similarly, HIV occurs as an opportunistic infection among individuals who are already suffering from complications arising due to HCV infections (Atina *et al.*, 2004).

The two viruses share similar modes of transmission; percutaneous route remains the most efficient mode of transmission of HCV (Chen 2014). Co infected individuals have been found to have a lower quality of life as compared to the HIV mono-infected individuals (Pereira & Fialho 2016). Despite the availability of a number of studies showing the prevalence of HIV/HCV co-infections, the mechanism of interaction between the two is not yet clearly understood. Different mechanisms of the synergistic effects of the two viruses have been previously evaluated, but the data is not yet sufficient enough to assist in the proper management of the co-infections. Some studies suggest that HIV up regulates cytokines which in turn cause fibrosis among HCV infected individuals. Presence of anti-HCV antibodies among those infected with HIV have shown pre-disposition to HCV infections (Nagu, Bakariand & Matee 2008).

HCV causes infection of hepatocytes that are a target for the activated effector cells, such as cytotoxic T lymphocytes (CTL) resulting in apoptosis. The T lymphocytes are key in fighting HIV infection, their presence in low quantities or complete absence in the body leaves an opportune window in which high HIV viral loads are reached at a fast pace. The presence of pro-inflammatory cytokines results in activation of hepatic stellate cells (HSC) to produce extracellular matrix. This induces a dysregulation of the imbalance between matrix metalloproteinase's (MMPs) and tissue inhibitors of matrix metalloproteinase's (TIMPs), leading to liver fibrosis. HIV may induce a direct effect on both hepatic stellate cells and hepatocytes by the interaction between viral proteins (gp 120) and C-C chemokine receptor type 5 (Claudio, Miriam, Claudia, Paola & Vincenzo 2014). Effects of HIV/HCV interaction on the hepatic cells play a critical role in the

development of hepatic steatosis and fibrosis progression. This may be one of the explanation behind the phenomena of rapid progression of liver fibrosis to cirrhosis and hepatocellular carcinoma among HIV-HCV co-infected individuals.

2.8 Intravenous drug use

Increased use of illicit drugs has been on the rise especially among developing countries due to increased levels of poverty, lack of employment opportunities, tendency of young people dropping out of school, prostitution among others. Nevertheless, there is a deficiency in studies that give the exact number of people who are dependent on drugs. This may be due to overt reporting.

Studies carried out among intravenous drug users showed that most of the drug users have habits that increase their vulnerability to infections that are blood borne and STIs. These habits include sharing needles, use of contaminated needles, some left their needles in common locations after intravenous the drug, lack of condom use during sex. A small percentage (6%) of drug users reported that they cleaned the needles before using them (Tan, Kapiga, Khoshnood & Bruce 2015). The genotypes that were previously occurring in HCV-endemic area are now found among people who inject drugs (Ruta & Cernescu 2015). Other studies carried out at the coast of Kenya and in Nairobi have also found out that the prevalence of HIV infections among people who inject drugs to be relatively high as compared to the general population (Kurth, Cleland, Des-Jarlais, Musyoki, Lizcano, Chun and Cherutich 2015, Mwatelah, Lwembe, Osman, Ogutu, Aman, Kitawi, 2015).

CHAPTER THREE

METHODOLOGY

3.1 Study site and population

This study was conducted in Malindi (Figure 3.1) on subjects recruited from the Comprehensive Clinical Care (CCC) center of the Malindi district hospital. Subjects were enrolled voluntarily after written informed consent as approved by the Scientific and Ethical Review Unit (SERU) of the Kenya Medical Research Institute (KEMRI). Relevant demographic and non-invasive personal data including age, gender, behavior risk e.g. drug use, residence and occupation were collected using structured questionnaires (written in both English and Kiswahili). This information was coded to delink from patient identity. Consenting subjects were asked to donate venous blood, which was later processed at the KEMRI laboratories.

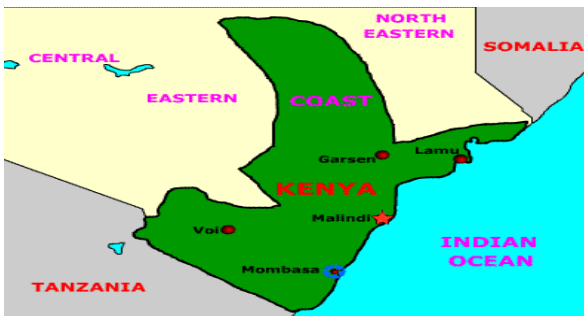


Figure 3.1: Map of Kenya showing the location Malindi sub county where the study was conducted.

(<http://www.mapzones.com>)

3.2 Study design

This was a descriptive cross-sectional study.

3.2.1 Inclusion criteria

1. All CCC patients who were above 18 in Malindi
2. Those consenting to participate.

3.2.2 Exclusion criteria

1. Those not consenting to participate.
2. Those that have adverse medical conditions that does not allow them to participate in the study.

3.3 Sample size determination

There is no prevalence data on either HCV mono infection or HIV/HCV co-infection in Kilifi county to form the basis for sample size calculation. Therefore, estimation of a prevalence with approximate 95% confidence level was used.

$$n_r = Z^2P(1-P)/d^2$$

n= required sample size.

p= Expected prevalence

Z=Level of confidence (95% level of confidence used, therefore Z value is 1.96)

d= Precision (0.05)

$$n = 1.96^2 * 0.05 * 0.05 / 0.05^2 = 385.$$

3.4 Sampling Method

Convenience sampling method was used to recruit the study participants, with all those appearing at the facility and meeting enrolment criteria qualifying for the upon consenting (Phillip 2013, Tyrer *et al.*, 2016).

3.5 Ethical approvals and consent

This study was official approved by the Scientific and ethical Review Committee of the Kenya Medical Research Institute and conducted under ERC/SSC #2477 and in

accordance with the Helsinki Declaration of 1975 (revised in 2000). Participants were informed adequately and their voluntary consent obtained in writing. Patient data was treated with strict confidence, including code labeling of questionnaires and specimen for confidentiality.

3.6 Qualitative procedures

In depth interviews were used where a questionnaire was administered to the individuals who consented to participate in the study. This tool was used to collect the demographic and behavioral data from the participants. Additional information of ARV regimen was retrieved from the participants' file.

3.7 Laboratory procedures

3.7.1 Blood collection.

Five milliliters of venous blood was drawn by a trained phlebotomist and collected into EDTA anticoagulated vacutainer tubes. The tubes were inverted ten times to mix the blood.

3.7.2 Separation of plasma.

The EDTA tube with blood was placed in a centrifuge and spun at 3000 rpm for 10 minutes to separate plasma from the whole blood. The resultant plasma was transferred into labeled cryovials in duplicate aliquots, transferred into labeled zip lock bags and transported to Nairobi for further processing. All transportation was by courier under dry ice in a Styrofoam box.

3.7.3 Ammonium chloride Extraction of PBMCs

Three to 5 mL cellular portion of blood after separating plasma were added to a falcon tube containing 30ml of 0.84% ammonium chloride and vortexed completely. The tubes containing the mixture were incubated at 37°C for 10 minutes. The tube was spun at 3000 rpm in a centrifuge for 10 minutes at room temperature and the supernatant was discarded. The procedure was repeated three times. Exactly 1ml of 0.84% ammonium chloride was added to the falcon tube and mixed by turning up and down a few times. Approximately 500µl of the mixture was added to two labelled micro centrifuge tubes

and spun at 3000rpm for 10 minutes. The supernatant was aspirated using a vacuum pump.

3.7.4 Rapid diagnostic test for HIV-1 and HCV

The screening for HIV-1 was conducted using vironostika kits (biomerieux, South Africa). The HIV-1 kits rely on the detection of anti-HIV-1 antibodies in plasma, including Group O. These antigens include inactivated, purified HIV-1 viral lysate proteins, purified viral envelope proteins, and a synthetic peptide with amino acid sequence corresponding to that of the transmembrane immunodominant domain of the HIV-1 Group O (ANT 70) isolate. Immune complexes are formed through the interaction between anti-HIV in the specimen and HIV antigens coated on the micro well producing a reaction.

HCV was screened using diaspot HCV kit (Bresta Perkasa, Indonesia). The HCV kit consists of recombinant HCV antigen-coated strips that react with anti-HCV antibodies in the test plasma.

3.7.5 RNA extraction procedure

Extraction of RNA from plasma samples was conducted following manufacturer's instructions using the QIAamp® Viral RNA Mini. Briefly, 310 microliters of buffer AVE was added to the tube containing 310µg of lyophilized carrier RNA to obtain a solution of 1 µg/µl. The carrier RNA was dissolved thoroughly and divided into conveniently sized aliquots and stored at -20°C. Carrier RNA was mixed with buffer AVL gently by inverting the tube 10 times, following manufacturer's instructions. Vortexing was not done to avoid foaming.

Five hundred and sixty microliters of prepared buffer AVL containing carrier RNA was pipetted into a 1.5 ml microcentrifuge tube. One hundred and forty microliters of plasma was added to the buffer AVL-carrier RNA in the microcentrifuge tube and mixed by

pulse-vortexing for 15 s. The mixture was then incubated at room temperature (15 – 25°C) for 10 min. The tube was briefly centrifuged to remove drops from the inside of the lid. Five hundred and sixty microliters of ethanol (96 – 100%) was added to the sample, and mixed by pulse-vortexing for 15 s. After mixing, the tube was briefly centrifuged to remove drops from inside the lid.

Six hundred and thirty microliters of the resulting lysate solution was carefully applied to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. The cap was tightly closed to avoid cross-contamination during centrifugation. The column was centrifuged at 6000 x g for 1 min. The QIAamp Mini column was then placed into a clean 2 ml collection tube and the tube containing the filtrate discarded. This step was repeated with the remaining lysate.

The QIAamp Mini column was carefully opened and 500 µl of Buffer AW1 added. The cap was closed and the column centrifuged at 6000 x g for 1 min. The QIAamp Mini column was placed in a clean 2 ml collection tube and the tube containing the filtrate discarded. The QIAamp Mini column was carefully opened and 500 µl of Buffer AW2 added. The cap was closed and the column centrifuged at full speed (20,000 x g) for 3 min. The QIAamp Mini spin column was then placed in a new 2 ml collection tube and the old collection tube with the filtrate was discarded. The column was centrifuged again at full speed for 1 min.

The QIAamp Mini spin column was again placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded. The QIAamp Mini spin column was carefully opened and 40 µl of distilled water (RNase, DNase free water) was carefully pipetted to the center of the membrane without touching it. The column was incubated at room temperature (15 – 25°C) for 2 min, and then centrifuged at 6000 x g for 1 min. The column was discarded and the 1.5ml microcentrifuge tube was tightly closed. To prevent frequent thawing that affects the integrity of the RNA, the RNA extracted was divided into three portions and placed in three separate microcentrifuge tubes and stored at -80°C until use.

3.7.6 DNA extraction procedure

DNA extraction was carried out using Qiaamp DNA Kit following instructions in the QIAamp® DNA Mini and Blood Mini Handbook. Briefly, Qiagen Protease (Qiagen, Maryland, USA) was prepared by adding the required amount of protease solvent to the tube containing the enzyme. Twenty microlitres of QIAGEN Protease (or proteinase K) was pipetted into the bottom of a 1.5 ml microcentrifuge tube and 200 µl sample (PBMCs mixed with PBS pH 7.4) was added to the microcentrifuge tube. Two hundred microlitres of buffer AL was then added to the sample and mixed by pulse-vortexing for 15 s. The mixture was then incubated at 56°C for 10 min. The 1.5 ml microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid.

Two hundred microlitres of ethanol (96 – 100%) was added to the sample and mixed again by pulse-vortexing for 15 s. After mixing, the 1.5 ml microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid. The mixture was then applied to the QIAamp Mini spin column (in a 2ml collection tube) without wetting the rim. The cap was closed and the mixture centrifuged at 6000 x *g* for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded. The QIAamp Mini spin column was carefully opened and 500 µl Buffer AW1 was added without wetting the rim. The cap was closed and the column centrifuged at 6000 x *g* for 1 min.

The QIAamp Mini spin column was then placed in a clean 2 ml collection tube and the collection tube containing the filtrate discarded. The QIAamp Mini spin column was carefully opened and 500 µl of buffer AW2 added without wetting the rim. The cap was closed and the column centrifuged at full speed (20,000 x *g*) for 3 min. The QIAamp Mini spin column was then placed in a new 2 ml collection tube and the old collection tube with the filtrate was discarded. The column was centrifuged again at full speed for 1 min. The QIAamp Mini spin column was then placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded. The QIAamp Mini

spin column was carefully opened and 40 µl of distilled water (RNase, DNase free) was carefully pipetted to the center of the membrane without touching it.

The column was incubated at room temperature (15 – 25°C) for 2 min, and then centrifuged at 6000 x g for 1 min. The column was discarded and the 1.5 ml microcentrifuge tube contained extracted DNA was tightly closed. To prevent frequent thawing that affects the integrity of the DNA, the DNA extracted was divided into two portions and placed in two separate microcentrifuge tubes and stored at -80°C until required.

3.7.7 Amplification of RNA for HCV virus

Plasma-derived RNA samples were reverse transcribed using Qiagen One-step RT-PCR kit (Qiagen, Maryland, USA). A two-round nested PCR amplification targeted a 365 bp non-structural protein 5B (NS5B) of the HCV genome as reported in the associated

publication(Theamboonlers , Chinchai, Bedi, Jantarasamee, Sripontong,& Poovorawa2002,

Sunanchaikarn , Theamboonlers, Chongsrisawat , Yoocharoen , Tharmaphornpilas , Warinsathien 2007).

First round PCR was performed by adding 5µl of cDNA to a final volume of 25 µl PCR reaction mixture containing 0.5µM each of NS5BF1 and NS5BR1 primers, 400nM dNTP, 2mM MgCl₂, and 0.025 Units/µl of Taq DNA polymerase. Samples were amplified under the following conditions: 15 min at 95°C for initial denaturation followed by 45 cycles of denaturation at 95°C for 30sec, annealing at 58°C for 30sec, and extension at 72°C for 30 sec. A final extension step was done at 72°C for 10min.

For the second round nested PCR, 4µl of first round PCR product was added to a reaction mixture identical to the first round, but using inner primer set NS5BF2 and NS5BR2 (0.5µM each) instead of outer primer sets. The thermocycling profile was

performed under conditions identical to the first round. The PCR products were analysed by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualized under UV light.

Table 3.1: Pool of HCV specific primers used for amplification of NS5B regions by polymerase chain reaction

HCV Region	Primer Sequence	Purpose	Citation
RNA dependent polymerase (NS5B, nt 7999–8020); 382 bp	NS5BF1_5'- TGA TAC CCG CTGYTT TGA CTC-3'	Round 1 PCR	Theamboonlers <i>et al</i> 2002, Sunanchaikarn <i>et al</i> 2007
	NS5BR1_5'- ACCTGGTCA TAG CCT CCGTGA-3'		
	NS5BF2_5'- ATA CCC GCT GYT TTG ACT CAN-3'	Nesting PCR/Sequencing	
	NS5BR2_5'- GTA CCT CAT AGC CTC CGT G-3'		

3.7.8 Amplification of RNA for HIV virus

HIV was amplified using both *env* primers (546bp product) and *Pol-RT* primers- (701bp product) on the resulting cDNA as previously described (Nzomo 2015, Khamadi 2008, Lwembe 2007).

For the RNA template, amplification followed One-Step RT-PCR protocol as outlined in the manufacturers kit (Qiagen, CA) followed by a nested PCR. For the One-Step RT-PCR part, the primers RT18 and KS104 were used for the forward and reverse

amplifications respectively. A final PCR reaction volume of 25 μ l included a 1x PCR buffer, 0.4mM dNTPs, 1.5mM MgCl₂, 5 μ l of RNA template, 1 μ l enzyme mix, 0.6 μ M of each primer and 5 units of RNaseOut. Reverse transcription was performed at 50°C for 30 minutes followed by the first round PCR that comprised 95°C denaturation for 15 minutes and 38 cycles of 94°C for 30sec denaturation, annealing at 55°C for 45sec and extension at 72°C for 1 minute. A final extension step was performed at 72°C for 10 min. Nested PCR was done using HotStar taq polymerase (New England Biolabs, Ipswich, MA) protocol in a reaction volume of 25 μ L comprising 1x PCR buffer, 0.2mM dNTPs, 2mM MgCl₂, 0.5 μ M forward and reverse primers, 0.625 units of the enzyme and 3 μ l of RT-PCR product as template. Primers KS101 and KS102 were used as forward and reverse nested PCR primers respectively.

Thermocycling conditions for nested PCR included denaturation at 95°C for 5 minutes followed by 38 cycles of denaturation at 95°C for 30seconds, annealing at 56°C for 45seconds and extension at 68°C for 45 seconds. A final extension was done at 68°C for 10 minutes.

For PCR amplification of the envelope region C2-V3 and HXB2 primers were used for the first round and nested PCR reactions respectively. First round amplification was performed in a total volume of 25 μ L comprised of 5 μ L of DNA template, 0.6 μ M of forward primer M5 and reverse primers M10 (positions 6975-7520), 0.4mM dNTPs, 1.5mM MgCl₂, and 1 μ L enzyme mix (Qiagen, CA).

Second round amplification was performed in a total volume of 25 μ L comprised of 5 μ L of DNA template, 0.6 μ M of forward primer M3 and M8, 0.4mM dNTPs, 1.5mM MgCl₂, and 1 μ L enzyme mix (Qiagen, CA). Thermocycling parameters were similar to that used for One-Step RT PCR except for the reverse transcription step that was omitted.

Table 3.2: Pool of HIV specific primers used for amplification of *env* and *pol RT* regions by polymerase chain reaction (PCR)

HIV-1 Region	Primer Sequence	Purpose	Citation
Envelope (C2-V3, nt 6975–7520)	M5_5'- CCAATCCCATACATTATTGTGCCCCAGCTGG -3' (F) M10_5'- CCAATGTCCCTCATATCTCCTCCTCCAGG -3' (R)	Round 1PCR	(S.A)
HXB2); 546bp	M3_5'- GTCAGCACAGTACAATGCACACATGG-3' (F) M8_5'- CCTTGGATGGGAGGGGCATACATTGC-3' (R)	Nested PCR/ sequencing	<u>Khamadi et al., 2008</u>
Pol-RT (nt 2480–3180)	RT18_5'-GGAAACCAAAAATGATAGGGGGAATTGGAGG -3' (F) KS104_5'- TGACTTGCCCAATTTAGTTTTCCCACTAA-3' (R)	Round 1 PCR	(Lwembe <i>et al.</i> , 2007)
HXB2); 701bp	KS101_5'-GTAGGACCTACACCTGTTCAACATAATTGGAAG-3' (F) KS102_5'-CCCATCCAAAGAAATGGAGGAGTTCTTTCTGATG -3' (R)	Nested PCR/ sequencing	

Key: (F) - Forward Primer; (R) - Reverse Primer

3.7.9 Gel Electrophoresis

A 1.6% agarose gel matrix was made by mixing 0.64 g of agarose powder (Invitrogen, USA) with 40 ml of 1X Tris EDTA electrophoresis buffer. This was heated in a microwave until the powder had dissolved and then cooled to about 50°C. Two µl of ethidium bromide was then added to the mixture. Ethidium bromide intercalates into the DNA and fluoresces under UV light and hence allows visualization of nucleic acid after electrophoresis.

The solution was poured into a casting tray containing a sample comb and was polymerized for 20 min at room temperature. The sample comb was removed once the gel had polymerized and the gel was inserted into an electrophoresis chamber and

covered with buffer solution. The first well was reserved for the DNA ladder. A total volume of 6 μl was used that comprised for the first well, 1 μl DNA ladder, 1 μl loading dye and 4 μl of distilled DNase free, RNase free water. The samples were loaded on the remaining wells by mixing 5 μl of the amplicon with 1 μl of loading dye. Since DNA is negatively charged, the gel was placed in the gel tank with the wells facing the cathode so that the current was run towards the anode (positive pole). A current of 100 volts was applied for 30 min.

The larger DNA fragments move more slowly through the agarose gel matrix than the smaller fragments and in this way, the DNA fragments are fractionated, The DNA ladder helps one to identify the size of the DNA fragments. The gel was visualized under UV light.

3.7.10 Purification and Sequencing

Amplified products were purified using QIAquick PCR purification kit (Qiagen, Maryland USA) so as to remove primers, nucleotides, enzymes and other impurities from DNA samples that could interfere with sequencing. Briefly, binding buffer was added directly to the PCR sample and the mixture applied to the QIAquick spin column. The binding buffer contains a pH indicator that allows for easy determination of the optimal pH for DNA binding. Nucleic acids were adsorbed to the silica membrane in the high-salt conditions provided by the buffer. Impurities were washed away and pure DNA was eluted with 40 μl of water. Two microlitres was examined for presence of DNA and purity using a nanodrop spectrophotometer using Nanodrop2000 looking out for A_{260}/A_{280} ratios of between 1.7 and 1.9. The purified products were sequenced using the Big Dye chain terminator DNA sequencing kit according to manufacturer's protocol (Invitrogen, U.S.A) at the Macrogen laboratory in Netherlands. The sequencing required of 10 μl of 20ng/ μl PCR product and 10 μl of 5pmol/ μl nested PCR primers. The sequences were obtained from an Applied Bio systems 3500, 8-capillary genetic analyser, assembled, aligned and edited using Bio Edit software. Multiple sequence

alignments were performed with Clustal X (v.1.83). Aligned sequences were examined by the BLASTN program (<http://www.ncbi.nlm.nih.gov>) Genotyping and Phylogenetic Analysis tool. Phylogenetic analyses were performed using the Neighbour-joining method with an integrated 6-Parameter bootstrapping (Shin-I, 2008)

3.8 Data management

3.8.1 Data Storage

All data including virus subtypes, drug resistance profiles and patient information were entered into excel, in a computer and later transferred to SPSS for analysis. The information was treated confidentially with access limited to only authorised personnel

3.8.2 Data Analysis

The generated sequences were aligned using CLUSTALW in the presence of specific subtype reference sequences retrieved from the Los Alamos database (<http://hiv-web.lanl.gov>). The aligned sequences were manually inspected and edited using BIOEDIT. The frequency of nucleotide substitutions was estimated by the Kimura two-parameter method and phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei 1987). Resulting trees were visualized using Tree view. Drug resistance was assessed using the bioinformatics portal of the Stanford drug resistance database for HIV, and the geno2Pheno (www.geno2pheno.org) for HCV. Appropriate statistical analyses were conducted to generate comparison matrices for the various outputs. Specifically, a chi-square test was used to compare genotype distribution, drug resistance (anti-HIV and anti-HCV)-associated mutations, across single and co-infection statuses. These statistical analyses were performed using the statistical software SPSS 20.0 for windows (SPSS, Chicago, IL, USA).

CHAPTER FOUR

RESULTS

4.1 Subject characteristics

A total of 452 subjects were enrolled into the study from the comprehensive care clinic (CCC) of the Malindi District Hospital. Details of the participant characteristics were recorded and analyzed (Table 4.1). Of the 452 subjects 41.2% were intravenous drug users (IDUs). There were more male drug users compared to the females 59.79% and 27.24% respectively. All the study participants were aware of their HIV status, whereas more females (70.03%) were aware of the HIV status of their sexual partners compared to the males (30.92%). More males had multiple partners as compared to the females.

Table 4.1: Subject characteristics

	Male (n=195)	Female (n=257)
Married (Customary/official)	41.02% [80/195]	46.69% [120/257]
Drug use	59.49% [116/195]	27.24% [70/257]
Knowledge of own HIV status	100% [195/195]	100% [257/257]
Knowledge of sexual partner HIV status	30.77% [60/195]	70.03% [180/257]
Other sex partners	23.07% [45/195]	11.67% [30/257]

4.2 Antiretroviral treatment and Substance use characteristics

All the study participants were enrolled in the ART program on different ARV regimen according to the 2013 WHO guidelines where treatment was initiated to patients with a CD4 count of <500 cells/mm³ (Table 4.2). There were more male than female injectors and more female than male non-injectors. The standard HIV treatment therapy in Kenya consists of 2 nucleoside reverse transcriptase inhibitors and one non-nucleoside reverse transcriptase inhibitors. In terms of treatment, all the IDUs were enrolled in antiretroviral treatment (ART). Among the IDUs, 28% were receiving first-line AZT+3TC+NVP/EFV regimen, 8.1% received D4T+3TC+NVP/EFV, and 24% were on maintenance septrin and cotrimoxazole therapy. Median duration on first line ART was 149 months. None of the injectors were reported to be on opioid substitution therapy. Among the non-injectors 40.9% were receiving first-line AZT+3TC+NVP/EFV regimen, 32.7% received D4T+3TC+NVP/EFV.

Table 4.2 Distribution of subjects into the various substance use and ART categories.

ARV Regimen			Total
	intravenous drug user	Non- intravenous drug user	
AZT+3TC+NVP/EFV	52 (28%)	133 (50%)	185 (46.02%)
D4T+3TC+NVP/EFV	15 (8.1%)	133 (50%)	148 (32.7%)
Septrin maintenance therapy	44 (24%)	0	44 (24%)
Adherence counselling	70 (38%)	0	70 (38%)
Not on ARV	3 (1.6%)	0	3 (1.6%)
Defaulters	2 (1.1%)	0	2 (1.1%)
Total	186 (41.2%)	266 (58.8%)	452

Key; AZT-zidovudine, 3TC-lamivudine, NVP-nevirapine, EFV-efavirenz. The table above shows the different antiretroviral therapy combinations of the participants.

4.3 Prevalence of Hepatitis C virus (HCV)

HCV testing was done for all the 452 subjects that included 186 injectors and 266 non-injectors (Table 4.3). The HCV infections were solely found among the injectors at 14% (26/186). Within specific groups of gender and age, the males had higher rates of HCV infection compared to females at 9.74% (19/195) and 2.7% (7/257) respectively. Those aged between 31-40 years had a higher prevalence of HCV 7.65% (14/183) compared to those aged <30, 6.02% (8/133). Within the age group with the highest HCV prevalence, the males 6.6% (12/183) had higher infection rates than females 1.09% (2/183). All the HCV positive results were obtained from those who injected drugs. For the 26 HCV positive injectors alone, 19 (73.1%) were males, while only 7 (26.9%) were females.

Thus, the intravenous drug users had significantly higher risk for HCV infections (χ^2 2.77), and this risk was more prominent among those aged between 31-40 years and below as well as among male injectors.

Table 4.3. Prevalence of HCV by age and gender of subjects.

HCV rapid		* SEX * Age group Cross tabulation			
Age group			SEX	Total	
			Female	Male	
<=30	HCV	Positive	4 (1.5%)	4 (2.06%)	8 (1.77%)
	rapid	Negative	80 (31.1%)	45 (22.96%)	125 (27.65%)
	Total		84 (32.68%)	49 (25%)	133 (29.42%)
31-40	HCV	Positive	2 (0.77%)	12 (6.12%)	14 (3.09%)
	rapid	Negative	106 (41.25%)	63 (32.14%)	169 (37.39%)
	Total		108 (42.02)	75 (38.27%)	183 (40.47%)
>=41	HCV	Positive	1 (0.38%)	3 (1.53%)	4 (0.88%)
	rapid	Negative	64 (24.9%)	68 (34.69%)	132 (29.20%)
	Total		65 (25.29%)	71 (36.22%)	135 (29.87%)
Total	HCV	Positive	7 (2.7%)	20 (9.8%)	27 (5.75%)
	rapid	Negative	250 (97.27%)	176 (89.79%)	426 (94.25%)
	Total		257 (56.8%)	195 (43.14%)	452

4.4 Genetic analysis of HIV-1 and HCV subtypes

4.4.1 Genetic analyses of HIV-1 subtypes

Out of the HIV positive samples and due to limited resources, 50 were randomly picked for HIV subtyping. Out of the 50 samples, 50% (25/50) were positive for integrated HIV provirus by PCR. A positive sample is described by presence of a 701base pair product appearing as a band on the agarose gel. A positive control was included which was derived from a previously known HIV infected and genotyped sample, while a no-template control in which water was used as template acted as a negative control (Figure 4.1). Of the 25 pro-virus positive samples assessed with the pol-RT primers, 60% were subtype A, with majority (86.66%) of these being of the A1 sub-subtype while the rest (6.67%) of the A viruses were sub-subtype A2D and AE respectively. Sixteen percent of the 25 isolates were subtype D and recombinant strains, while subtype C recombinants comprised 12% of all the genotyped viruses (Table 4.5). The subtype isolates were also examined for drug resistance mutation, and 4/25 (16%) harbored mutations that confer resistance to nucleoside reverse transcriptase inhibitors while 24% (6/25) had mutations that confer resistance to non-nucleoside reverse transcriptase inhibitors.

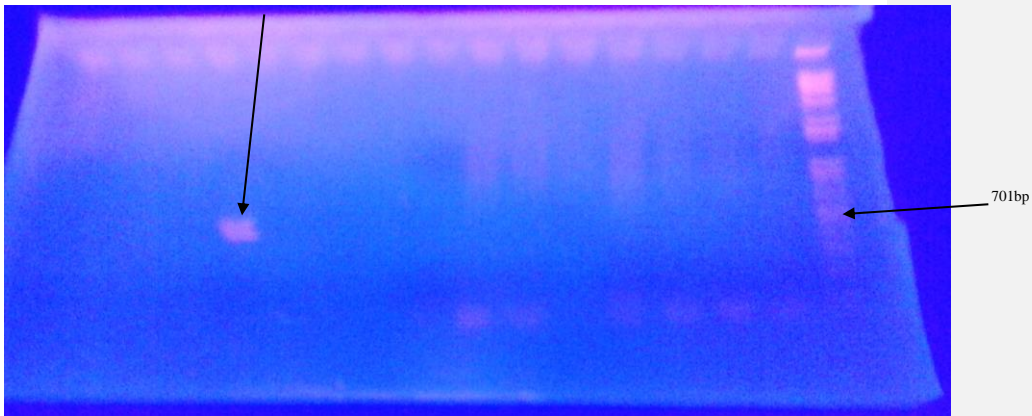
Of the 7 pro-virus positive samples assessed with the C2-V3 primers majority were genotype A1 at 57%(4/7). The other subtypes were A1D and A1A2 recombinants at 28.6% (2/7) and 14.3% (1/7) respectively (Table 4.6).

S1

Ladder



Patient ID	Age	Sex	HAART regimen	SUBTYPE	NRTI resistance	NNRTI resistance
MLD011	31	F	NR	CD	NONE	NONE
MLD183	27	F	ABC/3TC/NVP	A1	NONE	NONE
MLD191	34	M	TDF/3TC/NVP	CD	NONE	NONE
MLD198	26	F	D4T/3TC/NVP	A1	NONE	NONE
MLD021	41	M	TDF/3TC/NVP	D	NONE	NONE
MLD040	49	F	NR	A1	NONE	NONE
MLD541	53	M	D4T/3TC/NVP	AE	NONE	K103E
MLD545	52	F	AZT/3TC/EFV	A1	NONE	NONE
MLD548	46	F	D4T/3TC/NVP	N.C	NONE	NONE
MLD060	36	F	TDF/3TC/EFV	BC	M184V	V106M
MLD001	30	M	AZT/3TC/NVP	A1	NONE	NONE
MLD002	29	F	AZT/3TC/NVP	A2D	NONE	NONE
MLD003	44	F	AZT/3TC/EFV	A1	NONE	E138A
MLD008	45	M	D4T/3TC/NVP	A1	NONE	NONE
MLD009	47	M	AZT/3TC/NVP	A1	NONE	NONE
MLD010	22	F	None	BC	NONE	NONE
MLDU011	46	F	TDF/3TC/NVP	D	NONE	NONE
MLD012	56	F	D4T/3TC/NVP	A1	NONE	NONE
MLD013	39	M	D4T/3TC/NVP	A1	M184V, D67N	G190A
MLD014	50	M	None	A1	NONE	NONE
MLD016	46	F	NR	A1	M184V	K103A
MLD166	28	M	D4T/3TC/NVP	A1	NONE	NONE
MLD167	32	F	D4T/3TC/NVP	BC	NONE	NONE
MLD185	52	M	AZT/3TC/NVP	A1	M184V	K103N



Key: *SI-sample 1, 701bp- band size.* The figure above shows gel electrophoresis of a positive HIV band.

Figure 4.1 HCV positive bands on a gel photo

Table 4.5 *Pol-rt* region genetic and drug resistance information.

Key: *MLD-Malindi, F-female, M-male.* The table above gives a summary of *pol-RT* subtypes and drug resistance mutations from the study participants (NRTI=16%, NNRTI=24%).

Table 4.6 Summary of the envelope genotype profile of the virus isolates

Patient ID	AGE	SEX	SUBTYPE
MLD 185	52	M	A1
MLD 301	37	F	A1
MLD 258	40	M	A1
MLD 245	32	F	A1A2
MLD 016	46	F	A1
MLD 012	56	F	A1D
MLD 011	46	F	A1D

Key: *MLD-Malindi, F-female, M-male.* The table above shows the subtype profile of the envelope region.

4.4.2 HIV phylogenetic analysis

Phylogenetic sequence analysis was performed on all the PCR positive samples to infer evolutionary relationship with reference and other HIV sequences across the globe. Ninety-nine percent of the samples had strong genetic relationships to the African reference sequences (Figure 4.2). The remaining 1% had 62% sequence identity with reference sequence from China, while two isolates were unclassified.

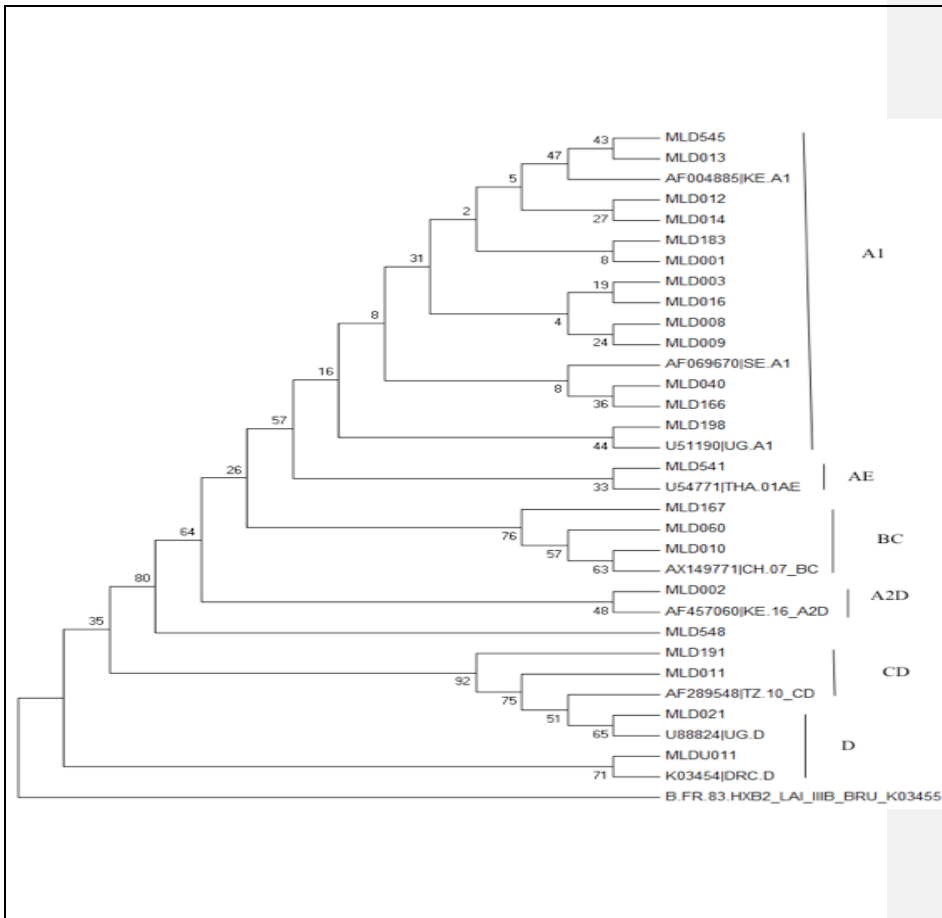
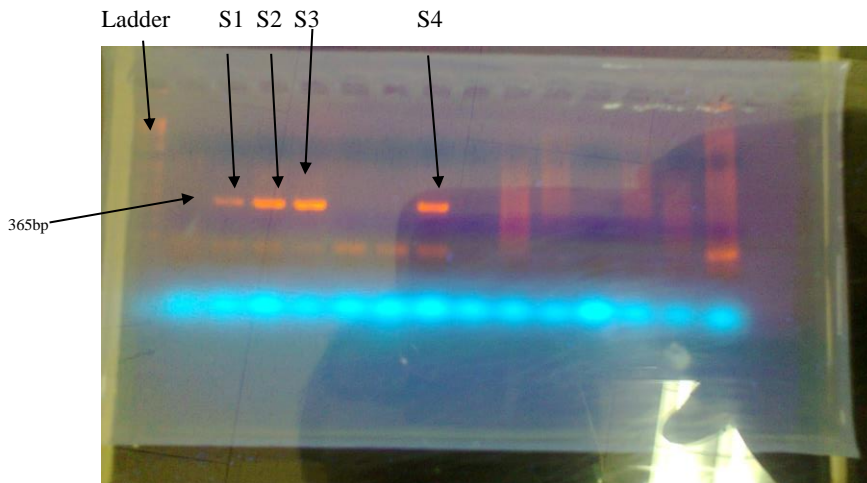


Figure 4.2 Phylogenetic relationships of HIV-1 pol-RT gene isolates

The evolutionary distances were inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Kimura 2-parameter method(Kimura,1980). The references that did not cluster with any of the samples were removed to reduce clutter.

4.4.3 Genetic analysis of HCV subtypes

All the samples that were HCV positive by rapid serological detection test were also subjected to genetic analysis and sequencing to infer evolutionary relationship with HCV sequences across the globe. Out of the 26 samples that were positive by serology, 4 were positive for integrated HCV provirus by PCR. A positive sample is described by presence of a band at 365 base pairs using a standard DNA ladder run alongside amplified samples on agarose gel (Figure 4.3). A previously genotyped sample of HCV was used as a positive control while a no-template control in which water was used as template acted as a negative control. Using phylogenetic analysis to assess the, seventy-five percent of the HCV samples were subtype 1a while 25% were subtype 4a (Figure 4.4). None of the samples had drug resistance mutations.



Key; S1-sample 1, S2-sample 2, S3-sample 3, S4-sample 4, 365bp-band size. The figure above shows gel electrophoresis of HCV samples.

Figure 4.3 HCV positive bands on a gel photo

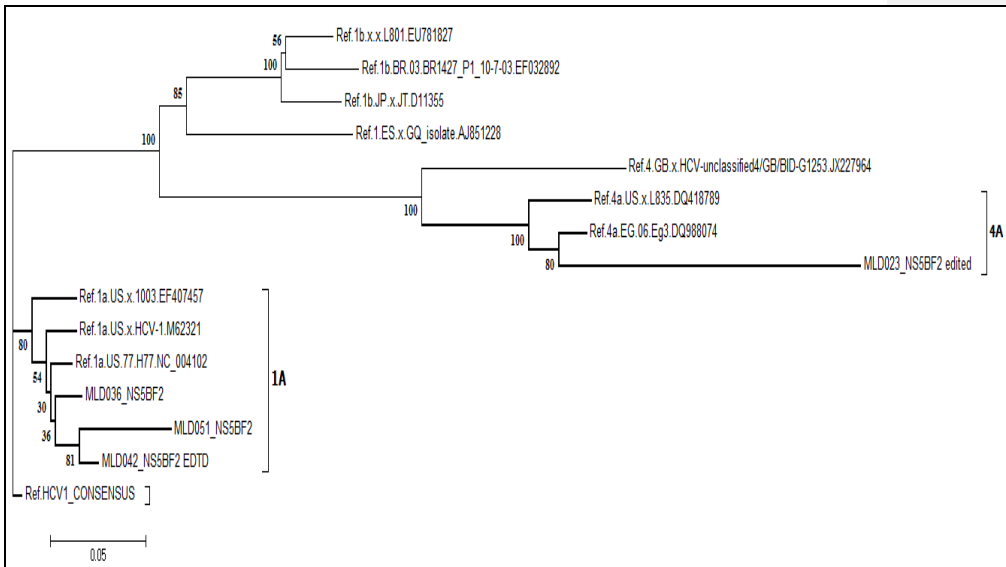


Figure 4.4 Phylogenetic relationships of HCV NS5B region

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum branch length= 1.00404697 is shown. The analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+noncoding.

CHAPTER FIVE

DISCUSSION

5.1 Subject characteristics

There were more female (56.85%) than male (43.41%) participants who were recruited into the study. Forty one point two percent of the population were intravenous drug users, of which 59.79% were males compared to 27.24% who were female. Similar carried out in Nairobi among drug users found out that there were more male drug users than female (Muasya et al., 2009). The The number of younger IDUs aged less than 30 years (73.5%) was more than older IDUs, similar observations have been made in studies that assessed the prevalence of drug use in Kenya and other parts of the world (Muasya *et al* 2009, Tan *et al* 2015). All the study participants were aware of their own HIV status whereas the females knew more about their partner's status compared to the males. The males had multiple affairs compared to the females, this trend is in line with the national surveys where the males were found to have more than one sexual partners (KAIS 2012).

Those on ART, were receiving the following treatment regimens (percentages given based on total number of patients): 46.02% on AZT+3TC+NVP, 32.7% on D4T+3TC+NVP/EFV and 24% on septrin maintenance therapy. There were 38% of the participants who were on adherence counselling, whereas 1.1% accounted for those that had defaulted. The recommended first line ART in Kenya is AZT+3TC+NVP/EFV or TDF+3TC+NVP/EFV(NASCOP, 2011). There were more IDUs on AZT+3TC+NVP/EFV (28%). Although the recommended treatment was the most common among patients receiving ART, the continued use of D4T in ART was contrary to existing WHO recommendation for D4T phase-out due to its association with toxicity and intolerance (WHO, 2009.)

5.2 HCV infections in Malindi

In this study HCV infections were found solely among intravenous drug users at 5.75%. This rate is higher than the known prevalence of HCV 0.2-0.9%. There were nearly twice as many males as females from the Malindi study population who were disclosed intravenous drug users (IDUs), and all injections were associated with heroin use. Both HIV-1 and HCV infections weigh heavily on health outcomes of many developing nations. We found individual and co-infection rates with HIV-1 and HCV that were high for the IDUs. In Kenya there are no structured or systemic HCV screening programs for people at risk, affected or infected with HIV, unless they also show signs and symptoms of liver disease. The consequence of this lack of HCV screening is that actual presence of a co-infection is not detected until later stages of disease, further affecting treatment response upon initiation of relevant antiretroviral medication.

Another study assessed the factors that affect early treatment of HCV. The results showed that delays in referrals to liver specialists and lack of HCV knowledge by service providers play a big role in delayed response of HCV treatment (Dore & Thomas 2005).

A study carried out by National Authority for Campaign against Alcohol and Drug Abuse (NACADA) showed that there is an increase in intravenous drug use in the coastal region, 60% of the drug users are less than 30 years old (Korir 2013). Socio-economic impediments like poverty and stigma continue to push the IDUs to sharing of needles (Njeru & Ngesu 2014), a practice that is credited with the escalation of infection among the community injectors. Most of the/these IDUs (not them) share needles since they cannot afford to buy new ones' due to their social economic status. HIV-1 and HCV infection rates were high as 16% among intravenous drug users whereas there were no HCV infections among the non- injectors. These data reveal for the first time, an astonishingly high burden of HCV infection among Kenyan intravenous drug users' population. There is no national data that can reliably show the trend if illicit drug use across the country, nor the associated burden of infection. A previous study by

independent investigators that focused on two country regions reported a high proportion of intravenous drug users in Malindi as compared to Nairobi (Harania, Karuru , Nelson & Stebbing 2008).

In this study, more than twice the number of male injectors was infected with HCV as compared to their female counterparts. The male IDUs also had 15% more HIV-1 infection rates than females, suggesting a disproportionate gender bias in the risk of infection that affects more males than females with similar behavioral risks. It is not immediately clear why more males than females who are both injectors, are at an increased risk of infection, but it is likely that male injectors are less likely to disclose their injecting behavior and seek remedial and preventative counseling than female injectors. Socio-cultural attributes that impede overt reporting of drug use by females may have contributed to the fewer number of female IDUs in the current study setting. A separate study of Canadian youths-at-risk showed no differences in rates of initiation into injection drug use between genders, but did find differences in gender-related risk factors that influenced initiation (Ahamad *et al.*, 2014).

Data arising from studies conducted in other countries as well as a few conducted in Kenya, have previously reported much lower prevalence of HIV and HCV infections among injectors (Strathdee, Hallett, Bobrova, Rhodes and Booth 2010, Sypsa, Paraskevis, Malliori , Nikolopoulos & Panopoulos 2014 & Liao, Kang, Tao, Cox & Qian 2014). Although IDU as a practice is not overtly prevalent in Kenya, it is reported to be one of the major drivers of the HIV epidemics in the country (Petersen , Myers , van-Hout , Pluddemann & Parry 2013). In addition to the observed high HIV-1 infection rates, 18% of all HIV-1 infected IDUs were co-infected with HCV while 96% of all HCV cases had HIV-1 infection. By comparison, only 5.3% of HIV-1 negative IDUs were infected with HCV. These coinfection rates were similarly much higher for younger IDUs (under 40 years old) and higher in males than females. None of the injectors was on opioid substitution therapy (OST), and only 36% of the IDUs were receiving antiretroviral therapy. A study carried out in USA showed that there is a high rate of HIV infected persons who are co-infected with HCV, most of them being current

or past drug users. These co-infection incidents are a major risk for worsened disease prognosis, particularly since most of the individuals infected with HCV may not be aware of their HCV status and thus not receiving or having initiated treatment in spite of receiving HIV treatment (Helen-Maria *et al.*, 2012). Initiating HCV treatment among those co-infected with both viruses has been shown to have a negative impact on adherence to HIV treatment in previous studies. (Roux, Lions, Cohen, Winnock, Salmon-Céron, Bani-Sadr 2012). In this study the prevalence of drug use among our subjects is 41.2%. This indicates that there is an upward trend in injection drug use in Kenya. These results are concurrent with a previous study carried out in the Coastal region which showed that the prevalence of drug use was at 60% (Korir *et al.*, 2013). Tourist hyperactivity combined with economic disparity in this region may be contributing to the increase in risky behavior.

5.3 HIV diversity and drug resistant mutations

From this study 68% of all the infecting HIV strains isolated from the subjects were subtype A. Majority of A strains were sub-subtype A1 at 88.24% while A2 were 11.76%. Of the remaining 32% of the HIV isolates; 12% were subtype C, 16% were subtype D. These data show that HIV subtype A remains the most dominant circulating genotype among Kenyans by analysis of both the *pol-RT* and *env* regions. These findings are consistent with recent report from the same Kenyan population setting and other studies from different Kenyan regions (Kitawi *et al.*, 2015, Nzomo *et al.*, 2015, Kantor *et al.*, 2014, Cheriro *et al.*, 2015). Subtype D has also been found to be the second most prevalent circulating genotype in different regions of Kenya (Cheriro *et al.*, 2015, Songok *et al.*, 2004). This concurs with results from this study. Subtype D has been found to be more virulent than the other subtypes. An independent study carried out prospectively in Kenyan women showed that those affected with subtype D had fast rates of CD4 decline compared to the others (McKinnon, Nico, Rupert, Souradet, Rupert, Apidi 2012).

Sequences from this study were much closely related to African reference isolates apart from two that were related to references from Thailand and China respectively. This may be an indication that the isolated may have been brought into the country by tourists or an individual that visited any of the two countries and brought back the isolates.

Both NRTI and NNRTI drug resistance mutations were found among the samples at. There were no drug resistance mutations found among PI. Several studies carried out in Kenya have shown that there are NRTI and NNRTI drug resistance mutations among both ART naïve and experienced patients (Kiptoo *et al.*, 2013, Onywera *et al.*, 2017).

5.4 HCV diversity and drug resistance mutations

In this study, subtype 1a was predominant at 75% followed by subtype 4a at 25% among the HCV positive samples. Although there is scanty research on HCV Geno-epidemiology especially in African countries, the most predominant genotype reported by other studies is genotype 4. Both subtype 1a and 4a from this study were closely related to reference sequence isolates from the U.S.A. Overall, genotypes 1 and 4 have been found to be the most prevalent genotypes among HIV/HCV co-infected patients (Berenguer *et al.*, 2016). In a study of IDUs in Nairobi, genotype 1 was found to still be predominant in local circulation (Muasya *et al.*, 2000), which is consistent with the findings of the present study. Genotype 1 and genotype 4, which were the two predominant subtypes found among the IDUs in this study, also are known to not respond well to interferon-based anti-HCV drug treatment compared to genotypes 2 and 3 (Chen 2012, Rose, Markov, Lam, Pybus 2013). Therefore, the likelihood of poor disease prognosis is significantly increased among the IDUs who are preferentially infected by these genotypes.

There were no drug resistance mutations found among the HCV isolates. Several studies have found minimal drug resistance mutations associated with the NS5B region, approximately less than 1% (Kliemann , Tovo, da-Veiga , de-Mattos, Wood 2016).

5.5 Conclusion.

1. The survey shows that there was high prevalence of intravenous drug users among the CCC patients (41.2%).
2. The prevalence of HCV was 5.8%. The infection was solely restricted to intravenous drug users. High burden of genotype 1a (75%) and 4a (25%) among the HCV isolates were reported. There were no drug resistant mutations among the amplified HCV specimen
3. Most prevalent HIV genotype circulating among the subjects is subtype A (60%). The prevalence of drug resistant mutations among the HIV specimen was relatively high and was restricted to NRTIs (16%) and NNRTIs (24%).

5.6 Recommendation.

There is insufficient data on HIV/ HCV co-infections and intravenous drug use both at molecular level and immunological level especially in Kenya therefore more research should be carried out to provide better understanding of the prevalence, how the two viruses interact and their outcomes due to drug response. There is an urgent need for *in vitro* and *in vivo* models of dual HIV/HCV infection to better understand the pathogenesis of co-infection.

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APPENDICES

Appendix 1A

CONSENT FORM.

PATIENT CONSENT SEEKING FORM.

PREVALENCE OF INTRAVENOUS DRUG USE and HEPATITIS C VIRUS (HCV) INFECTIONS AMONG PATIENTS VISITING MALINDI SUB COUNTY HOSPITAL COMPREHENSIVE CARE CENTRE, KILIFI COUNTY, KENYA.

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INTRODUCTION

Purpose of study: This study is aimed at determining the distribution of HIV/HCV drug resistant subtypes among HIV/HCV comprehensive care clinic patients in Malindi. The purpose of the study is to determine the prevalence of HIV/HCV subtypes and their antiretroviral drug resistance profiles among comprehensive care clinic patients in Malindi. This information will be useful to the government when developing viral epidemic management programs. The evidence generated from this study will be summarized and disseminated to responsible stakeholders' i.e. Ministry of Health and the field sites

Procedures: You have been selected at random. If you agree to be a participant in this study, we will take 2 drops of blood from your finger. This will take about a minute. We will use sterile and disposable instruments that are clean and safe. Your finger will be cleaned using a disinfectant before pricking. If your blood will test positive for hepatitis,

HIV or other viral infection, a further five milliliters of blood will be taken from your vein (hand) for analysis in the laboratory to determine the viral subtype that has infected you. In order to ensure complete confidentiality of the test results, no names will be attached to the blood samples, but an identification number assigned to you will be used to label the sample. The sample will be taken to the laboratory, analyzed and final report concluded in less than a year. This work will be done in the KEMRI HIV laboratory. The researchers will not disclose your HIV status to anyone else. Even if you decide not to participate in the study, you are still eligible for free counseling services.

Risks: Blood taking will expose you to no health risks except minimum discomfort associated with puncturing of your skin. The discomfort will last only few minutes. In the event of fainting when blood is being taken, the phlebotomist is as well qualified in administering first aid which will be for free.

Benefits in participating: This study will enable the participant to know his/her HIV and HCV infection status. Also, the participant will know whether he is infected with virus strains harboring antiretroviral drug resistance mutations, which will enable the clinicians to make wise decisions when changing treatment. The study will assist the government when planning management of viral epidemics to include all viral co infections.

Participant's Rights: Your participation in this study is voluntary and if you decline to participate, you will not be denied any services that are normally available to you. We will make every effort to protect your identity. You will not be identified in any report or publication of this study or its results. However, if you want to know the type/subtype of virus that has infected you, feel free to contact the medical officer of health in this institution and this will be availed to you. You are also encouraged to ask any questions that occur to you at this time or ask questions at any time in the course of your contact with the investigators. You will also be given a copy of this agreement for your own information. This research has been reviewed and approved by the KEMRI/ National Ethical Review Committee and Jomo Kenyatta University of Agriculture and Technology. These committees review research studies in order to help protect

participants. If you have any questions about your rights as a research participant you may contact:

- The Secretary KEMRI Ethical Review Committee at Tel. 020-272-2541, or
- The Director ITROMID, Jomo Kenyatta University of Agriculture and Technology, P.O. 62000, Nairobi

Consent to participate: You are free to accept or refuse to participate in this study. If you choose to refuse or withdraw, your rights to be attended to in this or other hospitals now or in future will not be affected. I have read the information stated above and have asked questions, all of which were answered satisfactorily. I hereby give consent for my participation as explained to me. The above details about the study and the basis of participation have been explained to me and I AGREE to take part in the study. I understand that I am free to choose to be part of the study. I also understand that if I do not want to go on with the study, I can stop at anytime. I GIVE MY CONSENT for my blood to be tested for viral antibodies and if infected to determine which subtype it is. I also give consent for my sample to be collected, stored and analyzed as guided by this proposal. Please sign here or put your right hand thumb mark if you agree Signature-----

----- Sample Code Date -----

I, the undersigned, have fully explained the relevant details of this study to the patient named above.

Technologist;

Name.....Signature.....

Witness

Name.....Signature.....Address

.....

Appendix 1B

FOMU YA IDHINI

PREVALENCE OF INTRAVENOUS DRUG USE and HEPATITIS C VIRUS (HCV) INFECTIONS AMONG PATIENTS VISITING MALINDI SUB COUNTY HOSPITAL COMPREHENSIVE CARE CENTRE, KILIFI COUNTY, KENYA.

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Taarifa kwa washirika

Utafiti huu unalengo la kuuangali aina ya viini vya Ukimwi na viini vya hepatitis C miongoni mwa wagonjwa wanaohudumiwa katika kliniki ya ukimwi katika hospitali ya Malindi. Iwapo mgojwa ana viini vya HIV na HCV mwilini kwa pamoja husababisha maadhara makubwa. Ujumbe huu utatumika na mashirika kadhaa ya serekali kuboresha huduma za afya kwa waadhiwa wa wagonjwa haya.

Tutakavyofanya Uchunguzi

Tutahitaji damu kiwango kidogo cha milliliter 5. Hiyo damu tutaipeleka kwa chumba cha utafiti ilitupime aina ya virusi vya HIV na HCV vinavyopatikana kwa damu yako. Utahisi uchungu kidogo wakati unadungwa shindano kwa madhumuni ya kutoa damu. Shughuli hii itachukua dakika moja. Tutatumia vifaa safi na ambavyo vinatupwa baada ya kutumika mara moja. Mahali ambapo damu itatolewa patasafishwa kabla ya

Field Code Changed

kudungwa. Majina hayatatumika kwenye sampuli hizo. Kila anayejihusisha katika utafiti huu atapelelezi nambari ya utafiti. Ufafiti huu utamalizika kwa mda wa mwaka mmoja na utafanywa katika maabaraya KEMRI. Watafiti wahusika hawatazungumza habari yoyote kuhusu utafiti huu kwa watu wasiohusika.

Adhari za utafiti huu

Utahisi uchungu kidogo wakati sindano inaingizwa mwilini kwa madhumuni ya kutoa damu. Iwapo mhusika atazirai wakati damu inatolewa, muuguzi atampa mshiriki huduma ya kwanza.

Ni faida gani utapata kutoka kwa uchunguzi huu?

Yale matokeo tutakayopata kutoka kwa upelelezi huu yatamsaidia mgonjwa kujua iwapo ameadhiriwa na viini vyote hivi kwa pamoja. Ujumbe huu utamwezesha muadhiriwa kupata matibabu kwa haraka.

Haki ya washiriki

Kushiriki kwa utafiti huu ni kwa hiari yako. Usiposhiriki katika utafiti huu hautakatazwa kupata huduma za afya kutoka kwa hospitali hii. Majina yako hayata tumuka mahali popote kwenye reporti ya utafiti huu. Unapotaka kujua ni aina ipi ya kiini inayokuadhiri unaweza kumpigia simu mdudumu wa afya katika hospitali hii ama kumtembelea ili kupata maelezo zaidi.

Utakapohitaji kuuliza maswali wakati wowote ule, wanautafiti wako tayari kukujibu. Pia unaweza kupiga simu wakati wowote kupitia nambari ifuatayo.

Ruth Sada: 0733915717

Makubaliano

Tafadhali onyesha kwa alama hapa chini chaguo lako.

Unakubalia au unakataa kuhusika na Ufafiti huu?

Ninakubali Sahihi:.....Nambari ya
utafiti:.....Tarehe:.....

Mhudumu

Jina:.....Sahihi:.....

Shahidi

Jina.....Sahihi.....

Appendix 2A

QUESTIONNAIRE.

PREVALENCE OF INTRAVENOUS DRUG USE and HEPATITIS C VIRUS (HCV) INFECTIONS AMONG PATIENTS VISITING MALINDI SUB COUNTY HOSPITAL COMPREHENSIVE CARE CENTRE, KILIFI COUNTY, KENYA.

Jomo Kenyatta University of Agriculture and Technology Institute of Tropical Medicine and Infectious Diseases

Dear participant,

You are kindly requested to fill in the questionnaire.

All the information collected will be kept and at no time will it be made in reference to you.

The results will be used for research purposes only.

Unique study

No.....Nationality/origin.....

Age.....

Sex

Occupation.....

Residence

1. Do you consider yourself as a Kenyan?

Yes.....

No.....

2. Do you travel often to countries other than Kenya?

Yes.....

No.....

- If Yes, which one(s).....
- Which country did you last visit.....when.....

3. Do you travel often to regions in Kenya outside where you live now?

Yes.....

No.....

1. If Yes, which one(s)

2. Which county/region did you last visit.....

when

4. Are you married? Yes..... No..... (if No, proceed to 5)

If married, do you currently live together with your spouse?

Yes.....

No.....

If not living together, do you ever come into contact?

Yes.....

No.....

How regular is the contact?

5. Do you consider your relationship intimate?

Yes.....

No.....

6. Do you have other sex partner(s)?

Yes.....

No.....

- If Yes, how many?.....
- If Yes, how regular?.....

7. Have you taken HIV test before?

Yes.....

No.....

- If no, are you willing to take one?

8. Do you know your HIV status?

Yes.....

No.....

- If no, do you want to know?

9. Does your partner/spouse know your HIV status? Yes.....No.....

- If No, do you want them to know?

Yes.....

No.....

- If Yes, do you need help to discuss with them your status?

Yes.....

No.....

10. Has your partner/spouse taken HIV test before?

Yes.....

No.....

- If Yes, do you know their status?

Yes.....

No.....

- If No, do you continue to have sexual contact together?

Yes.....

No.....

11. How long has it been since you first knew about your HIV status?

12. Are you taking any HIV drugs?

Yes.....

No.....

- If Yes, how long since you started?

13. Have you had to change the drugs because of complications?

Yes.....

No.....

- If Yes, how many times?

14. Have you had to stop taking the drugs temporarily for personal reasons?

15. Have you ever forgotten or postponed taking the drugs?

- How often?

16. Do you take them regularly now?

Yes..... (Skip question 17)

- No.....
17. If not taking HIV drugs, do you know you can get them free?
Yes.....
No.....
18. Do you want to start taking HIV drugs?
Yes.....
No.....
19. Do you use any hard drugs?
Yes.....
No.....
20. If yes which one?.....
21. How do you take the drugs?
a. Injecting the drug
b. smoking/sniffing the drug
22. Do you agree to extended storage of your blood/derivative samples for future analyses?
Yes.....No.....
23. Is there anything you want to tell me or questions you wish to ask?

Appendix 2B

MAHOJIANO

PREVALENCE OF INTRAVENOUS DRUG USE and HEPATITIS C VIRUS (HCV) INFECTIONS AMONG PATIENTS VISITING MALINDI SUB COUNTY HOSPITAL COMPREHENSIVE CARE CENTRE, KILIFI COUNTY, KENYA.

Chuo kikuu cha Jomo Kenyatta University of Agriculture and Technology Institute of Tropical Medicine and Infectious Diseases

Mshirika mpendwa,

Unahitajika kujibu maswali yaliyomo katika kurasa hizi.

Majibu yatakayo kusanywa yatawekwa vizuri na hayatatumika kwa wakati wowote kukutambulisha.

Majibu haya yatatumika kwa utafiti tu.

Nambari

ya

Utafiti.....Utaifa/Asili.....

Umri..... Mume/Mke

Kazi..... Makao

1. Je, wewe in Mkenya?

Ndio.....

La.....

2. Je, wewe husafiri mara nyingi kwa nchi nyingine kuliko Kenya?

Ndio.....

La.....

- Kama ndio, we husafiri wapi?
- Je, mwisho ulitembelea nchi gani?.....lini?.....

3. Je, wewe husafiri ndani ya nchi pasi na mahali unapokaa?

Ndio.....

La.....

- Kama ndio, wapi?
- Nchi gani ama sehemu gani ulisafiri.mwisho?.....lini?

4. Umeoa/Umeolewa?

Ndio..... La..... (kama La, endelea #5)

- Je, kwa sasa unaishi pamoja na mwenzio?
Ndio....
La....
- Je, wewe unauhusiano wa kimapenzi na mwenzio?

Ndio.....

La

- Kama hamuishi pamoja, mna uhusiano wa kimapenzi?

Ndio.....

La.....

- Mara ngapi mnajihusisha kwa kitendo cha kujamiana?

5. Je mna uhusiano wa kimwili na mpenzi wako?

Ndio.....

La.....

6. Je una uhusiano wa kimapenzi na watu wengine?

Ndio.....

La.....

- Kama ndio, wangapi?.....
- Kama ndio, mara ngapi?.....

7. Ume wahi kupimwa viini vya Ukimwi?

Ndio.....

La.....

- Kama la, utakubali kupimwa?

8. Unajua hali yako ya ukimwi?

Ndio.....

La.....

- Kama la, wataka kujua hali yako?

9. Je, Mwenzi/Mpenzi wako ajua hali yako ya ukimwi?

Ndio.....

La.....

- Kama la, unataka/unakubali wajulishwe?

Ndio.....

La.....

- Kama ndio, unahitaji ushahidi siku ya kuwajulisha hali yako?

Ndio.....

la.....

10. Je, Mwenzi/Mpenzi wako amewahi kupimwa kwa ajili ya Ukimwi?

Ndio.....

La.....

Sijui.....

- Kama ndio, unajua hali yake/yao?

Ndio.....

La.....

- Kama la, wewe unaendelea kuwa naye kimwili?

Ndio.....

La.....

11. Ni muda gani tangu ujue kuhusu hali yako ya Ukimwi ?

12. Je, unatumia dawa ya kutibu Ukimwi?

Ndio.....

La.....

- Kama ndio, umetumia kwa muda gani?

13. Je, umewahi kubadilishiwa madawa haya kwa ajili yeyote?

- Kama ndio, mara ngapi umebadili madawa haya?

14. Je, umewahi kusimamisha utumishi wa madawa haya kwa sababu yoyote?

15. Umewahi kusahau ama kuahirishwa utumizi wa haya madawa popote?

- Mara ngapi?

16. Je, kwa sasa unatumia dawa hizo mara kwa mara?

Ndio.....

La.....

17. Je, kama hutumii dawa ya Ukimwi, unajua waweza kuzipata bure?

Ndio.....

La.....

18. Je, unataka kuanza kutumia dawa ya kutibu Ukimwi?

Ndio.....

La.....

19. Je unatumia madawa ya kulevya?

20. Unatumia dawa (madawa) ipi ya kulevya?

21. Je waitumiaje dawa hiyo ya kulevya?

a. Kutumia sindano

b. Kuvuta au Kunusa

22. Je, unakubaliana na kuhifadhi kwa damu/sampuli yako kwa ajili ya utumizi baadaye?

23. Je, kuna maswali unataka kuuliza?

Appendix 3

Ethics review committee
approval.



KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1

April 15, 2013

TO: WASHINGTON OCHIENG (PRINCIPAL INVESTIGATOR)

THROUGH : DR. JUMA RASHID; *Forwarded 16/09/2013*
DIRECTOR, CCR

RE: **SSC PROTOCOL NO. 2477 – REVISED (*RE-SUBMISSION*): GENETIC CHARACTERIZATION OF HIV-1 STRAINS CIRCULATING IN KENYA AND DETERMINATION OF KNOWN MUTATIONS CONFERRING RESISTANCE TO ANTIRETROVIRAL DRUGS**

Make reference to your letter dated 4th April, 2013, Received on April 4, 2013.

We acknowledge receipt of the Revised Study Protocol.

This is to inform you that the Ethics Review Committee (ERC) reviewed the document listed above and is satisfied that the issues raised at the 211th meeting held on 26th November, 2012 have been adequately addressed.

The study is granted approval for implementation effective this **15th day of April 2013**. Please note that authorization to conduct this study will automatically expire on **April 14, 2014**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **March 4, 2014**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC. You are also required to submit any proposed changes to this protocol to the ERC prior to initiation and advise the ERC when the study is completed or discontinued.

You may embark on the study.

Sincerely,

**Dr. Elizabeth A. Bukusi,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE**

Appendix 4
SSC committee approval



KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
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E-mail: director@kemri.org info@kemri.org Website: www.kemri.org

ESACIPAC/SSC/101264

13th December, 2012

Bernhards Ogutu

Thro'
Director, CCR
NAIROBI

*forwarded
17/12/2012*

REF: SSC No. 2477 (Revised) – Genetic characterization of HIV-1 strains circulating in Kenya and determination of known mutations conferring resistance to antiretroviral drugs

Thank you for your letter received on 7th December, 2012 responding to the comments raised by the KEMRI SSC.

I am pleased to inform you that your protocol now has formal scientific approval from SSC.

The SSC however, advises that work on the proposed study can only start after ERC approval

Sammy Njenga
Sammy Njenga, PhD
SECRETARY, SSC

In Search of Better Health

Appendix 5

ERC waiver letter

5/10/2014.

TO
THE COORDINATOR,
ITROMID,
P.O.BOX 62000-00200,

Re: Waiver of ERC and SSC Approval Ruth Sada Mwatelah.

Ruth S. Mwatelah is a student working on her thesis project titled '*Co-prevalence and genetic diversity of human immunodeficiency virus type 1 (HIV-1) and hepatitis C virus (HCV) among Kenyan subjects*'. Ruth's thesis is covered primarily under my SSC#2477; 'Genetic Characterization of HIV-1 Strains and Determination of Known Mutations Conferring Resistance to Antiretroviral Drugs in Kenya' and by a collaborating project SSC #; 101264.

I request you to waive the requirement for her proposal to go through the SSC/ERC procedures on ground that the parent protocol has been granted approval and there is no need for duplicate approval requests.

Yours sincerely,



Dr. Washington Ochieng'

PI and Scientist, CVR-KEMRI

