COMPARISON OF HEPATITIS B VIRUS SERO-MARKERS, CLINICAL STAGES AND GENOTYPES BETWEEN HIV-1 INFECTED AND UNINFECTED INJECTING SUBSTANCE USERS IN MOMBASA CITY, KENYA

BY

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DECLARATION

I declare that this thesis is my original work and has not been presented to any other University or Institution for a degree or any other award.

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May God bless you all!

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DEDICATION

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ABSTRACT

Globally, it is estimated that 1.2 million injecting substance users are HBV infected while 1.7 million live with HIV. Africa is home to about 1.02 million injecting substance users of whom 12.7% are HBV infected while 12.1% live with HIV. In Kenya, the population of injecting substance users is approximately 50,000 most of whom reside in Nairobi and Mombasa cities. Nairobi and Mombasa cities are home to about 23,000 and 27,000 injecting substance users, respectively. The prevalence of HBV infection among injecting substance users is 3% in Nairobi city and 13.8% in Mombasa city. In addition, HIV prevalence in injecting substance users is 18.7% in Nairobi city and 43.9% in Mombasa city. HBV diagnosis and clinical staging requires concurrent testing of hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), hepatitis B pre-core antigen (HBeAg), hepatitis B pre-core antibody (HBeAb) and hepatitis B core antibody (HBcAb). HBV exist as ten genotypes (A to J) which vary by geographic area. HIV infection alters host response to HBV leading to significant variations in the pattern of HBV sero-markers, clinical stages and genotypes. In addition, injecting substance use alters host immune response by reducing CD4+ T cell proliferation. HBV sero-markers, clinical stages and genotypes have been determined in HIV-1 infected injecting substance users in Mombasa city, Kenya. However, comparison of HBV sero-markers, clinical stages and genotypes between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya, remains unknown. This study, therefore, compared HBV sero-markers, clinical stages and genotypes between HIV-1 infected (n=157) and uninfected (n=214) injecting substance users in Mombasa city, Kenya. In a cross-sectional study, the injecting substance users were recruited via snowball method and their socio-demographic data recorded on questionnaires. The CD4+ T cells were enumerated using flow cytometer and categorized according to the Centre for Disease Control and Prevention immunological staging criteria. Plasma samples were sero-tested for HBsAg, HBsAb, HBeAg, HBeAb and HBcAb using the HBV-5 panel rapid test cassettes. Clinical staging was based on serological profile of the five HBV sero-markers. Phylogenetic analysis was used to determine HBV genotypes in acute and chronic clinical stages. Age and CD4+ T cell count were compared between the study groups using Mann-Whitney U test. Gender, CD4+ T cell immunological stages and HBV sero-markers were compared between the study groups using Chi-square test. HBV clinical stages and genotypes were compared between the study groups using Fisher's exact test. The frequency of HBsAg (P=0.004) and HBcAb (P=0.008) sero-markers were higher while that of HBsAb (P=0.019) sero-marker was lower in HIV-1 infected group compared to the uninfected group. The frequency of acute (P=0.033) and chronic (P=0.021) clinical stages were higher while that of vaccine type response stage (P=0.008) was lower in the HIV-1 infected group compared to the uninfected group. Only HBV genotype A clusters was detected, with higher frequency in the HIV infected group compared to uninfected group (P=0.009). In conclusion, HBsAg and HBcAb sero-markers and acute and chronic clinical stages are higher while HBsAb sero-marker and vaccine type response clinical stage are lower in HIV-1 infected compared to uninfected injecting substance users in Mombasa city. In addition, genotype A clusters is higher in HIV-1 infected compared to uninfected injecting substance users in Mombasa city. Therefore, HIV-1 infected injecting substance users in Mombasa city should be vaccinated against HBV. Medical follow-up programmes and harm reduction measures should be initiated to lower the burden of HBV infection and transmission among HIV-1 infected injecting substance users in Mombasa city.

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ACRONYMS AND ABBREVIATIONS

ART	Antiretroviral therapy
ARV	Antiretroviral
AU	African Union
Buffer AE	DNA extraction elution buffer
Buffer AL	Lysis buffer
Buffer AW1	Ethanol-based stringent wash solution
Buffer EB	DNA purification elution Buffer
Buffer PBI	Binding Buffer
Buffer PE	Wash Buffer
CDC	Centers for Disease Control
HBcAb	Hepatitis core antibody
HBeAb	Hepatitis B pre-core antibody
HBeAg	Hepatitis B pre-core antigen
HBsAb	Hepatitis B surface antibody
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HIV	Human Immunodeficiency virus
IDUs	Injecting substance users
KNBS	Kenya National Bureau of Statistics
NACADA	National Campaign Against drug Abuse Authority
NACC	National AIDS Control Council
NASCOP	National AIDS and STI Control Programme
Non-IDUs	Non-injecting substance users
PCR	Polymerase Chain Reaction
PEPFAR	U.S. President's Emergency Plan for AIDS Relief and the Interfaith Health Program
UNAIDS	United Nations Programme on HIV/AIDS
WHO	World Health Organization

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OPERATIONAL TERMS

Injecting substance user	A person who gives himself or herself substances like heroin parenterally. The injecting of substance is rarely performed aseptically and may result in the spreading of HBV and HIV.
Substance	A drug whose use is controlled or made illegal by law.

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CHAPTER ONE: INTRODUCTION

1.1 Background Information

Substance is a drug whose use is controlled or made illegal by law (UNODC, 2014). An injecting substance user is a person who gives himself or herself substances like opiates and opioids parenterally (UNODC, 2014). The injecting of substance is rarely performed aseptically and may result in the spreading of HBV and HIV (UNODC, 2014). Globally, approximately 12.7 million people are injecting substance users of whom 1.2 million live with hepatitis B virus (HBV) while 1.7 million are infected with human immunodeficiency virus (HIV) (UNODC, 2014). Africa is home to about 1.02 million injecting substance users of whom 12.7% are HBV infected while 12.1% live with HIV (Nelson *et al.*, 2011; UNODC, 2014). In Kenya, the population of injecting substance users is approximately 50,000 most of whom reside in Nairobi and Mombasa cities (NASCOP, 2012). Nairobi and Mombasa cities are home to about 23,000 and 27,000 injecting substance users, respectively (NASCOP, 2012). An estimated 18.7% of injecting substance users in Nairobi and 43.9% of injecting substance users in Mombasa city are living with HIV infection (Deveau *et al.*, 2006; Tun *et al.*, 2015). In addition, about 3% and 13.8% of the injecting substance users in Nairobi and Mombasa cities, respectively, live with HBV (Muasya, 2009; Kibaya *et al.*, 2015). Injecting substance users are predisposed to HBV and HIV infections due to unprotected sexual activities and needle and syringe sharing while injecting substances (Beckerleg *et al.*, 2005). Taken together, HBV and HIV infections are important co-morbidities of injecting substance users.

Accurate diagnosis of hepatitis B virus (HBV) requires concurrent testing for the presence of the five hepatitis B virus sero-markers namely hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), hepatitis B pre-core antigen (HBeAg), hepatitis B pre-core

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antibody (HBeAb) and hepatitis B core antibody (HBcAb) (Mahoney, 1999; Krajden *et al.*, 2005; Mast *et al.*, 2006). HBsAg represent active HBV infection, HBeAg indicate high viral replication and persistence while HBsAb and HBeAb signify hepatitis B resolution (Mahoney, 1999; Krajden *et al.*, 2005). HBcAb is a non-protective antibody and is of class IgM and IgG denoting acute-window and past infection, respectively (Krajden *et al.*, 2005). Co-infection with HIV changes HBV sero-marker profile. HIV and HBV co-infected individuals rarely seroconvert from HBsAg, HBeAg, HBcAg to HBsAb, HBeAb and HBcAb, respectively (Ranjbar *et al.*, 2011), and this is largely linked to lower CD4+ T cell counts (Gaglio *et al.*, 2007; Li *et al.*, 2011). HBsAg sero-marker has been determined in HIV-1 infected injecting substance users in Mombasa city, Kenya (Kibaya *et al.*, 2015). However, the five hepatitis B virus sero-markers (HBsAg, HBsAb, HBeAg, HBeAb and HBcAb) between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya, have not been compared.

Hepatitis B clinical stages guide genotyping, clinical management as well as predicting disease reactivation and exacerbation (Rotman *et al.*, 2009; Walsh and Locarnini, 2012). HIV affects HBV clinical stages. After acute HBV clinical stage, HIV infected individuals are up to six-fold likely to develop chronic HBV clinical stage than are HIV uninfected individuals (Gatanaga *et al.*, 2000). In addition, HIV infected persons who acquire vaccine type or natural immunity to HBV remain at risk for loss of immunity and develop acute hepatitis B clinical stage (Thio, 2009). The loss of vaccine type and natural immunity to HBV is associated with lower CD4+ T cell counts resulting from HIV-1 pathogenesis (Thio, 2009). Injecting substance users in Mombasa city have a longer duration of injecting substance because they are initiated into injecting substance at an early age of about 10 years (NACADA, 2012). Longer duration substance use weakens CD4+ T cell immunity, reducing

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the probability of clearing HBV and HIV infections upon exposure (Thio *et al.*, 2002; Brown *et al.*, 2007; Reimer *et al.*, 2007; Riss *et al.*, 2012). Chronic hepatitis B virus clinical stage has been reported among HIV-1 infected injecting substance users in Mombasa city, Kenya (Day *et al.*, 2013). However, hepatitis B clinical stages between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya, have not been compared.

So far, ten HBV genotypes designated A, B, C, D, E, F, G, H, I and J have been reported with Africa having mainly genotype A and to a varying extent genotypes C, D and E (Zhang and Cao, 2011). In Kenya, molecular epidemiological studies have demonstrated the co-existence of genotypes A, D and E (Mwangi *et al.*, 2008; Kim *et al.*, 2011; Day *et al.*, 2013; Kwange *et al.*, 2013; Ochwoto *et al.*, 2013). HIV infection alters host response leading to significant variations in the pattern HBV genotype infection (van der Kuyl *et al.*, 2013). HBV genotype A is circulating among HIV-1 infected injecting substance users in Mombasa city, Kenya (Kibaya *et al.*, 2015). However, HBV genotypes between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya, have not been compared. As such, the current study compared the hepatitis B virus sero-markers, clinical stages and genotypes between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya.

1.2 Problem statement

As described above, injecting substance use weakens immune response by reducing CD4+ T cell proliferation. In addition, HIV alters host immune response to HBV leading to significant variation in HBV sero-markers, clinical stages and genotypes. Hepatitis B virus sero-markers, clinical stages and genotypes have been determined in HIV-1 infected injecting substance users in Mombasa city, Kenya. However, hepatitis B virus sero-markers, clinical stages and genotypes between HIV-1 infected and uninfected injecting substance users in

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Mombasa city, Kenya, have not been compared. Therefore, the current study compared hepatitis B virus sero-markers, clinical stages and genotypes between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya.

1.3 Objectives

1.3.1 General objective

To compare hepatitis B virus sero-markers, clinical stages and genotypes between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya.

1.3.2 Specific objectives

1. To compare hepatitis B virus sero-markers between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya.
2. To compare hepatitis B virus clinical stages between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya.
3. To compare hepatitis B virus genotypes between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya.

1.4 Research questions

1. How does hepatitis B virus sero-markers compare between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya?
2. How does hepatitis B virus clinical stages compare between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya?
3. How does hepatitis B virus genotypes compare between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya?

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1.5 Significance of the study

Establishment of the HBV sero-markers, clinical stages and genotypes aids practicing physicians determine ideal anti-viral therapy for those at risk of severe disease outcome. The knowing of hepatitis B genotypes will aid in the designing vaccines for most-at-risk populations. In addition, the determination of HBV sero-markers, clinical stages and genotypes guides molecular epidemiological surveillance studies. The findings of this study will be used by service providers and policy makers in prevention of HBV among injecting substance users and the general population. The results of this study will guide diagnosis and clinical staging of HBV.

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CHAPTER TWO: LITERATURE REVIEW

2.1 Hepatitis B virus molecular biology

In 1974, Robinson and co-workers purified HBV DNA from plasma samples of chronic HBsAg carriers (Robinson *et al.*, 1974). Later in1978, forerunners of molecular biology research from France (Charnay *et al.*, 1979), USA (Valenzuela *et al.*, 1979) and United Kingdom (Pasek *et al.*, 1979), successively sequenced cloned HBV DNA. The three pioneers of molecular biology further showed that HBV DNA was circular with four open reading frames (ORF) of surface (S), core (C), polymerase (P) and X genes (Charnay *et al.*, 1979; Pasek *et al.*, 1979; Valenzuela *et al.*, 1979) encoding for HBsAg (larger, medium and small HBsAg), core proteins (both HBeAg and HBcAg), polymerase and hepatitis B X antigen (HBxAg), respectively (Appendix 1) (Liang, 2009). HBsAg contributes to immunopathogenesis of HBV persistent infection (Kondo *et al.*, 2013). Even though the role of HBcAg is unknown, its secretory form HBeAg enhances viral replication and persistence (Carey, 2009). Polymerase possesses DNA polymerase, reverse transcriptase and RNaseH attributes necessary for HBV replication (Schadler and Hildt, 2009). The reverse transcriptase uses the viral pregenomic RNA (pgRNA) as the template to synthesize minus-strand viral DNA via its RNA-dependent DNA polymerization activity (Jones and Hu, 2013). The minus-strand DNA is then used by DNA polymerase as the template for plus-strand DNA synthesis (Jones and Hu, 2013). RNase H is required to degrade the pgRNA template during minus-strand DNA synthesis (Jones and Hu, 2013). Finally, HBxAg is required to initiate and maintain virus replication after infection (Lucifora *et al.*, 2011).

2.2 Diagnosis of hepatitis B virus

Viral markers of HBV infection includes viral antigens (HBsAg, HBcAg and HBeAg), viral antibodies (HBsAb, HBeAb and HBcAb) and viral DNA (Bonino *et al.*, 2010). The current

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diagnostic repertoire of HBV serum markers include detection of viral antigens and antibodies by immunoassays and viral DNA measured by PCR based amplification tests (Krajden *et al.*, 2005). These tests establish the HBV disease stage as well as monitor for effective vaccination and treatment (Mahoney, 1999; Krajden *et al.*, 2005; Bonino *et al.*, 2010).

HBcAg is not detectable in the serum because it is totally degraded in serum (Krajden *et al.*, 2005). However, a non-protective HBcAb (antibody to HBcAg) of class immunoglobulin M (HBcAb-IgM) and immunoglobulin G (HBcAb-IgG) are detected in serum (Mahoney, 1999; Mikulska *et al.*, 2014). HBcAb-IgM may be the only marker detected during the acute-window period but may also be detected in a subset of chronic carriers (Bonino *et al.*, 2010). HBcAb-IgG denotes past infection and it persists throughout lifespan (Mahoney, 1999).

The presence of detectable HBeAg in serum is associated with high levels of HBV replication, greater infectivity and an increased risk of hepatic fibrosis (Mahoney, 1999; Krajden *et al.*, 2005; Bonino *et al.*, 2010). HBeAb (antibody to HBeAg) denotes a less infectious state and a partial resolution of HBV infection (Bonino *et al.*, 2010). Occasionally, carriers can demonstrate both HBeAg and HBeAb positivity (Krajden *et al.*, 2005). Mutations in the core and pre-core gene regions of HBV lead to undetectable HBeAg, but this may not affect the course of acute or chronic infection (Ochwoto *et al.*, 2013).

In virus infected liver cells, HBsAg is produced in excess and secreted into the blood, where it serves as a marker of active infection and infectivity (Krajden *et al.*, 2005). The disappearance of serum HBsAg and appearance of HBsAb (antibody against HBsAg) represent recovery in both acute and chronic infection but HBV DNA persists in the liver

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inducing oncogenic effect (McMahon, 2009b). In the absence of HBsAg, serum HBsAb indicates protective immunity against HBV acquired by vaccination (isolated HBsAb positive) or natural infection (HBsAb and or HBeAb positive) (Bonino *et al.*, 2010). Mutations in the *Surface*-gene of HBV results in occult infection, defined as the presence of HBV DNA in the liver with detectable or undetectable HBV DNA in the serum of HBsAg negative individuals, drives HBV epidemiology (Raimondo *et al.*, 2008). HBV vaccination may solve the problem of overt HBV infection but may favour occult hepatitis B infection (Gerlich *et al.*, 2010).

2.3 Epidemiology of hepatitis B virus and HIV in injecting substance users

Substance is a substance whose use is controlled or made illegal by law (UNODC, 2014). An injecting substance user is a person who gives himself or herself substances like opiates and opioids through injecting mode (UNODC, 2014). The injecting of substance is rarely performed aseptically and may result in the spreading of HBV and HIV (UNODC, 2014). Globally, more than 12.7 million people inject substances (UNODC, 2014). Of these, about 1.2 million people live with chronic hepatitis B, indicated by HBsAg and 6.4 million are positive for HBcAb, symbolizing exposure to HBV, while 1.7 million are infected with HIV (Nelson *et al.*, 2011; UNODC, 2014). There are about 1.02 million injecting substance users in Africa of whom about 12.7% and 12.1%, respectively, are infected with HBV and HIV (Nelson *et al.*, 2011; UNODC, 2014). In Kenya, it is estimated that there are 50,000 injecting substance users who are concentrated in specific geographic areas like Nairobi and Mombasa cities (NASCOP, 2012). Most importantly, about 23,000 and 27,000 injecting substance users are residents of Nairobi and Mombasa cities, respectively (NASCOP, 2012). Approximately, 18.7% and 43.9% of injecting substance users in Nairobi and Mombasa cities, respectively, are HIV-infected (Deveau *et al.*, 2006; Tun *et al.*, 2015). In addition,

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about 3% and 13.8% of the injecting substance users in Nairobi and Mombasa cities, respectively, live with HBV (Muasya, 2009; Kibaya *et al.*, 2015).

Injecting substance users in Mombasa city engage in unprotected sex, commercial sex and share needle and syringes while injecting substances hence they are at risk of HBV and HIV infections (Beckerleg *et al.*, 2005; Oyaro and Wylie, 2012). The prohibition of injecting substance use in Kenya has led to imprisonment of injecting substance users. Injecting substance users interact with infected and uninfected convicts in Kenya's prisons where risky behavior is high with no HBV and HIV-1 prevention strategies (NASCOP, 2012). For instance, risky sexual practices, injecting substance use, syringe and needle sharing and tattooing occur in Kenya's prisons (NASCOP, 2012). To reduce HBV and HIV-1 transmission among injecting substance users, the World Health Organization (WHO) has called for implementation of a comprehensive package of harm reduction measures which promotes needle and syringe exchange programmes (NSPs) and allows only lawful arrest of injecting substance users among the world's countries (WHO, 2010).

2.4 Hepatitis B virus sero-markers

In 1963, the first HBV sero-marker was discovered and named Australian antigen (AuAg) (Blumberg *et al.*, 1984). In the following years, AuAg was linked to HBV infection and renamed hepatitis B surface antigen (HBsAg) (Gerlich, 2013). To date, HBsAg is a major diagnostic marker for HBV infection in resource limited countries like Kenya (Barth *et al.*, 2010).

It is estimated that 360 million people worldwide are HBsAg sero-positive, of whom between 8% to 20% are in Africa (WHO, 2009). In Kenya, the prevalence of HBsAg sero-marker

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ranges from 8% to 30% in various populations (MOH, 2013). For instance, a transversal study in Trans-Nzoia, Kenya, reported a 10.5% HBsAg sero-prevalence among febrile patients visiting health facilities (Demba *et al.*, 2013). The sero-prevalence of any of the three viral markers (HBsAg, HBcAb or HBsAb) is approximately 56.2% among outpatients attending three distinct hospitals in Mombasa, Kilifi, and Malindi, Kenya (Hyams *et al.*, 1989). In areas such as Turkana in North-Western Kenya, HBsAg sero-prevalence stands at 8.8% among asymptomatic nomads (Mutuma *et al.*, 2011). A study involving pregnant women in former Kenya's six provinces including Nairobi Province estimated a sero-positivity of 9.3% for HBsAg, 8.8% for both HBsAg and HBeAg and 30.2% for HBsAb (Okoth *et al.*, 2006).

Previous studies have also reported prevalence of HBV sero-markers among HIV-1 infected Kenyans. Two separate studies conducted in Nairobi city, Kenya, showed that about 6% of HIV-1 infected individuals were HBsAg positive (Harania *et al.*, 2008; Muriuki *et al.*, 2013). A randomized clinical trial in Nairobi city, Kenya, estimated that 6.9% and 11%, respectively, of HIV-1 infected individuals on antiretroviral therapy are HBsAg and HBeAg positive (Kim *et al.*, 2011). A prospective study involving HIV-1 infected female sex workers in Mombasa city, Kenya, indicated a sero-positivity of 6.9% for HBsAg, 53% for HBcAb and 3.8% for both HBsAg and HBeAg (Day *et al.*, 2013). About 53% of HIV-1 infected individuals attending liver clinics were reported to test positive for HBsAg in Kisumu city, Kenya (Otedo, 2004).

In most-at-risk populations such as injecting substance users in Nairobi and Kisumu cities of Kenya, it has been reported that about 4.4% of them were HBsAg positive (Oyaro and Wylie, 2012). A cross-sectional study of injecting substance users in Nairobi reported a 4.5%

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HBsAg prevalence (Odek-Ogunde *et al.*, 2004). Another study estimated that 3% of injecting substance users residing in Nairobi were HBsAg positive, 0.6% of whom were co-infected with HIV-1 (Muasya, 2009). In Mombasa, it is estimated that 13.8% of injecting substance users are HBsAg sero-positive (Kibaya *et al.*, 2015).

Accurate diagnosis of HBV requires concurrent testing for the presence of HBsAg, HBsAb, HBeAg, HBeAb and HBcAb sero-markers in an individual's serum (Bonino *et al.*, 2010). HIV influence HBV sero-marker profile. HIV promotes persistence of HBsAg, HBcAg and HBeAg by infecting CD4+ T cells responsible for priming B cells to produce HBsAb, HBcAb and HBeAb which neutralizes HBsAg, HBcAg and HBeAg, respectively (Chang and Lewin, 2007). HIV infection can complicate the diagnosis of HBV because spontaneous reverse seroconversion marked by the disappearance of HBsAb and reappearance of HBsAg occur in HIV infected individuals (Thio, 2009). HBsAg sero-marker has been determined in HIV-1 infected injecting substance users in Mombasa city, Kenya (Kibaya *et al.*, 2015). However, the five hepatitis B virus sero-markers (HBsAg, HBsAb, HBeAg, HBeAb and HBcAb) between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya, have not been compared. As such, the current study compared the five HBV sero-markers (HBsAg, HBsAb, HBeAg, HBeAb and HBcAb) between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya.

2.5 Hepatitis B virus clinical stages

Immunization with hepatitis B vaccine is the most effective way to prevent hepatitis B infection (Liaw and Chu, 2009). However, mutations within the HBV *surface*-gene results in HBsAg negative mutants that hinder the success of HBV vaccine (Gerlich, 2006). HBV vaccine unresponsiveness is also linked to reduced CD4+ T cell proliferation resulting from

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injecting substance use (Riss *et al.*, 2012; Kamath *et al.*, 2014). A mathematical modelling estimated that without vaccination, 64.8 million would become HBV-infected and 1.4 million would succumb to HBV-related illness worldwide (Goldstein *et al.*, 2005).

Hepatitis B incubation period (window period) lies between 1 to 6 months and it correlates with the level of inoculum (Barker and Murray, 1972). Several host factors such as age of infection, gender, immune status, genotype and transmission route influence the course of acute hepatitis B infection (Croagh and Lubel, 2014). Most (about 90%) of acute infection in the immune competent adult results in complete recovery with subsequent development of natural immunity (Rizzetto, 1999). However, positive prognosis of acute hepatitis B infection is not assured. Nearly 1-2% develop fulminant hepatitis B acute liver failure (Lee, 2012), while 5% progress to a chronic hepatitis B state (Rizzetto, 1999). Meanwhile, over 90% of HBV infections (perinatal HBV transmissions in babies and horizontal transmissions in children develop to chronic hepatitis B (Heller and Valencia-Mayoral, 2007). This higher prevalence of chronic hepatitis B in children is due to HBeAg, also serving as an immune tolerogen *in utero*, thus inducing immune tolerance to this receptive underdeveloped immunity (Walsh and Locarnini, 2012). Taken together, immune competent individuals successfully resolve acute hepatitis B, compared to susceptible immunocompromised individuals such as HIV-1 infected and babies (Puoti *et al.*, 2006; Walsh and Locarnini, 2012).

Chronic hepatitis B clinical phases are conceptualized in terms of the state of balance between the host immune system and the hepatitis B virus and have been labelled: immune tolerant, immune clearance, inactive and/or non-replicative, reactivation and immune escape (Walsh and Locarnini, 2012; Croagh and Lubel, 2014). Immune tolerant phase is ascribed to

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perinatal transmission and is defined by HBeAg positivity, HBsAg positivity, and minimal liver damage in spite of high HBV DNA viremia (Croagh and Lubel, 2014). However, during immune tolerant phase, the viral-host DNA integration occurs increasing the chances of hepatocellular carcinoma (Walsh and Locarnini, 2012). Even though the stimulation of host immune system is unclear, immune clearance phase is associated with liver inflammation, hepatocyte death and HBeAg sero-conversion to HBeAb (McMahon, 2009b). At this point, HBeAg immunotolerant effect is lost and there is emergence of basal core or pre-core promoter mutations actually induced by immune pressure (Walsh and Locarnini, 2012). Inactive phase is then marked by little or no liver damage, HBeAg negative, HBeAb positive and low HBV DNA level (Walsh and Locarnini, 2012; Croagh and Lubel, 2014). However, patients in the inactive phase can revert back to HBeAg positive immune clearance phase (Walsh and Locarnini, 2012).

The reactivation phase, commonly known as immune escape or HBeAg negative chronic hepatitis B, is associated with increased hepatocyte damage, liver inflammation, and the appearance of pre-core/core and/or G1896A pre-core stop codon mutant viruses (Croagh and Lubel, 2014). It has been reported that about 1% of chronic hepatitis B patients progress to immune control phase characterized by undetectable or very low HBV DNA levels (Walsh and Locarnini, 2012; Croagh and Lubel, 2014). During the immune control phase, however, occult HBV infection persists in the liver inducing oncogenic effect (Raimondo *et al.*, 2008; McMahon, 2009b). Indeed, development of liver cirrhosis and hepatocellular carcinoma correlate positively with length of chronic hepatitis B infection (McMahon, 2009b).

Hepatitis B clinical stages guide clinical management as well as predicting disease reactivation and exacerbation (Rotman *et al.*, 2009; Walsh and Locarnini, 2012). HIV

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influences hepatitis B prognosis by infecting CD4+ T cells responsible for priming HBV specific cytotoxic CD8+ T cells, successively clearing acute hepatitis B (Sobao *et al.*, 2002; Miao *et al.*, 2013). Furthermore, CD4+ T cells prime HBV specific memory B cells to produce antibodies responsible for hepatitis B resolution and immunity by natural infection or vaccination (Pasricha *et al.*, 2006). Therefore, loss of vaccine type and natural immunity to HBV occurring among HIV infected individuals is associated with lower CD4+ T cell counts (Thio, 2009). Injecting substance users at Mombasa city have a longer injecting substance use duration because they are initiated into substance injecting at an early age of about 10 years (NACADA, 2012). Longer duration substance use cause immune suppression, which reduces the probability of clearing HBV and HIV upon exposure (Thio *et al.*, 2002; Brown *et al.*, 2007; Reimer *et al.*, 2007; Riss *et al.*, 2012). Hepatitis B virus chronic clinical stage has been determined in HIV-1 infected injecting substance users in Mombasa city, Kenya (Day *et al.*, 2013). However, hepatitis B virus clinical stages between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya, have not been compared. Therefore, the current study compared hepatitis B virus clinical stages between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya.

2.6 Hepatitis B virus genotypes

The annotation of the HBV genome paved way for HBV genotyping and sub-genotyping. Hepatitis B virus genotyping and sub-genotyping, respectively, is based on intergroup divergence of >8% and >4% ≤ 8% in the complete genome sequence (Schaefer, 2007). However, in certain circumstances, the novel HBV genotype is proposed based on intergroup divergence of 7% ± 0.04% in the complete genome and epidemiological significance (Tatematsu *et al.*, 2009). In 1988, the first four HBV genotypes (A-D) were identified (Okamoto *et al.*, 1988). To date, six more genotype designated E, F (Norder *et al.*, 1994), G,

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H (Stuyver *et al.*, 2000) I (Tran *et al.*, 2008) and J (Tatematsu *et al.*, 2009) have been identified based on inter-group divergence of >8% in the complete genome sequence except for genotype I which differed by 7% ± 0.04% from genotype C (Tran *et al.*, 2008). Intra- and inter-genotypic recombination frequently occurring within the surface (S) and core (C) genes is the genesis and evolution of these currently classified hepatitis B virus genotypes (Simmonds and Midgley, 2005).

Genotyping has been used primarily to demonstrate the geographic spread and clinical outcome of hepatitis B virus genotype (Zhang and Cao, 2011). Genotype A is associated with high vaccine escape and lamivudine resistance mutation rates (Sheldon *et al.*, 2007) and is predominant in Northern Europe and Africa (McMahon, 2009a). Genotype B and C have a longer immune tolerance phase because of their higher HBeAg titers promoting virus persistence and chronicity and is prevalent in Asia (Walsh and Locarnini, 2012). Thus, genotypes B and C are the most virulent contributing significantly to the global hepatocellular carcinoma fatalities (McMahon, 2009a). Virus specific basal-core and pre-core promoter (Bc/BcP) mutations and or G1896A frequently detected during hepatitis B virus reactivation (commonly known as immune escape phase) is prevalent in genotype D (Couto *et al.*, 2013), and therefore the European and African regions including Kenya (Zhang and Cao, 2011; Kwange *et al.*, 2013). Genotype E is uniquely found in West Africa, East Africa and Madagascar, F and H are predominant in Alaska, Central America and Pennsylvania (McMahon, 2009a; Zhang and Cao, 2011) while I and J were identified in Vietnam (Tran *et al.*, 2008) and Japan (Tatematsu *et al.*, 2009), respectively.

In Kenya, molecular epidemiological studies have demonstrated the co-existence of genotypes A, D and E. For example, study involving blood donors in Nairobi detected

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genotypes A, D and E (Mwangi *et al.*, 2008). Another study isolated genotypes A and D from blood donors residing in Nairobi, Kisumu, Embu, Eldoret and Mombasa (Kwange *et al.*, 2013). A molecular epidemiology study detected genotypes A and D among liver disease patients in Nairobi (Kwange *et al.*, 2013). A longitudinal study involving HIV-1 patients on antiretroviral therapy at Nairobi detected only genotype A (Kim *et al.*, 2011). Only genotype A was also isolated from HIV-1 infected commercial sex workers and injecting substance users in Mombasa city, Kenya (Day *et al.*, 2013; Kibaya *et al.*, 2015).

HBV genotype infections vary by risk-population (Teshale *et al.*, 2011). For instance, HBV genotype A has been restricted to risk-population like injecting substance users and commercial sex workers while HBV genotype G is restricted to homosexual men (Sanchez *et al.*, 2007; van der Kuyl *et al.*, 2013). HIV infection influences HBV genotype infection (van der Kuyl *et al.*, 2013). For example, HBV genotype G infection are noticeable after infection with a "helper" HBV strain, such as genotype A and H, and especially during HIV-1 co-infection that decreases HBV immune control and increases HBV replication (Benhamou, 2007). HBV genotype A is circulating among HIV-1 infected injecting substance users in Mombasa city, Kenya (Kibaya *et al.*, 2015). However, HBV genotypes between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya, have not been compared. Thus, the current study compared hepatitis B virus genotypes between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya.

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CHAPTER THREE: METHODOLOGY

3.1. Study site

The present study was conducted at Bomu hospital in Mombasa city, Kenya. Bomu hospital is located on latitude -4.0434771 and longitude 39.6682065 West of Mombasa city, Kenya, an area of high rate of HIV and HBV infection and substance use (PEPFAR, 2012). Bomu hospital is a social enterprise whose purpose is to provide quality healthcare for all regardless of their economic status'. Apart from providing affordable health care service for the poor, Bomu hospital has a substance rehabilitation center and community-based outreach programmes that supports over 75% of injecting substance users in the west of Mombasa city, Kenya (PEPFAR, 2012). There are about 27,000 injecting substance users of whom 43.9% and 13.8% live with HIV and HBV, respectively in Mombasa city, Kenya (Deveau *et al.*, 2006; NASCOP, 2012; Kibaya *et al.*, 2015). Injecting substance use starts at an early age of between 10-12 years in Mombasa city, Kenya (Kahuthia-Gathu *et al.*, 2013). Injecting substance users in Mombasa city are predisposed to HBV and HIV because they engage in unprotected sex with multiple sex partners and share needle and syringes while injecting substance (Beckerleg *et al.*, 2005).

3.2. Study design and study population

This was a cross-sectional study targeting injecting substance users resident in Mombasa city, Kenya. Injecting substance users were defined as those who had injected substance during the previous month before the start of the study (UNODC, 2014). The specific criterion for ascertaining injecting substance users' status was checking for the presence of needle scars. Injecting substance users were recruited via snowball sampling method. Injecting substance users aged at least 18 years, exhibiting needle scars, reporting injecting substance use and provided written informed consent were recruited into the study. The injecting substance

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users were stratified into either HIV-1 infected or uninfected group based on their HIV-1 status. Sociodemographic information such as age, gender, injecting substance history, antiretroviral history and HIV infection status were recorded on a questionnaire (appendix 2).

3.3 Sample size

Sample size will be calculated using the following formula (Salganik, 2006):

$$n = D \frac{Z^2_{1-\alpha} P (1-P)}{d^2}$$

n = sample size of injecting substance users

$Z_{1-\alpha}$ = 95% Confidence interval usually 1.96

P = Prevalence of HBV using the five sero-markers in IDUs, about 21.0% (Lum *et al.*, 2008)

d = margin of error which is 5% (0.05)

D = Design Effect = 2

$$n = 2 \frac{2.58 \text{ x } 0.21 \text{x } 0.79}{0.0025} \quad 342.41$$

At least 343 injecting substance users will be recruited.

3.4 Laboratory techniques

3.4.1 Human immunodeficiency virus-1 testing

Each participant underwent pre- and post-HIV test counselling. Approximately, 3 mL of venipuncture blood was collected in anticoagulant tubes (Becton, Dickinson and Company, Franklin Lakes, USA) from each study participant. A drop of the blood was used for HIV-1 serological testing. The HIV-1 sero-positivity was determined using HIV rapid test strip (Abbott Laboratories, Tokyo, Japan) according to the Kenya national guidelines for HIV testing and counselling (NASCOP, 2010).

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3.4.2 CD4+ T cell measurements

CD4+ T cell counts were established using flow cytometer (Becton-Dickinson™, Franklin Lakes, USA). The anti-CD3, anti-CD4 and anti-CD45 fluorescent-tagged monoclonal antibodies were used in the CD4+ T cell enumeration. About 5.0 µl of blood samples were placed in a tube and red blood cell lysis buffer added. After 5 minute incubation, the cells were washed and fluorescent-tagged antibodies (anti-CD3, anti-CD4, and anti-CD45) were added. The cells were incubated for 30 minutes after which the samples were washed and the CD4+ T cells enumerated on the flow cytometer according to the manufacturer's recommendation (Becton-Dickinson™, Franklin Lakes, USA). CD4+ T cell counts were categorised into categories 1 (≥500 cells/µL), 2 (≥200<500 cells/µL) and 3 (<200 cells/µL) using the Centers for Disease Control (CDC) immunological staging (CDC, 1992).

3.4.3 Plasma separation

Plasma samples were separated according to previously established standard operating procedure (Tuck *et al.*, 2009). About 3.0 mL of blood was collected into heparinized tubes and centrifuged (2000 rpm) at 4°C for 20 minutes. After centrifugation, 1.0 ml of plasma was placed into 1.5 ml screw cap tubes using a clean pipette and labelled with study participant's identification number. Plasma samples in screw cap tubes were frozen immediately at -80°C freezer.

3.4.4 Determination of Hepatitis B virus sero-markers

The five hepatitis B virus sero-markers (HBsAg, HBsAb, HBeAg, HBeAb and HBcAb-IgM) were determined by testing of plasma samples using HBV-5 panel rapid test cassette (appendix 3) according to the manufacturer's instructions (Healthaw Medical limited, Hangzhou, China). About, 5 µl of plasma was added to the five sample wells (HBsAg,

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HBsAb, HBeAg, HBeAb and HBcAb-IgM sample wells) followed by two drops of buffer. The assay was let to stand for 20 minutes after which the sero-reactivity was recorded. A coloured band at both test and control zones indicated positive results for HBsAg, HBsAb and HBeAg. Coloured band at either control or test zone indicated negative or invalid results, respectively, for HBsAg, HBsAb and HBeAg. On the other hand, a coloured band at both test and control zone indicated negative results for HBeAb and HBcAb. Coloured band at either control or test zone indicated positive or invalid results, respectively, for HBeAb and HBcAb.

3.4.5 Determination of Hepatitis B virus clinical stages

Hepatitis B clinical staging was based on interpretation of the five HBV sero-markers (HBsAg, HBsAb, HBeAg, HBeAb and HBcAb) reactivities (Table 1) (Mahoney, 1999; Krajden *et al.*, 2005; Mast *et al.*, 2006).

Table 1: Hepatitis B clinical staging

Hepatitis B Clinical stage	HBsAg	HBsAb	HBcAb	HBeAg	HBeAb
Acute infection	+/-	-	+/-	-	-
Chronic infection	+	-	+/-	-	+
Vaccine type response	-	+	-	-	-
Resolved infection	-	+/-	+/-	-	+/-
Uninfected	-	-	-	-	-

Table legend: The clinical classification of the hepatitis B infection status was adopted from (Mahoney, 1999; Krajden *et al.*, 2005; Mast *et al.*, 2006). +, positive sero-marker; -, negative sero-marker; HBsAg, hepatitis B surface antigen; HBsAb, hepatitis B surface antibody; HBeAg, hepatitis B pre-core antigen; HBeAb, hepatitis pre-core antibody; HBcAb, hepatitis B core antibody.

3.4.6 DNA extraction

Hepatitis B virus DNA was extracted from HBV sero-positive plasma samples using viral DNA extraction kit according to the manufacturer's protocol (QiaAmp™, Valencia, USA). About, 200µL of plasma was added to 20µL protease in a micro-centrifuge tube. About

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200µL of buffer AL was then added to the sample, mixed and incubated at 56°C for 10 minutes. About 200µL of ethanol (96-100%) was added to the sample, mixed and centrifuged briefly. The mixture was then transferred to spin column, centrifuged at 8000 rpm for 1 minute and the filtrate discarded. About 500 µL of buffer AW1 was added to the spin column placed in a fresh 2mL collection tube, centrifuged at 8000rpm for 1 minute and the filtrate discarded. The spin column was then placed in a fresh collection tube and centrifuged at 18000 rpm for 1 minute and the filtrate discarded. Finally, DNA elution was performed by addition of 200 µl buffer AE to spin column in a fresh 2mL collection tube, followed by incubation at room temperature for 1 min, and then centrifugation at 8000 rpm for 1 minute.

3.4.7 DNA amplification

The HBV *PreS1*-gene region of acute and chronic hepatitis B virus infected injecting substance users were amplified by nested polymerase chain reaction (PCR). Nested PCR was performed using two sets of primers in two successive runs of PCR; with the second set used for amplifying a secondary target within the first run product. Therefore, the product from the second PCR amplicon had minimal contamination from unwanted products of primer dimers, hairpins, and alternative primer target sequences. Each PCR reaction contained 12.5 µL of 2 × PCR master mix, 1.25 µL of each primer and 5 µL of the HBV DNA to be tested in a final volume of 25 µL by adding molecular grade water. The amplification was performed with primers HBPr1 (position: 2850-2868, 5'-GGGTCACCATATTCTTGGG-3') and HBr135 (position: 803-822, 5'-CAAAGACAAAAGAAAATTGG-3') for the first round, and HBPr2 (position: 2867-2888, 5'-GAACAAGAGCTACAGCATGGG-3') and HBPr3 (position: 3226-3246, 5'-CCACTGCATGG CCTGAGGATG-3') for the second round (Stuyver *et al.*, 2000).

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The first and second PCR were performed under same conditions. Initialization step, essential for DNA polymerase heat activation, was achieved by heating the reaction to a temperature of 94°C for 9 minutes. Denaturation step consisted of heating the reaction to 94°C for 30 seconds. Denaturalization step caused DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules. The annealing step reaction temperature was lowered to 50°C for 30 seconds allowing annealing of the primers to the single-stranded DNA template. Stable DNA-DNA hydrogen bonds were formed only when the primer sequence very closely matched the template DNA sequence. The DNA polymerase bound to the primer-template hybrid and began DNA formation. At the extension/elongation step, temperature was increased to 72°C for 1 minute. At this step, the DNA polymerase synthesized a new DNA strand complementary to the DNA template strand by adding deoxynucleotide triphosphates (dNTPs) that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent DNA strand. Final elongation was performed at a temperature of 72°C for 9 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA was fully extended. Final hold, for short-term storage of the reaction, was performed at 4°C for 10 minutes.

To check whether the PCR generated the anticipated DNA fragments, 1% agarose gel electrophoresis was employed for size separation of the PCR products. The size of PCR products were determined by comparison with DNA ladder (a molecular weight marker), run on the gel alongside the PCR products. The presence of 360bp fragments in PCR products indicated positive results for HBV DNA.

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3.4.8 DNA purification

Amplified DNA products were cleaned using viral DNA Purification Kit according to the manufacturer's protocol (QIAquick™ Inc., Valencia, USA). About 75 µl of Buffer PBI was added to PCR product and mixed. The mixture was transferred to spin column placed in a 2 mL collection tube, centrifuged for 1 minute and the filtrate discarded. About 0.75 ml Buffer PE was then added to the spin column in the 2 mL collection tube, centrifuged at 1800rpm for 1 minute and the filtrate discarded. DNA elution was done by addition of 30 µL Buffer EB (10 mM Tris-Cl, pH 8.5) to the spin column in a fresh 1.5 ml micro-centrifuge tube, followed by centrifugation for 1 minute.

3.4.9 Genotyping of hepatitis B virus DNA

Direct sequencing was performed on the resulting purified PCR amplicons using sequencing kit (BigDye™ Terminator version 1.3, Foster City, USA) on genetic analyzer (ABI PRISM™ 3100, Foster City, USA). Forward and reverse sequencing reactions were performed using 1.25 µL of primers HBPr2 and HBPr3, respectively. The resulting forward and reverse chromatograms were scanned for base mis-calls and edited using ChromatoGate (CG) software version 1.2 (Alachiotis *et al.*, 2013). Pairwise alignment and consensus sequences (appendix 4) were generated using DNA Baser Sequence Assembler version 4.20.0 (Heracle Software, Germany). Homologies to the consensus sequences were identified in GenBank using Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990). Contiguous sequences were then aligned with complete HBV genotypes A-J reference sequences from the Genbank using ClustalW (Thompson *et al.*, 1997). Phylogenetic tree was constructed from the multiple sequence alignment using Neighbor-Joining method based on 1000 bootstrap replicates in MEGA version 6.0 (Hall, 2013; Tamura *et al.*, 2013). Hepatitis B genotypes were determined by interpreting the phylogenetic tree. HBV consensus sequences

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reported in this study can be accessed using GenBank accession numbers: KP407579, KP407581, KP407582, KP407583, KP407584, KP407585, KP407586, KP407587, KP407588, KP407591, KP407592, KP407593, KP407597, KP407599, KP407600 and KP407601.

3.5 Data analysis

Age and CD4+ T cell counts were compared between the HIV-1 infected and uninfected injecting substance users using Mann-Whitney U test. The CD4+ T cell immunological stages and gender were compared between the HIV-1 infected and uninfected injecting substance users using Chi-square test. Hepatitis B virus sero-markers were compared between the HIV-1 infected and uninfected injecting substance users using Chi-square test. Hepatitis B virus clinical stages were compared between the HIV-1 infected and uninfected injecting substance users using Fisher's exact test. Phylogenetic analysis was used to determine the HBV genotypes circulating among HIV-1 infected and uninfected injecting substance users. HBV genotype frequencies were compared between HIV-1 infected and uninfected injecting substance users using Fisher's exact test. All statistical tests were two-tailed and statistical significance was determined using *P*-value of less than 0.05.

3.6 Inclusion criteria

Injecting substance users who were 18 years or older and signed an informed consent form were included in the study

3.7 Exclusion criteria

Injecting substance users who were mentally unstable were excluded from the study.

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3.8 Ethical considerations

This study was part of a larger HIV-1 cross-sectional survey of injecting substance users that was ethically approved by Kenyatta University Ethical Review Committee number PKU019/116 of 2012 (appendix 5). This study was conducted according to Helsinki Declaration (Parsa-Parsi *et al.*, 2014). Written informed consent (appendix 6) was obtained from each participant before enrolment. All the study participants were provided with health education on sexually transmitted infections (STIs) including HIV, hepatitis B and C, tuberculosis, hygiene and nutrition. Participants testing positive for HIV were referred to the comprehensive care centers at Bomu Hospital for treatment, care and support.

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CHAPTER FOUR: RESULTS

4.1 Demographic and laboratory measurements of study participants

The demographic and laboratory measurements of the study participants are presented in Table 2. A total of 371 individuals were enrolled in to this study comprising of 157 (42.3%) HIV-1 infected and 214 (57.7%) HIV-1 uninfected injecting substance users. Gender distribution differed significantly between the study groups, such that the HIV-1 infected injecting substance users had a higher frequency of females compared to the HIV-1 uninfected injecting substance users (54.8% vs. 6.5%; $P<0.0001$). However, age distribution was similar between the study groups (median, 30.60; IQR, 6.50 vs. 31.71; 9.13 years; $P=0.271$). Most importantly, the HIV-1 infected injecting substance users presented with significantly lower CD4+ T cell counts relative to the HIV-1 uninfected injecting substance users (median, 456; IQR, 449 vs. 905; 641 cells/μl; $P<0.0001$). Consistent with the reduced CD4+ T cell counts, the frequency of CD4+ T cell counts <200 cells/μl (38.2% vs. 13.6%) and CD4+ T cell counts ≥200<500 cells/μl (15.9% vs. 8.4%) were significantly higher in the HIV-1 infected compared to the HIV-1 uninfected injecting substance users ($P<0.0001$).

Table 2: Demographic and laboratory measurements

Characteristic	HIV-1 uninfected,	HIV-1 infected,	*P*-value
Gender, n (%)			
Females	14 (6.5)	86 (54.8)	**<0.0001[a]**
Male	200 (93.5)	71 (45.2)	
Age (IQR), yrs.	31.71 (9.13)	30.60 (6.50)	0.271[b]
CD4+ T cells×10^3/μL	905 (641)	456 (449)	**<0.0001[b]**
<200[c]	29 (13.6)	60 (38.2)	
≥200<500[d]	18 (8.4)	25 (15.9)	**<0.0001[a]**
≥500[e]	167 (78.0)	72 (35.9)	

Legend: CD4+ T cells were categorized based on CDC categorization ([c] Category 3; [d] Category 2; [e] Category 1) (CDC, 1992). [a] Chi-Square test. [b] Mann Whitney U test.

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4.2 HBV sero-markers between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya

Comparison of HBV sero-markers between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya, is presented in Table 3. The frequency of HBsAg sero-marker was significantly higher in the HIV-1 infected injecting substance users compared to HIV-1 uninfected injecting substance users (9.6% vs. 2.3%; *P*=0.004). In contrast, the frequency of HBsAb sero-marker was significantly lower in the HIV-1 infected injecting substance users relative to the HIV-1 uninfected injecting substance users (8.3% vs. 16.8%; *P*=0.019). Although none of the study participants in HIV-1 infected and uninfected injecting substance users were reactive to the HBeAg sero-marker, HBeAb positivity was similar between the groups (13.4% vs. 12.1%; *P*=0.754). Consistent with the pattern observed for HBsAg reactivity, a significantly higher HBcAb positivity was observed in the HIV-1 infected injecting substance users compared to the HIV-1 uninfected injecting substance users (10.2% vs. 3.3%; *P*=0.008).

Table 3: HBV sero-markers between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya

Hepatitis B sero-marker	HIV-1 uninfected, n=214	HIV-1 infected, n=157	*P*-value
HBsAg	5 (2.3)	15 (9.6)	**0.004**[a]
HBsAb	36 (16.8)	13 (8.3)	**0.019**[a]
HBeAg	0 (0.0)	0 (0.0)	-
HBeAb	26 (12.1)	21 (13 .4)	0.754[a]
HBcAb	7 (3.3)	16 (10.2)	**0.008**[a]

Legend: Number (n) and proportions (%). HBsAg, hepatitis B surface antigen; HBsAb, hepatitis B surface antibody; HBeAg, hepatitis B pre-core antigen; HBeAb, hepatitis pre-core antibody; HBcAb, hepatitis B core antibody.
[a] Chi-square test.

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4.3 HBV clinical stages between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya

Comparison of HBV clinical stages between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya, is presented in Table 4. Significantly higher frequency of acute infection in the HIV-1 infected injecting substance users compared to the HIV-1 uninfected injecting substance users (5.7% vs. 1.4%; *P*=0.033). Likewise, a significantly higher frequency of chronic HBV infection was observed in the HIV-1 infected injecting substance users compared to the HIV-1 uninfected injecting substance users (5.1% vs. 0.9%; *P*=0.021). A significantly lower frequency of vaccine type response was observed in the HIV-1 infected injecting substance users relative to the HIV-1 uninfected injecting substance users (6.4% vs. 15.4%; *P*=0.008). The frequencies of past/resolved (8.3% vs. 11.3%; *P*=0.385) and HBV uninfected statuses (74.5% vs. 71.0%; *P*=0.482) were similar between the HIV-1 infected and uninfected injecting substance users.

Table 4: HBV clinical stages between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya

Hepatitis B Clinical stage	HIV-1 uninfected, n=214 (%)	HIV-1 infected, n=157 (%)	*P*-value
1	3 (1.4)	9 (5.7)	**0.033**[a]
2	2 (0.9)	8 (5.1)	**0.021**[a]
3	33 (15.4)	10 (6.4)	**0.008**[a]
4	24 (11.3)	13 (8.3)	0.385[a]
5	152 (71.0)	117 (74.5)	0.482[a]

Legend: 1=Acute (HBsAg+/-, HBcAb+/-); 2=Chronic (HBsAg+, HBeAb+, HBcAb-IgM+/-); 3=Vaccine type response (isolated HBsAb+); 4=Resolved infection (HBsAb+/-, HBeAb+/-, HBcAb-IgM+/-); 5=Susceptible (HBsAg-, HBsAb-, HBeAg-, HBeAb-, HBcAb-IgM-) (Mahoney, 1999; Krajden *et al.*, 2005; Mast *et al.*, 2006).
[a] Fisher's exact test.

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4.4: HBV genotypes between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya

Hepatitis B virus DNA was detected in 3 (100%), 2 (100%), 8 (88.9%) and 8 (100%) of acute hepatitis B HIV-1 uninfected, chronic hepatitis B HIV-1 uninfected, acute hepatitis B HIV-1 infected and chronic hepatitis B HIV-1 infected study participants, respectively. Sequencing of the *PreS1*-gene was successfully conducted for 16 (HIV-1 infected, n=12 and HIV-1 uninfected, n=4) out of the 21 hepatitis B virus DNA positive individuals. BLAST results illustrated that 6 (37.5%), 4 (25.0%), 1 (6.2%) and 5 (31.3%) of the sequences obtained in this study were closely homologous to HBV genotype A previously identified in Uganda, Haiti, Caribbean Island of Martinique and Central African Republic, respectively.

The phylogenetic analysis of the HBV genotypes isolated from HIV-1 infected and uninfected injecting substance users is presented in Figure 1. According to the phylogenetic analysis, all the 16 HBV isolated from acute and chronic hepatitis B infected injecting substance users were genotype A. The sub-genotype of the HBV genotype was A1, a predominant genotype in Africa. The HBV genotype reference sequences obtained from GenBank showed clear differences in genetic diversity. Most of the HBV genotype isolated from HIV-1 infected and uninfected injecting substance users clustered closely to each other with little or no genetic diversity.

Comparison of HBV genotypes between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya, is presented in Table 5. A significantly higher frequency of HBV genotype A infection was observed in the HIV-1 infected injecting substance users compared to the HIV-1 uninfected injecting substance users (75% vs. 25%; P=0.009).

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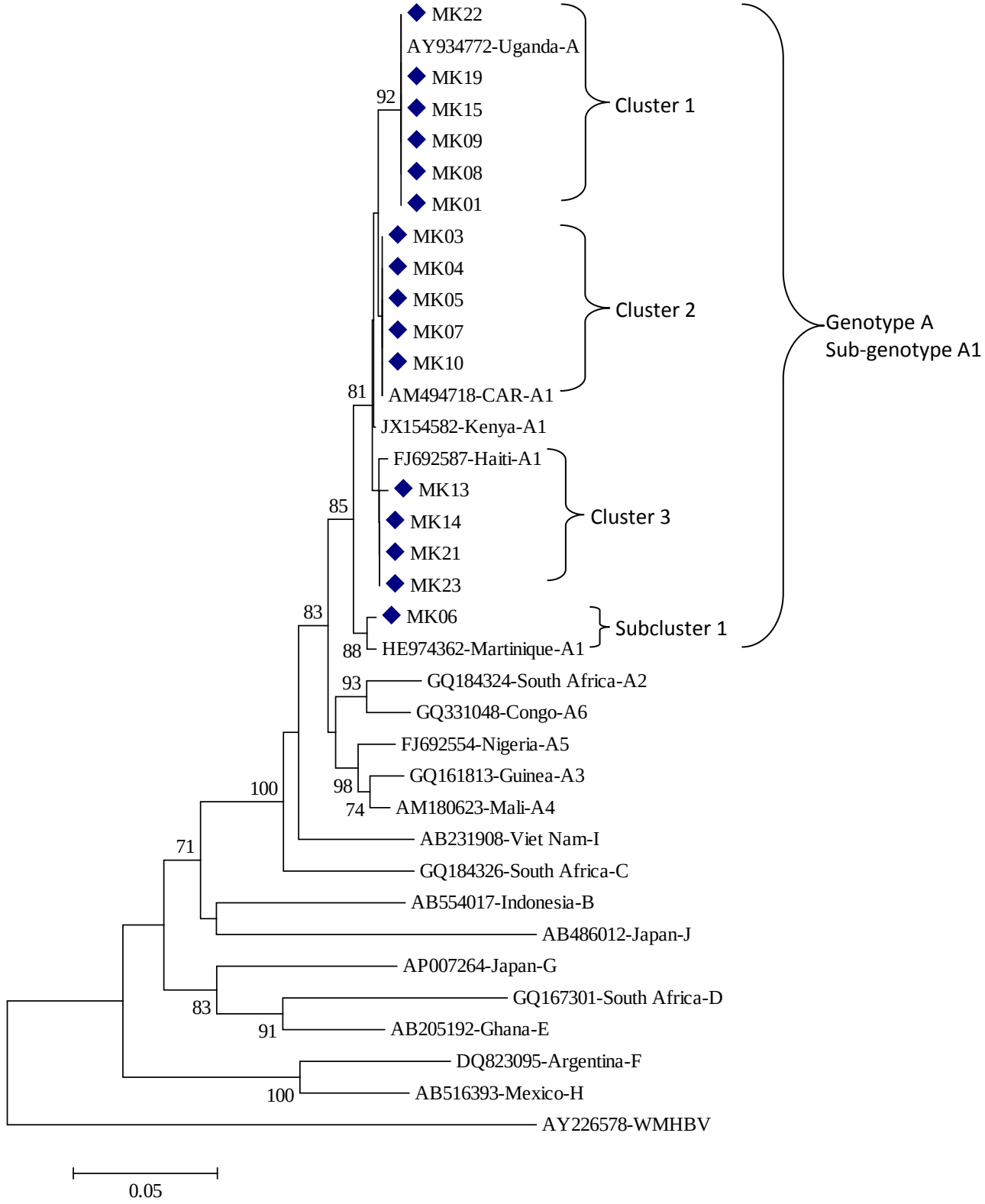

Figure 1: Phylogenetic analysis of HBV genotypes from injecting substance users.
Legend: Neighbor-Joining method based on 1000 bootstrap replicates using p-distances (Tamura *et al.*, 2013). HBV genotype sequences from GenBank together with their country of origin and accession numbers are presented. Wooly monkey HBV (AY226578-WMHBV)

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used as the out-group. Relevant bootstrap values are indicated. HBV isolates from study participants are indicated by diamond signs.

Table 5: HBV genotypes between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya

Genotype	HIV-1 uninfected, n=214	HIV-1 infected, n=157	*P*-value
A	4 (1.9)	12 (7.6)	**0.009**

Legend: Number (n) and proportions (%). HIV-1, human immunodeficiency virus type 1.
[a] Fisher's exact test.

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CHAPTER FIVE: DISCUSSION

5.1 Introduction

This study compared hepatitis B virus sero-markers, clinical stages and genotypes between the HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya. Results revealed that HBsAg and HBcAb sero-markers were higher in the HIV-1 infected compared to uninfected injecting substance users. However, HBsAb sero-marker was lower among the HIV-1 infected relative to the uninfected group. Acute and chronic HBV clinical stages were higher in the HIV-1 infected compared to the uninfected individuals. Vaccine type response clinical stage was lower in the HIV-1 infected relative to the uninfected group. HBV genotype A, that cluster together, was higher in the HIV-1 infected compared to the uninfected individuals.

5.2 HBV sero-markers between HIV-1 infected and uninfected substance users in Mombasa city, Kenya

The higher HBsAg and HBcAb sero-markers with concomitant lower HBsAb sero-marker detected in the HIV-1 infected injecting substance users indicates increased exposure and susceptibility to hepatitis B virus. These findings are consistent with previous studies illustrating higher HBsAg and HBcAb sero-markers and lower HBsAb sero-marker among HIV-1 infected compared to uninfected injecting substance users (Rodriguez-Mendez *et al.*, 2000). In this study, HIV-1 infected injecting substance users presented with lower CD4+ T cell counts. CD4+ T cells induce B cell production of HBsAb which neutralizes HBsAg (Chang and Lewin, 2007). However, higher HBcAb, a non-protective antibody to HBcAg, has been associated with HIV-1 related CD4+ T cell decline (Cohen Stuart *et al.*, 2009; Mikulska *et al.*, 2014). HIV-1 infected individuals have elevated naive B cells (Chong et al., 2004). Consequently, HBcAg bind and activate naive B cells to elicit HBcAb via CD4+ T

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cell independent pathways (Cao et al., 2001). Taken together, HBsAg and HBcAb sero-markers are higher while HBsAb sero-marker is lower among the HIV-1 infected compared to the HIV-1 uninfected injecting substance users in Mombasa city, Kenya. Therefore, injecting substance users in Mombasa city, Kenya, should be provided with vaccination to reduce hepatitis B infection.

5.3 HBV clinical stages between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya

The higher acute and chronic clinical stages with concomitant lower vaccine type response in HIV-1 infected injecting substance users indicate increased risk of acquiring acute infection and subsequent progression to chronic infection. The findings of this study are similar to previous study that showed higher acute and chronic clinical stages and lower vaccine type response among HIV-1 infected substance users (Tien *et al.*, 2004). A possible explanation for this observation is that HIV-1 infected injecting substance users in this study, presented with lower CD4+ T cell count. CD4+ T cells play an important role within the immune system by priming HBV specific cytotoxic CD8+ T cells, successively clearing acute hepatitis B infection (Sobao *et al.*, 2002; Miao *et al.*, 2013). Furthermore, CD4+ T cells are responsible for priming specific memory B cells to produce HBsAb following vaccination against HBV (Pasricha *et al.*, 2006). In contrast to the robust virus-specific CD4+ T cell response present in patients with self-limiting acute HBV infection, patients with chronic infections have reduced HBV specific T cell response (Sobao *et al.*, 2002; Chang *et al.*, 2005). Taken together, Acute and chronic HBV clinical stages are higher while vaccine type response clinical stage is lower among the HIV-1 infected compared to the HIV-1 uninfected injecting substance users in Mombasa city, Kenya. Therefore, initiating and up-scaling of screening, treatment and intensive medical follow-up programmes are essential for lowering

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the burden of hepatitis B infection among HIV-1 infected injecting substance users in Mombasa city, Kenya.

5.4 HBV genotypes between HIV-1 infected and uninfected injecting substance users in Mombasa city, Kenya

HBV genotype A, that clustered together, was higher in the HIV-1 infected than uninfected injecting substance users. This finding is consistent with previous studies involving injecting substance users from Netherlands (van Houdt *et al.*, 2007) and United States (Torbenson *et al.*, 2004) that detected only genotype A. Interestingly, the finding of this study is also similar to previous studies involving HIV-1 infected adults and commercial sex workers on antiretroviral therapy in Kenya (Kim *et al.*, 2011; Day *et al.*, 2013) that detected only genotype A. The HBV genotype A clusters observed in this study suggest high rate of transmission/re-infection among the injecting substance users via unprotected sex with multiple sex partners and needle and syringe sharing while injecting substance. This hypothesis is corroborated by previous studies that reported that injecting substance users at Mombasa city engage in unprotected sex with multiple sexual partners and share needle and syringe while injecting substance (Beckerleg *et al.*, 2005), which are efficient routes of acquiring HBV genotype A infection (Walsh and Locarnini, 2012). Taken together, HBV genotype A cluster is higher in the HIV-1 infected compared to uninfected injecting substance users in Mombasa city, Kenya. Therefore, harm reduction programmes such as needle and syringe exchange programmes (NSPs) and sexual education which emphasizes the use of protective devise such as condoms should be initiated so as to reduce HBV transmission/re-infection among HIV-1 infected and uninfected injecting substance users at Mombasa city, Kenya.

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CHAPTER SIX: SUMMARY OF FINDINGS, CONCLUSION AND RECOMMENDATIONS

6.1 Summary of Findings

HBsAg and HBcAb sero-markers were higher among the HIV-1 infected compared to uninfected injecting substance users. However, HBsAb sero-marker was lower among the HIV-1 infected relative to the uninfected group. Acute and chronic HBV clinical stages were higher among the HIV-1 infected compared to the uninfected individuals. Vaccine type response clinical stage was lower among the HIV-1 infected relative to the uninfected group. HBV genotype A, that clustered together, was higher among the HIV-1 infected compared to the uninfected individuals.

6.2 Conclusion

In summary, these results indicate that:

i. HBsAg and HBcAb sero-markers are higher while HBsAb sero-marker is lower among the HIV-1 infected compared to the HIV-1 uninfected injecting substance users in Mombasa city, Kenya.
ii. Acute and chronic HBV clinical stages are higher while vaccine type response clinical stage is lower among the HIV-1 infected compared to the HIV-1 uninfected injecting substance users in Mombasa city, Kenya.
iii. HBV genotype A cluster is higher in the HIV-1 infected compared to uninfected injecting substance users in Mombasa city, Kenya.

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6.3 Recommendation

i. HIV-1 infected injecting substance users in Mombasa city, Kenya, should be provided with vaccination to prevent hepatitis B infection.

ii. Initiating and up-scaling of screening, treatment and intensive medical follow-up programmes are essential for lowering the burden of hepatitis B infection among HIV-1 infected injecting substance users in Mombasa city, Kenya.

iii. Harm reduction programmes such as needle and syringe exchange programmes (NSPs) and sexual education which emphasizes the use of protective devise such as condoms should be initiated so as to reduce HBV transmission among HIV-1 infected injecting substance users in Mombasa city, Kenya.

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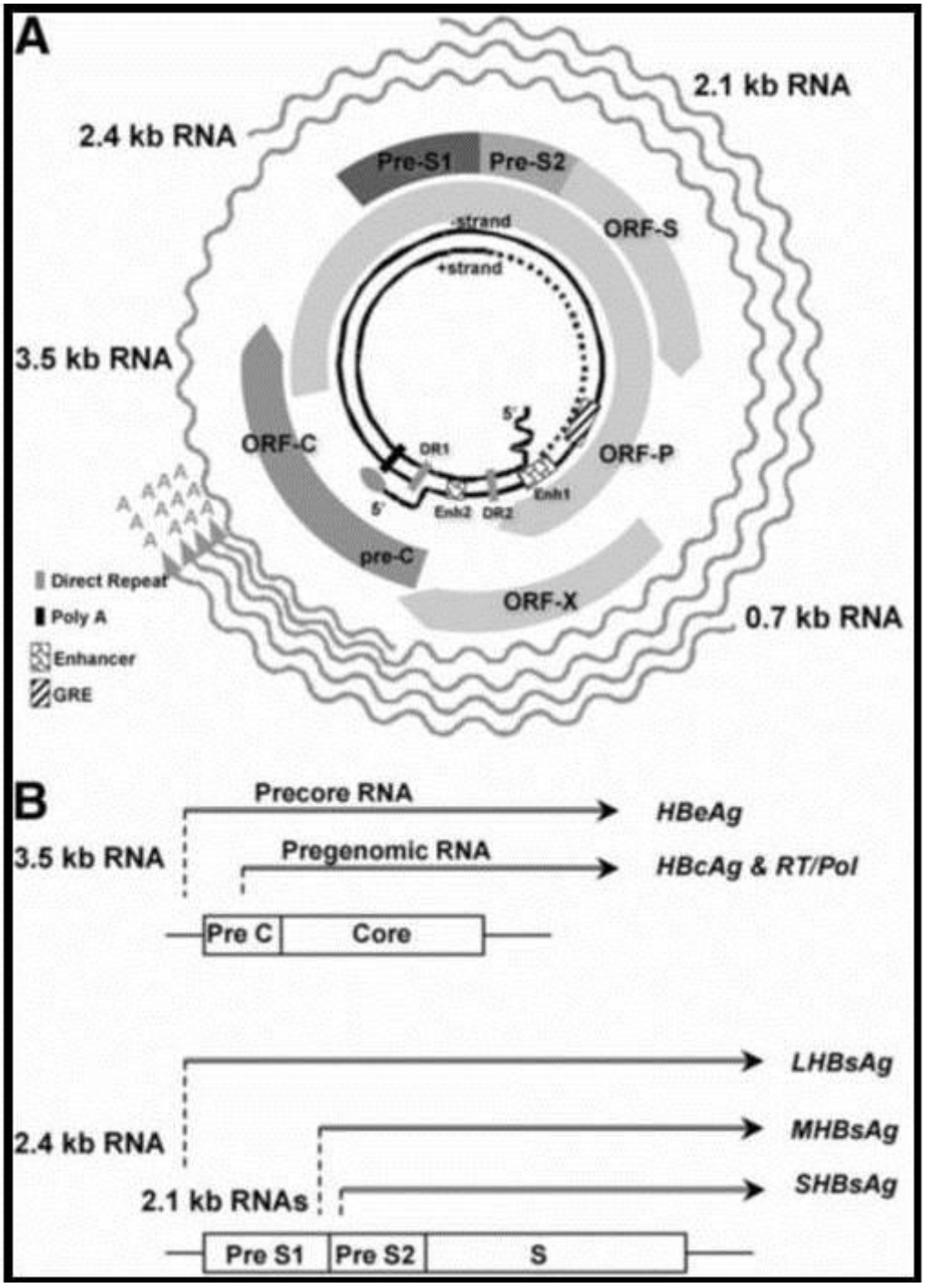
APPENDICES

Appendix 1: The HBV genome



(A) The genomic organization, partially relaxed circular double stranded DNA, RNA transcripts and gene products are shown. (B) The transcription start sites of various HBV transcripts and the proteins they encode. DR1 and DR2 are the direct repeats. Adopted from (Liang, 2009).

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Appendix 2: Study questionnaire

English Version

Title: HIV/Pulmonary TB Co-Infection amongst Intravenous Substance Users in Mombasa, Kenya.

Study participant code _____

Interview date_____/_____/_____

1. Age _____(Years) Birth date_____/_____/_____

2. Gender: ☐Male ☐Female

3. Do you inject yourself with substance?
☐Yes ☐No

4. How long have you injecting yourself with substance?_____ (Months)

5. Do you share needles are syringes with your friends while injecting substance?
☐Yes ☐No

6. Do you practice blood flashing?
☐ Yes ☐ No

7. Do you know your HIV status?
☐Yes ☐No

8. Are you on antiretroviral therapy?
☐Yes ☐No

Kiswahili version

Mada Ya Utafiti: Uambukizo pamoja wa virusi vya HIV na Kifua kikuu kati ya watumiaji wa mihadarati kwa kujidunga, Mombasa Kenya

Nambari ya siri ya mhusika _____

Siku ya kutahiniwa_____/_____/_____

`

1. Umri_____(Miaka) Tarehe ya kuzaliwa_____/_____/_____

2. Jinsia: ☐ Mme ☐Mke

3. Je unajidunga madawa ya kulevya kama substancei?
☐Ndio ☐ La

4. Umejidunga na substancei kwa muda gani?_____ (Miezi)

5. Je munatumia sindano kwa pamoja wakati mnajidunga substancei?
☐Ndio ☐ La

6. Je unajidunga na damu ya mwenziye aliyejidunga substancei?
☐ Ndio ☐ La

7. Je unajua hali yako kuhusu kirusi cha ukimwi?
☐Yes ☐No

8. Je unatumia madawa ya tiba thidi ya ukimwi?
☐Ndio ☐La

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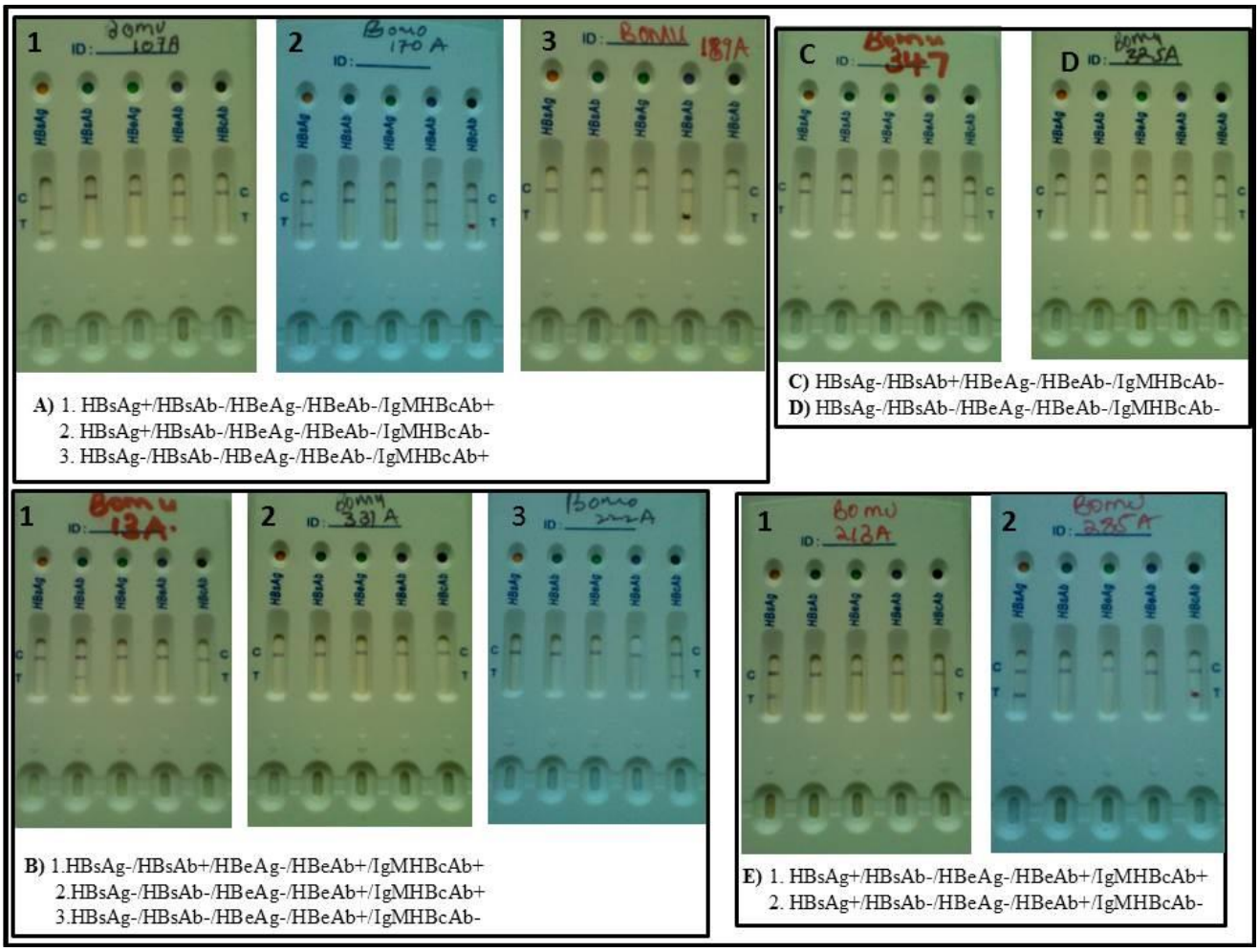
Appendix 3: Sero-reactive HBV-5 panel rapid test cassette



(A) Acute infection, (B) Disease resolution/Past infection/Acquired immunity, (C) Vaccine type response, (D) Uninfected (E) Chronic infection.

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Appendix 4: Study participants' hepatitis B virus fasta nucleotide sequences

>MK01
AAGGCATGGGGACAAATCTTTCTGTTCCCAATCCTCTGGGATTCTTTCCCGATCA
TCAGTTGGACCCTGCATTCGGAGCCAATTCAAACAATCCAGACTGGGACTTCAAC
CCCATCAAGGACCACTGGCCACAAGCCAACCAGGTAGGAGTGGGAGCATTCGGC
CCAGGGTTCACTCCCCCACACGGAGGTGTTTTGGGGTGGAGCCCTCAGGCTCAGG
GCATATTGGCTACAGTGCCAGCAGTGCCTCCTCCTGCCTCCACCAATCGGCAGTC
AGGAAGGCAGCCTACTCCCATCTCTCCACCTCTAAGAGACAGTCATCCTCAGGCC
A
>MK03
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CCCATCAAGGACCACTGGCCACAAGCCAACCAGGTAGGAGTGGGAGCATTCGGG
CCAGGGTTCACTCCCCCACACGGAGGTGTTTTGGGGTGGAGCCCTCAGGCTCAGG
GCATATTGGCTACAGTGCCAGCAGTGCCTCCTCCTGCCTCCACCAATCGGCAGTC
AGGAAGGCAGCCNACTCCCATCTCTCCACCTCTAAGAGACAGTCATCCTCAGGCC
A
>MK04
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AGGAAGGCAGCCTACTCCCATCTCTCCACCTCTAAGAGACAGTCATCCTCAGGCC
A
>MK05
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A
>MK06
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A
>MK07
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`

AGGAAGGCAGCCTACTCCCATCTCTCCACCTCTAAGAGACAGTCATCCTCAGGCC
A
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A
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A
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>MK21
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A
>MK22
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AGGAAGGCAGCCTACTCCCATCTCTCCACCTCTAAGAGACAGTCATCCTCAGGCC
A

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Appendix 5: Ethical Approval letter

KENYATTA UNIVERSITY
ETHICS REVIEW COMMITTEE

Fax: 8711242/8711575
Email: kuerc.chairman@ku.ac.ke
kuerc.secretary@ku.ac.ke
Website: www.ku.ac.ke

P. O. Box 43844,
Nairobi, 00100
Tel: 8710901/12

Our Ref: KU/R/COMM/51/32-4

Date: June 6th, 2012

Valentine Budambula
School of Public Health,
Kenyatta University
P.O. Box 43844, Nairobi.

Dear Ms. **Valentine**

APPLICATION NUMBER PKU019/I16 of 2012 - **'HIV/Pulmonary TB co-infection amongst intravenous drug users in Mombasa, Kenya. Version 4.**

1. IDENTIFICATION OF PROTOCOL

The application before the committee is with a research topic 'HIV/**Pulmonary TB co-infection amongst intravenous drug users in Mombasa, Kenya'**, Version 4. Dated 19th May, 2012.

2. APPLICANT

Valentine Budambula
School of Public Health,
Kenyatta University
P.O. Box 43844, Nairobi.

3. SITE

Mombasa County, Kenya.

4. DECISION REACHED.

The committee has considered the research protocol in accordance with the Kenyatta University Research Policy (section 7.2.1.3) and the Kenyatta University Ethics Review Committee Guidelines, and is of the view that against the following elements of review,

i. Scientific design and conduct of study,
ii. Recruitment of research participant,
iii. Care and protection of research participants,
iv. Protection of research participant's confidentiality,
v. Informed consent process,
vi. Community considerations.

AND APPROVED that the research may proceed for a period of ONE year from 6th June, 2012.

5. ADVICE/CONDITIONS

i. Progress reports are submitted to the KU-ERC every six months and a full report is submitted at the end of the study.

ii. Serious and unexpected adverse events related to the conduct of the study are reported to this board immediately they occur.

iii. Clearance must be obtained for transportation of any biological material out of the country i.e. Kenya.

iv. Notify the Kenyatta University Ethics Committee of any amendments to the protocol.

When replying, kindly quote the application number above.

If you accept the decision reached and advice and conditions given please sign in the space provided below and return to KU-ERC a copy of the letter.

PROF. NICHOLAS K. GIKONYO
CHAIRMAN ETHICS REVIEW COMMITTEE

I ..Valentine Budambula..... accept the advice given and will fulfill the conditions therein.

Signature..... Dated this day of.....6th June..... 2012.

cc. Vice-Chancellor
Director: Institute for Research Science and Technology

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Appendix 6: Informed consent form

English Version

Title: HIV/Pulmonary TB Co-Infection amongst Intravenous substance Users in Mombasa, Kenya.

Dear participant:

You are invited to take part in this research study because you have a history of intravenous substance use. This form tells you why this research study is being done, please read then you can decide if you want to join this study or not. The Investigators in this study are Kenyatta University and Technical University of Mombasa. A study team will be working closely with the investigators and the study will run for 3 years.

The purpose of this study is to determine the factors associated with HIV and pulmonary TB co-infections among intravenous substance users. If you choose to participate in this study, the team will require 3ml of blood (HIV voluntary testing and Complete Blood Count) and three early morning sputum (for TB testing) from you. No drug or chemical will be introduced into your body.

You can decide whether to take part in this study or not. You are free to say yes or no. If you say no, your regular medical care will not change. Even if you join this study, you do not have to stay in it, you may stop at any time. It is important to note that there is no financial benefit for participating in this study at the same time there will be no any cost implications to you. Participation in this study is important as the findings of the study have the potential of being used to lobby for funding for antiretroviral drugs (ARVs) and primary healthcare for substance users.

The risks in this study include possible discomfort due to questions on health and personal behaviour/history. In addition, discomfort may be experienced while a blood sample is being obtained. Every effort will be made to keep your study records confidential but we cannot guarantee it. No funds have been set aside to pay any costs if you are harmed because of this study. If you think that you were harmed because of this study, contact the Principle or co-Investigator.

By signing my name below, I confirm the following:

I have read (or been read to) this entire consent document. All of my questions have been answered to my satisfaction. The study's purpose, procedures, risks and possible benefits have been explained to me. I agree to let the study team use and share the health information and other information gathered for this study. I voluntarily agree to participate in this

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research study. I agree to follow the study procedures as directed. I have been told that I can withdraw from the study at any time.

Participant's Name..... Signature.....Date.....

Principal Investigator..... Signature.....Date..... Or supervisor

Note: Below are some of the key contacts

Principle investigator – Dr Tom Were 0720326127; Co-Investigator – Valentine Budambula 07222822448; KU-ERC kuerc.chairman@ku.ac.ke

Kiswahili Version

Mada Ya Utafiti: Uambukizo pamoja wa virusi vya HIV na Kifua kikuu kati ya watumiaji wa mihadarati kwa kujidunga, Mombasa Kenya

Kwako mhusika:

Unaalikwa kushiriki kwenye utafiti kwa sababu uko na historia ya utumiziaji wa mihadarati kama substancei kwa kujidunga shindano. Fomu hii inakuelezea kwa sababu gani utafiti huu unafanywa. Tafadhali soma fomu hii halafu uamue kama utashiriki kwenye utafiti huu. Watafiti ni wanatoka Chuo kikuu cha Kenyatta na Chuo cha utalamu cha Mombasa. Timu ya utafiti itafanya kazi kwa karibu na watafiti wakuu, na utafiti mwenyewe utachukua muda wa miaka miwili.

Nia hasa ya utafiti huu nikutathimini au kuamua sababu zinazohusishwa na uambukizo pamoja wa virusi vya HIV na kifua kikuu kati ya watumiaji wa mihadarati kwa kujidunga. Ukichagua kushiriki kwenye utafiti huu, hii timu ya watafiti itahitaji kiasi cha mililita 3 za damu kutoka kwako (kwa ajili ya upimaji wa hiyari wa virusi vya HIV na hesabu ya kiwango cha damu) na pia watahitaji makohozi ya asubuhi

(kwa ajili ya upimaji wa maambukizi ya kifua kikuu). Hakuna dawa ama kemikali zozote zitakazoekwa kwa mwili wako.

Unaweza kutoa uamuzi wa kushiriki kwenye utafiti huu au la, pia uko huru kusema ndio ama la. Ukisema la matibabu yako ya kawaida hayataathirika. Si lazima kubaki kama mshiriki

`

unaweza ukakatiza kushiriki wakati wowote. Ni muhimu kufahamu kwamba hakuna faida za kifedha kwa kushiriki kwenye utafiti huu. Wakati huohuo hautagaramika kivyovyote kifedha kwa kushiriki. Kushiriki katika utafiti huu ni muhimu kwa sababu, uvumbuzi ama majibu ya utafiti huu yatasaidia kupigania au kushawishi misaada ya kifedha kwa dawa za kuvunja makali ya virusi na pia afya ya msingi kwa watumiaji wa mihadarati.

Hatari zinazoambatana na kushiriki katika utafiti huu ni kama usumbufu kutokana na maswali ya kiafya na ya kibinafsi hasa tabia na historia yako. Kadhalika utahisi usumbufu hasa wakati wakutolewadamu. Juhudi zote zitafanywa kwa ajili ya kuhifadhi habari na jumbe zako zote kwa njia ya usiri wa hali ya juu. Lakini hatuezi kuhakikisha hili. Hakuna fedha ambazo zimehifadhiwa kwa ajili yakukufidia endapo utadhurika kutokana na utafiti huu. Kama unafikiria kwamba ulidhurika kutokana na utafiti huu wasiliana na mtafiti mkuu.

Kwa kuweka sahihi jina langu nathibitisha yafuatayo:

1) nimesoma (ama nimesomewa) karatasi hii ya kutoa idhini ya kukubali, na maswali yangu yote yamejibiwa na nimeridhika; 2) nia, mitindo, hatari pamoja na faida zinazoambatana na utafiti huu zimeelezwa kwangu; 3) nakubali na kuruhusu timu ya utafiti kutumia na kugawa habari za kiafya ama aina yoyote ya habari zitakazo kusanywa kutokana na utafiti huu; 4) nimekubali kwa hiyari kushiriki kwenye utafiti huu. Nakubali kufuata mitindo ya utafiti huu; 5) nimeelezwa kwamba ninaweza kukoma kushiriki wakati wowote.

Jina la mshiriki..... Sahihi..... Tarehe.....

Mtafiti mkuu/Msaidizi..... Sahihi..... Tarehe.....

Zaidi; wasiliana na wafuatao

Mtafiti mkuu – Dr. Tom Were 0720326127; Mtafiti msaidisi - Valentine Budambula nambari ya rununu, 07222822448; KU-ERC kupitia barua pepe kuerc.chairman@ku.ac.ke