DETERMINATION AND CHARACTERIZATION OF OCCULT HEPATITIS B IN HIGH-RISK POPULATIONS IN KENYA

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DECLARATION

This thesis is my original work and has not been presented for the attainment of a degree in any other university or institution.

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DEDICATION

I dedicate this work to my father, Mr. Solomon Kiptoon, My mum, Mrs Peris Ronguno, My daughter, Michelle Jemtai, and my two boys, Daniel Kibet and Raymonds Kipkorir. Your support and understanding during the years I have worked on this project are appreciated.

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ACRONYMS AND ABBREVIATIONS

ALT	Alanine transaminase
anti-HBc	Antibodies to Hepatitis core antigen
anti-HBe	Antibodies to Hepatitis e antigen
anti-HBs	Antibodies to Hepatitis surface antigen
ARV	Antiretroviral therapy
AST	Aspartate aminotransferase
BCP	Basal core promoter
BSC	Biosafety cabinet
cccDNA	Covalently-closed circular DNA
CBHI	Chronic Hepatitis B infection
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide trisphosphate
EIA	Electrochemiluminescence immunoassay
ENHI	Enhancer I
ENHII	Enhancer II
GGHs	Ground glass hepatocytes
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B virus
HBsAg	Hepatitis B surface antigen
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HIV	Human immune-deficiency virus
IDU	Injection drug use
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IRB	Institutional Review Board
KAVI	Kenya Aids Vaccine Initiative
kb	kilobase
LS	Large surface
mRNA	Messenger ribonucleic acid

MHR	Major hydrophilic region
MS	Middle surface
MSM	Men having sex with men
MSM-SW	Men having sex with men sexual workers
NAT	Nucleic acid amplification technology
NML	National Microbiology Laboratory
OBI	Occult hepatitis B infection
ORF	Open reading frame
preC RNA	preCore RNA
pgRNA	pregenomic RNA
PCR	Polymerase chain reaction
PWID	People who inject drugs
rcDNA	Relaxed circular DNA
RNA	Ribonucleic acid
rt-PCR	Real-time PCR
SS	Small surface
TB	Tuberculosis
WHO	World Health Organization

ABSTRACT

Occult hepatitis B virus (OBI) refers to the detection of HBV DNA in the serum or liver of individuals lacking the HBV surface antigen (HBsAg). It is often described among individuals having antibody to the core antigen as the sole serological marker of infection ("anti-HBc only"). However, it has also been observed in the absence of any serological markers ("seronegative OBI"). In cases of "seropositive" OBI, the presence and quantity of anti-HBs may provide an indication of putative risk for OBI, as isolated studies have shown absent or low levels (≤100 mIU/mL) of anti-HBs are more frequently associated with OBI. This has important consequences for blood donation screening and vaccination policy. The lack of HBsAg detection maybe attributed to the window period during acute infection, the virus's low replication phenotype, or a flaw in the expression or release of HBsAg. Mutations within the surface antigen-coding region have also been associated with loss of immunoassay detection for HBsAg. OBI often results in a non-symptomatic or benign infection, although it has been allied to serious hepatic disease, particularly in those co-infected with HCV or HIV, in which occult HBV is more frequently detected. The prevalence and characteristics of OBI in high-risk groups remain unknown in Kenya. Relying solely detection of HBV surface antigen (HBsAg) may lead to under-diagnosis of OBI cases, emphasizing the need for a comprehensive investigation to inform targeted public health interventions and strategic policies to combat HBV in Kenya. This study aimed at investigating the prevalence and molecular characterization of OBI in high-risk Kenyan populations. Sera from 65 HBsAg-negative patients presenting with jaundice at Kenyan medical facilities and 99 male sex workers (MSM-SW), along with 13 non-MSM men having HIV-positive partners were analyzed. Testing included HBV serological markers and additional hepatitis C virus (HCV) screening for MSM-SW patients. HBV DNA was extracted and analyzed using real-time polymerase chain reaction (rt-PCR). Of the 166 HBsAg-negative samples, 31 (18.7%; 95% CI 13.5 – 25.3) tested positive for HBV DNA, classifying them as OBI positive with an overall prevalence of 18.7%. Notably, 64.5% (20/31) of these cases were found to have HBV core protein antibody (anti-HBc positive). Among the MSM-SW cohort, a 10.1% (10/99; 95% CI 5.6 – 17.6) HBsAg positivity rate was observed. All samples tested negative for HCV. HBV genotype A predominated, with distinct genetic clustering patterns observed among the study cases, indicating potential common networks or non-African sources. The high prevalence of OBI across examined cohorts, along with the occurrence of chronic HBV infection in the MSM-SW population, highlights the necessity for targeted screening programs to enable preventive measures, including HBV vaccination, and ensure access to treatment and care.

CHAPTER ONE: INTRODUCTION

1.1 Background Information

Hepatitis B infection poses a significant threat to global health, particularly to citizens of developing countries (Zheng et al., 2011). Hepatitis B virus (HBV) is the principal causative agent of hepatitis B infection, which is spread via direct exposure to contaminated blood, like in the case of injection drug use (IDU), within families, between sexual partners, and from mother-to-child during pregnancy or at birth (Loarec et al., 2022). Based on serological assays for viral antigens and resultant antigens, numerous divergent outcomes have been described conventionally following HBV infection, encompassing chronic and acute hepatitis that may advance to liver cirrhosis, hepatocellular carcinoma (HCC), and rarely, fulminant hepatitis (Li et al., 2020). The Centers for Disease Control and Prevention (CDC) approximates that at least 296 million people around the world have HBV infection, including more than six million children below five years (CDC, 2022). Besides, it is attributed to >820,000 mortalities annually mainly from cirrhosis and HCC complications (CDC, 2022; WHO, 2018). The WHO estimates that 14 million, 18 million, and 60 million of the general population live with the disease in Europe, South-East Asia, and Eastern Mediterranean, respectively, while 5 million of adults in the Americas region are infected (WHO, 2018).

The prevalence and incidence of HBV infection is greatest in parts of Sub-Saharan Africa and Western Pacific regions, where at least 80 million and slightly above 100 million residents, respectively, are chronically infected (WHO, 2021). A recent investigation that conducted pooled analysis and systematic review approximated that the occurrence of chronic hepatitis B infection (CBHI) was 8.8% (82 million) in Africa, representing 25% of the infected population globally (Schweitzer et al., 2015; Sonderup & Spearman, 2022). The number of chronic carriers was

estimated to have doubled by 2017, with the infected having >20% odds of developing cirrhosis or HCC depending on the age of infection (Breakwell et al., 2017). East Africa is considered a high-risk region for HBV infection, with findings from a recent meta-analysis reporting a prevalence rate of 8.5% in Kenya and Uganda (Kafeero et al., 2021). Ly et al. (2016) analyzed data from HIV negative individuals in Kenya and found that out of the 1,091 respondents aged between fifteen and sixty-four years (equivalent to 31.5%), (n=344) had been exposed to HBV, while 2.1% (n=23) had CBHI. These figures correspond to the approximated 6.1 million and 398,000 Kenyans exposed to HBV and those with CBHI, respectively (Makokha et al., 2023).

Other studies have reported disparities in the occurrence of HBV infections across diverse geographical areas of Kenya. For instance, Mutuma et al. (2011) found an occurrence rate of 8.8% among patients seeking medical care for asymptomatic HCC in Turkana County. In another observational study, Okoth et al. (2006) evaluated HBV serological markers in n=2,241 pregnant women attending antenatal clinics in various clinics across Kenya. The findings showed that 8.8% (n=18) were positive for HBV e antigen (HBeAg), 9.3% (n=205) had HBsAg, while 30.2% (n=669) had been either vaccinated against HBV or exposed to the virus owing to the presence of anti-HBsAg (anti-HBs). A sero-positivity of HBsAg of >8% was reported by Wahome et al. (2022) in a study involving school-going first-time blood donors at Kwale Satellite Blood Transfusion Center (KSBTC). The results from 50 included studies in Makokha et al. (2023) systematic review and meta-analysis with a total sample size of 108,448 revealed an overall pooled prevalence estimate of 7.8%. These empirical findings show that HBV infections remain a public health concern in Kenya, and its prevalence ranges between 1% - 8 % in the general population (‘low-risk population’) (Ginzberg et al., 2018). The findings underscore the

substantial burden of HBV in Kenya, emphasizing the urgent need for public health interventions and strategic policies to bring the disease under control and ultimately achieve its elimination

Investigations involving high-risk populations, including HIV positive individuals, men having sex with men (MSM), injecting drug users (IDUs), jaundiced patients seeking medical care, and female sex workers, show remarkably elevated incidences and prevalence of HBV. Muriuki et al. (2013) focused on the prevalence HCV and HBV co-infections among 300 Kenyans HIV-positive individuals in Nairobi and reported a frequency of 16% HIV-HBV co-infection. Another study that involved screening of blood samples of n=1,308 residents of Korogocho and Viwandani slums aged between 15 and 54 for anti-HCV, HBsAg, and anti-HIV-1 found that n=10 (0.76%), n=174 (13.3%), and n=268 (20.4%) of the participants were HCV, HBV, and HIV positive respectively (Kerubo et al., 2015). Of the 268 HIV positive subjects, 0.46% (n=6) and 4.26% (n=56) had HCV and HBV respectively. Comparative findings were described by Harania et al. (2008) study, which reported 1% of n=378 HIV infected participants had HCV while 6% were HBV positive in Nairobi, Kenya. Similarly, subgroup analysis in Makokha et al. (2023) meta-analysis showed higher prevalence in patients with jaundice (41.7%) and lower prevalence in blood donors (4.1%). Notably, prevalence in HIV-infected individuals was 8.2%. The findings emphasize the critical need for targeted interventions and policies to combat HBV in these high-risk populations.

Chronic HBV infection is characterized by the persistent detection of HBsAg in serum, which remains as the principal diagnostic parameter in the screening for and confirmation of HBV infection in a large share of low-income nations (Asli et al., 2016). However, the existence of HBV core antigen antibodies (anti-HBc) IgM is an important serological marker in the window period in the resolution of an severe infection between the appearance and disappearance of

antibodies to the HBsAg (anti-HBs) (Song & Kim, 2016). The HBV genome is the foremost traceable indicator of an acute infection, and confirmatory tests for HBV DNA are especially valuable in the diagnosis of acute HBV infection before measurable levels of HBsAg in serum mount (Song & Kim, 2016). Previously, the elimination of HBsAg expression in chronic HBV patients was regarded as an indication of clinical remission and viral DNA clearance (Ferreira et al., 2014). However, HBsAg clearance followed by detectable low levels of HBV DNA in serum and liver tissues has been described in chronic or self-limited HBV infection; a phenomenon described as “occult” HBV (OBI) or “silent” HBV infection (Gachara et al., 2017; Saitta et al., 2022). OBI is defined as the detection of HBV DNA in the serum or liver of HBV exposed patients without the HBsAg, and it is often described in those with antibody to the core antigen as the sole serological marker of infection ("anti-HBc only") (Raimondo et al., 2007). However, it has also been observed in the absence of any other serological markers ("seronegative occult HBV") (Makvandi, 2016). In cases of "seropositive" occult HBV, the presence and quantity of anti-HBs may provide an indication of putative risk for occult HBV, as isolated studies have shown absent or low levels (≤100 mIU/mL) of anti-HBs are more frequently associated with occult HBV (Said, 2011).

1.2 Statement of Problem

Yearly, more than 820,000 people die due to HBV-associated conditions, such as chronic hepatitis, liver cirrhosis, and HCC (Sunbul, 2014). In Sub-Saharan Africa, > 8.8% of adult population harbor the virus, with 1.7 million of the infected acquiring the infection via unsafe injection practices and risky sexual behaviors (Sonderup & Spearman, 2022; WHO, 2015). Just like overt HBV, OBI) is more commonly detected in populations with a higher risk of HBV transmission than in those with lower risk of HBV transmission. These include individuals who

are HIV-infected, men who have sex with men (MSM), sex workers, injecting drug users (IDUs), as well as in regions with an endemic prevalence of HBV exceeding 8% (Bart et al., 2013; Su et al., 2017). HBV is not only highly prevalent throughout sub-Saharan Africa, it has also been suggested as among neglected tropical diseases disproportionately affecting the region (O'Hara et al., 2017). Understandably then, OBI is fairly prevalent throughout Africa, varying from nearly 7% - 10% in 'low-risk' populations comprising of blood donors or healthcare workers, to approximately 6% to 30% in HIV-infected populations (Bivigou-Mboumba et al., 2018a; N'Dri-Yoman et al., 2010a; Gachara et al., 2017; Sondlane et al., 2016; Oluyinka et al., 2015).

The prevalence of HBV infection in Kenya is projected to be between 1%-5%, and as outlined earlier, the rates among groups with elevated risk, such as HIV co-infected, IDUs, jaundiced patients seeking medical care are much higher (Ginzberg et al., 2018; Kerubo et al., 2015; Kilongosi et al., 2015; Muriuki et al., 2013; Ochwoto et al., 2016; Wahome et al. 2022). The incidence of OBI in high risk groups in Kenya is not known. Besides, the reliance on the detection of HBV surface antigen (HBsAg) as the principal diagnostic marker for HBV in low-income settings may potentially lead to under-diagnosis of OBI cases (Salyani et al., 2021). This is particularly concerning in regions where OBI prevalence is likely to be higher among high-risk groups. Therefore, the main objective of the current study was to investigate and characterize OBI in two groups at increased risk of HBV infection in Kenya, namely, jaundiced patients seeking medical care in various hospitals and MSMs. The secondary objective was to examine the association with anti-HBs quantitative levels. Understanding the prevalence and characteristics of OBI in these groups is critical for effective public health interventions and strategic policies to combat HBV in Kenya.

1.3 Justification of the Study

Infections with OBI is significant in diverse clinical settings. First, it can be spread via haemodialysis, organ transplantation, encompassing orthotopic liver transplantation, or blood transfusion (Kilongosi et al., 2015; Kwak & Kim, 2014). Second, OBI often results in a non-symptomatic or benign infection, although it has been allied to serious liver disease, particularly in those co-infected with HCV or HIV, in which occult HBV is more frequently detected (Kerubo et al., 2015; Gachara et al., 2017). Third, OBI infection may fasten the process from liver disease and HCC by incidentally contributing to persistent fibrosis and inflammation of the liver, as well as via its direct proto-oncogenic effect (Said 2011). Fourth, OBI may galvanize and cause acute HBV infections in individuals receiving chemotherapy or immunocompromised patients (Kwak & Kim, 2014). Ensuring timely linkage to care for individuals testing positive for OBI strategies within high-risk groups in Kenya is paramount in curbing the transmission of the virus and safeguarding public health. This initiative involves seamlessly connecting OBI-positive individuals with specialized healthcare facilities for tailored treatment and monitoring, while concurrently implementing targeted vaccination programs to provide long-term protection against HBV, ultimately fortifying the resilience of these vulnerable populations. Timely and strategic antiviral treatment, along with regular monitoring of the hepatitis B virus (HBV) DNA levels, can prevent the recurrence of HBV activity and subsequent worsening of a patient's clinical condition. The above reasons underscore the requirement for HBV DNA testing in high-risk population groups for the timely discovery and treatment of liver diseases to thwart progression to liver cirrhosis and HCC.

1.4 Null Hypotheses

1. H0: There is no significant difference in the prevalence of occult HBV infection (OBI) among high-risk population groups in Kenya.
2. H0: There is no significant variation in the genotypes of occult HBV infection (OBI) within the high-risk population groups in Kenya.
3. H0: Individuals lacking or having a low level (≤100 mIU/mL) of anti-HBs will have a higher rate of occult HBV than individuals having higher (>100 mIU/mL) anti-HBs levels.

1.5 Main Objective

To determine and characterize OBI in two groups of high risk populations in Kenya

1.6 Specific objectives

1. To determine the prevalence of OBI in high-risk population groups in Kenya
2. To determine the genotypes of the OBI from the high-risk population groups in Kenya
3. To establish the association between anti-HBs antibodies levels and OBI in the high-risk population groups in Kenya.

1.7 Significance of the Study

The research has addressed the lack of information on HBV and OBI among high-risk populations in Kenya by shedding light into the pathogenesis and diagnosis of OBI in patients presenting with jaundice and the MSM-SW group. The findings of prevalence offered new insights into the number of OBI cases, as well as the burden of HBV among the selected study groups. The results of the study will help to inform decisions of policymakers and medical practitioners in the healthcare sector in developing local protocols and guidelines for testing,

surveillance, diagnosis, monitoring, management, prevention, and control of OBI and overt HBV.

CHAPTER TWO: LITERATURE REVIEW

2.1 Hepatitis B Molecular Characteristics

HBV is a small virus that belongs to the *Hepadnaviridae* family of DNA viruses. It measures approximately 42 nm in diameter. The virus has a partly double-stranded (ds) circular DNA genome of 3.2 kb comprising of four overlapping open reading frames (ORFs) (Hossain & Ueda, 2017). HBV replicates via an RNA intermediate, and after an infection, its genome is transferred in to the nucleus of the host hepatocytes, where the partially dsDNA is transformed into a complete, covalently closed circular DNA (ccDNA). The latter utilizes the host cell replicative systems to create four HBV messenger RNAs (mRNAs), namely, the 2.1 kb and 2.4 kb surface antigen mRNAs, 0.7 kb X protein mRNA, 3.5 kb preCore mRNA, and pregenomic RNA (pgRNA/preC RNA) (Hossain & Ueda, 2017). The preC RNA encodes the preCore protein that is post-translationally processed into mature viral e-antigen (HBeAg), while the pgRNA encodes both polymerase enzyme and core protein, and it also acts as the template for DNA replication (Yao et al., 2018). The 2.1 kb and 2.4 kb surface antigen mRNAs encode viral small surface protein (SS), middle surface protein (MS), and large surface protein (LS). Thus, a mature infectious virion particle, aka Dane particle, is enclosed by the above surface proteins, which share a mutual C-terminal S domain containing a common termination codon at each start site (Yao et al., 2018). The HBsAg comprises 226 amino acids (aa), and the area between aa 99 – aa 169 is the major hydrophilic region (MHR) (Hossain & Ueda, 2017). The "α" determinant located on one of the extra-membranous loop within the MHR between aa 101 and aa 159, and it is considered as a hotspot for HBsAg mutations.

2.2 Transmission Mechanisms and Risk Factors of HBV and OBI

In the Kenyan context, the transmission dynamics of HBV are particularly complex, reflecting a diverse range of potential routes, including unprotected sexual encounters, sharing of contaminated needles among IDUs, and, notably, perinatal transmission from an infected mother to her newborn during childbirth (Downs et al., 2023). Moreover, unsterilized medical equipment, a concern in many healthcare settings, can serve as an unfortunate conduit for transmission (Salyani et al., 2021). However, the risk of HBV transmission to the general population in Kenya is significantly amplified due to the presence of specific high-risk groups within the population. For instance, MSMs in Kenya may engage in intimate sexual practices that heighten their risk of exposure to infected bodily fluids, similarly, IDUs in the country face an escalated risk due to the sharing of needles, a behavior that is unfortunately not uncommon (Downs et al., 2023). Commercial sex workers, operating within diverse and often high-risk environments, may be at increased risk due to the nature of their work, and PLWHIV are at a heightened risk due to their compromised immune systems, potentially rendering them more susceptible to HBV infection (Gachara et al., 2017). This convergence of high-risk groups significantly amplifies the potential for HBV transmission to the broader population in Kenya. Therefore, targeted interventions within these specific Kenyan populations are pivotal, not only for the health and well-being of these communities but also as a critical component in preventing the wider dissemination of HBV in the country. Implementing comprehensive strategies, including culturally sensitive education, accessible testing, widespread vaccination campaigns, and harm reduction practices, is essential in curtailing the spread of HBV within these communities in Kenya and, consequently, safeguarding the overall public health of the nation.

The risk factors for both OBI and CHBI are influenced by a range of socio-economic, cultural, and healthcare-related factors. OBI is particularly prevalent among individuals with compromised immune systems, such as those co-infected with HIV or undergoing immunosuppressive therapy (Lau et al., 2021; Smalls et al., 2019). Besides, past HBV infections can lead to OBI, and in Africa, where blood screening practices may not be uniformly comprehensive, transfusions can pose a risk (Downs et al., 2023). CHBI, on the other hand, is significantly associated with vertical transmission from mother to child, unprotected sexual contact, sharing of contaminated needles (especially among IDUs), and occupational exposure, particularly in healthcare settings (Makokha et al., 2023). Given the high prevalence of HBV in Kenya, particularly in certain regions, the risk of transmission is amplified, making prevention strategies, including vaccination and targeted screening, critical in mitigating the impact of both OBI and chronic HBV within the country. Additionally, culturally sensitive education and improved access to healthcare services play pivotal roles in addressing these risk factors and curbing the spread of HBV in Kenya (Makokha et al., 2023).

2.3 Mechanisms of Occult Hepatitis B Infection

Infection with HBV is associated with many forms of chronic outcomes, including HCC, cirrhosis, CBHI, and asymptomatic carriers (Ferreira et al., 2014). Recommendations from empirical studies indicate that even though the implementation of screening tests for HBsAg has remarkably decreased the transmission of the virus among blood donors, the available diagnostic techniques fail to detect "silent" or "occult" cases; the ongoing occurrence of HBV genomes in the hepatic tissue (with or without detectable DNA in serum) of people lacking HBsAg (Asli et al., 2016; Makvandi, 2016; Raimondo et al., 2007). OBI was first described in the 1970s when a novel type of HBV infection was detected in a person presenting with symptoms of acute

hepatitis testing positive for anti-HBc immunoglobulin G (IgG) antibodies but lacking HBsAg (Makvandi, 2016). Consequently, the clinical entity of OBI was characterized after the arrival of highly sensitive molecular techniques (Grob et al., 2000; Hu, 2002).

Patients test positive for both HBV DNA and HBsAg markers in classic cases of CBHI (Torbenson & Thomas, 2002). As a result, the failure to detect HBsAg has been considered as an equivalent marker for the absence of active viral replication and viral genome. Therefore, the detection of DNA of HBV in serum of individuals negative for HBsAg led to the description of OBI (Makvandi, 2016). In many cases, OBI is linked with antibodies to the core antigen (anti-HBc) in the absence of both anti-HBs and HBsAg, which resulted in the introduction of the concept of "anti-HBc alone" (Grob et al., 2000; Raimondo et al., 2007). Nonetheless, reports of seronegative OBI cases have been documented, where neither anti-HBc nor anti-HBs antibodies are detected in the serum or liver samples of the suspected patients (Raimondo et al., 2008; Saitta et al., 2022). Kwak and Kim (2014) reviewed studies on OBI status with regards to its description, detection, epidemiology, and mechanism. The researchers paid attention to the clinical significance of OBI by examining the spread, reactivation, prevalence, and its function in the evolution of liver fibrosis and HCC.

The findings showed that in OBI, viral DNA can be either traceable or untraceable, and in the former, the quantity is often <200 IU/mL (Kwak & Kim, 2014). False anti-HBc negativity and positivity in the diagnosis of OBI should be taken into account when it is described in terms of presence of anti-HBc alone, since some anti-HBc antibodies positive serum samples lack viral DNA (Madhavan et al., 2021). Besides, anti-HBc-negative status does not exclude seronegative OBI. Seropositive OBI can manifest in diverse situations, including HBsAg negative tests following recovery from past HBV infection, CBHI, and acute infection (Kwak & Kim, 2014).

Explicitly, “anti-HBc alone” can be reported when the titre of anti-HBs antibodies is significantly low in false positive cases, the “window period” in co-infection with HIV or hepatitis C virus (HCV), an acute HBV infection, and during a long period after HBV infection (Gachara et al., 2017; Gibney et al., 2008; Knöll et al., 2006). Conversely, seronegative OBI is attributed to primary OBI from the start of the contagion due to progressive loss of anti-HBs or mutations (Raimondo et al., 2007; Y. Kim, 2013).

Malagnino et al. (2018) analysed the records of a research medical facility in France to examine the occurrence of OBI in sample of 3,966 individuals concurrently investigated for HBV serology and DNA within a seven-year term. Results from serological assays showed that 32.9% had anti-HBs antibodies alone (post-immunization profile), 47.3% were sero-negative, and 9.5% samples had both anti-HBs and anti-HBc antibodies but lacked HBsAg indicating a resolute past infection (Malagnino et al., 2018). In addition, 5.5% of the patients had the surface antigen and anti-HBc antibodies but were negative for anti-HBs antibodies, suggesting an ongoing HBV infection, whereas 0.07% were positive for all the above serological markers, and 4.6% exhibited a sequestered positivity of anti-HBc antibodies. A concomitant quantification of HBV viral load was performed in 10% of the samples with serological profiles. While all the samples with positive anti-HBs profile tested negative for HBV DNA, 70% of HBsAg-positive samples had traceable HBV DNA. The patients with all the HBV serological markers had detectable HBV viral load, which confirms an unresolved infection irrespective of traceable anti-HBs antibodies. Of the n = 2283 HBsAg-negative samples, which were tested for HBV viral load, 2.33% was formerly positive with a substantial level of viremia >20UI/mL in 37 patients and <20 UI/mL in 14 patients. Retest results, however, illustrated that 4/14 had untraceable HBV DNA, suggesting that 1.2% (n = 47) of the patients were positive for OBI (Malagnino et al., 2018). Madhavan et

al. (2021) used the immunosorbent ELISA assay to determine the frequency of anti-HBc total antibodies in samples testing negative for HBsAg and PCR technique to examine the presence of HBV DNA in anti-HBc-positive patients. The results showed that 133/910 of the analysed serum samples turned positive for anti-HBc and 20/910 contained HBV DNA (Madhavan et al., 2021). How patients maintain a low but stable level of the active hepatitis B virus has been attributed to a diversity of mechanisms. These encompass the presence of mutations in the HBV S region, assimilation of the viral DNA into the host genome, altered host immunological response, and infection of peripheral blood mononuclear cells by the virus.

2. 3.1 Mutations

Available empirical evidence have shown that gene mutations contribute to the failure in detection of the viral surface antigen by viable diagnostic assays by influencing the antigenicity, secretion, and expression of the HBsAg (Zhu et al., 2016). Any alteration in the pre-S/S region may result in the inhibition of anti-HBs production and changes of the HBsAg antigenicity (Hu, 2002). Although sequencing of HBV nucleotide has demonstrated a broad array of HBV-genome mutations in pre-S/S region, investigations have reported that a great proportion of alterations appear arbitrary and nonsense with indeterminate consequences (Chen, 2018; Zheng et al., 2011). But, certain types of pre-S/S alterations are “hot spots” that could determine the occurrence of OBI, particularly within the immunodominant “α” determinant of S protein, by inhibiting the immunogenicity and antigenicity of HBsAg or viral secretion and replication (Zhu et al., 2016). Hu (2002) outlines that conformational arrangement of the S protein following alteration of the HBsAg gene may cause HBsAg untraceable by commercially available serological kits. For instance, nonsense mutations result in formation of premature stop codons in critical regions associated with HBsAg production, leading to the generation of truncated and

nonfunctional surface antigens (Rajput, 2020). Notably, the "α" determinant of the HBsAg comprises of 124-147 amino acids within a 2-loop structure full of cysteine residues that are essential in the formation of disulfide bonds and conformation maintenance in the region. Thus, substitution of a solitary aa in the “α” region may modulate an escape from the release of circulating antibodies to the HBsAg, which causes the development of OBI or an active infection after HBV immunization (Zhu et al., 2016).

Elkady et al. (2017) examined the occurrence and virological characteristics of OBI infections in individuals presenting with hematological cancers in Egypt. The study involved serological analyses and quantification of HBV DNA from 165 serum specimens. The findings revealed two escape mutations S143L and P120T associated with the occurrence of OBI infections in the tested serum samples (Elkady et al., 2017). In another study, Chen (2018) appraised the role of the pre-S/S area and the associate variants in the onset and advancement of liver diseases. From the review, the researchers revealed five pre-S/S variants based on the various types of mutations, namely, pre-S/S nonsense mutation, C-terminus S point mutation, pre-S1 splice variant, pre-S point mutation, and pre-S deletion. The obliteration in C-terminal and S-promoter half of the pre-S1 were substantially predominant in subjects with enduring hepatitis and hepatic disease (Chen, 2018). Observably, pre-S deletion mutations have been attributed to liver cirrhosis and HCC by stimulating endoplasmic reticulum (ER) stress, which caused the induction of Akt/mammalian target of rapamycin signaling in ground glass hepatocytes (GGHs) and elevated production of vascular endothelial growth factor-A (Chen, 2018). Zhang et al. (2016) examined the frequency of OBI in households with HBV infection in China and sought to clarify the relationship between S gene genetic diversity and the occurrence of OBI. The S gene from OBI or CBHI samples was amplified, cloned, and sequenced.

In addition, HBsAg-positive patients were used as controls to enhance the comparability and reliability of findings as they had elevated odds of having a shared source of infection. Besides, OBI patients often share similar genetic and living backgrounds; thus, allowing researchers to offer a meaningful evaluation between the test and control cohorts. The assay illustrated that, a large share of the clones from 15 OBI samples were comparable to their HBV positive family members, suggesting that there is elevated odds for most OBI patients to acquire the infections from similar sources. In the above study, deletion/insertion mutations were attributed to OBI in 4.4% and stop codon mutations in 13.6% of patients, respectively, compared to 1.9% and 1.5% in the control cohort respectively. Based on the above observations, Zhang et al. (2016) resolved that the alterations only affected HBsAg secretion, expression, and/or antigenicity in a marginal of the OBI subjects since they occurred only in 18% of the cloned sequences. HBsAg aa residues spawning between 170 – 179 in the test cohort were highly likely to be altered compared to those in control group. Besides, the regularity of aa mutations in the MHR, C-terminal, and N-terminal, encompassing the "α" determinant region, of the S gene in the experimental cluster were not substantially disparate from those in the comparison cohort (Zhang et al., 2016). In particular, single point alterations in the "α" determinant region and MHR that may influence the antigenic activity of HBsAg, like T123N, D144A, Q129N, and G145R, were hardly noted in the sequenced clones, suggesting that point alterations in the S gene serves no to little function in the occurrence of OBI.

In an Indian study, 68 gene modifications were noted, including in the S and its overlapping P genes (Saha et al., 2017). Of the numerous gene alterations detected, sQ129R was the principal mutation in the surface gene that was strongly correlated with the occurrence of OBI, which has been previously documented to be allied to perinatal transmission and diagnostic escape (Yao et

al., 2013). Furthermore, a study involving a case of *de novo* HBV infection in a HBV naïve organ recipient described HBV infection reactivation with altered HBsAg carrying sQ129R following liver transplantation from an absolutely seronegative person (Blaich et al., 2012). The sQ129R mutation affects the structure and properties of the surface antigen, potentially leading to reduced antigenicity. As a result, individuals with this mutation may have low or undetectable levels of HBsAg in their blood, making it challenging to diagnose the infection using conventional methods. Indeed, a lab-based investigation that involved analysis of mutations on the HBV surface protein in the occurrence of OBI among people donating blood in China revealed that sQ129R substantially diminished the expression of HBsAg from liver cells, thereby offering a potential reason underlying the absence of HBsAg in the OBI populations (Huang et al., 2012).

The above observations are inconsistent with the findings of a recent study involving patients co-infected with HIV in Cameroon (Gachara et al., 2017). In the latter research, G159A mutation was the principal mutation occurring in the "α" determinant area within the MHR region observed in 80% of the serum samples compared to 41.3% in typical HBV infections. Kim et al. (2017) compared the molecular features of OBI infections in anti-HBc only versus 20 control individuals with typical HBV infections in South Korea. The study involved testing for anti-HBc, anti-HBs, and HBsAg in all the sera specimens, and those with anti-HBc positive results were further quantified for viral load. The findings revealed 19 point mutations of the MHR from n = 5 samples, including M133T, P127S/T, and S114T. Numerous aa substitutions were noted in the MHR from aa 100 – aa 160 in patients with anti-HBc alone OBI. Tian et al. (2007) investigated the occurrence and clinical characteristics of OBI and the associated mutations in blood donors. The study revealed a high regularity of alterations between aa119-aa123. In

particular, as noted in OBI positive samples from patients with hematological malignancies in (Elkady et al., 2017) study, Tian et al. (2007) attributed P120T and T123A (aa120-aa123) to OBI in one patient. Notably, substitution of aa of the "α" determinant area in the MHR results in conformational alterations that influence attachment of the neutralizing antibody; consequently, resulting in a false negative HBsAg assay (Tian et al., 2007). In particular, alterations in the "α" determinant area result in the abolition of the two loops, which, in turn, cause changes in the acidity, electrical charge, or hydrophilicity of the loop. Therefore, it is anticipated that patients with isolated anti-HBc sero-positive status with a high viral load often contain HBsAg mutants (Launay et al., 2011).

2.3.2 HBV DNA Integration

During both acute and chronic HBV infections, HBV genome can be incorporated into the host chromosomes, and both episomal and assimilated HBV molecules can be found in OBI patients (Britchot et al., 1996). Pollicino and Raimondo (2014) add that although assimilation of HBV DNA plays no function in the viral multiplication process, it may result in lasting persistence of the virus in the infected hepatocytes, independent of the HBsAg negative or positive status. However, Pollicino and Raimondo (2014) warn that the detection of incorporated viral genome in HBsAg negative individuals should not be straightforwardly regarded as OBI, as the latter is essentially associated with intrahepatic persistence of whole, episomal, replication competent HBV DNAs. Nonetheless, a study by Saitta et al. (2015) investigating and characterizing HBV genome integration in HCC specimens from OBI subjects noted that 75.5% (37/49) of the OBI-positive HCC tissues had integrated HBV DNA. Furthermore, none of the OBI-negative HCC specimens had HBV DNA incorporated into the patient chromosomal genome. In another study, researchers examined the frequency of OBI in individuals diagnosed with HCC and related

hepatic ailments, as well as identify cases of HBV DNA assimilation into the their hepatic cells' DNA (Wong et al., 2020). The scholars emphasized that OBI patients displayed an elevated frequency of HBV DNA integration, which is a potential contributor of host cell transformation and advancing malignancy. Thus, although more investigations are required to validate the HBV DNA integration mechanism, it is a possible explanation for the occurrence of OBI.

2.3.3 Co-infection with Other Viruses

Many studies published in the 90s have well-documented cases of co-infection of hepatitis B and C (Chan et al., 1991; Chen et al., 1990; Fattovich et al., 1991). The findings of these investigations illustrated that reduced cases of HBV replicative intermediates were predominant in people co-infected with HCV. Another study by Cacciola et al. (1999) found that 33% of the subjects with hepatic disease due to chronic HCV have measurable HBV genome but no detectable HBsAg in serum. In addition, these scholars noted a substantial correlation between OBI and HCV-associated cirrhosis. These studies suggest that the presence of both HCV and HBV causes mutual viral interference between the viruses making the affected patients show delayed appearance, reduced level, and shortened expression of serum antigenemia.

In Malagnino et al. (2018) study, HIV co-infection was predominant in the OBI patients (18/47), with an inclination towards an elevated regularity in the anti-HBc only cohort (14/26) versus the anti-HBs/anti-HBc group (4/21). The appearance anti-HBs in OBI specimens is congruent with the observations made in a recent investigation involving patients co-infected with tuberculosis, which found that 50% of the patients with OBI had both anti-HBc and anti-HBs markers (Trigo et al., 2016). Mphahlele et al. (2006) examined the effect of HIV infection on the routine detection of HBsAg in HIV-positive and HIV-uninfected specimens in South Africa in a retrospective, unmatched case control study. The results showed that both cohorts had equal

chance of HBV exposure; however, HIV-infection alone was noted as a key risk factor for OBI. In particular, 33.3% of sera with anti-HBc alone seromarkers from HIV-positive/HBsAg-negtaive had positive HBV DNA compared to 0% in the HIV-negative cohort. The potential elucidations for the above scenarios (anti-HBs, HBV DNA, anti-HBc positive) in HBsAg-negative samples is that the anti-HBs immunoglobulins were incapable of neutralizing the virion particles owing to the loss of antigenicity, thereby enabling the altered viruses to evade neutralization even when the amount of anti-HBs is at protective levels (Levicnik-Stezinar et al., 2008; Oluyinka et al., 2015; Trigo et al., 2016).

In another a cross-sectional retrospective research, Tramuto et al. (2013) evaluated the prevalence, causal factors, and genotypic features of HIV-positive/HBsAg-negative/HBV DNA-positive patients in Sicily, Italy. It is hypothesized that infection with HIV has a significant influence on the occurrence of OBI by lowering $CD4^+$ counts. Immunosuppression triggers HBV reactivation and can result in elevated viremia devoid clinical manifestations. However, their findings revealed that even though anti-HBc-positive/HBV DNA-positive samples was correlated with elevated levels of HIV RNA, there was insignificant disparity in the mean $CD4^+$ counts between HIV negative and HIV infected subjects, and the incidences of OBI were relatively similar between cohorts irrespective of their $CD4^+$ count in the plasma (Tramuto et al., 2013). In an investigation conducted in India, researchers analyzed n = 441 HBsAg-negative serum samples to determine the incidence of OBI among HIV-positive individuals (Saha et al., 2017). The results showed that 6.3% of the 441 cases were positive for OBI, including 2.2% seronegative and 17.8% anti-HBc positive cases, a rate that is comparatively lower than the 11.3% HIV/HBV co-infection reported in a previous investigation done in Eastern India (Saha et al., 2013). In addition, the overall elevated frequency of OBI in HIV positive individuals in

India, mainly among anti-HBc-positive individuals, is relatively consistent with the occurrence described in Sub-Saharan Africa, including frequencies observed in Cameroon and South Africa (Bell et al., 2012; Gachara et al., 2017; Lukhwareni et al., 2009; Mphahlele et al., 2006).

HCV co-infection was noted in 6.4% of the OBI positive samples, but there was no difference in serological markers between cohorts in Malagnino et al. (2018) investigation. The above finding is supported by Fabris et al. (2008) research, which noted a significant correlation between OBI and HCV co-infections, but inconsistent with the outcomes observed in Tramuto et al. (2013) study. The latter found uncommon OBI cases among HCV-positive subjects, with the consistent use of antiretroviral therapy (ARV) in the HIV-positive participants possibly contributing to the decreased incidence of OBI (Tramuto et al., 2013). Similarly, Khattab et al. (2005) reported an converse association between HBV DNA and HCV RNA levels, with only n = 4/53 subjects classified as OBI-positive having co-infection with HCV. Interestingly, all of the four patients with both OBI and hepatitis C infection did not respond to treatment for HCV, possibly owing to OBI escape mutants.

2.4 Prevalence of Occult Hepatitis B

Although the global occurrence of overt CHBI is well described, the geographic variability in the occurrence of OBI has not been carefully investigated (Torbenson & Thomas, 2002). Nonetheless, a topical meta-analysis examining the frequency of OBI in Northern America and Western Europe reported an overall prevalence of 34% among 2,729 HBsAg – negative subjects enrolled in 34 studies (Pisaturo et al., 2019). When data from individual studies published between the late 90s and early 2000s are considered, it appears that the rates of occurrence of OBI in Asia are higher compared to in other parts of the globe. For instance, Koike et al. (1998) recorded 95% positive cases of OBI in chronic HCV patients in Japan, and in a similar

investigation involving HCV co-infected subjects described 86.7% positive incidences, representing n=26/30 Japanese patients (Uchida et al., 1997). Similarly, other researches performed by Fukuda et al. (2001) and Nirei et al. (2000) among Japanese chronic HCV patients recorded OBI occurrence rates of 49% (N=45) and 37% (N=49), respectively. A Yuen et al. (2010), however, reported a low prevalence of 0.13% in n=3044 blood donors in North Eastern China, while Li et al. (2008) recorded 0.1% OBI-positive cases in n=10,727 Taiwanese blood donors, and Song et al. (2009) noted 0.7% of n=1,047 HBsAg negative were OBI positive. Nevertheless, it is challenging to match statistics from single studies since the investigation populations vary substantially in antecedents that possibly affect the occurrence of OBI, such as the HBV genome diagnostic tool employed and HBV risk factors in the study groups.

In Africa, a region that is considered high intermediate endemic for HBV, the prevalence is estimated to be approximately between 5–8%, and has been suggested to be a neglected tropical disease disproportionately affecting the region (O'Hara et al., 2017). Understandably then, OBI is fairly prevalent throughout Africa both in high risk and low risk cohorts. Most studies investigating the occurrence of OBI in sub-Saharan Africa have focused on high risk populations, and as expected, they have recorded higher rates than in low risk cohorts (blood donors). For example, Fopa et al. (2019) examined more than 1,000 blood samples donated between August 1st 2016 – March 31st 2017 in Yaoundé, Cameroon to establish the occurrence of OBI in the country's HBV low-risk population. The authors found a frequency of anti-HBc and HBsAg reactivity of nearly 50% and 8%, respectively. Furthermore, approximately 1% of the 522 anti-HBc positive, HBsAg negative subjects met the description of OBI, implying that one in 200 blood units distributed for transfusion in various Cameroon hospitals contain potentially infectious HBV DNA (Fopa et al., 2019).

N'Dri-Yoman et al. (2010) investigated the occurrence of OBI in 495 untreated HIV-1-positive adults in Côte d'Ivoire with <500 cells/mm^3 $CD4^+$count. 10% (n=51) of the ART-naïve patients had detectable HBV DNA in a cross-sectional study. Bivigou-Mboumba et al. (2018) analysed samples from HIV-infected patients receiving ART treatment for HBV serological markers in Gabon. The researchers also determined the HBV viral load by rt-PCR and conducted molecular characterization of the HBV DNA positive specimens via sequencing and phylogenetic analysis. The study recorded a higher OBI prevalence of 17.5% compared to that of 5.9% reported by Gachara et al. (2017) among HIV-infected subjects in Cameroon, but lower than the 26.5% recorded among HIV-positive Botswana's patients receiving tenofovir/emtricitabine therapy (Ryan et al., 2017). The disparities are possibly linked to the discrepancy in the sensitivities of the amplification techniques employed by the two investigations, the less sensitive nested PCR in the Cameroonian study versus the more sensitive rt-PCR in Botswana's case.

Nonetheless, whereas one patient had anti-HBc only OBI in the Bivigou-Mboumba et al. (2018) study, and the most pervasive genotype was A3 tailed by genotype E in Gachara et al. (2017) study. In ten samples were regarded anti-HBc alone, genotype E was more common than genotype A. Likewise, Allain et al. (2009) screened samples for HIV-1, HCV, and HBV genomes from n = 109 individuals donating blood in South Africa. Specimens containing HBV DNA but negative for HBsAg were tested for confirmation using rt-PCR, nested PCR, and positive samples were tested for anti-HBs and anti-HBc. The authors then amplified the pre-S/S region, performed whole genome sequencing, and analyzed and matched to 73 HBsAg positive strains. In addition, genotypes were determined by phylogenetic examination. Of the 109 blood units, 54 were OBI positive (49.5%), 14/109 were regarded as within the window period of pre-

seroconversion, and 20/109 were considered false-positive OBI cases. The most commonly circulating genotype A1 was more prevalent (n=23) than its counterpart genotype D (n=7).

Similar observations were made in Sudan, where both OBI and overt HBV cases are rampant. For instance, Mudawi et al. (2014) investigated the occurrence of OBI and overt HBV infection among ART-naïve HIV-positive patients in Khartoum, Sudan through a cross-sectional study. HIV, HCV, and HBV infections were assayed using immunoassays for anti-HIV, anti-HCV, and anti-HBc antibodies, respectively, while rt-PCR was employed to quantify HBV DNA viral load. Confirmation of HBV infection was discovered in 62.8% of the HIV-co-infected subjects, including 15.1% OBI and 26.8% overt HBV cases. From the above studies involving HBV high-risk populations, there is a necessity for OBI screening to enhance patient outcomes. In Burkina Faso, Diarra et al. (2018) analysed blood samples for anti-HBc, anti-HBs, and anti-HBe serological markers from n=219 HBsAg-negative individuals in Burkina Faso. The authors also amplified and sequenced the pre-S/S region after determining quantitative viral loads using rt-PCR.

The OBI was prevalent in 7.3% of the studied population, with genotype E being the most predominant in Burkina Faso. In the southern part of Africa, the prevalence of OBI is comparatively as high as it is reported in the western, central, and northern Africa. Carimo et al. (2018) carried out a cross-sectional analysis of blood samples of n=518 ART-naïve HIV-positive patients to explore the rates of OBI and frequency of anti-HBc alone OBI in Maputo, Mozambique. The outcome illustrated that 471/518 (90.9%) were negative for HBsAg, 45.2% of whom had antibodies to both the core and surface antigens, while 8.3% OBI cases were regarded anti-HBc alone. Similarly, the results of Mphahlele et al.'s (2006) retrospective analysis showed a frequency of 2.4% of OBI in HIV-uninfected and 22.1% in HIV-infected individuals from

specimens previously screened for both HIV and HBsAg. These findings are much higher than the 9.6% reported among healthcare workers who had previously received vaccination against HBV in South Africa (Sondlane et al., 2016).

In a more recent publication, Eltom et al. (2020) performed a systematic review and meta-analysis to determine the occurrence of OBI among high-risk populations in Sudan. A quantitative synthesis of the 11 articles that met the study's eligibility criteria found 15.51% overall pooled frequency of OBI. Unexpectedly, the pooled incidence of OBI was relatively low among the subjects considered having an elevated risk for occult HBV infection (12.59% in febrile patients and 13.39% in haemodialysis patients) than the 16.48% reported in one of the study included in this Eltom et al. (2020) systematic review involving examination of 100 sera samples retrieved from the Sudanese Central Blood Bank, which tested negative for HBV surface antigen, reported an extremely high OBI rate.. In summary, the pooled prevalence of OBI was 15.51% in high-risk cohorts, with subgroup analysis showing an occurrence rate of 12.59% in patients presenting with febrile symptoms, 13.26% in hemodialysis patients, and 16.48% among low-risk group of blood donors (Eltom et al., 2020).

In sum, the occurrence of Occult Hepatitis B Infection (OBI) exhibits notable geographic variability, with higher prevalence rates observed in certain regions. Studies from Asia, particularly in Japan, have reported substantial occurrences of OBI, indicating a significant public health concern. In Africa, where Hepatitis B is endemic, OBI is prevalent in both high-risk and low-risk populations, underscoring the importance of comprehensive screening protocols. Investigations in countries like Cameroon, Côte d'Ivoire, Gabon, and Sudan have demonstrated the presence of OBI among HIV-positive individuals, emphasizing the need for vigilant screening in co-infected populations. Furthermore, studies in Burkina Faso and

Mozambique have highlighted the prevalence of OBI, shedding light on the scope of this hidden form of HBV infection in Southern Africa. The systematic review in Sudan reaffirmed the presence of OBI in high-risk groups, with varying prevalence rates, providing valuable insights for public health interventions. These findings collectively emphasize the significance of OBI screening in both high-risk and low-risk populations to ensure comprehensive management and prevention strategies for Hepatitis B.

2.5 Circulating Genotypes of Occult HBV Infections in Sub-Saharan Africa

The HBV genome is exceedingly diverse and can be categorized into eight genotypes, A-H; however, recent analyses have led to the designation of two novel genotypes, I and J (Forbi et al., 2013; Matos et al., 2013). The aforementioned genotypes develop during replication due to impaired proofreading capacity of viral polymerase resulting in nucleotide misincorporation. Besides, viral genotypes alongside other viral and host factors, including specific gene modifications, viral load levels, and host environment influence clinical course and outcomes of the disease (Sunbul, 2014). Diverse genotypes are linked to disparate modifications in the HBV core and pre-core promoter gene regions, which, in turn, influence their pathogenicity and disease progression to liver cancer (Wang et al., 2019). Genotype A has a predisposition for chronicity, while gene alterations are often seen in genotype C, while both regularity of gene modifications and chronicity are predominant in genotype D (Sunbul, 2014). Liver cirrhosis and HCC are more frequently observed in individuals diagnosed with HBV genotypes C and D when compared with other variants (Zampino et al., 2015). Furthermore, HBV genotypes is not only prognostic of clinical course but are also narrowly associated with optimal ART plan, especially for individuals presenting with symptoms of chronic HBV (Sunbul, 2014).

The diverse genotypes vary with geographic spread, sensitivity to antiviral treatment, and disease severity (Cao, 2009; Forbi et al., 2013). Whereas some are limited to specific geographic regions, others are widely spread across the globe. Sunbul (2014) reported genotype A to be common in Northern Europe, sub-Saharan Africa, and Western Africa, D is predominant in India, Africa, and Europe, while genotypes C and B are widespread in Asia. Furthermore, genotype G is dominant in Mexico while H is localized to South and Central America (Pujol et al., 2009). Past epidemiological studies have reported the predominance of HBV genotype E in West Africa, and that it has an extremely low intra-genotype variance, signifying that it emerged recently (Forbi et al., 2010; Olinger et al., 2006). The most recent genotypes J and I were characterized among Japanese and Vietnamese, correspondingly (Sunbul, 2014).

Genotype A is further classified into A1, A2, and A3, with the first two having been observed regularly in South Africa (Allain et al., 2009; Kimbi et al., 2004; Pujol et al., 2009). Contrastingly, A3 is common in West and Central African countries (Forbi et al., 2010; Kimbi et al., 2004). A1/A2 is mainly endemic in Europe and northern America owing to the escalating movement of young Asian adults to these nations (Pujol et al., 2009). Hübschen et al. (2011) examined the genetic disparity of HBV genotype A in Africa by assaying 263 HBV strains from Cameroon and Nigeria. The results of the phylogenetic evaluation of S fragment sequences showed that 33.5% were genotype A while the remaining were genotype E. In line with Forbi et al. (2010) findings, 94.5% of samples from Nigeria were of genotype E, while A was predominant in Cameroon (Hübschen et al., 2011). Similarly, Kabamba et al. (2020) sought to explore the sero-prevalence and characterization of HBV strains circulating in Congolese donating blood in Lumbubashi, Democratic Republic of Congo (DRC). The outcomes of the sero-prevalence study showed that 53.1% of the HBV strains were of genotype E, 41.8%

genotype A, and 1.3% and 3.8% were genotypes A1/E and A3/E, respectively (Kabamba et al., 2020). The above findings are in line with those recorded by Fopa et al. (2019) and Atipo-Ibara et al. (2015), which found that a large share of individuals donating blood in DRC and Cameroon are infected with HBV genotypes E and A, respectively. More novel variants of HBV/A have been reported in Africa, for instance, A4 and A5 were identified in Nigeria and Mali, A7 among Cameroonian patients, A6 among African-Belgian patients, and the newest variant A8 in Lumbubashi, DRC (Forbi et al., 2013; Kabamba et al., 2020). The above observations display a greater genetic diversity of HBV type A in Africa compared to in other parts of the globe, signifying the necessity for additional phylogenetic analyses of HBV strains to comprehend the routes of viral transmission globally.

In East Africa, studies have found genotype A and D to be the most prevalent HBV strains. Ambachew et al. (2018) conducted a cross-sectional, laboratory-based analysis of n=103 HBsAg positive blood donor samples to establish the serological, genetic, and mutation characteristics of HBV isolates in Southern Ethiopia. The authors performed quantitative rt-PCR to measure the viral loads, and successfully amplified the S-gene of 85 samples using nested PCR. Of these samples, 68.2% (n=58) were characterized as genotype A and the remaining 31.8% (n=27) were genotype D. In a similar investigation, Patel et al. (2020) examined the seroprevalence and genetic variability of OBI among HIV-positive patients in Gondar, Ethiopia. OBI was observed in approximately 20% of HIV-positive anti-HBc-positive specimens, with 81% of the OBI patients presenting with genotype D variant (Patel et al., 2020).

In another Kenyan study, Mwangi et al. (2009) analyzed plasma drawn from blood donor samples to characterize the circulating HBV genotypes. Sixty-five percent of the 80 HBsAg positive specimens containing HBV-DNA were amplified, sequenced, and phylogentically

analyzed. Genotypes A (A1 and A2), E, and D were noted to be predominant in Kenya with a prevalence rate of 88.5%, 7.7%, and 3.8%, respectively. Kilongosi et al. (2015) study involving IDUs and HIV-1 infected individuals in the costal Kenya reported the dominance of genotype A1 with minimal human diversity, a finding that is supported by Day et al. (2013) study. The latter which described A1 in HIV-1 positive adults on ART in Nairobi and HIV-1 infected female commercial sex workers in coastal Kenya (Day et al., 2013). The above findings are consistent with Aluora et al. (2020) observations, which reported exclusive predominance of A1 in voluntary blood donors in Kenya. Likewise, mutations allied to HBV reactivation, HBV drug resistance, and HCC risk were detected in 14%, 23%, and 45.5%, of subjects, in that order in a recent Kenyan study (Aluora et al. 2020).

2.6 Preventive Measures and Treatment Regimes Available for OBI

The studies by Zobeiri (2013), Lledó et al. (2011), and Raimondo et al. (2014) collectively underscore the critical importance of preventive measures in managing occult hepatitis B virus infection (OBI). Zobeiri (2013) highlights the necessity of administering preventive treatment to inhibit the development of hepatitis and reduce mortality, particularly in cases of HBV reactivation despite lamivudine treatment. This approach is crucial in patients receiving chemotherapy and immunotherapy, where antiviral prophylaxis should be continued for at least six months post-treatment. Furthermore, Lledó et al. (2011) advocate for the implementation of HBV nucleic acid amplification testing and multivalent anti-HBs antibodies for accurate OBI detection, especially in cases involving organ transplantation. The study emphasizes the uncertainty surrounding the effectiveness of prior hepatitis B immunization in preventing OBI transmission through transplantation. Raimondo et al. (2014) highlight the significance of identifying patients prone to reactivation, particularly those undergoing immunosuppressive

therapy or chemotherapy. This underscores the necessity for tailored preventive strategies to mitigate the risk of OBI-related complications.

The literature reviewed provides essential insights into the treatment strategies for OBI. Zobeiri (2013) suggests that initiating antiviral treatment before the onset of hepatitis is crucial, as delayed treatment may be insufficient to control HBV reactivation. This is particularly relevant for patients lacking anti-HBs antibodies who are at higher risk for reactivation prior to chemotherapy. Furthermore, Raimondo et al. (2014) stress the importance of promptly initiating antiviral therapy in patients prone to reactivation, particularly those undergoing immunosuppressive therapy or chemotherapy. They emphasize that timely intervention can halt HBV reactivation and prevent its clinical sequelae. Additionally, Makvandi (2016) recommends the use of highly sensitive molecular methods for screening HBV DNA in high-risk groups, such as patients undergoing chemotherapy, organ transplant recipients, and healthcare workers. This approach ensures early detection and timely initiation of antiviral therapy, thereby minimizing the risk of OBI-related complications and progression to severe liver diseases.

2.7 Diagnostic Challenges of OBI

Detecting occult hepatitis B virus infection (OBI) poses a substantial challenge due to various factors highlighted in the literature. The reliance on serological tests, as demonstrated by El-Adly et al. (2020), is one of the key obstacles. While widely used, these tests have shown a detection rate for OBI below 20%, indicating their unreliability for accurate diagnosis. This discrepancy is further exacerbated by the cost constraints associated with highly sensitive nucleic acid amplification tests (NAAAT), as discussed by Wang et al. (2023). Although liver histology is considered a gold standard with a higher detection rate, it is invasive and lacks standardization, limiting its practicality. Salyani et al. (2021) emphasize that even in the context of high

prevalence in specific populations, the detailed worldwide prevalence patterns of OBI remain largely unknown. The study suggests that further research is necessary to comprehend the mechanisms driving OBI development and contribute to global efforts in eliminating viral hepatitis.

In regions with high prevalence, like Upper Egypt, where HBV is a major health concern, accurate diagnosis becomes even more crucial. El-Adly et al. (2020) highlight the prevalence of HBV among Assiut governorate patients and emphasize the need for effective monitoring of liver disease progression in carriers through a combination of molecular, biochemical, and serological markers. de Almeida & de Paula (2022) emphasize that OBI, characterized by the presence of HBV DNA without detectable HBsAg, is maintained by a complex interplay of host, immunological, viral, and epigenetic factors. The lack of a validated detection test further complicates diagnosis. This review suggests that OBI's global prevalence remains poorly understood, and it is most prevalent in specific high-risk groups. The difficulties in detecting and treating OBI challenge the World Health Organization's goal of eliminating viral hepatitis by 2030. This emphasizes the need for continued research and efforts to better understand and address OBI.

2.8 Gaps in Literature

From the reviewed literature, it is apparent that HBV is a prevalent infection in Kenya posing serious public health challenges. Nonetheless, irrespective of the increasing number of publications on HBV, none have described the occurrence of OBI in any population. This is even worsened by the widespread dependence of serological markers as the hallmark of HBV detection, the high number of HIV and HCV patients and those seeking medications for liver diseases, and the escalating population index in Kenya (Ochwoto et al., 2016; Ochwoto et al.,

2017). In addition, there is an increasing but neglected HBV high-risk cohort in Kenya of men having sex with men (MSM), whose HBV status is unknown. Furthermore, nearly all of the studies on the genotypic characterization conducted in East Africa, mainly in Kenya, involve either low risk populations or HBsAg-positive samples. Thus, the current dissertation sought to fill the above research gaps by investigating the seroprevalence and circulating genotypes of OBI among two high risk groups, namely, jaundiced patients seeking medical care at various hospitals and MSMs. In sum, this study sheds light on the prevalence of HBV and its occult form among high-risk populations in Kenya, specifically MSM-SW and jaundiced patients. The two groups of participants closely interact with individuals in the general population, at times donating blood without knowledge of their OBI status. Thus, the findings highlight the importance of targeted interventions and vaccination campaigns to safeguard the general population by reducing the prevalence of HBV and OBI in these at-risk populations.

CHAPTER THREE: METHODOLOGY

3.1 Study Design

This study employed a cross-sectional retrospective study design involving the analysis of archived sera.

3.2 Specimen Collection

The samples analyzed in the present study were archived serum from a cohort of male sex workers primarily having sex with men (MSM-SW) in Nairobi described by McKinnon et al. (2014), and archived sera from jaundiced patients seeking medical care at four selected hospitals in Kenya, namely, Kenyatta National Hospital (Nairobi), Moi Teaching and Referral Hospital (Eldoret), New Nyanza Provincial General Hospital (Kisumu), and Coast General Hospital (Mombasa) described by Ochwoto et al. (2016). MSM-SM were recruited prospectively between 2009 – 2012 as part of an open cohort at a clinic in Nairobi central region that offers them an array of medical services, encompassing HIV/STI testing and therapy, demonstration of effective condom use, provision of lubricants and condoms, and health education. The use of snowball and hot spot-based sampling technique led to the recruitment of 127 subjects; however, only 99 archived sera had adequate volume for investigation for OBI. Therefore, the basis for selection of serum for analysis was the sufficiency of serum volume after the previous assays. In addition, another sample of 20 men, who indicated to never participating in receptive anal intercourse (non-MSM) and were in a serodiscordant sexual relationship with HIV-positive female partners, was enlisted to serve as controls. Only 13 specimens from the non-MSM with adequate serum volume were included in the follow up investigation for OBI.

On the other hand, the jaundice specimens had been tested to investigate the frequency, geographic spread, and molecular characterization of hepatitis viruses among individuals

requiring clinical care in hospitals in Kenya. Participants were recruited prospectively between January 2012 and April 2013. Only patients with no history of laboratory and clinical diagnosis of viral hepatitis and presentation of jaundice were enlisted to participate in the research. In both studies, 4mL of whole blood were drawn from those patients, who met the research inclusion criteria, and their demographic information, such as age and gender was recorded. 164 HBsAg-negative samples were analyzed, 65 of which had adequate serum volume for OBI investigation. All the samples had been assayed for HCV in the original investigation and were reported to be negative (Ochwoto et al., 2016). The remaining sera were then kept frozen at -80^0c to avoid the deterioration of the viral DNA at the National Microbiology Laboratory (NML), Winnipeg, Canada, where the initial assays were carried out.

3.3 Serological Testing

All serological tests were assayed by Electrochemiluminescence immunoassay technique (EIA) using the COBAS e411 analyzer (Elecsys; Roche Diagnostics, Quebec, Canada). The EIA is a highly sensitive and specific technique widely employed in serological analysis that relies on the interaction between specific antibodies and antigens to detect and quantify analytes of interest. The serological assay utilized in this study employed a one-step double antibody sandwich assay, following a 2-point calibration method. The interpretation of results was categorized based on the COI values: a COI of less than 0.9 was considered non-reactive, while values between 0.9 and less than 1.0 fell within the gray zone, and a COI of 1.0 or higher was deemed reactive. Serum samples collected using standard sampling tubes or tubes containing separating gel were utilized for analysis. The cobas e 411 analyzer module required a sample volume of 50 µL.

3.3.1 MSM Cohort

A 50µl aliquot of all the samples from the archived sera was first tested for anti-HCV. Another 40µl was tested for anti-HBc by EIA. 50µl of the specimens that tested positive for anti-HBc positive were further assayed for HBsAg. Viral DNA from 600µl of the anti-HBc positive, HBsAg negative (aHBc+/HBsAg-) samples was extracted and amplified for quantitative HBV viral load. Further, 40µl of all the samples were tested for anti-HBs antibodies.

3.3.2 Jaundiced Samples

The archived sera from Ochwoto et al. (2016) study with known HBsAg results and no HBV DNA results were ordered and inventoried, and those with volumes of approximately 800µl-1000µl were selected for analysis during the inventory process. The samples with adequate sera were placed in a separate box during analyses and placed back in the inventory boxes after the assays. Like in the MSM cohort, 50µl and 40µl of all the specimens were tested for anti-HCV and ant-HBc, respectively by EIA (Elecsys; Roche Diagnostics, Quebec, Canada). Viral DNA from 600µl of the anti-HBc+/HBsAg- specimen was extracted and amplified for quantitative detection, and 40µl of all the remaining sera were tested for anti-HBs antibody (Elecsys; Roche Diagnostics, Quebec, Canada).

3.4 HBV DNA Extraction and Amplification

In a biosafety cabinet, 600µl of sera were eluted into a 1.5ml microcentrifuge tube with previously added 50µl of Proteinase K (20mg/ml) Invitrogen. The proteinase K digests any contaminant protein in the specimens (Qamar et al., 2017). Then, 600µl of Acrometrix was used as the negative control, while a 1:1 dilution of Acrometrix positive control with low HBV DNA and negative control served as positive control. The contents of the microcentrifuge were vortexed, followed by a quick spin. The preparations were then incubated at 56^{0}c for 15 minutes

while centrifuging at 1250 revolutions per min (rpm). After the incubation, the mixture was quickly spanned to condense any sample off the lids. The whole volume of treated sample in the microcentrifuge was added into easyMag strip wells (NucliSENS easyMag, bioMerieuxInc, Saint-Laurent, QC) with lysis buffer in a biosafety cabinet accordingly. The mix was incubated for 10 minutes at room temperature before 140µl of NucliSENS bead solution was added using a single-channel micropipette. After proper mixing of the solution, the strips were moved from the BSC to the easyMag machine, where the strip barcodes were re-scanned. The easyMag machine was programmed to start the automatic nucleic acid extraction for an hour after which the strips were placed into metal holders and transferred to the BSC, where the entire target 60µl of eluent for each sample were pipetted into centrifuge tubes and stored at -80^{0}c for DNA amplification. The primers used in this process were multiplex, allowing for the simultaneous amplification of multiple target sequences.

3.5 HBV DNA Detection

Real-time PCR was performed to detect the three HBV DNA target regions, namely, the surface/polymerase (PreS), Enhancer I (ENHI), and X/Enhancer (XENH). In brief, the master plate and the master mix were prepared in a BSC in the clean room to avoid the risk of contamination. The preparation of the master mix involved labeling three PCR tubes PreS, ENH1, and XENH. For 20 µl of HBV-specific master mix per tube, 4.0 µl QuantiTect Virus Master Mix (5x) (Qiagen, Toronto, ON), 1.0 µl 20x primer-probe mix specific for the PreS coding region (nt 228-371, based on GenBank reference sequence AY128092), or the ENHI regulatory region (nt 1178-1281), or the X/ENHII genomic region (nt 1549-1671), and 0.4 µl 50x ROX dye solution, was be added into the labelled tubes (see Table 1). The mixture was vortexed thoroughly before 5 µl was added to the FastPlate wells.

The master plate was transferred to the PCR room, where 15 µl of the DNA extract was added in a BSC and sealed. The ABI 7500 (ThermoFisher Scientific, Burlington, ON) thermocycler program was set for Fast 7500, Standard Quantification, Standard run (~2 hours). The thermocycling conditions involved 50 cycles of denaturation at 95^{o}C for 5 minutes, annealing at 95^{o}C for 15 seconds, and extension at 60^{o}C for 45 seconds. The samples were considered positive if two or more target regions of the genome were detected (Osiowy et al., 2013). The cycle threshold (Ct) cut-offs, below which the specimen were regarded to be positive for HBV DNA as detected for each genomic region, were determined following validation and were as follows: 37.77 (HBsAg-coding region), 40.82 (ENHI regulatory region), 38.33 (X/ENHII genomic region).

Table 3.1: Primer and probe sequences for HBV DNA detection and sequence analysis

Primer or Probe name	5'—3' sequence	Approximate genomic region[a]
Surface-FWD primer	TCCTCACAATACCRCAGAGT	228–247
Surface-REV primer	GATARCCAGGACAARTTGGAG	371–351
Surface real time PCR probe	AAAATTCGCAGTCCCCAACCTCCA	306–329
ENHI-FWD primer	AAGTGTTTGCTGACGCAA	1178–1195
ENHI-REV primer	GAGTTCCGCAGTATGGATC	1281–1263
ENHI real time PCR probe	CCATCRGCGCATGCGYGGAA	1224–1243
X/ENHII-FWD primer	CCGTCTGTTCCTTCTCATCTG	1549–1569
X/ENHII-REV primer	GTCCAAGAGTCCTCTTATGYAAG	1671–1649
X/ENHII real time PCR probe	TGCACTTCGCTTCACCTCTGCAC	1580–1602
HBPr134 first stage FWD	TGCTGCTATGCCTCATCTTC	414–433
HBPr135 first stage REV	CARAGACAAAAGAAAATTGG	822–803
HBPr75 nested stage FWD	CAAGGTATGTTGCCCGTTTGTCC	455–477
HBPr94 nested stage REV	GGYAWAAAGGGACTCAMGATG	795–775

[a]According to GenBank Accession AY128092 nucleotide numbering.

The primers and probes were prepared by Integrated DNA Technologies (Katana, Ontario), with the oligonucleotide probes having a double quencher (ZEN/3'IB) and a 5'6-FAM fluorescent reporter dye combination.

3.6 HBV DNA Amplification and Sequencing

The amplification of the HB pol region was performed using nested PCR and it was anticipated to generate a 342bp product. In the first round reaction, a total volume of 50µl containing 10µl of DNA extract was added to 23.1µl high-pressure liquid chromatography-grade water, 8.0µl of 1.25mM deoxynucleoside tris-phosphates (dNTPs), 5µl of1 10x buffer (Qiagen), 1µl of magnesium chloride ($MgCl_2$), 0.4µl of Qiagen Hot Star polymerase, and 1.25µl of each primer (20uM HBPr134 and 20uM HBPr135 shown in table 1) described by (Stuyver et al., 2000). The ABI 7500 thermocycling (ThermoFisher Scientific, Burlington, ON) conditions involved 40 cycles of denaturation at 94^{o}C, annealing at 45^{o}C, and extension at 72^{o}C for 30 seconds each. 5µl of the first round PCR product served as the template for the nested PCR under similar reaction conditions.

An aliquot of 1.25µl of each of the amplicons positive by nested PCR were gel purified using the Qiagen Gel Purification kit. Purified DNA was quantified with Nanodrop, where the 1.25µl aliquots of positive amplicons were loaded onto the Nanodrop to automatically measure absorbance at 280 nm wavelength. DNA concentration was calculated using a 260/280 ratio around 1.8 indicating pure DNA. The pure DNA amplicons were sequenced using Applied Biosystems 3730 XL DNA Analyzer (ThermoFisher Scientific). Resulting Sequences were aligned and trimmed using Clustal X v2.0.11 (Larkin et al., 2007) and BioEdit v7.2.5 (Hall, 1999), respectively. Maximum likelihood analysis of the partial HBsAg coding-region (trimmed to 327 bp representing nt 458 to 784, based on GenBank accession AY128092) was done using

DIVEIN software (Deng et al., 2010) by the K2+γ model determined as the most appropriate substitution model for the alignment. Phylogenetic tree construction was optimized by the adjoining neighbor interchange and sub-tree pruning and regrafting with branch support computed by the approximate likelihood-ratio test based on a Shimodaira-Hasegawa-like procedure (Anisimova & Gascuel, 2006). Nucleotide sequence alignments were translated to amino acid to determine the presence of mutations influencing immune recognition of the HBsAg protein and proneness to antiviral treatments.

3.7 Statistical Analysis

All the collected data were recorded and entered into the SPSS® version 20 (IBM, Chicago, IL, USA). The association of anti-HBc with OBI and the association of DNA positivity with MSM-SW demographic and behavioral characteristics was analyzed by Fisher's exact test (two-tailed). Chi-square was applied to determine the relationship of educational level with HBV DNA positivity. Confidence intervals of prevalence estimates were calculated by computing the confidence interval of a proportion by the Wilson/Brown method. *P* values <0.05 were considered significant.

3.8 Ethical Consideration

The ethical approval for collection and investigation of specimens from MSM-SW and non MSM-SW was obtained through institutional review boards (IRBs) at the Kenyatta National Hospital and the University of Manitoba (McKinnon et al., 2014). Both IRBs authorized the research of infectious pathogens in agreeing respondents, encompassing the detection and genotyping of HBV and HCV infectious agents. The ethical approval for collection and investigation of specimens from patients seeking medical care for jaundice was attained from the Kenya Medical Research Institute's National Ethical Review Committee (approval number SSC

2436, Appendix 1) (Ochwoto et al., 2016). Signed consent was collected from each participant prior to sample and demographic information collection in the original study.

CHAPTER FOUR: RESULTS

4.1 Demographic Information

4.1.1 MSM-SW and Non-MSM

The behavioral and demographic characteristics, as well as the HIV serostatus of both the test and control groups were gathered during the original investigations. For the MSM cohort, including the MSM control group, the participants were aged between 18 – 56 years, with the mean age of 27.9 years. The average age of the respondents' first sexual encounter was 15.5 years. Information on marital status was available for 90.9% (90/99) of the respondents, with 74.5% being single and 25.5% married. Eighty-three (92.2%) of the participants provided information on their level of education, and 39/83 (49%) completed post-secondary education, 36/83 (43.38%) finished high school, and 8/83 (9.4%) had only a primary certificate. Behaviorally, more than half of the 83 respondents (46/83) actively engaged in commercial sex work, with a large share of them participating in at least two of the following practices: insertive vaginal, insertive anal, and receptive anal sex, and >20% engaged in all the above sexual behaviors. More than 35% of the active sex workers did not engage in vaginal sex but reported participating in both anal insertive and anal receptive sex.

Roughly a third of the subjects engaged in two or more types of sex including vaginal, and at least three-quarters practiced two or more types of sexual activities. An evaluation of baseline characteristics shows numerous disparities between men engaging in heterosexual activities, and men solely having sex with men. Although a large share of the regular sex workers reported that

they either ‘never’ (58.62%) or ‘sometimes’ (24.14%) engage in oral sex, the percentage of those who ‘always’ engage in oral sex was higher among those practicing commercial sex, with 10.34% in the MSM cohort always engaging in oral sex versus 27.27% in the paid sex group. A reverse trend was observed with regards to condom use, with 64.29% of the 28 men having sex with regular sex partners reporting that they ‘always’ use condoms in either insertive anal, insertive vaginal, or receptive anal sex compared to those who ‘never’ used condom.

In terms of vaccination and HIV status, 46/99 (46.5%) of the MSM-SW men were HIV-positive, and as started earlier, the 13 non-MSM men in the control group were HIV-negative while their female partners are HIV positive. Of the 46 HIV-positive respondents, 41 were on anti-retroviral therapy (ARVs) and only 5 reported not on the ARVs. Furthermore, 32 MSM-SW men, including one HIV-positive and 31 HIV-negative, had received HBV vaccine without their knowledge of HBV status during a simulated HIV-1 vaccine viability trial conducted by KAVI Institute of Clinical Research. The levels of alanine aminotransferase levels of the subjects in either groups were unavailable for analysis in this OBI study (McKinnon et al., 2014).

4.1.2 Jaundiced Patients

As outlined previously, samples from 65 jaundiced patients seeking clinical care from four hospitals in Kenya were included in the present investigation. The average age of the respondents was slightly higher than in the MSM-SW cohort (38.7-years), with the youngest aged 16 years and oldest 84 years. Geographically, more than half of the participants 44/65 (52.31%), were drawn from Nairobi, 11/65 (16.92%) from Kisumu, and 10/65 (15.38%) from Embu. In total, there was insignificant disparity with regards to gender, as there were 34 males and 31 females. Unlike in the MSM-SW sample, other demographic information on the

behavioral attributes, HIV status, or vaccination status were unavailable for the jaundice samples.

4.2 Prevalence of Occult HBV Infection

4.2.1 Prevalence of OBI in the MSM Cohort

Unlike the jaundiced samples, specimens from the MSM-SW and non-MSM controls had not previously been tested for overt HBV. Therefore, both CBHI, which is determined by HBsAg positivity, and OBI were assayed in these groups, while all the jaundice samples were HBsAg-negative. Ten samples of the MSM-SW tested positive for HBsAg, seven of which contained HBV DNA, thus establishing a CBHI prevalence of 10.1% among MSM-SW (10/99, 95% CI 5.6-17.6). HBV DNA was detected in 10 MSM-SW and 1 non-MSM HBsAg-negative men indicating an OBI prevalence of 11.2% (10/89; 95% CI 6.2 – 19.5) and 8.3% (1/12; 95% CI 0.4-35.4), respectively (Figure 4.1). HIV serostatus of the non-MSM and MSM-SW men were previously tested in the original cohort investigation (McKinnon et al., 2014).

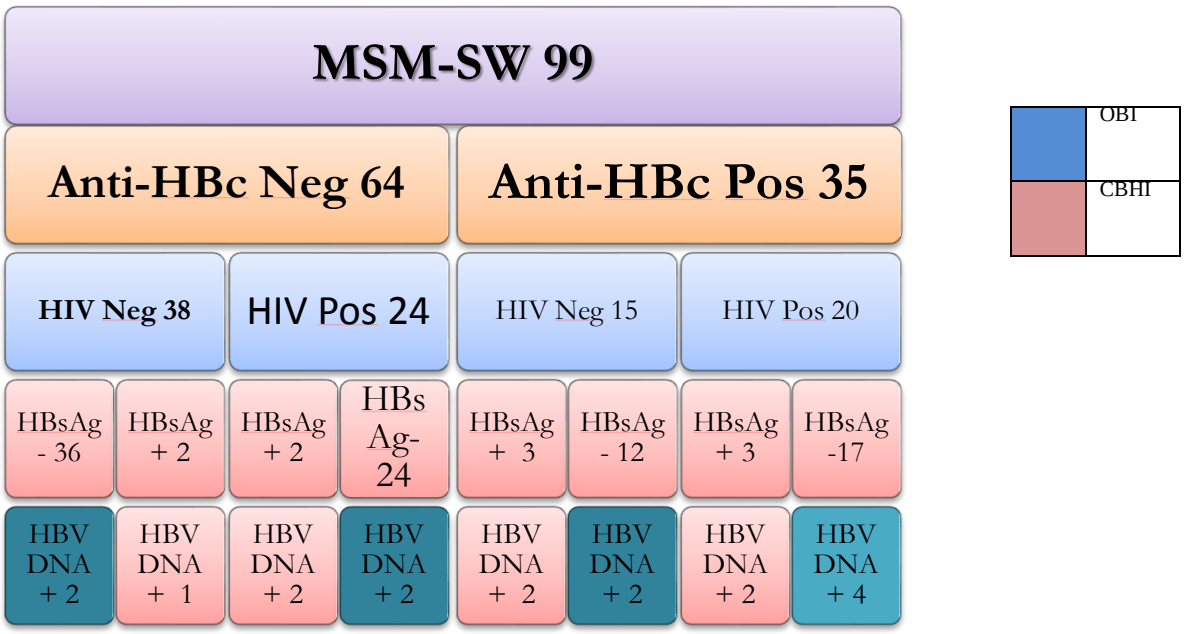


Figure 4.1: Flow chart of OBI testing of the MSM-SW specimens and results

In the present study, 70.58% of all MSM-SW samples with detectable HBV DNA originated from unvaccinated individuals, except 2 cases of HBsAg-positive and 3 OBI incidences in vaccinated HIV-negative MSM-SW specimens (Figure 4.2). HIV-co-infection was present in 58.8% HBV DNA-positive MSM-SW subjects (10/17, 95% CI 36.0 - 78.4) although there was no considerable variance observed between HBV DNA positivity and HIV positivity among the MSM-SW cohort (P = 0.2952). There was no case of HCV co-infection among the OBI positive individuals, since all the MSM-SW samples had no traceable anti-HCV markers.

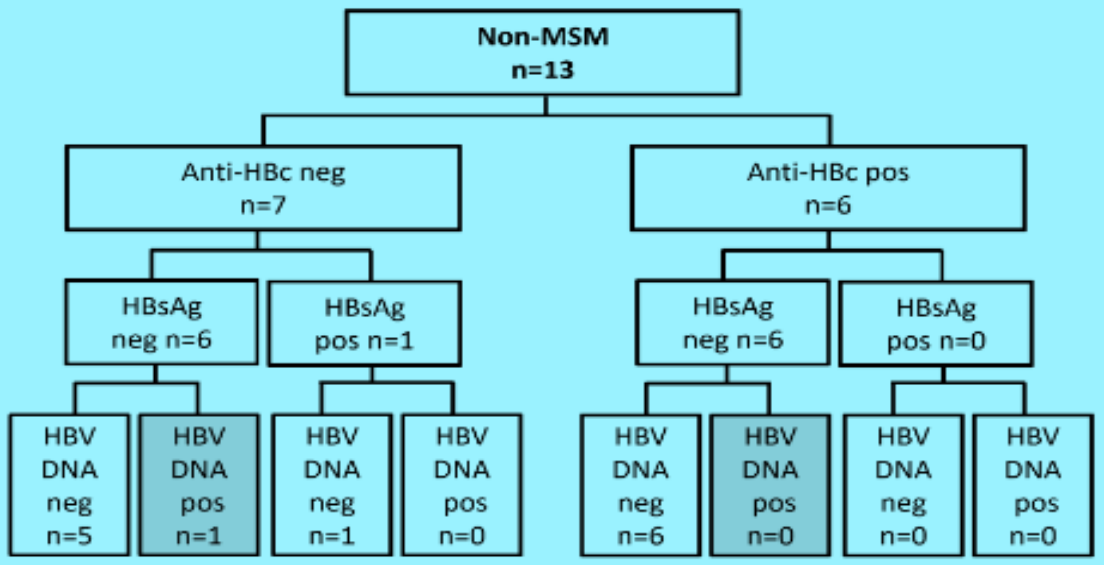


Figure 4.2: Flow chart of OBI testing of the non-MSM specimens and results

In addition, as illustrated in Table 4.1 below, there was no substantial connection between HBV DNA positivity and behavioral, treatment, and demographic features of the MSM-SW except for HIV antiretroviral therapy, which had a positive correlation with OBI (P = 0.0042; Fisher's exact test).

Table 4.1: The association between HBV DNA positivity and behavioral, and demographic features

Variable	HBV DNA positive[a]	HBV DNA negative[a]	*P* Value[b]
Marital status (single)	14/16 (87.5%)	54/75 (72.0%)	0.02253
Education			
Primary or less	1/15 (6.7%)	7/68 (10.3%)	o.8986
Secondary	7/15 (46.7%)	29/68 (42.6%)	(Chir-
Postsecondary	7/15 (46.7%)	32/68 (38.2%)	square)
Antiretroviral treatment	12/15(80.0%)	26/68 (38.2%)	**0.0042**
Oral sex			
Often-always[c] with a regular partner	3/15 (20%)	11/66 (16.7%)	0.7165
Often-always with a casual partner	2/11 (18.2%)	8/54 (14.8%)	0.6732
Insertive anal sex			
Often-always with a regular partner	8/15 (53.3%)	37/66 (56.1%)	>0.9999
Often-always with a casual partner	5/12 (41.7%)	28/53 (52.8%)	0.5372
Receptive anal sex			
Often-always with a regular partner	7/15 (46.5%)	18/66 (27.3%)	0.2140
Often-always with a casual partner	7/12 (58.3%)	16/52 (30.8%)	0.1965

[a]Not all MSM-SW participants in the study answered all interview questions. [b]Fisher's exact test unless otherwise indicated. [c]In comparison to never-sometimes. Bold indicates *P* value <0.05

4.2.2 Prevalence of OBI in the Jaundiced Patients

As exhibited in Figure 4.3, 56.9% (37/65; 95% CI 44.8 – 68.2) of the HBsAg-negative samples from jaundiced patients were anti-HBc positive. Twenty specimens, including 14 samples containing anti-HBc antibodies and 6 negative for anti-HBc, had HBV DNA by real-time PCR in at least two different HBV genomic regions. The 20 jaundiced samples containing HBV DNA suggested a 30.8% OBI positivity rate (95% CI 20.9 – 42.8). A substantial correlation between OBI and anti-HBc antibody positivity among all the high-risk groups was noted (P = 0.0153;

Fisher's exact test), with the highest observed when jaundiced and MSM-SW groups are taken into account (P = 0.007; Fisher's exact test).

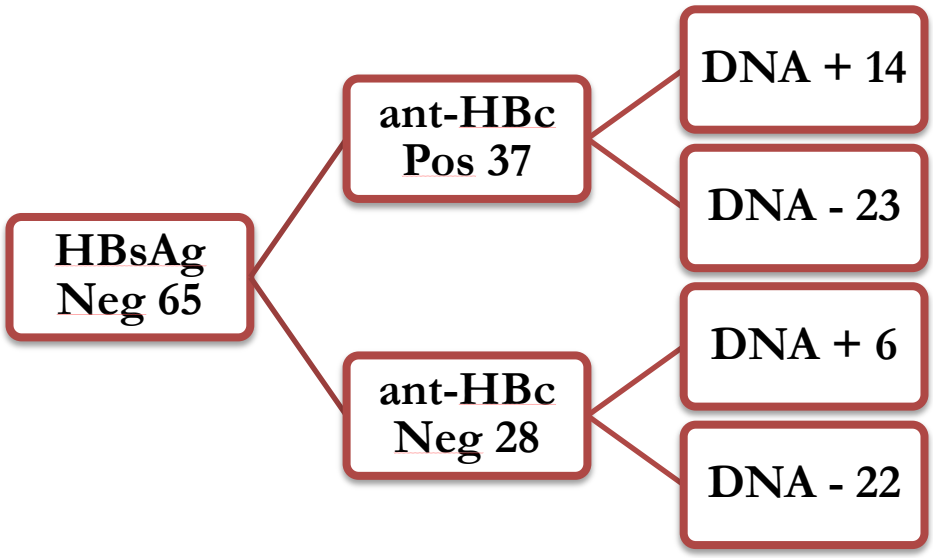


Figure 4.3: Flow chart of OBI testing of the jaundiced specimens and results

4.3 Association between Anti-HBs Levels and OBI

As shown in Table 4.2 below, four anti-HBc-positive samples of the 10 containing HBV DNA from the MSM-SW cohort had anti-HBs levels >100mIU/Ml, while five anti-HBc- negative and 1 anti-HBc-positive had anti-HBs levels ≤100 mIU/mL. Similarly, 14 anti-HBc-positive and 4 anti-HBc-negative samples of the 20 HBV DNA positive samples had anti-HBs levels ≤ 100 IU/Ml in the jaundiced cohort, and only 2 anti-HBc-positive HBV DNA- positive samples had anti-HBs levels >100 IU/Ml.

Table 4.2: Association between Anti-HBs and OBI in the analyzed samples

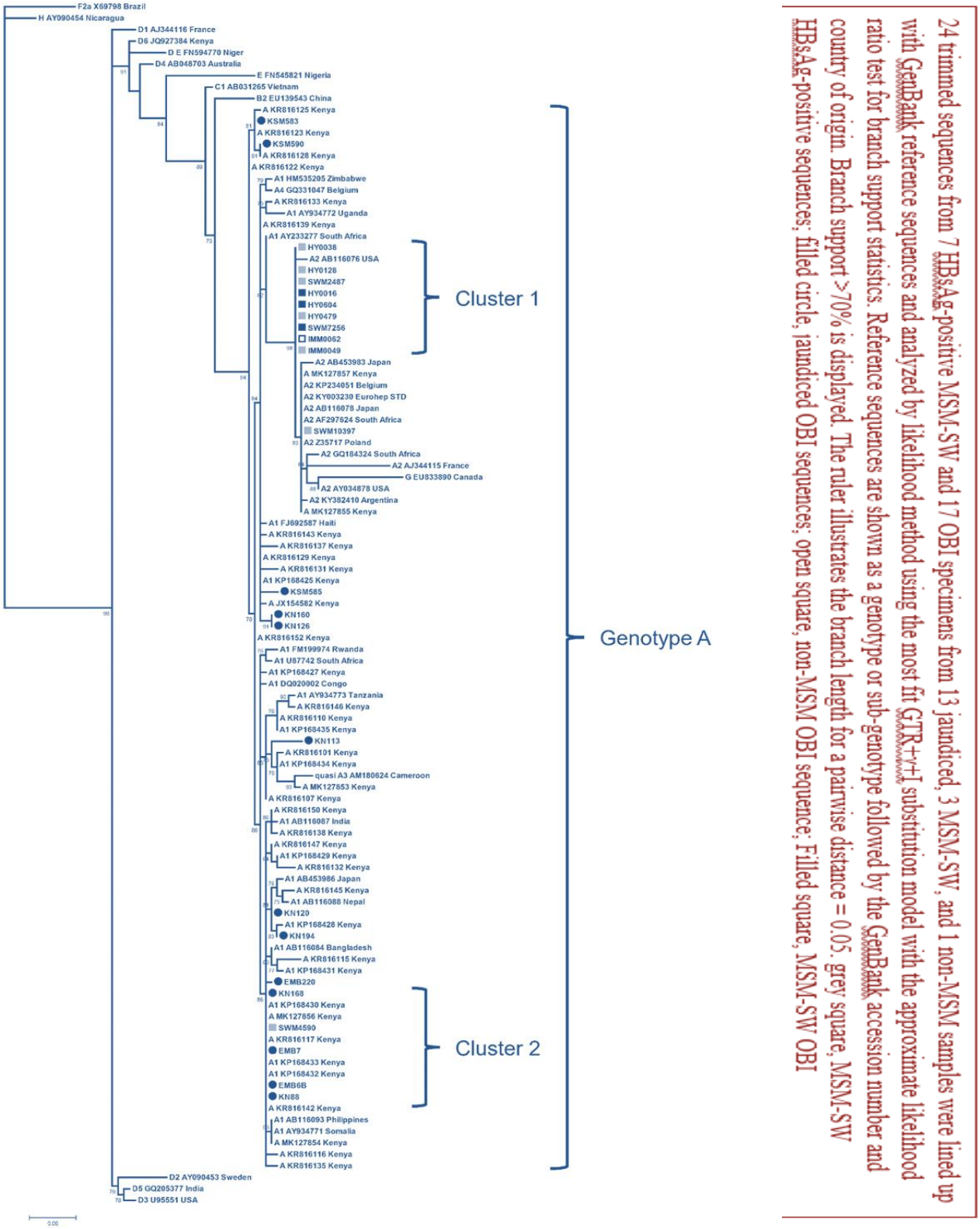
	Anti-HBc+/rt-PCR +	**Anti-HBc-/rt-PCR +**	**Total**
Anti-HBs ≤100 mIU/Ml	15	9	24
Anti-HBs >100 mIU/Ml	4	0	4

4.4 Genotypes of the OBI in High-Risk Populations of Kenya

Thirty-eight (n = 38) specimens with HBV DNA by rt-PCR were re-amplified by a nested PCR for sequence analysis. The latter led to 24 samples testing positive by nested PCR with at least 327 base pairs long genome for phylogenetic analysis. Seven CBHI sequences from 7 HBsAg-positive MSM-SW specimens and seventeen OBI sequences from 13 jaundiced subjects, 3 MSM-SW, and 1 non-MSM specimens were aligned with GenBank reference sequences depicting HBV sub-genotypes, encompassing 42 Kenyan HBV reference sequences before they were subjected to maximum likelihood phylogenetic analysis. As illustrated in Figure 4.4, all the analyzed sequences were determined to belong to HBV genotype A (GenBank Accession No - MK487133—MK487155, MN972524). MSM-SW segments were mainly grouped together, including with a single non-MSM OBI sequence (Cluster1) and exhibited complete sequence identity over 327 nucleotides. Sequences of jaundiced patients were not clustered, even though a second smaller constellation (Cluster2) within the phylogenetic tree, composed of a combination of jaundice and MSM-SW sequences, as well as numerous Kenyan reference sequences, demonstrated full sequence identity over 327 base pairs.

Although decisive categorization of sub-genotype of all the genetic constitutions could not be determined owing to the cost constraints associated with sub-genomic region analysis; nonetheless, based on tree topology, sequences from the patients presenting in various hospitals with jaundice appeared analogous to sub-genotype A1. On the other hand, sequences from the MSM-SW samples were classified entirely in Cluster 1 which appeared closely similar to sub-genotype A2 with 98% branch support. Alterations allied to impaired surface antigen, virion secretion, and immune escape mutants were rarely noted in the amino acid alignments of OBI sequences. In particular, there were no recognized nucleotide analog resistance alterations along

the amino acid alignments of the polymerase reading frame (rtl111 – rtM218). Three amino acid alterations occurred in the surface antigen major hydrophilic regions of three samples from the patients presenting with jaundice. D144E was observed in KN113 (accession number MK487153) and both T118A and T116N were noted in KN160 and KN126 (accession numbers MK487142 and MK487143, respectively).



24 trimmed sequences from 7 HBsAg-positive MSM-SW and 17 OBI specimens from 13 jaundiced, 3 MSM-SW, and 1 non-MSM samples were lined up with GenBank reference sequences and analyzed by likelihood method using the most fit GTR+γ+I substitution model with the approximate likelihood ratio test for branch support statistics. Reference sequences are shown as a genotype or sub-genotype followed by the GenBank accession number and country of origin. Branch support >70% is displayed. The ruler illustrates the branch length for a pairwise distance = 0.05. grey square, MSM-SW HBsAg-positive sequences; filled circle, jaundiced OBI sequences; open square, non-MSM OBI sequence; Filled square, MSM-SW OBI

Figure 4.4: Phylogenetic analysis of the HBsAg-coding region sequence

CHAPTER FIVE: DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Prevalence of CHBI among MSM-SW Samples

Sub-Saharan Africa remains a high intermediate endemic region for HBV infection, with recent estimates from systematic reviews showing an overall prevalence of >6% of overt HBV (Schweitzer et al., 2015). The odds of HBV infection are significantly elevated in specific high-risk populations, like MSM, female sex workers, and individuals presenting with fever and jaundice (Burns & Thompson, 2014; Kafeero et al., 2020; Katusiime et al., 2016; Kilonzo et al., 2018; Msomi et al., 2020; Muriuki et al., 2013). The present study involved a cohort of 177 heterogeneous populations of individuals with high risk for HBV exposure. The sample included 65 jaundiced patients seeking medical care in four major hospitals across Kenya, 99 MSM-SW, and 13 non-MSM-SW. The results showed a high prevalence of overt HBV (10.1%) among the MSM-SW faction. Even though the proposal protocol of the current investigation was not intended to approximate the occurrence rate of CBHI among the above-mentioned high-risk groups, the samples of MSM-SW and non-MSW-SW cohorts had not been screened for HBsAg. Therefore, none of the samples with adequate specimen from the MSM-SW and non-MSM-SW cohorts were excluded, and the findings of the study with regards to the occurrence of CBHI remain valid.

The HBsAg prevalence in the MSM-SW and non-MSM-SW cohort is comparable to the 11.2% reported in a longitudinal follow-up study involving 779 MSM in West Africa, with the highest rates occurring in MSMs who had six or more sexual partners (Dah et al., 2019). The HBV incidence was also similar to the prevalence of 11% described by cross-sectional investigations of MSM in Brazil and 10% in Lagos, Nigeria (Adebajo, 2016; Soares et al., 2014). The observed

incidence was significantly less than the 17.4% described by Karoney et al. (2020) in an investigation involving participants drawn from an heterogeneous population with increased risk for HBV exposure in the western part of the country, including female sex workers, MSM, substance users, patients presenting with indications of chronic hepatic disease, and residents living with HIV. It is worth noting that the 10.1% prevalence in the present study is significantly higher when considering the overall sample size of 99 MSM-SW individuals compared to the sample sizes of the other cited studies, highlighting the heightened risk within this population. The 10.1% HBsAg positivity rate in the present study was higher than 6% described in Salyani et al. (2021) cohort study that involved a Kenyan cohort of 208 HIV-positive ART naïve adults, as well as the prevalence reported in other parts of sub-Saharan Africa that ranged between 8.7% - 9.9% (Bell et al., 2012; Carimo et al., 2018; Ryan et al., 2017). The above findings support the CDC's report that 20% of new cases of HBV occur principally among adults identifying as MSMs, and are likely to have HIV co-infection possibly because HBV and HIV share similar transmission mechanisms (Schillie, 2018).

5.1.2 Prevalence of OBI in the Jaundiced and MSM-SW Subjects

This study offers the first description of OBI in Kenya, which, as previously outlined, is defined by the detection of HBV DNA in the serum or liver in the absence of notable HBsAg either in the absence or presence of other serological markers of HBV exposure or infection (Burns & Thompson, 2014; Wu et al., 2017). OBI is typically allied to extremely low amounts of HBV DNA, frequently < 200 IU/mL in the serum, which is in line with the subclinical infection where the quantity of the surface antigen may drop below the edge of analytical detection (Said, 2011; Satoh et al., 2008; Wu et al., 2017). Even though mutations in the surface antigen coding region may also contribute to false positive OBI, they were uncommonly noted among the OBI-

specific HBV sequences in the present cross-sectional investigation. Besides, the odds for OBI is influenced by numerous determinants, encompassing HBV-HIV coinfection and population pervasiveness; nonetheless, the detection of OBI is also contingent on test specificity and sensitivity (Zhu et al., 2016).

According to the 2008 Taormina conference on OBI, experts commended that rt-PCR or nested PCR, which are extremely sensitive DNA amplification protocols, should be utilized in the detection of OBI (Raimondo et al., 2008). The analyses in the present investigation were performed according to the above-mentioned expert commendations and confirmed OBI by a positive result in at least two genomic regions by rt-PCR followed by nested PCR to get a sequence evidence. The frequency of OBI in this cross-sectional retrospective study was 18.7% (31/166; 95% CI 13.5 – 25.3), which was congruent with the intermediate endemicity of HBV in sub-Saharan Africa, where OBI frequencies of more than six percent have been documented. In particular, the 18.7% prevalence of OBI was slightly higher than 15.1% observed among HIV-infected individuals in Sudan and 17% among Nigerian blood donors (Oluyinka et al., 2015; Yousif et al., 2014). It was substantially higher than the 5.6% recorded in among HIV-infected adults in Kenya not initiated on ART, and the 5.9% and 6.9% among HIV-positive cohorts examined in two studies conducted in Cameroon, and 8.7% reported among blood donors in Nigeria (Akintule et al., 2018; Gachara et al., 2017; Salpini et al., 2015; Salyani et al., 2021). The high prevalence of OBI in the Kenyan heterogeneous population examined in the current study, which is consistent with the 26.5% observed among 272 HIV-infected persons seeking ART treatment in a hospital in Botswana, confirm the hyperendemicity of HBV exposure and infection in sub-Suharan Africa (Ryan et al., 2017).

As outlined previously, the detection of OBI is contingent on the studied population, sensitivity of the applied assay, and the demographic features investigated participants (Raimondo et al., 2007; Zhu et al., 2016). Thus, the relatively low prevalence reported by Gachara et al. (2017) and Salyani et al. (2021) compared to the high prevalence recorded in such studies as Ryan et al. (2017a) and Yousif et al. (2014) could be attributed to the difference in the NAT assays used. The low prevalence investigations used conventional PCR techniques, while the present study and those reporting high OBI incidences employed the expert recommended rt-PCR and/or nested-PCR. Although Salpini et al. (2015) and Akintule et al. (2018) adopted similar analytic approaches as this investigation, the disparity in the prevalence of OBI in the former could be ascribed to demographic features, while the high prevalence in the latter can conceivably be attributed to the unsurprising elevated seroprevalence of HBV in Nigeria ranging between 9% – 39% (Emechebe et al., 2009), with anti-HBc incidence of >50% of blood donor population (Allain & Candotti, 2009).

In this research, a disparity in the regularity of OBI between the jaundiced patients and MSM-SW cohorts was noted, with the MSM-SW cohort displaying an approximately 63.3% lower frequency of OBI (10/89; 11.2%, 95%, CI 6.2-19.5) compared to the jaundiced patients (10/65; 30.8%, 95%, CI 20.9 – 42.8). The above group difference may be contributed by the effects of risk factors for HBV transmission. Ndiritu et al. (2006) explain that Kenya launched a pentavalent inoculation comprising of the HBV, Haemophilus influenza type b, and Diphtheria-Tetanus-Pertussis antigens in November 2001 administered at 6, 10, and 14 weeks of age. Before the enforcement of the immunization campaign, elevated frequencies of CBHI in sub-Saharan Africa occurred mainly among children aged below five years, which contributed to between 30 – 50 % higher risks for CHBI among children (Spearman et al., 2017). Thus, irrespective of

the amplified risk for HBV spread via high-risk sexual practice among the MSM-SW group, the odds for acquiring CHBI in adulthood is diminished by up to 15% vis-à-vis the development of CHBI in early years of life. In the above light, whereas the jaundiced patients and the MSM-SW cohorts were recruited from the Kenyan general population, it was anticipated that the individuals with indications of fever of unknown origin were more possibly carry subclinical and overt HBV infection. The above is confirmed by the aspect that the HBsAg positivity rate in the original investigation involving the patients presenting with jaundice and fever of unknown origin was slightly above 50% (Ochwoto et al., 2016) versus 10.1% in the MSM-SW cohort reported in this research. In conclusion, the implementation of a pentavalent inoculation in Kenya in 2001, targeting infants at specific intervals, has significantly reduced the incidence of chronic HBV infection (CHBI) in early childhood, mitigating the risk of acquiring CHBI in adulthood. While the MSM-SW group faces an amplified risk of HBV transmission through high-risk sexual practices, their overall odds of acquiring CHBI in adulthood have been reduced by up to 15% compared to the risk in early childhood. This study underscores the importance of vaccination campaigns in curtailing the prevalence of CHBI. Additionally, the varying HBsAg positivity rates observed between the jaundiced patients and the MSM-SW cohort further highlight the diverse epidemiological profiles within different populations.

In the above view, older age has been identified as predictor of OBI in published investigations (Khamduang et al., 2013; Liang et al., 2010). Khamduang et al. (2013) examined the risk factors, prevalence, and influence of sequestered anti-HBc and OBI in expectant women in Thailand testing positive for HIV. The findings showed that the frequency of isolated anti-HBc heightened with their age, with the highest OBI prevalence occurring in subjects aged above 35 years. Similarly, Liang et al. (2010) conducted a cross-sectional investigation to explore the causal

factors for isolated anti-HBc among HIV positive and HIV-uninfected subjects from a HBV hyperendemic region. The anti-HBc was observed in 12.1% of the participants (193/1,598), in which OBI occurred in 1.6% of the samples. Participants with OBI were substantially older (≥40.7 years; P<0.0001, CU 99%, 27.1 – 44.9), a finding that may partly elucidate the 3-fold augmented frequency of OBI among symptomatic patients, given that the average age of the participants in the MSM-SW cohort was nearly 10 years lower (28.6 years) than the mean age of subjects in the jaundiced cohort (37.8 years). Similarly, de Almeida Pereira et al. (2006) reported a statistically significant association between older age with increased prevalence of HBV attributing it to intensified risk of exposure with time.

HIV infection was identified as a risk factor for OBI in the current study although the correlation was not statistically significant. In particular, HIV co-infection was present in 58.8% (10/17; 95% CI: 36.0 – 78.4) of HBV DNA positive MSM-SW subjects, a finding that is consistent with upshots of many studies involving a diversity of subjects mainly in HBV hyperendemic regions. For example, Platt et al. (2020) performed a systematic review and meta-analysis to determine the pervasiveness of HBV-HIV coinfection among patients across the globe. The authors searched EMBASE, MEDLINE and other databases for studies focusing on HIV-HBV co-infections published between 2002 and 2018. Consistent with estimations of this study, the meta-analysis reported a 7.6% or 2.7 million cases of overt HBV-HIV co-infections from 80/195 countries worldwide (interquartile range (IQR) 5.6%- 12.1%), with 1.9 million of the cases (69%) occurring in sub-Saharan Africa (Platt et al., 2020). The 58.8% frequency of HBV DNA positivity in the MSM-SW cohort is substantially higher than the 6.3% OBI rates among anti-HBc positive subjects reported in an Indian study (Saha et al., 2017), the 23.8% noted in a study done in South African (Bell et al., 2012), and the 31.5% anti-HBc positive specimens from HIV-

infected patients analyzed in Eastern India, in which more than half of the samples had CD4 counts below 200 cells/mm^3 (Panigrahi et al., 2012). Existing evidence confirm that OBI is a common occurrence in HIV-positive patients, and its pervasiveness differs substantially in diverse geographic locations (Gachara et al., 2017; Ramezani et al., 2012). Thus, as illustrated in other studies, the substantially high HBV DNA positivity among the HIV-infected MSM-SW cohort could be attributed to immunosuppression caused by HIV infection resulting in diminished antibody reaction to HBV surface antigen test, and HBV reactivation (Burnett et al., 2005; Lukhwareni et al., 2009b). Resultantly, people living with HIV should get their blood tested for HBV DNA before ART initiation to ensure that HBV therapy is also included in their plan of care.

It is well established that blood containing anti-HBc in the lack of HBsAg is symptomatic of an ongoing or previous infection that can be infectious (Shastry & Bhat, 2011). There was a statistically significant link between anti-HBc positivity and occult HBV among all the high-risk groups (Fisher's exact test; $P<0.05$), with 30.8% of the 37 anti-HBc-positive samples showing OBI positivity in the jaundiced cohort. The above incidence is higher than the 6% reported in eastern Ethiopia among anti-HBc-positive HIV-infected participants on ART (Ayana et al., 2020). Findings of published studies across the globe have highlighted an increased risk of isolated anti-HBc among individuals co-infected with HCV or HIV attributing the latter to the interference or down-regulation of HBsAg secretion (Helmy & Al-Sebayel, 2006; Gibney et al., 2008; Wu et al., 2017). While the above is true, it does not explain the insignificant relationship between the presence of anti-HBc antibodies and occurrence of OBI among HIV-positive MSM-SW in this research.

There were no statistically noteworthy associations between behavioral, treatment, or demographic features and HBV DNA positivity among the MSM-SW group apart from the association with HIV ART ($P = 0.0042$, Fisher's exact test). The positive relationship between HIV ART and measureable HBV DNA can be attributed to a diversity of factors, including a recent initiation of ART among the respondents as reported by an investigation involving HIV patients co-infected with HBV in Cameroon (Liégeois et al., 2020), reduced CD4 counts, and elevated HIV viral load (≥300 copies/Ml) as reported in Hoffmann and Thio, (2007) study. As per Hoffmann and Thio (2007), there are numerous essential etiologies of an increase in liver enzymes among individuals with HBV/HIV co-infection on ART that can decrease the tolerability of the antiretroviral medications. Notably, the elevation in hepatic enzymes can be attributed to three causes: CHBI-associated and ART-related etiologies. HBV-associated causes include foreshadowed HBeAg seroconversion by flashes of aspartate aminotransferase (AST) and alanine transaminase (ALT); increased risk for HDV among individuals with CHBI, and emergence of drug-insensitive HBV variants or occurrence of fulminant or acute liver failure when HBV multiplication is abruptly unmitigated following withdrawal of active antiviral medications (Hoffmann & Thio, 2007).

ART-associated causes of raised liver enzymes are a product of drug toxicity (Hoffmann & Thio, 2007). Observably, ART may harm the liver via distinctive reactions, like those that arise with abacavir and nevirapine (Kaplowitz, 2004), inhibition of DNA polymerase-γ in the mitochondria, and direct toxicity. HIV co-occurrence with CHBI enhances the odds for hepatotoxicity from ART medications 3 – 5-fold (Livry et al., 2003; Puoti et al., 2003). Besides investigations examining HIV co-occurrence with HBV in Taiwan and Thailand reported an elevation in hepatotoxicity in 15-16 cases /100 person years in individuals with HIV and CHBI compared to

4.5 – 8.0 cases/100 person years in subjects with HIV only (Law et al., 2003; Sheng et al., 2004). Besides, there is mounting concern regarding the polypharmacy of hepatotoxic medications, particularly to treat tuberculosis (TB) in HIV/HBV patients in HBV endemic regions, such as Africa (Kaplowitz, 2004). The combination of ART and TB treatment has the potential of raising hepatic enzymes in individuals infected with both HIV and HBV than either treatment alone (Wong et al., 2000). In individuals receiving anti-TB medications and ART, HBV DNA positivity enhances the odds for severe ALT and AST elevations by 300% more than that for either ART or anti-TB treatment (Perrillo, 2001; Puoti et al., 2003).

An issue of particular importance to the MSM-SW cohort with respect to ART treatment is the possibility for CHBI to depress immune recovery following commencement of ART (Hoffmann & Thio, 2007). Available empirical evidence is inconsistent with respect to the impact of CHBI on CD4 T-cells recovery. A higher median rise in CD4 T-cell count was noted in HIV-mono-infected subjects (62 cells/μL) versus with both HBV and HIV (29 cells/μL) infections in a Thailand study after 4 – 8 weeks of ART among HIV-NAT. However, after 48 weeks, the rises in CD4 count were same irrespective of HBV status (Law et al., 2004)). Findings from an Italian study reported mounting diversity of mean CD4 T-cell count among subjects with and without CHBI, with those with CHBI posting lower CD4 recovery (De Luca et al., 2002). Similarly, in a Nigerian investigation, the suppression of HIV RNA and absolute rise in CD4 count up to 36 weeks was comparable between subjects with overt HBV infections and HBV-uninfected individuals on ART. Thus, it is possible that the substantial association between HBV DNA positivity and OBI among the MSM-SW samples may be attributed to the impact of CHBI on CD4 count recovery following ART.

Another predictor of OBI in the general population is the engagement in risky sexual behaviors. The demographic features of the MSM-SW revealed that a significant portion of the respondents reported irregular condom use, having multiple sexual partners, and engaging in unprotected oral and vaginal sexual activities, however, the association between the risky sexual behavior and the occurrence of OBI was not statistically significant. Inoue and Tanaka (2016) submit that HBV is predominantly reported among MSM-SW and non-MSM-SW since many sexual partners are prevalent in this cohort. Besides, penetrative anal sex is typically extremely hurtful than vaginal penetration, leading to enhanced odds for exposure to infected blood. In summary, the study provides evidence that there is a difference in the prevalence of OBI among high-risk population groups in Kenya. The prevalence of OBI varies between different groups, with jaundiced patients having a higher frequency compared to MSM-SW individuals; therefore, H0 for the hypothesis that there is no significant difference in the prevalence of occult HBV infection (OBI) among high-risk population groups in Kenya is rejected**.**

5.1.3 Association between Anti-HBs Levels and OBI

The relationship between anti-HBs and OBI is complicated. Available empirical evidence suggest that in some cases, patients with OBI may still produce anti-HBs antibodies, indicating they may have developed immunity to HBV; however, in many other cases, individuals with occult HBV may not produce anti-HBs despite past exposure or vaccination, which can complicate diagnostic assays used to detect HBV infection (Jiang et al., 2021). Twenty-four of the OBI positive samples with detectable DNA had anti-HBs levels ≤100. The above may be attributed to the aspect that viral replication was extremely weak for the host to mount a strong humoral immune response (Yan et al., 2022). The above authors also explain that HBV DNA levels of serological positive OBI samples are often <20,000I IU/ml. Thus, positive anti-HBs

suggests the host has elicited a robust immune reaction to the viral replication which binds with circulating HBsAg leading to formation of circulating immune complexes (CICs) (HBsAg-CICs), which, in turn, cause undetectable HBsAg (Yan et al., 2022). The ultimate strategy to cope with transfusion-transmitted HBV infection caused by transfusion of blood from donors with historical HBV infection is to reject all donors having anti-HBV core antigen (anti-HBc). However, this strategy would result in a huge loss of blood donors and subsequent blood inventory collapse. On the other hand, anti-HBc-positive blood is reportedly not infectious when containing more than 100 mIU/mL of anti-HBV surface antigen (anti-HBs) (Yoshi et al., 2019). This finding provides valuable insights for blood safety protocols and helps strike a balance between safeguarding against transfusion-transmitted HBV infection and maintaining a stable blood supply. It highlights the importance of a nuanced approach to donor selection and screening, taking into consideration both serological markers and quantitative measures of antibody levels. In sum, the study provides evidence that individuals lacking or having a low level of anti-HBs do not necessarily have a higher rate of occult HBV compared to individuals with higher levels of anti-HBs. The relationship between anti-HBs levels and OBI is complex, and some individuals with OBI may still produce anti-HBs antibodies. Therefore, H0 for the hypothesis that individuals lacking or having ≤100 mIU/mL of anti-HBs will have a higher rate of occult HBV than individuals having >100 mIU/mL anti-HBs level is rejected**.**

5.1.4 HBV Circulating Genotypes in the MSM-SW and Jaundiced Patients

The 24 HBV strains sequenced in this study, including 7 HBsAg positive MSM-SW specimens and 17 OBI consisting sequences from 13 jaundiced participants, 1 non-MSM, and 3 MSM-SW9 and showed the circulation of genotype A. It has been illustrated that HBV genotype A is highly prevalent in high-risk populations in Kenya. Similar genetic characterization of HBV have been

documented in an investigation that examined the genetic multiplicity of HBV in individuals using drug in three Kenya cities, namely Kisumu, Nairobi, and Mombasa (Oyaro et al., 2018), as well as in Mabeya et al. (2017) and Greer et al. (2018) who focused on HIV/HBV co-infected patients, and Ochwoto et al. (2016) investigated jaundiced patients. Three subgenotypes of genotype A have been characterized, namely: genotype A1, A2, and A3. In the present study, although all of the sequenced samples belonged to genotype A, there were unanticipated disparities in the phylogenetic assembling of OBI sequences among jaundiced and MSM-SW cohorts. Sequences from the jaundiced OBI cases were spread throughout the genetic tree within subgenotye A1 reference sequences. In contrast, a large share of the sequences from the MSM-SW patients with CHBI and all OBI form the MSM-SW clustered with HBV subgenotype A2 reference sequences, with a 98% branch support.

Numerous studies have reported the predominance of HBV subgenotype A1 in the African populations, including studies conducted in Kenya (Hübschen et al., 2011; Gachara et al., 2017; Kramvis, 2018; Lago et al., 2014; Ochwoto et al., 2016; Webale et al., 2015). Subgenotype A1 has been allied to extremely elevated frequencies of HCC in sub-Saharan Africa (McMahon, 2009). Kew (2010) reviewed the topical progresses in the pathogenesis and causes of hepatocellular carcinoma among Black Africans. The findings showed the occurrence of genotype A1 in 86.5% of the participants and genotype D in 8.1%, and not only were the patients with subgenotype A1 at elevated odds of developing HCC when matched to those with genotype D but also their risk was associated with younger age (Kew, 2010). Subgenotype A2 is often observed outside Africa, mainly in Europe (Tamada et al., 2012).

Surprisingly, all the MSM-SW sequences assembling with A2 reference sequences demonstrated complete sequence identity with over 327 bp of alignment, which is indicative of a

shared network or source of transmission possibly originating from a non-African source circulating within the MSM-SW population. Nonetheless, it is essential to take into account that the comparatively short HBV surface antigen coding region alignment employed in the analysis is impartially conserved and unplanned clustering may arise, as it is demonstrated by sequence identity of numerous research samples within GenBank reference sequences, encompassing numerous with Kenya origins. Consequently, full genome sequence phylogenetic analysis is necessary to completely delimit case associations and address subgenotype characterization. Unlike A1, subgenotype A2 has been found to be less aggressive with respect to the occurrence of HCC, and those HCC-associated A2 cases predominantly occur among much older patients (McMahon, 2009). A prospective investigation examining the virologic events among Spanish patients with subgenotype A2 infection found a significant correlation between HBsAg clearance, HBV DNA clearance, and a higher cumulative frequency of chronic biochemical remission among patients with CHBI than in individuals diagnosed with other HBV genotypes. In sum, the study identifies significant variation in the genotypes of occult HBV infection within the high-risk population groups. It notes unexpected disparities in the phylogenetic assembling of OBI sequences among the jaundiced patients and the MSM-SW cohorts; therefore, the HO for the hypothesis that **t**here is no significant variation in the genotypes of OBI within the high-risk population groups in Kenya is rejected**.**

5.1.5 HBV Genetic Mutations Observed in the MSM-SW and Jaundiced Patients

Genetic mutations influence the antigenicity, secretion, and expression of the HBsAg, leading to the inability of available diagnostic assays to detect the HBsAg marker (Zhu et al., 2016). In this study, three genetic alterations occurring in the HBsAg-coding area, namely D144E, T118A, and T116N, are possibly allied to immune escape were noted in a large share of the jaundiced cohort

(Ochwoto et al., 2016). The above have been reported in other investigations, including a comprehensive review of HBV vaccine and drug escape mutations in Africa and a cross-sectional study conducted in Netherlands (Cremer et al., 2018; Mokaya et al., 2018). Of the three modifications, T116N and D144E has been broadly linked with the manifestation of OBI. Svicher et al. (2012) explain that the correlation between T116N and OBI is ascribed to the aspect that the mutation present a novel N-linked glycosylation site that can diminish HBsAg immunogenicity and impair virion secretion. Huang et al. (2012) documented the incidence of occult HBV infections among 61/38,499 voluntary blood donors in South China and attributed them to four mutations in α determinant region, including D144E, K141E, C124Y, and C124R, which significantly reduced the sensitivity of seven commercial HBsAg immunoassays. Mutation T118A has been allied to vaccine resistance (Sitnik et al., 2004).

While the observed mutations in the current study have been attributed to immune escape, the immunization status of the jaundiced subjects was unknown, and other potential causes of immune pressure that might have contributed to the emergence of the alterations remain unknown. Nonetheless, as explained by Colagrossi et al. (2018), indiscriminate alterations in both ORFs may result in changes in the immunogenicity, release, or expression of HBsAg, thereby influencing vaccine response since the HBsAg-coding region is overlaid by the reverse transcriptase region of the HBV polymerase gene. In sum, the data in this investigation does not offer enough proof to determine whether immune escape or drug resistant mutations pose an enhanced risk to Kenya's HBV high-risk populations. Nonetheless, it would be judicious to screen and monitor HBV infection in all patients diagnosed with HIV before commencement of ART.

5.1.6 Limitations of the Study

There are numerous limitations that may have affected the findings and their analyses reported herein. First, the specimens were sampled from archived sera from two previous investigations examining the occurrence of HIV infections among MSM-SW in Nairobi (McKinnon et al., 2014) and the prevalence of overt HBV among patients presenting with jaundice in various hospitals in Kenya (Ochwoto et al., 2016). The selection of the samples was grounded on the sufficiency of the remaining volume of sera after the original analyses, an action that might have presented selection bias. Existing pieces of evidence indicate that selection bias, which can arise when selection, recruitment, or continued participation in an investigation is somewhat reliant on the odds for having the outcome or exposure of interest, can result in underestimation or overestimation of the association between the study variables (Geneletti et al., 2009) (Talari & Goyal, 2020). Thus, it is possible that the prevalence of the OBI among the high-risk cohorts might have either been over- or under-estimated. Nonetheless, the observed findings are coherent with the described prevalence in various sub-Saharan Africa.

Second, even though the samples were analyzed by an authenticated rt-PCR method targeting three unique HBV genome (HBsAg- coding region, ENH regulatory region, and X/ENHII genomic region), samples that tested negative for OBI were not re-tested or re-extracted using another technique. The re-testing was impossible due to the insufficiency of specimen volume; consequently, there is a likelihood that occurrence rates of OBI reported in the study was under-reported. Third, some information about the jaundiced patients was lacking making it difficult to compare the risk factors for OBI between the jaundiced patients and the MSM-SW cohorts. Fourth, the insufficiency of the specimen volumes made it impossible to perform serological test for anti-HBs levels leaving the determination of the association between anti-HBs and OBI

unanswered. Lastly, inadequate respondent numbers were available for statistical analysis, therefore, only major associations were observed. Thus, even though a substantial relationship between OBI and behavioral variables or mutations was not noted in the study, it might exist in a study with appropriate power. However, this is the first investigation to report the pervasiveness of OBI among high risk populations of Kenya and provides important data that is helpful in policy formulation and for future studies.

5.2 Conclusions and Recommendations

5.2.1 Conclusions

The principal aim of this study was to examine and describe OBI in individuals at increased odds of HBV exposure and infection, namely, MSM-SW and jaundiced patients seeking medical care in four hospitals in Kenya.

From the analysis, the findings of the research resulted in the following conclusions:

1. The overall prevalence of OBI in the cross-sectional retrospective study was 18.7%. Furthermore, a higher prevalence of OBI was observed in jaundiced patients (30.8%) compared to MSM-SW cohorts (11.2%).
2. All 24 HBV genotypes sequenced in the study belonged to genotype A, indicating its high prevalence in high-risk populations in Kenya. Notably, while all sequenced samples fell under genotype A, the genetic tree analysis revealed a diverse distribution within subgenotype A1 reference sequences in jaundiced OBI cases, which aligns with common patterns in the African population. Conversely, sequences from MSM-SW patients with OBI were clustered with GenBank subgenotype A2 reference sequences, suggesting a potential non-African source circulating within this cohort.

3. The study did not directly address the association between anti-HBs levels and OBI. However, it is essential to note that the prevalence of OBI was not significantly correlated with risky sexual behaviors, highlighting the complex nature of OBI transmission within high-risk populations. Additionally, older age emerged as a probable causal factor for OBI, potentially contributing to the elevated frequency observed in the jaundiced patient group.

5.2.2 Application of Research Findings

1. OBI is a predictor for HBV transmission and the development of HCC. Therefore, it is necessary for Kenya's Ministry of Health to recognize the burden of infection signified by OBI and come up with national mitigation and control measures.
2. Since OBI in the present study was associated with anti-HBc positivity among individuals presenting with jaundice with increased odds for HBV infection , the Ministry of Health should recommend the use of highly sensitive methods to screen suspect cases of viral hepatitis to detect the low HBV DNA levels associated with OBI (Locarnini & Raimondo, 2019).
3. Owing to the high risk of HBV infection in sex workers, the Ministry of Health should also roll-out targeted screening and HBV immunization of vulnerable individuals in the MSM-SW cohorts to inhibit the spread of the virus and facilitate referral to care for individuals testing positive for OBI or CBHI.
4. National public health campaigns should be conducted across Kenya to highlight the risk of HIV – HBV co-infections on the health status of individuals living with HIV. Besides,

a need exists for testing patients infected with HIV for HBV and/or OBI before initiating ART.

5. In clinical practice, the finding that blood containing more than 100 mIU/mL of anti-HBV surface antigen (anti-HBs) from donors with a positive anti-HBc status is reportedly not infectious provides valuable guidance for blood safety protocols. Therefore, there is a need for policy formulation in blood donation programs, emphasizing the need for a nuanced approach to donor selection and screening. This policy should incorporate not only traditional serological markers but also quantitative measures of anti-HBs antibody levels to ensure both donor safety and a stable blood supply.

5.2.3 Recommendations for Further Research

Additional cross-sectional and longitudinal cohort studies involving large sample sizes are necessary to determine the current weight of OBI among both low- and high risk groups in Kenya. Additional investigations will help in addressing the limitations encountered in the current study.

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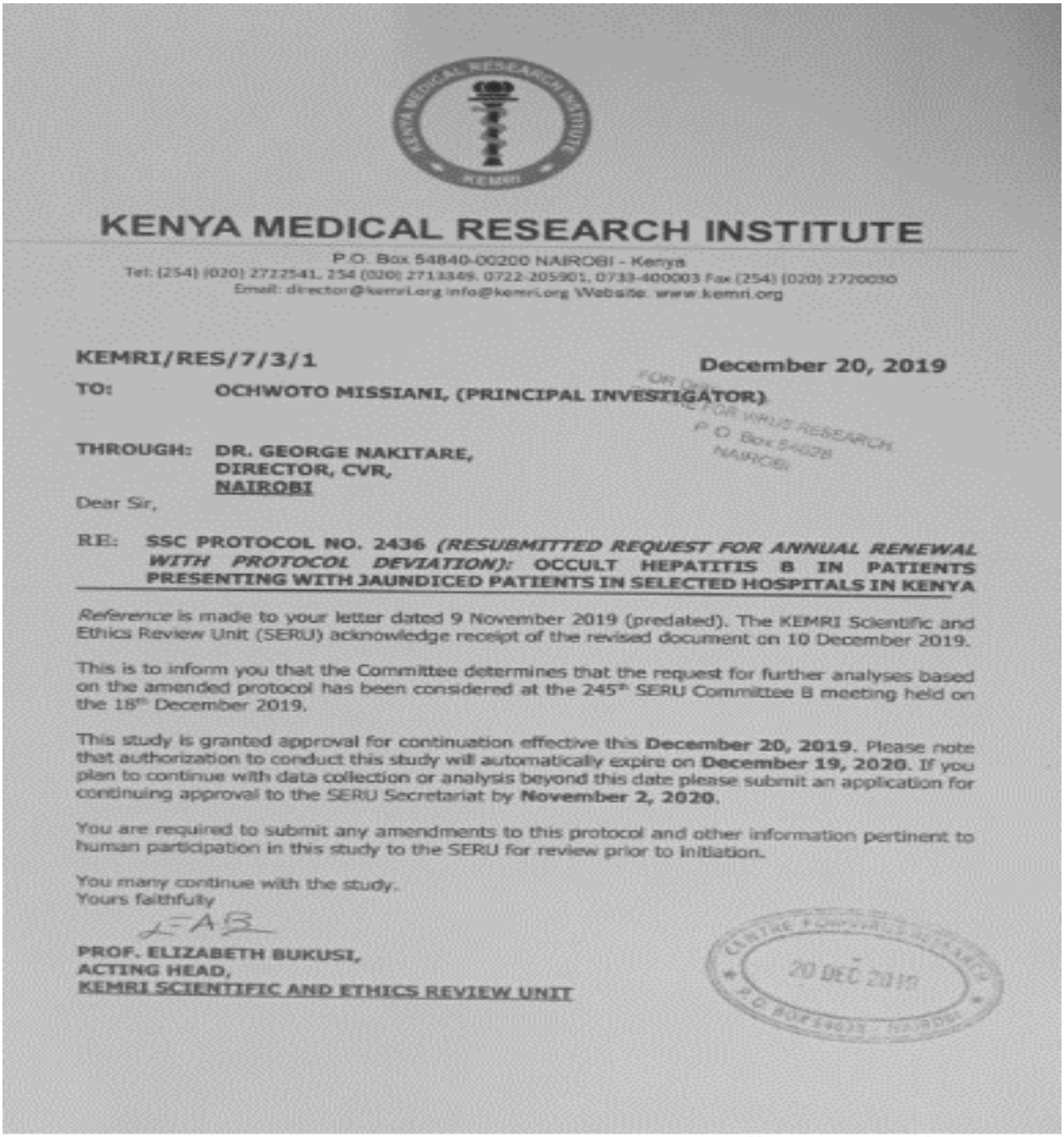
APPENDICES

Appendix 1: Ethical Approval Letter



KENYA MEDICAL RESEARCH INSTITUTE

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Email: director@kemri.org info@kemri.org Website: www.kemri.org

KEMRI/RES/7/3/1 **December 20, 2019**

TO: **OCHWOTO MISSIANI, (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. GEORGE NAKITARE,**
DIRECTOR, CVR,
NAIROBI

Dear Sir,

RE: **SSC PROTOCOL NO. 2436** ***(RESUBMITTED REQUEST FOR ANNUAL RENEWAL WITH PROTOCOL DEVIATION):*** **OCCULT HEPATITIS B IN PATIENTS PRESENTING WITH JAUNDICED PATIENTS IN SELECTED HOSPITALS IN KENYA**

Reference is made to your letter dated 9 November 2019 (predated). The KEMRI Scientific and Ethics Review Unit (SERU) acknowledge receipt of the revised document on 10 December 2019.

This is to inform you that the Committee determines that the request for further analyses based on the amended protocol has been considered at the 245th SERU Committee B meeting held on the 18th December 2019.

This study is granted approval for continuation effective this **December 20, 2019**. Please note that authorization to conduct this study will automatically expire on **December 19, 2020**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the SERU Secretariat by **November 2, 2020**.

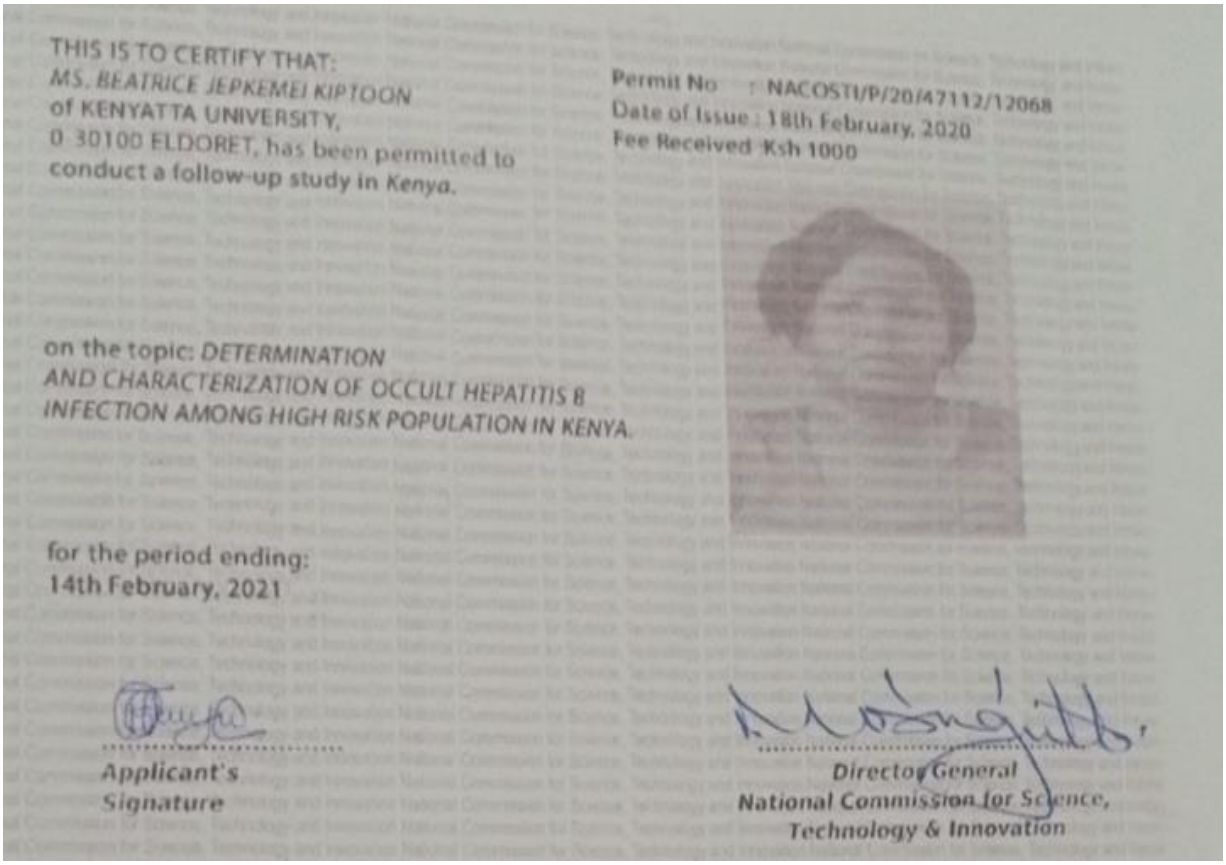
You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SERU for review prior to initiation.

You many continue with the study.
Yours faithfully

PROF. ELIZABETH BUKUSI,
ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

20 DEC 2019

Appendix 2: NACOSTI Approval



THIS IS TO CERTIFY THAT:
MS. BEATRICE JEPKEMEI KIPTOON
of KENYATTA UNIVERSITY,
0-30100 ELDORET, has been permitted to
conduct a follow-up study in Kenya.

Permit No : NACOSTI/P/20/47112/12068
Date of Issue : 18th February, 2020
Fee Received :Ksh 1000

on the topic: DETERMINATION
AND CHARACTERIZATION OF OCCULT HEPATITIS B
INFECTION AMONG HIGH RISK POPULATION IN KENYA.

for the period ending:
14th February, 2021

Applicant's
Signature

Director General
National Commission for Science,
Technology & Innovation

Appendix 3: Publication in *PLoS ONE Journal* Arising from the Study

PLOS ONE

RESEARCH ARTICLE

Characterization of occult hepatitis B in high-risk populations in Kenya

Kiptoon Beatrice Jepkemei[1¤a], Missiani Ochwoto[2], Ken Swidinsky[1], Jacqueline Day[1], Henok Gebrebrhan[3], Lyle R. McKinnon[3,4,5], Anton Andonov[1¤b], Julius Oyugi[3,4], Joshua Kimani[3,4], George Gachara[6], Elijah Maritim Songok[2,3], Carla Osiowy[1,3]*

1 National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba, Canada, **2** Kenya Medical Research Institute, Nairobi, Kenya, **3** Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, Manitoba, Canada, **4** Department of Medical Microbiology, University of Nairobi, Nairobi, Kenya, **5** Centre for the AIDS Programme of Research in South Africa (CAPRISA), Durban, South Africa, **6** Department of Medical Laboratory Sciences, Kenyatta University, Nairobi, Kenya

¤a Current address: Department of Medical Laboratory Sciences, Kenyatta University, Nairobi, Kenya
¤b Current address: Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, Manitoba, Canada
* carla.osiowy@canada.ca

OPEN ACCESS

Citation: Jepkemei KB, Ochwoto M, Swidinsky K, Day J, Gebrebrhan H, McKinnon LR, et al. (2020) Characterization of occult hepatitis B in high-risk populations in Kenya. PLoS ONE 15(5): e0233727. https://doi.org/10.1371/journal.pone.0233727

Editor: Jason Blackard, University of Cincinnati College of Medicine, UNITED STATES

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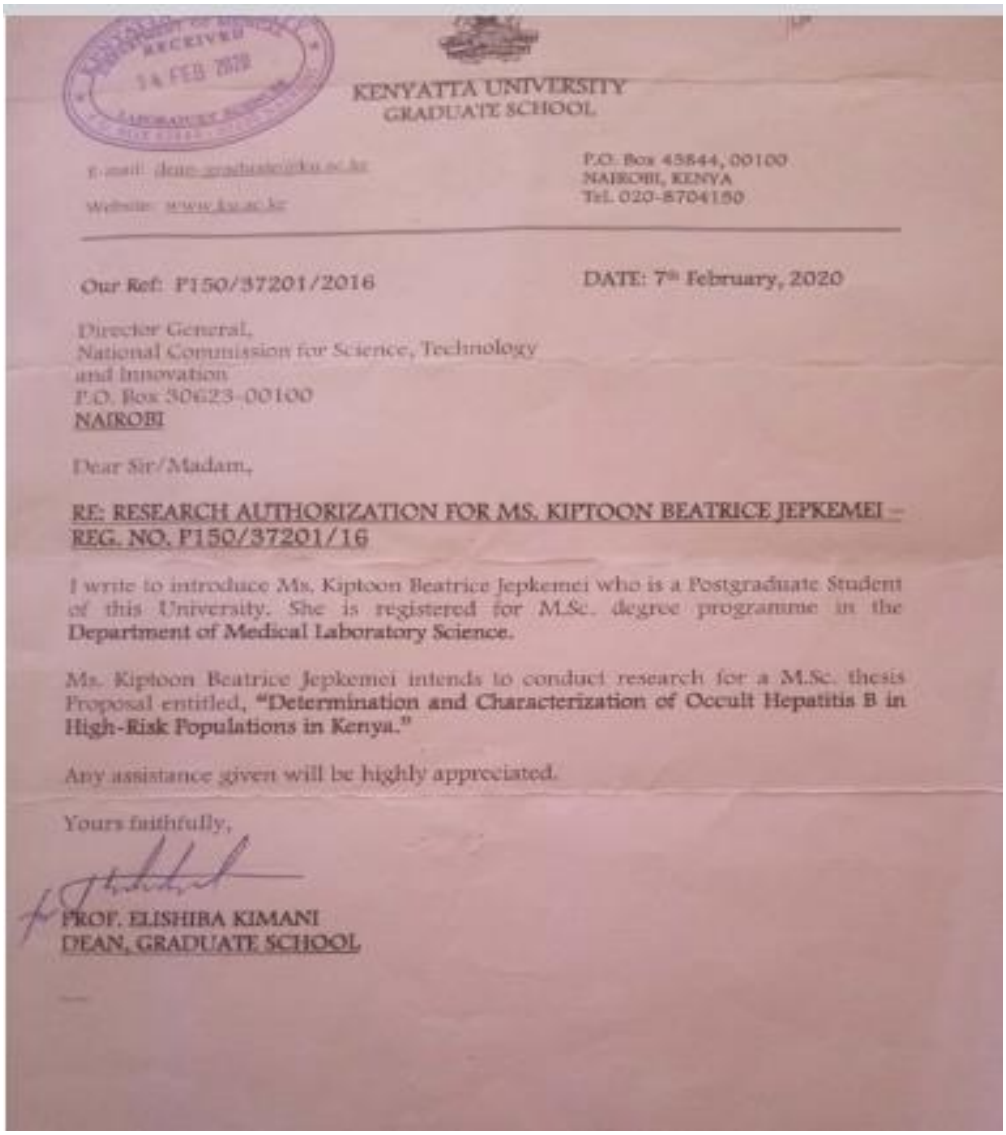
Abstract

Occult hepatitis B infection (OBI) is defined as the presence of hepatitis B virus (HBV) DNA in the liver or serum in the absence of detectable HBV surface antigen (HBsAg). OBI poses a risk for the development of cirrhosis and hepatocellular carcinoma. The prevalence of OBI in Kenya is unknown, thus a study was undertaken to determine the prevalence and molecular characterization of OBI in Kenyan populations at high risk of HBV infection. Sera from two Nairobi cohorts, 99 male sex workers, primarily having sex with men (MSM-SW), and 13 non-MSM men having HIV-positive partners, as well as 65 HBsAg-negative patients presenting with jaundice at Kenyan medical facilities, were tested for HBV serological markers, including HBV DNA by real-time PCR. Positive DNA samples were sequenced and MSM-SW patients were further tested for hepatitis C virus (HCV) infection. Of the 166 HBsAg-negative samples tested, 31 (18.7%; 95% confidence interval [CI] 13.5–25.3) were HBV DNA positive (i.e., occult), the majority (20/31; 64.5%) of which were HBV core protein antibody positive. HCV infection was not observed in the MSM-SW participants, although the prevalence of HBsAg positivity was 10.1% (10/99; 95% CI 5.6–17.6). HBV genotype A was predominant among study cases, including both HBsAg-positive and OBI participants, although the data suggests a non-African network transmission source among MSM-SW. The high prevalence of HBV infection among MSM-SW in Kenya suggests that screening programmes be instituted among high-risk cohorts to facilitate preventative measures, such

Appendix 4: Research Authorization Letter



KENYATTA UNIVERSITY
GRADUATE SCHOOL

RECEIVED
14 FEB 2020

e-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 020-8704150

Our Ref: P150/37201/2016

DATE: 7th February, 2020

Director General,
National Commission for Science, Technology
and Innovation
P.O. Box 30623-00100
NAIROBI

Dear Sir/Madam,

RE: RESEARCH AUTHORIZATION FOR MS. KIPTOON BEATRICE JEPKEMEI – REG. NO. P150/37201/16

I write to introduce Ms. Kiptoon Beatrice Jepkemei who is a Postgraduate Student of this University. She is registered for M.Sc. degree programme in the **Department of Medical Laboratory Science.**

Ms. Kiptoon Beatrice Jepkemei intends to conduct research for a M.Sc. thesis Proposal entitled, **"Determination and Characterization of Occult Hepatitis B in High-Risk Populations in Kenya."**

Any assistance given will be highly appreciated.

Yours faithfully,

for

PROF. ELISHIBA KIMANI
DEAN, GRADUATE SCHOOL

Appendix 5: Approval of Research Proposal

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 020-8704150

Internal Memo

FROM: Dean, Graduate School

DATE: 7th February, 2020

TO: Ms. Kiptoon Beatrice Jepkemei
C/o Department of Medical Laboratory Science

REF: P150/37201/2016

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

This is to inform you that Graduate School Board, at its meeting on 29th January, 2020, approved your Research Proposal for the M.Sc. Degree entitled, **"Determination and Characterization of Occult Hepatitis B in High-Risk Populations in Kenya."**

You may now proceed with your Data collection, subject to clearance with the Director General, National Commission for Science, Technology & Innovation and Director, Ethical Committee, Kenyatta University.

As you embark on your data collection, please note that you will be required to submit to Graduate School completed Supervision Tracking and Progress Report Forms per semester. The forms are available at the University's Website under Graduate School webpage downloads.

Thank you.

EDWIN OBUNGU
FOR: DEAN, GRADUATE SCHOOL

CC: Chairman, Department of Medical Laboratory Science
Supervisors:
1. Dr. George Gachara
C/o Department of Medical Laboratory Science
Kenyatta University

Dr. Carla Osiowy
National Microbiology Laboratory, Public Health Agency of Canada
C/o Department of Medical Laboratory Science
Kenyatta University