GENOTYPES OF HEPATITIS B VIRUS AMONG VOLUNTARY BLOOD DONORS IN KINSHASA, DEMOCRATIC REPUBLIC OF CONGO

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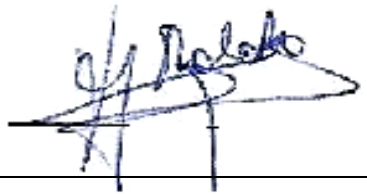
ABSTRACT

Hepatitis B represents a major global health problem. Despite the high endemicity of hepatitis B in Sub-Saharan Africa, little is known about the epidemiology and genotypes of hepatitis B virus (HBV) in Africa. The few published studies that describe the genetic characteristics of HBV in the Democratic Republic of Congo (DRC) involved a very small adult population size. In the present study, epidemiological and molecular characteristics of HBV infection were studied among voluntary blood donors (n=582) at the National Blood Transfusion Centre in Kinshasa, DRC. After obtaining an informed consent, a questionnaire was administered to collect socio-demographic data, followed by blood collection. Sampled blood was screened for hepatitis B surface antigen (HBsAg) using enzyme-linked immunosorbent assay (ELISA). The prevalence of HBsAg among voluntary blood donors in Kinshasa was found to be 6.9 % (40/582). Blood donors aged between 28 and 37 years had the highest HBsAg prevalence. Using PCR targeting the partial S gene, HBV-DNA was detected in 50% (20/40) of HBsAg positive samples and afterwards sequenced in order to perform phylogenetic analysis. Nine (60.0 %), four (26.6 %) and one (6.7 %) out of the 15 HBV nucleotide sequences clustered into genotypes A, E and D, respectively. One (6.7 %) HBV nucleotide sequence did not cluster with any of the known genotypes and could be a novel genotype, subgenotype or recombinant HBV. The present study confirms that HBV infection is endemic in Kinshasa and demonstrates that HBV genotype A predominates in adults. Further studies are needed to assess detailed virological and clinical characteristics of HBV genotypes, as well as the existence of HBV recombinant genotypes in DRC, which may have an impact on clinical outcomes, drug effect and vaccination efficacy.

DECLARATION

I, Patience Maindo Pati Moloko, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.



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DEDICATION

To my spouse Didier Mudwahefa Anzenza and our children Amanda and Olivia Mudwahefa, who patiently accepted to have me away for my studies, and to my beloved parents, Mr. Sylvestre Maindo Papée and Mrs. Colette Lieke Mopembu, who brought me up as a potential researcher, I dedicate the present work.

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LIST OF ABBREVIATIONS AND ACRONYMS

BCP	Basal core promoter
BLASTn	Basic local alignment tool for nucleotides
cccDNA	Covalently closed-circular DNA
CA	California
CNTS	National Blood Transfusion Centre (Centre National de Transfusion Sanguine)
CPDA	Citrate phosphate dextrose adenine
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HBc Ag	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBIG	Hepatitis B immune globulin
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HIV	Human immunodeficiency virus
Ig M	Immunoglobulin M
MEGA	Molecular evolutionary genetics analysis
ORF	Open reading frame
PCR	Polymerase chain reaction

RNA	Ribonucleic acid
USA	United States of America
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Hepatitis B virus (HBV) infection represents a major global health problem. According to the World Health Organization (WHO), 400 million people live with chronic hepatitis B infection (WHO, 2014), which is the persistence of hepatitis B surface antigen (HBsAg) in the blood in a period of more than 6 months (Pujol *et al.,* 2009). Up to 15-40 % of HBV infected patients develop cirrhosis, liver failure, or hepatocellular carcinoma (HCC) (Lok, 2002; Hou *et al.*, 2005). Every year, more than 780 000 people die from end-stage liver disease or HCC (WHO, 2014). Hepatocellular carcinoma is ranked as the fifth cause of cancer and third cause of death by cancer worldwide (Mutimer and Oo, 2011).

The global prevalence of chronic HBV infection, defined as the prevalence of HBsAg, varies widely worldwide. While Western Europe, North America and Australia exhibit the lowest rate, i.e. 2 %, Sub-Saharan Africa, East Asia and the Amazon exhibit the highest rate of hepatitis B, since more than 8 % of the population in these regions have chronic HBV infection. Eastern and Southern Europe, the Middle East, Japan, and part of South America have the intermediate rate of 2-7 % chronic carriers (Mulders *et al.*, 2004; Hou *et al.*, 2005; Hwang and Cheung, 2011; WHO, 2014).

Owing to the high genetic variability of HBV, eight confirmed HBV genotypes designated A to H, two tentatives genotypes named I and J, and around forty subgenotypes have been described (Pourkarim *et al.*, 2014). HBV genotypes and subgenotypes are characterized by a minimum sequence divergence of 7.5 and 4 % of the entire genome, respectively (Kramvis *et al.,* 2008; Kurbanov *et al*., 2010; Pourkarim *et al.*, 2014). Different HBV genotypes and subgenotypes display differences in transmission routes. Many studies have shown that some genotypes are associated with particular prognoses, such as progression to HCC, acute forms of hepatitis B or response to antiviral treatment. Thus, the knowledge of HBV genotype can help in predicting clinical outcome and planning suitable treatment (Miyakawa and Mizokami, 2003; Pujol *et al.,* 2009; Tatematsu *et al.*, 2009; Pourkarim *et al*., 2014; Sunbul, 2014). In addition, according to geographical distribution, HBV genotypes vary between continents, geographical regions, countries, and even between geographical regions within countries (Sunbul, 2014).

Yet, information on HBV genotypes circulating in Sub-Saharan Africa and especially in the Democratic Republic of Congo (DRC) is very limited. Mulders *et al.* reported, in a cohort study conducted from 2000 to 2003, that HBV genotype E was the most prevalent in six West Africa countries and DRC. HBV in DRC particularly showed a genetic diversity by displaying A and D genotypes in three adults, and E genotype in children (Mulders *et al.,* 2004). However, data on HBV genotypes circulating in adults in DRC is nearly inexistent. The present study investigated HBV genotypes found in voluntary blood donors in Kinshasa, DRC.

1.2 Problem statement and justification

The current genetic diversity of HBV in adults living in DRC is unknown, and might be different from the one described ten years ago. Indeed, the study performed by Mulders *et al.* (2004) mainly involved children and only three adults. Moreover, HBV genotypes differ according to transmission routes (Pujol *et al.,* 2009; Pourkarim *et al*., 2014; Sunbul, 2014). Since adults may also be infected with HBV via sexual intercourses, which is not common in children, HBV genotypes found in adults may differ from HBV genotypes found in children in DRC. Based on the reasons given above and in order to study on a greater number of adults, the present study was conducted to determine the genotypes of HBV in voluntary blood donors attending the National Blood Transfusion Centre (CNTS), in Kinshasa, DRC.

1.3 Objectives

1.3.1 General objective

The main objective of the present study was to determine the genotypes HBV in voluntary blood donors in Kinshasa, DRC.

1.3.2 Specific objectives

The specific objectives of the present study were:

(i) to determine the prevalence of HBsAg in voluntary blood donors in Kinshasa by using enzyme-linked immunosorbent assay (ELISA),

(ii) to establish the socio-demographic factors associated to HBsAg prevalence in voluntary blood donors in Kinshasa, and

(iii) to examine the genotypes of HBV in voluntary blood donors in Kinshasa by polymerase chain reaction (PCR), nucleotide sequencing and phylogenetic analysis.

1.4 Research questions

The research questions of the preset study were:

i. What is the prevalence of HBsAg in voluntary blood donors in Kinshasa?
ii. What are the socio-demographic factors associated to HBsAg prevalence in voluntary blood donors in Kinshasa?
iii. What are the genotypes of HV in voluntary blood donors in Kinshasa?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Hepatitis B virus

2.1.1 HBV classification

HBV is a hepadnavirus (hepa stands for liver and dna for deoxyribonucleic acid) classified into the *Hepadnaviridae*, a family of enveloped and hepatotropic viruses with a genome consisting of partially double-stranded DNA of 3.2 kilobase pairs (Siegel, 2004; Schaefer, 2007; Zhang and Cao, 2011). HBV, the hepadnavirus infecting humans, belongs to the genus of *Orthohepadnavirus* (ortho stands for mammalian). This genus also includes woodchuck hepatitis virus, Beechey ground squirrel hepatitis virus, Arctic ground squirrel hepatitis virus, Woolly Monkey HBV, Gorilla HBV, Orangutan HBV and Chimpanzee HBV (Hu *et al*., 2000; Schaefer, 2007).

2.1.2 The viral structure

The intact virion comprises the viral DNA surrounded by a nucleocapsid (core protein or antigen) and an outer layer constituted by the HBV surface protein or antigen (HBsAg) (Fig. 1). The HBsAg is composed of three proteins, including the small, medium and large proteins (Pujol *et al.*, 2009; Mutimer and Oo, 2011). The HBV genome (Fig. 2) is circular and contains four overlapping open reading frames (ORF), including the S, C, P and X ORFs. The S ORF can be divided into the pre-S1,

pre-S2, and S regions, which encode the proteins of the viral surface envelope, the HBsAg.

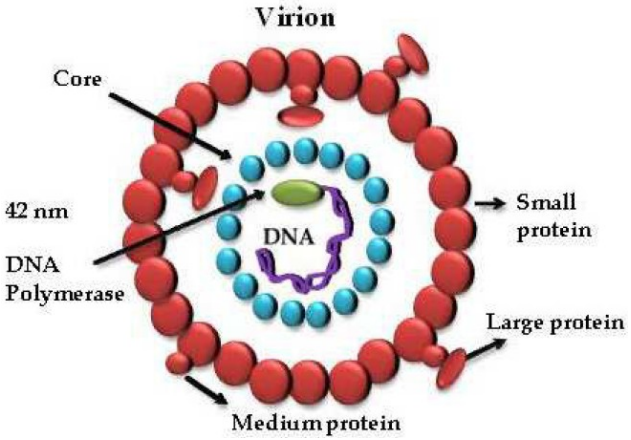

Figure 1: HBV virion. The virion has a size of approximately 42 nm. The three proteins of HBV surface antigen (small, medium and large) are embedded in the lipid envelope covering the core antigen (HBcAg). HBV DNA, which is covalently linked to the DNA polymerase, interacts with HBcAg. Source : Pujol *et al.* (2012).

Hepatitis B surface antigen circulates in blood as spherical and tubular particles (Van Damme *et al.,* 2010). The core or C ORF contains the precore and core regions and encodes hepatitis B core antigen (HBcAg) and hepatitis B e antigen (HBeAg) (Liang, 2009; Pujol *et al.*, 2009). In addition, two viral enhancers positively regulate transcription of the HBV promoters, including basal core promoter (BCP) that controls the transcription of both, the pre-core and core regions (Pujol *et al.*, 2009). Hepatitis B e antigen is clinically used as an indicator of active viral replication and has been associated with an increased risk of HCC (Zhang and Cao, 2011). The P

ORF encodes the polymerase (pol), a large protein (about 800 amino acids) comprising three domains: the terminal protein domain, which is involved in encapsidation and initiation of minus-strand synthesis; the reverse transcriptase domain, which catalyzes genome synthesis; and the ribonuclease H domain, which degrades pregenomic ribonucleic acid (RNA) and facilitates replication. Hepatitis B virus X ORF encodes the hepatitis B x antigen, a 16.5 kilodalton protein with multiple functions, including signal transduction, transcriptional activation, DNA repair, and inhibition of protein degradation (Liang, 2009). The overlapping structure of the coding regions makes the utility of the HBV genome more than 150 % (Zhang and Cao, 2011).

2.1.3 HBV replication

The initial phase of HBV infection involves the fusion of viral surface proteins with host cell membrane, allowing the nucleocapsid core protein to enter the cell (Brough, 2007). Mechanisms of viral disassembly and intracellular transport of the viral genome into the nucleus are not well understood and probably involve modification of the nucleocapsid core protein, which is transported into the nuclear membrane of the host cell (Liang, 2009). After entry of the viral genome into the nucleus, the single-stranded gap region in the viral genome is repaired by the viral polymerase protein to form a covalently closed-circular DNA (cccDNA). The cccDNA is the stable component of the replication cycle that is relatively resistant to antiviral action and immune clearance (Brough, 2007; Liang, 2009). The cccDNA serves as the transcriptional template for host RNA polymerase II. This enzyme generates a series of genomic and subgenomic transcripts or RNAs. All viral RNA is transported to the

cytoplasm, where its translation yields the viral envelope, core, and polymerase proteins, as well as the X and pre C polypeptides. Next, nucleocapsids are assembled in the cytosol, and during this process a single molecule of genomic RNA is incorporated into the assembling viral core. Once the viral RNA is encapsidated, the synthesis of viral partially double stranded DNA by reverse transcription of the viral RNA begins. Some core proteins bearing the mature partially double stranded DNA are transported back to the nucleus, where their newly minted DNA can be converted to cccDNA to maintain a stable intranuclear pool of transcriptional templates. Most core proteins, however, bud into regions of intracellular membranes bearing the viral envelope proteins. In so doing, they acquire lipoprotein envelopes containing the viral L, M, and S surface antigens and are then exported from the cell (Ganem and Prince, 2004).

2.1.4 Transmission patterns and HBV epidemiology

HBV is generally transmitted through contaminated body fluids via different routes of transmission, including sexual intercourse, unsafe injections, blood transfusions from infected patients and mother-to-neonate transmission (Pourkarim *et al.*, 2014). Tears, urine, saliva, sweat, bites and broken skin have recently become accepted as probable modes of transmission (Komatsu *et al.*, 2012; Pourkarim *et al.*, 2014). Indirect transmission through contaminated surfaces and object has been also reported, since HBV is able to survive on surfaces for at least seven days. However, the transmission routes largely depend on the regional prevalence of chronic carriers of HBV-infected individuals as described below (Shepard *et al.*, 2006).

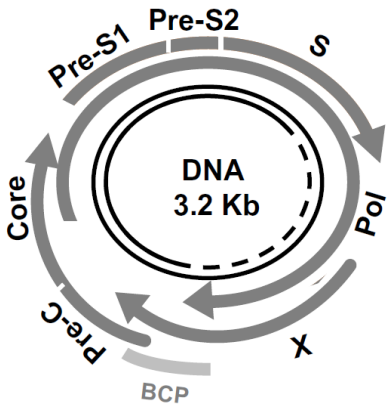

Figure 2: HBV genome organization.

The partially double-stranded, circular HBV DNA is indicated in black lines and the overlapped open reading frames (ORFs) in dark grey lines. In clear grey line the basal core promoter (BCP) is shown. The BCP controls the transcription of both, the pre-core and core regions. The precore and core regions encode hepatitis B core antigen (HBcAg) and hepatitis B e antigen (HBeAg). The S ORF is divided into the Pre-S1, pre-S2, and S regions, which encode the large, medium and small proteins of the viral surface envelope, the HBsAg. Pol encodes the polymerase. The X ORF encodes the hepatitis B x antigen. The arrows show the direction of transcription. Source: Pujol *et al.* (2009).

The prevalence of chronic HBV infection varies markedly throughout regions and can be categorized as high, intermediate and low endemicity (Fig. 3). The age at the time of infection is associated with the endemicity of HBV infection (Hou *et al*., 2005). Hepatitis B is highly endemic in South East Asia, China, Sub-Saharan Africa and the Amazon Basin, where HBsAg prevalence is ≥8 %. In those regions, 70-95 % of the population had evidence of HBV infection. In addition, most infections occur

during infancy or childhood, vertically at the time of birth from chronically infected mothers or horizontally (exposure to chronically infected household members) in those areas. The rates of chronic liver disease and liver cancer in adults are high, though most infections in children are asymptomatic (Hou *et al*., 2005; Shepard *et al*., 2006; Hwang and Cheung, 2011). In Russia, Eastern and Southern Europe, Central and South America, HBV prevalence is 2-7 %, which is considered intermediate. In these regions, between 10 and 60 % of the population show past or present serological evidence of HBV infection. Adolescents and adults are mainly infected in these areas. Additionally, the transmission patterns in these regions are mixed, including infant, early childhood and adult transmission (Hou *et al*., 2005; Hwang and Cheung, 2011). The endemicity of HBV is considered low in most developed areas, such as North America, Northern and Western Europe and Australia. In these regions, 5-7 % of the population are infected, and only 0.5-2 % of the population are chronic carriers (Hou *et al*., 2005). In addition, most HBV infections occur in adolescents and young adults in relatively well-defined high-risk groups, including injection drug users, homosexual males, health care workers and patients who require regular blood transfusion or hemodialysis (Hou *et al*., 2005; Shepard *et al.,* 2006; CDC, 2013).

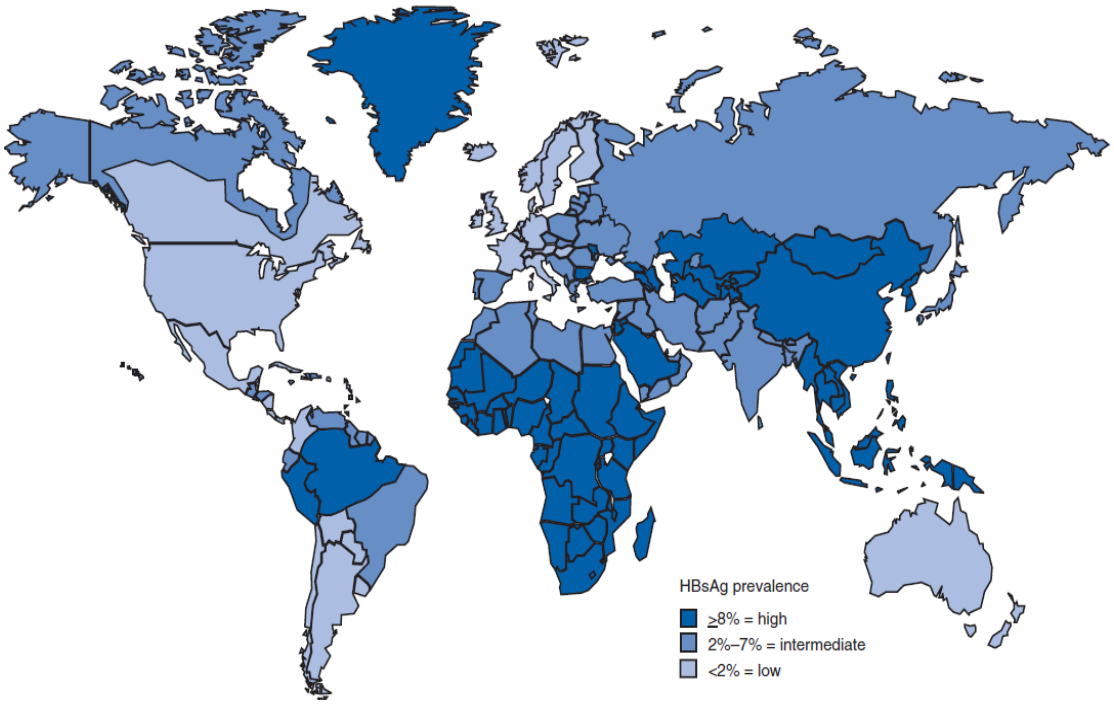

Figure 3: Geographic distribution of chronic HBV infection worldwide, 2006. Regions of high endemicity, where HBsAg prevalence is ≥8 %, are indicated in dark blue colour (■). Intermediate endemicity areas, where HBV prevalence is 2-7 %, are indicated in blue colour (■). The endemicity of HBV is considered low (HBsAg prevalence ‹ 2 %) in regions indicated in weak blue colour (■). (Source: CDC, 2008).

2.1.5 HBV genotypes

The reverse transcriptase enzyme lacks proofreading activity. Thus, the nucleotide substitution rate for HBV is higher than that of other DNA viruses. During persistent, long-term HBV infection and under different selective pressures, variants of HBV can emerge (Pourkarim *et al.*, 2014). There are eight confirmed HBV genotypes, A through H, and two tentative genotypes, including I and J (Pourkarim *et al.*, 2014; Sunbul, 2014). Hepatitis B virus genotypes are defined by a minimum sequence divergence of 7.5 % over the full-length genome. HBV genotype I has been questioned due to complex recombination. Genotype J is closer to gibbon/orang-utan genotypes than to human genotypes in the P and large S genes and closest to Australian aboriginal strains and orangutan-derived strains in the S gene, whereas it is closer to human than ape genotypes in the C gene (Tatematsu *et al.*, 2009). Within the same genotype, subgenotypes are subgroups having a nucleotide divergence between 4 % and 7.5 % of the whole genome and a high phylogenetic bootstrap support (Kramvis *et al.,* 2008; Kurbanov *et al*., 2010). Genotypes A-D and F are subdivided into subgenotypes, while no subgenotypes have been defined for E, G and H genotypes (Schaefer, 2007; Kurbanov *et al*., 2010; Hwang and Cheung, 2011; Sunbul, 2014). Subgenotypes are subdivided into clades, defined by whole genome sequence divergence less than 4% (Kramvis *et al.*, 2008; Kurbanov *et al*., 2010; Pourkarim *et al.*, 2014). Classification of HBV strains was based on the immunological heterogeneity of HBsAg prior to molecular analyses (genotype, subgenotype and clades), leading to the categorization into different HBV serotypes (subtypes). This serotype-based classification is still used, and epidemiological

studies describe associations between serologic subtypes and genotypes (Kramvis *et al*., 2008; Pourkarim *et al.*, 2014).

2.1.6 Geographical distribution of HBV genotypes

Different genotypes and subgenotypes show distinctive geographical distribution. Genotype A is dominant in North America, Northern and Western Europe. Some genotype A strains have been described in Hong Kong, Philippines and in some parts of Asia and Africa (Schaefer, 2007; Pourkarim *et al.*, 2014). Genotype B and C are most common in Southeast Asia. They have been also described in the Pacific Island. Genotype D can be found all over the world, though it is endemic to the Mediterranean region and Eastern Europe (Schaefer, 2007; Hwang and Cheung, 2011; Pourkarim *et al.*, 2014). Genotype D is detectable among intravenous drug users worldwide (Pourkarim *et al.*, 2014). Genotype F is found in South and Central America. Genotype G has been described in Germany, France, Mexico, Central America, and the United States. It has been recently reported in Belgium (Hwang and Cheung, 2011; Pourkarim *et al.*, 2014; Sunbul, 2014). Genotype H has been reported in Japan and America (Pourkarim *et al.*, 2014). Genotype I has recently been reported in Vietnam and Laos, while J has been described in the Ryukyu Islands in Japan (Sunbul, 2014). The pattern of genotype distribution changes according to the pattern of global migration (Kurbanov *et al*., 2010).

2.1.7 HBV genotypes in DRC

Few studies exist on HBV genotypes circulating among adults in DRC in general and in Kinshasa in particular. One study was performed on serum samples collected

between 2000 and 2002 from a cohort of 17 and 34 children reporting to hospitals in Lower Congo and Kinshasa respectively. The HBV genotype found belonged to genotype E. In addition, two HBsAg-positive patients from Kinshasa, as well as one patient from the DRC from whom samples were obtained in 1992 in Luxembourg were included in the study. The genotypes found in those adults were the A and D HBV genotypes (Mulders *et al.,* 2004). Hannoun *et al*. (2005) reported HBV genotype A, subtype or subgenotype A1, in one hepatitis-positive patient from Congo. Pourkarim *et al.* (2010) analyzed serum samples from two HBV-positive African-Belgian patients originally from Congo, which were collected in 1998 and 2001, respectively. The two patients were dually infected with human immunodeficiency virus (HIV) and HBV. HBV full length genomes were identified as HBV genotype A, subgenotype A6.

2.2 Hepatitis B

2.2.1 Natural history and clinical manifestations of hepatitis B

The spectrum of clinical manifestations of HBV infection varies in both acute and chronic disease. During the acute phase, manifestations range from subclinical or anicteric hepatitis to icteric hepatitis and, in some cases, fulminant hepatitis. During the chronic phase, manifestations range from an asymptomatic carrier state to chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Extrahepatic manifestations can occur in both acute and chronic infection (Mohr *et al*., 2014).

2.2.1.1 Acute hepatitis

After HBV transmission, the incubation period averages two to three months and can range from 1 to 6 months. A prodromal or preicteric phase may appear before acute hepatitis develops (Liang, 2009). During this period a serum sickness-like syndrome may develop. This syndrome manifests with extrahepatic signs including fever, skin rash, arthralgia and arthritis. During the prodromal phase, alanine aminotransferase levels rise and high levels of HBsAg and HBV DNA are detectable. This phase will usually cease with the onset of jaundice or dark urine, nausea and other unspecific constitutional symptoms. At least 70 % of patients will then have subclinical or anicteric hepatitis, while less than 30 % will develop icteric hepatitis. In case of coinfection with other hepatitis viruses or other underlying liver disease, the clinical course may be more severe. The symptoms including jaundice generally disappear after one to three months while HBsAg is cleared and viral levels decrease. However, some patients have prolonged fatigue even after normalization of serum aminotransferase concentrations. Persistent elevation of serum alanine aminotransferase for more than six months indicates progression to chronic hepatitis (Liang, 2009; Mohr *et al*., 2014). Hepatitis B virus DNA may persist lifelong in the form of cccDNA in patients who recover from acute hepatitis B. This latent infection maintains the T cell response that keeps the virus under control (Yotsuyanagi *et al*., 1998; Mohr *et al*., 2014). Complete eradication rarely occurs. Thus, immunosuppression can lead to reactivation of the virus, for instance, after organ transplant or during chemotherapy.

Fulminant hepatic failure is unusual, occurring in approximately 0.1-0.5 % of patients. Reasons and risk factors for fulminant hepatitis B are not well understood (Garfein *et al.*, 2004). This may correlate with substance use or coinfections with other viruses. Fulminant hepatitis B is believed to be due to massive immune-mediated lysis of infected hepatocytes. This is why many patients with fulminant hepatitis B have no evidence of HBV replication at presentation. In adults, the likelihood of fulminant hepatitis B is less than one % (Mohr *et al*., 2014).

2.2.1.2 Chronic hepatitis

The HBV chronicity rate is around 5 % or less in adult-acquired infection. In perinatally acquired infection it is estimated to be approximately 90 %, and 20-50 % for infections between the ages of one and five years (Ganem and Prince, 2004; McMahon *et al*., 1985). Most patients will not have a history of acute hepatitis. Most patients with chronic hepatitis B are clinically asymptomatic. Some may have nonspecific symptoms such as fatigue. In most instances, significant clinical symptoms will develop only if liver disease progresses to decompensated cirrhosis. In addition, extrahepatic manifestations, such as polyarteritis nodosa and glomerular disease, may occur in 10-20 % of patients with chronic hepatitis B and cause symptoms (Han, 2004). Accordingly, a physical exam will be normal in most instances. In advanced liver disease there may be clinical signs of chronic liver disease such as splenomegaly, spider angioma, caput medusae, palmar erythema, testicular atrophy, gynecomastia, etc. In patients with decompensated cirrhosis, jaundice, ascites, peripheral edema, and encephalopathy may be present (Mohr *et al*.,

2014). Early during infection, HBeAg, HBsAg, and HBV DNA are usually present in high titres, and there are mild to moderate elevations in serum aminotransferase levels. With time, however, the disease activity can resolve either with persistence of high levels of HBeAg and HBV DNA (the "immune tolerance phase") or with loss of HBeAg and fall of HBV DNA to low or undetectable levels ("inactive carrier state"). Other patients continue to have chronic hepatitis B, although some lose HBeAg and develop anti-HBe (HBeAg-negative chronic hepatitis B). These patients with HBeAg-negative chronic hepatitis may have residual wild type virus or HBV variants that cannot produce HBeAg due to precore or core promoter variants (Liang, 2009; Mohr *et al.*, 2014). The natural course of chronic HBV infection is determined by the interplay of viral replication and the host immune response. Other factors that may play a role in the progression of HBV-related liver disease include gender, alcohol consumption, and concomitant infection with other hepatitis virus(es). The outcome of chronic HBV infection depends upon the severity of liver disease at the time HBV replication is arrested. Liver fibrosis is potentially reversible once HBV replication is controlled. Very few patients with chronic HBV infection become HBsAg-negative in the natural course of infection (Mohr *et al.*, 2014). The annual rate of HBsAg clearance has been estimated to be less than 2 % in Western patients and even lower (0.1-0.8 %) in patients of Asian origin (Liaw *et al*., 1991) following an accelerated decrease in HBsAg levels during the 3 years before HBsAg seroclearance (Chen *et al*., 2012). If loss of HBsAg occurs, prognosis is considered favorable. However, clearance of HBsAg does not exclude development of cirrhosis or HCC in some patients, although the exact rate of these complications is unknown. This

phenomenon is thought to be linked to the fact that HBV DNA may still be present in hepatocytes despite HBsAg loss (Mohr *et al.*, 2014).

2.2.2 Clinical relevance of HBV genotypes and subgenotypes

Many studies suggest that HBV genotyping is important for determining clinical outcome and designing appropriate antiviral treatment. Several reports showed that genotype A evolves more rapidly in patients than genotype D does, which poses problems for treatment. Also, patients infected with genotype C progressed to end stage liver disease earlier than those infected by genotype B (Sumi *et al.*, 2003; Pourkarim *et al.*, 2014). A study performed in Brazil showed that patients infected with genotype F have higher mortality rates than those infected with genotype A or D (Gomes-Gouveâ *et al.*, 2009). In India, genotype D is associated with more severe liver complications than other genotypes (Thakur *et al.,* 2002). In the United States, it has been reported that genotype D is an independent risk factor for fulminant hepatitis (Wai *et al.*, 2005). However, patients infected with genotype F have a higher rate of liver-related mortality than those infected with genotype D (Sánchez-Tapias *et al.*, 2002; Pourkarim *et al.*, 2014). Genotype C and D generally tend to be related to more severe liver disease than genotype A and B and are more frequently associated with HCC. Moreover, the response to interferon-α treatment in patients with genotypes A and B is better than that in patients with genotypes C, D, and mixed genotypes, though no specific mutations have been associated to this immunomodulator (Pujol *et al.*, 2012; Shi, 2012). It has been shown that genotype B is more frequently associated with lamivudine-resistant variants than genotype C. Likewise, in some studies, it has been observed that genotype A develops antiviral

resistant variants earlier than genotype D (Kramvis *et al.*, 2005; Pourkarim *et al.*, 2014). There are very limited data on the association of genotype E with its clinical relevance. HBV genotypes E, F, and H appear to be sensitive to Interferon-α treatment (Cao, 2009).

There are also clinical differences among subgenotypes. Studies in Taiwan showed that a high proportion of younger patients with HCC had HBV genotype B compared to genotype C (Kao *et al.*, 2000). In contrast, a study done in the Okinawa Islands of Japan showed that genotype B in Japan was rarely associated with the development of HCC, and only in older age (Nakayoshi *et al.*, 2003). This disparity could be explained by the difference in sub-genotypes, because Japan is infected with sub-genotype B1, whereas Taiwan is predominantly infected with sub-genotype B2 (Hwang and Cheung, 2011).

2.2.3 Laboratory diagnosis of hepatitis B

Laboratory diagnosis of HBV infection focuses on the detection of HBsAg, which is a marker of HBV infection. WHO recommends that all blood donations be tested for this marker to avoid transmission to recipients. During acute HBV infection, HBsAg and immunoglobulin M (IgM) antibody to the core antigen (HBcAg) are present in the blood. HBeAg is present during the initial phase of infection. Chronic infection is characterized by the persistence of HBsAg beyond 6 months (with or without concurrent HBeAg). Persistence of HBsAg is the principal marker of risk for developing chronic liver disease and HCC later in life. Hepatitis B e antigen is a serum marker of active viral replication. Its presence indicates that the blood and

body fluids of the infected individual are highly contagious (Ganem and Prince, 2004; WHO, 2014). There are many detection techniques of HBV infection markers such as ELISA and Rapid Immunochromatographic Tests (Khan *et al*., 2010).

Hepatitis B virus DNA testing can be helpful in the assessment of level of viral replication. The current real-time PCR–based assay (TaqMan) has a low limit of detection of 5–10 HBV DNA copies/ml. With this degree of sensitivity, HBV DNA can be detected early during the course of infection, arising before the appearance of other serological markers, such as HBsAg or anti-HBc. Thus, HBV DNA testing has now become routinely used in blood product screening (nucleic acid testing) and monitoring of patients with HBV during treatment. Persistently high levels of HBV DNA following resolution of hepatitis may be indicative of a failure to control the infection and an evolution into chronic infection (Liang, 2009).

2.2.4 Treatment of hepatitis B

2.2.4.1. Prevention of HBV infection

Three main strategies are available for the prevention of HBV infection, including behavior modification, passive immunoprophylaxis, and active immunization (Hou *et al.*, 2005).

2.2.4.1.1 Behavior modification

Changes in sexual practice and improved screening measures of blood products have reduced the risk of transfusion-associated hepatitis. Behavior modification might be

more beneficial in developed countries than in developing countries, where neonates and children in early childhood are at the greatest risk of acquiring infection. In these groups, both passive and active immunoprophylaxis will be more effective (Hou *et al.*, 2005).

2.2.4.1.2 Passive immunoprophylaxis

Passive immunoprophylaxis is provided by hepatitis B immune globulin (HBIG). HBIG is a sterile solution of ready-made antibodies against hepatitis B. Hepatitis B immune globulin is prepared from human blood provided by selected donors who already have a high level of antibodies to hepatitis B virus. Passive immunoprophylaxis is used in four situations, including newborns of mothers infected with hepatitis B, after needlestick exposure, after sexual exposure, and after liver transplantation (Hou *et al.*, 2005).

For infants born to HBsAg positive mothers, current dosing recommendations are 0.13 ml/kg HBIG immediately after delivery or within 12 hours after birth in combination with recombinant vaccine. The combination results in a higher-than-90 % level of protection against perinatal acquisition of HBV (Stevens *et al*., 1987). However, between 3.7 % and 9.9 % of infants still acquire HBV infection perinatally from HBV infected mothers, despite immunoprophylaxis. Failure of passive and active immunoprophylaxis in this setting may be the result of in utero transmission of HBV infection, perinatal transmission related to a high inoculum, and/or the presence of surface gene escape mutants (Xu *et al*., 2002; Hou *et al*., 2005).

A passive-active immunization is recommended as post-exposure prophylaxis for all non-vaccinated persons. The first dose of active immunization should be given as early as possible, in combination with one dose of HBIG if the source is known to be HBsAg-positive. The latest time point for effective post-exposure prophylaxis is 12 hours after the exposure. Vaccinated individuals with a documented response do not need post-exposure prophylaxis (Mohr *et al*., 2014).

Hepatitis B immune globulin remains a central component of prophylaxis in HBV-infected patients undergoing liver transplantation. The current protocol is combination of HBIG with a nucleoside analog after liver transplantation. These combination protocols have reduced the rate of virologic breakthrough to 10 % or less (Terrault and Vyas, 2003).

2.2.4.1.3. Active immunization

Prevention of primary infection by vaccination is an important strategy to decrease the risk of chronic HBV infection and its subsequent complications. The first-generation of hepatitis B vaccine, an inactive plasma-derived vaccine, and the second generation of hepatitis B vaccine, a DNA recombinant vaccine, are currently available. Both vaccines were proven to be safe and efficacious in preventing HBV infection (Hou *et al.*, 2005). Although the eradication of hepatitis B by means of universal vaccination seems technically achievable, this task is made difficult by the fact that hundreds of millions individuals are already chronically infected with HBV. The elimination of hepatitis B will only be successful when this group of chronically

infected patients is cured naturally or through antiviral treatments (Pourkarim *et al.*, 2014).

2.2.4.2 Curative treatment of hepatitis B

The current treatment for HBV involves the use of interferon and/or antiretroviral drugs, since some of the anti-HIV reverse transcriptase drugs can also inhibit the HBV polymerase. Five nucleoside and nucleotide analogues inhibit HBV reverse transcriptase: Adefovir, Entevavir, Lamivudine, Telbivudine and Tenefovir (Pujol *et al.*, 2012). Antiviral treatment of patients with acute hepatitis B usually is not recommended. In adults, the likelihood of fulminant hepatitis B is less than 1 %, and the likelihood of progression to chronic hepatitis B is less than 5 %. Therefore, treatment of acute hepatitis B is mainly supportive in the majority of patients (Mohr *et al*., 2014). Antiviral treatment with HBV polymerase inhibitors can be considered in certain subsets of patients, including patients with a severe or prolonged course of hepatitis B, patients coinfected with other hepatitis viruses or underlying liver diseases, patients with immunosuppression, or patients with fulminant liver failure undergoing liver transplantation (Kondili *et al.*, 2004; Tillmann *et al*., 2006). In chronic hepatitis B, antiviral drugs can delay the progression of cirrhosis and reduce the incidence of HCC (WHO, 2014).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

Blood collection was conducted in the four districts of Kinshasa, namely Lukunga, Tshangu, Mont-Amba, and Funa, from November to December 2014. Kinshasa, the capital city of DRC, lies on longitude 15° 19' 20'' East of Greenwich and latitude 4° 19' 30'' South of Equator (Annuaire Mairie, 2015).

3.2 Study design and sample size

The present study was designed as an analytical cross-sectional study conducted from November 2014 to December 2014. Sample size was estimated using the formula from Kothari (2004) for estimating prevalence in unknown population size, i.e. $n = Z^2 p (1-p)/e^2$, where n = size of sample, z = Student's t-value at 95 % confidence level (1.96), p (1-p) = the variance of population, p = prevalence of HBsAg among blood donors and e = acceptable error (0.05). The prevalence of HBsAg among blood donors in Kinshasa has previously been reported to be 9.2 % (Mbendi *et al.,* 2001). Using this formula, the estimated minimum sample size was determined to be 128 blood donors. The sample size was increased by 10 % of the estimated minimum sample size (n = 128) in prediction of probable sample loss and improper responses in questionnaires. Thus, at least 140 blood donors were required to be enrolled in this study.

3.3 Study population and sampling

All blood donors who voluntarily presented themselves at CNTS during the study period were included in the present study. In addition, voluntary blood donors who presented themselves when CNTS staff went out in the field for blood collection during the study period were also enrolled. To be included in the present study, a blood donor had to be aged between 18 and 65 years, weigh at least 50 kg, have a hemoglobin concentration above 12.5 g/dl, an axillary temperature between 36 and 37.4 °C, a cardiac frequency between 60 and 100 beats per minute, and a normal systolic (90-130 mm Hg) and diastolic (60-90 mm Hg) blood pressure. In addition, all blood donors enrolled had to give an informed consent. Blood donors who had a history of transfusion and all those who belonged to a high-risk group, including drug addicts, commercial sex workers and those with multiple sexual partners, were excluded from blood donation. Blood donors who had jaundice in the past were excluded from the study as well.

3.4 Data collection and laboratory analyses

3.4.1. Collection of socio-demographic data

A questionnaire was administered to collect socio-demographic data including age, sex, address, marital status and profession (Appendices 1 and 2).

3.4.2. Laboratory analyses

3.4.2.1 Detection of HBsAg in blood donors

Ten millilitres of blood from each blood donor were collected aseptically into labelled vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) vacutainer tubes. In addition, 250 or 450 ml of blood from each blood donor were also collected aseptically into labelled blood bags containing citrate phosphate dextrose adenine anticoagulant (CPDA). Blood collected into EDTA vacutainer tubes was screened for HBsAg using ELISA (Hepanostika® HBs, Biomérieux, France) following manufacturer's instructions. Then, blood from HBsAg positive samples was drawn from either EDTA vacutainer tubes within 72 hours, or corresponding blood bags containing CPDA within 7 days, and spotted into cards (FTA® classic cards Whatman® 3 mm). Afterwards, FTA® classic cards were dried and stored at room temperature until used for PCR.

3.4.2.2 Detection of HBV DNA in blood donors

Hepatitis B virus DNA was extracted from FTA® classic cards using QIAamp nucleic acid extraction kits (Qiagen Sciences, Maryland, USA), following manufacturer's instructions. Afterwards, HBV DNA detection was performed by amplification of the partial S-gene, using a nested PCR that was expected to yield a product of approximately 400 base pairs. The following primer combinations were used: HBV_S1F with HBV_S1R as outer primers, and HBV_SNF with HBV_SNR as inner primers, as previously reported by Forbi *et al*. (2010), with some modifications (Table 1). After an initial denaturation step (10 min at 95 °C), DNA amplification

was performed for 40 cycles at 95 °C for 30 sec, 55 °C for 45 sec and 72 °C for 45 sec, and a final extension at 72 °C for 10 minutes using a Veriti 96-well Thermal Cycler (Applied Biosystems, Jurong, Singapore). Then, PCR amplicons were visualized via a GelRed–stained agarose gel using a gel documentation system (Gel Doc Ez Imager, Bio-Rad, California, USA).

Table 1: DNA sequences of the primers for partial S-gene amplification of HBV

Primer	Sequence (5'→3')	Genomic position
HBV_S1F*	CTA GGA CCC CTG CTC GTG TT	179
HBV_S1R*	CG AAC CAC TGA ACA AAT GGC ACT	704
HBV_SNF	GTT GAC AAG AAT CCT CAC AAT ACC	217
HBV_SNR	GA GGC CCA CTC CCA TA	658

* An F after the primer position stands for sense primers while R stands for anti-sense primers.

3.4.2.3 HBV genotyping and phylogenetic analysis

Polymerase chain reaction products were then subjected to dideoxynucleotide cycle sequencing PCR using BigDye Terminator Cycle Sequencing Kit version 3.0 (Applied Biosystems, Foster City, CA, USA). The sequencing was carried out by Macrogen Incorporation (Macrogen Europe, Amsterdam, The Netherlands). The HBV_SNF and HBV_SNR were used as primers for sequencing of the partial S gene. Products from the dideoxynucleotide cycle sequencing reaction were purified by ethanol precipitation and separated on a 3500 Genetic Analyzer (Applied

Biosystems). The nucleotide sequences obtained were subjected to a similarity search at GenBank using the Basic Local Alignment Search Tool for nucleotides (BLASTn) (Altschul, 1997). Together with the most similar sequences found at GenBank and representatives of HBV genotypes (A–H), they were aligned using Clustal W (Appendix 3). Afterwards, a phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis software (MEGA 6.06) by Maximum likelihood method and Tamura three-parameter option of nucleotide substitution. Bootstrap testing of phylogeny was inferred following 1000 replications to assess the reliability of the clusters. Values equal to or greater than 50 were indicated on the branches.

3.5 Statistical analyses

The data were computerized using Excel and statistical analyses were performed using R software version 3.1.3. The measures of variability as regarded to socio-demographic factors (age, sex, etc.) were determined. Chi-squared and Fisher's Exact tests were used when appropriate to compare proportions. The results were considered significant when $p < 0.05$ at 95 % confidence interval.

3.6 Ethics

The approval and ethical clearance to carry out this study was sought from the ethics review committee of the Public Health School of DRC (Approval certificate number ESP/CE/027/2015, Appendix 4). An informed consent was obtained from each selected blood donor. Confidentiality of participants was ensured through the use of identity codes to conceal their identity. The forms for written informed consent are attached in Appendices 5 and 6.

CHAPTER FOUR

4.0 RESULTS

4.1 Hepatitis B surface antigen prevalence among blood donors in Kinshasa

In the present study, the presence of HBsAg was detected in blood donors, using ELISA. A total of 582 blood donors including 503 men (86.4 %) and 79 (13.6 %) women were enrolled. Among the 582 blood donors, 40 (6.9 %) were positive for HBsAg. There was no significant difference in HBsAg prevalence (p = 0.84, Chi-squared test) between male and female blood donors who were enrolled in the present study (Table 2).

The median age of blood donors was 28 years (range from 18-64 years). Hepatitis B surface antigen seropositivities in different age groups are indicated in Table 3. Proportions of HBsAg positive blood donors were statistically different between different age groups (p=0.01, Fisher's Exact test). The highest rate of HBsAg carriers was found in blood donors aged between 28 and 37 years, followed by the age group of 58-67 years (Table 2).

Majority of the 582 blood donors (70.3 %) came from Funa (201/582) and Tshangu (209/582) districts. Minority of blood donors were living in Lukunga and Mont-Amba districts (Table 4). Though most HBsAg positive blood donors were from Funa and Tshangu districts (Table 4), HBsAg prevalences between different districts were not statistically different (p=0.08, Fisher's Exact test).

Table 2: Proportion of HBsAg positive blood donors by gender.

	HBsAg (%)		
Sex	Negative	Positive	**Total**
Male	468 (93.1 %)	35 (6.9 %)	503
Female	74 (93.7 %)	5 (6.3 %)	79
Total	**542**	**40**	**582**

Table 3: Proportion of positive HBsAg blood donors by age groups.

	HBsAg (%)		
Age (year)	Negative	Positive	**Total**
18 - 27	265 (96.0 %)	11 (4.0 %)	276
28 - 37	112 (86.8 %)	17 (13.2 %)	129
38 - 47	84 (92.3 %)	7 (7.7 %)	91
48 - 57	52 (94.5 %)	3 (5.5 %)	55
58 - 67	8 (88.9 %)	1 (11.1 %)	9
*NS	21 (95.5 %)	1 (4.6 %)	22
Total	**542**	**40**	**582**

*NS stands for not stated.

Table 4: Proportion of positive HBsAg blood donors by district of Kinshasa

	HBsAg (%)		
District	Negative	Positive	**Total**
Funa	182 (90.5 %)	19 (9.5 %)	201
Lukunga	89 (94.7 %)	5 (5.3 %)	94
Mont-Amba	76 (98.7 %)	1 (1.3 %)	77
Tshangu	194 (92.8 %)	15 (7.2 %)	209
*NS	1 (100 %)	0 (0 %)	1
Total	**542**	**40**	**582**

*NS stands for not stated

4.2 Hepatitis B virus DNA detection and genotyping

Out of the 40 HBsAg-positive samples, 36 were subjected to HBV-DNA extraction. The four other samples could not be traced in the freezers after HBsAg testing. Hepatitis B virus DNA was also extracted from four samples used as HBsAg positive controls by the CNTS. These positive controls were samples from blood donors, which were strongly positive in HBsAg serological detection and were then used as positive controls to subsequent ELISA. The detection of HBV DNA was successful in 50% (18/36) of HBsAg positive samples and 2/4 of HBsAg positive controls. From the 18 DNA samples, 14 were selected for sequencing, based on quality of PCR product. Of the 14 PCR products subjected to nucleotide sequencing, 13 were successfully sequenced. Both DNA detected in the two positive controls were also successfully sequenced. Thus, a total of 15 nucleotide sequences were obtained and were named beginning with "DRC-Kin" followed by study identification numbers or letters, e.g. DRC-Kin-9126519 (Table 5). After phylogenetic analysis, four out of the 15 (26.6 %) nucleotide sequences obtained from blood donors enrolled in the present study clustered into genotype E strains, while 1 (6.7 %) into genotype D, subgenotype D7, and 1 (6.7 %) nucleotide sequence (DRC-Kin-8140610) clustered into none of the known genotypes. Nine (60.0 %) nucleotide sequences clustered into genotype A, with 1 sequence clustering into subgenotype A1, 4 into subgenotype A3, 1 into subgenotype A4, 2 into subgenotype A6, and 1 into none of the known subgenotypes (Fig. 4). The nucleotide sequences obtained from the present study were then submitted to GenBank and were given the accession numbers from KR535608 to KR535622 (Table 5).

Table 5: HBV strains sequenced in the present study.

HBV strain	Country	Collection year	Accession number	Source
DRC-Kin-9126519	DRC	2014	KR535611	Human
DRC-Kin-8140610	DRC	2014	KR535612	Human
DRC-Kin-6148689	DRC	2014	KR535613	Human
DRC-Kin-5148698	DRC	2014	KR535614	Human
DRC-Kin-4120599	DRC	2014	KR535615	Human
DRC-Kin-1120976	DRC	2014	KR535616	Human
DRC-Kin-PC1	DRC	2014	KR535608	Human
DRC-Kin-1146462	DRC	2014	KR535609	Human
DRC-Kin-4146474	DRC	2014	KR535610	Human
DRC-Kin-3146662	DRC	2014	KR535617	Human
DRC-Kin-9120817	DRC	2014	KR535618	Human
DRC-Kin-PC3	DRC	2014	KR535619	Human
DRC-Kin-6120407	DRC	2014	KR535620	Human
DRC-Kin-2120412	DRC	2014	KR535621	Human
DRC-Kin-7120432	DRC	2014	KR535622	Human

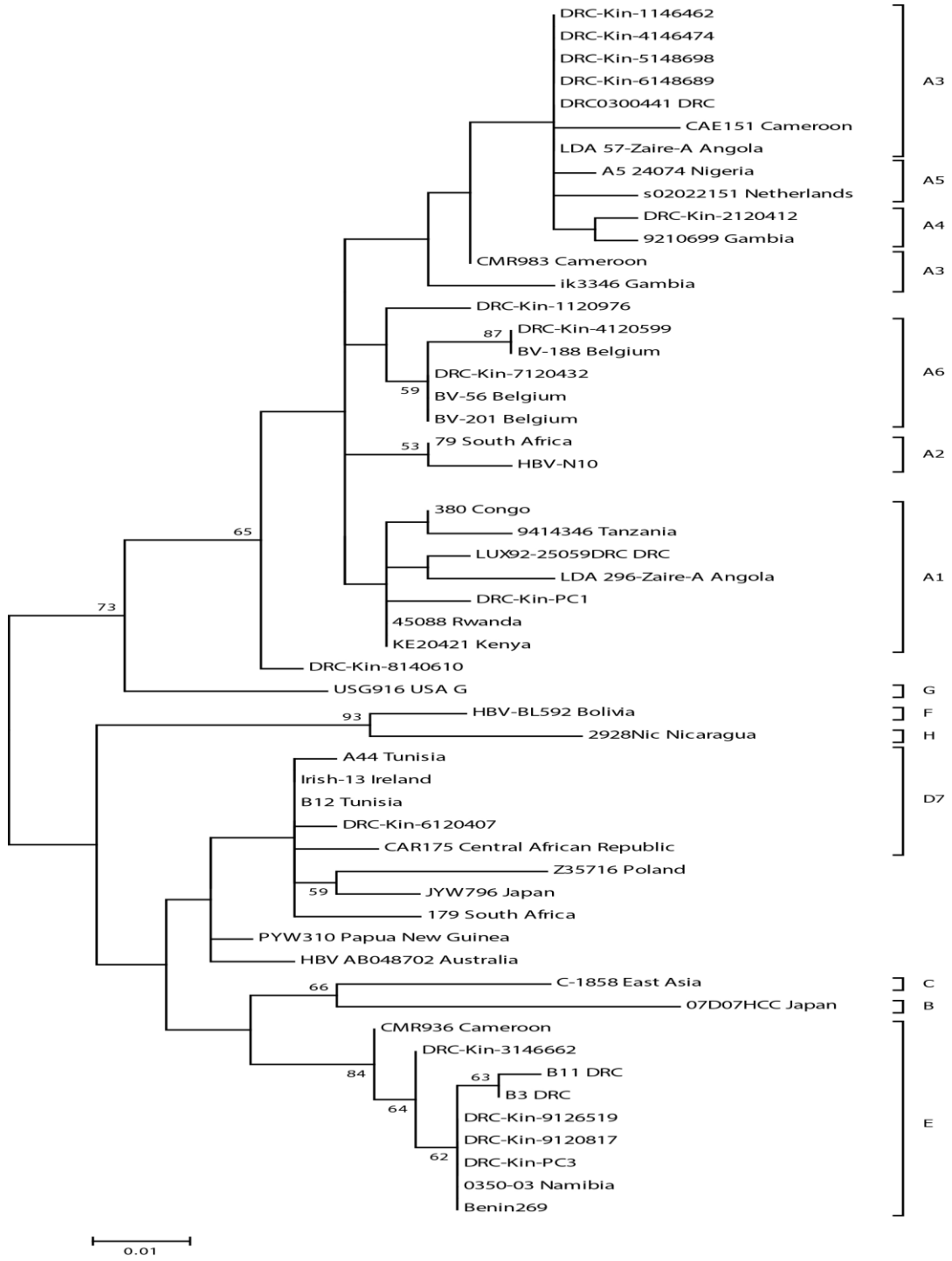

Figure 4: Genetic relationship of HBV in blood donors of Kinshasa. Maximum likelihood method was used to infer phylogenetic relationship, with the Kimura-3 parameters model and bootstrapping of 1000 replicates as implemented in MEGA6.06 software. Relevant bootstrap values, using a

cut-off value of 50 %, are shown. Sequences from this work are named by DRC-Kin" followed by numbers or letters. Sequences retrieved from GenBank are represented by their isolate and country names. The scale bar indicates the number of nucleotide substitutions per site.

CHAPTER FIVE

5.0 DISCUSSION

The present study aimed at determining HBsAg prevalence, associated risk factors and HBV genotypes among voluntary blood donors in Kinshasa, DRC. This is the first study, to our knowledge, conducted on genotyping HBV virus involving a large number of adults in DRC.

The results of the present work revealed that HBsAg prevalence among voluntary blood donors was 6.9%. This prevalence is similar to previous reports presented by the "Programme National de Lutte contre le SIDA" (PNLS), which showed that in 2010 HBsAg prevalence in Kinshasa among voluntary blood donors was 6.2 %, while it was 7.0 % among voluntary and non-voluntary blood donors, i.e. familial and remunerated blood donors (PNLS, 2014). The similarity between HBsAg prevalence among voluntary blood donors in 2014 and blood donors in 2010 calls for strenghtening blood donor selection criteria in order to reduce contamination risks to blood recipients. Moreover, this is incentive to reinforcement of HBV prevention programs in adults in order to reduce the transmission of the virus in the community.

Males were more enrolled than females in the present study. This finding corroborates previous reports indicating more restriction factors to blood donation in women than men, including menstruations, pregnancy, breastfeeding (Mbendi *et al.,* 2001; WHO, 2012). There was no statistically significant difference in HBsAg

prevalence between genders in the present work. Differences in HBsAg prevalences between genders were found in some populations, but not in others (Valente *et al*., 2010). A study conducted in Eastern DRC reported a higher HBsAg prevalence in males than in females (Kabinda *et al.*, 2014). This discordance in the results may be due to differences in risk factors in the study populations. Kabinda *et al.* (2014) enrolled blood donors from rural populations in their study, and HBV contamination during men circumcision might be higher in rural than in urban areas, exposing males to a higher risk of HBV infection than females in rural areas. In the present study, no blood donor originated from rural populations.

The median age was 28 years, which is similar to the median age of 26 years found among blood donors in Eastern DRC (Kabinda *et al*., 2014). This is explained by the fact that the majority of blood donors are youths. The high HBsAg prevalence found in the age group of 28-37 years may be favoured by a high HBV transmission via the sexual route. HBsAg rate was also predominant in the 58-67 year age group. This can be explained by the very low number of subjects in that age group, which has led to bias.

Differences in HBsAg prevalence between different districts of Kinshasa was not statistically significant. That may be attributed to the homogeneity of the population in Kinshasa and movements of subjects between different districts, which may contribute to the homogeneity in horizontal HBV transmission modes in the city.

Hepatitis B virus DNA was detected in 55% of HBsAg-positive samples in the present study. This rate was comparable to the 53 % obtained by Valente *et al.,* (2010), in Angola. This is lower than the 100 % obtained by Forbi *et al.* (2010) in Nigeria. Differences in HBV viral loads might explain this discrepancy. Fifteen HBV DNA samples from adult blood donors which were chronic carriers of HBsAg were genotyped in the present research, contrary to Mulders *et al.* (2004), Pourkarim *et al.* (2010) and Hannoun *et al*. (2005), who studied HBV genotypes in only three, two and one adults originating from DRC, respectively.

The HBV genotype A was the most prevalent genotype found in 60.0 % of the genotyped samples from the present study, followed by the genotype E (26.6 %), and genotype D (6.7 %). These results support the idea that genotype A is the most prevalent genotype in southern, eastern and central Africa (Kramvis and Kew, 2007). Within Africa, genotype E has a higher prevalence in Western and Central African countries, while genotype D is the dominant genotype in Northern Africa (Kramvis and Kew, 2007; Valente *et al*., 2010). Genotype D has also been found in South Africa (Kramvis and Kew, 2007). The possibility that the genotype D found in our sample has been imported from Northern Africa or South Africa has not been assessed, since we did not have data on maternal origin province, sexual partner origin or previous travel(s). This can also be supported by the fact that Kinshasa is a cosmopolitan city, like many capital cities worldwide. Therefore, a study with greater number of HBV strains is needed to evaluate the prevalence of genotype D in DRC. In the present study, three genotypes (A, E, and D) were found in adults living in DRC, contrarily to previous studies which reported only genotypes A and D (

Mulders *et al.,* 2004) or only genotype A (Hannoun *et al.,* 2005; Pourkarim *et al*., 2010) in adults originating from DRC. The very small sample size used by these authors can justify the absence of genotype E in adults. However, Mulders *et al.* (2010) reported genotype E in three HBV strains from children. The present study did not include children. Thus, further studies are needed in a larger number of children, in order to assess whether HBV genotype distribution is the same in children as in adults, because transmission modes differ in these two age groups and HBV variants can emerge during persistent long-term HBV infection (Pourkarim *et al.*, 2014).

One HBV strain was an outlier (DRC-Kin-8140610) and clustered with no published genotype. This might be a mixed, recombinant or new genotype, which is worthy of further analyses through whole genome sequencing (Pourkarim *et al.*, 2014). Furthermore, molecular characterization of complete HBV nucleotide sequences is needed in order to assess the genetic variability and clinical significance of HBV genotypes in DRC.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

The present study aimed at determining the HBV genotypes circulating in DRC. The results showed a high overall prevalence of HBsAg, similar to the one reported four years ago and a high HBsAg prevalence in blood donors aged between 28 and 37 years. This emphasizes the need of strengthening blood donor selection criteria and measures of reducing horizontal HBV transmission. In the present study, adults were found to display three HBV genotypes, namely A, D and E. This can have an impact on the choice of antiviral drugs to be administered, and HBV strains to be used in vaccination programs in DRC.

6.2 Recommendations

From the results obtained in the present study, there is a need of:

(i) Conducting further studies to investigate mutations in HBV strains found in DRC, in order to detect vaccine escape mutants and HBsAg mutations that may reduce the sensitivity of HBsAg detection tests;

(ii) Carrying out further molecular study to assess the impact of HBV diversity on clinical outcomes, drug effect, and

(iii) Determining the genotypes of HBV circulating in children in DRC in order to understand patterns of HBV transmission routes in children.

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APPENDICES

Appendix 1: Questionnaire administered to blood donors in Kinshasa (English version)

Study title: "Genotypes of hepatitis B virus among voluntary blood donors in Kinshasa, DRC".

Date :…….. Site name:……

Blood donor ID : …… Number of donation : …….

Socio-demographic data

Address (including district): …… Date of birth: …….

Sex : ☐ M ☐ F

Marital status : ☐ Married /cohabiting couple ☐ Single

☐ Divorced /Separated ☐ Widow (er)

☐ Other : ……

Occupation : ☐ Pupil-student ☐ Housewife ☐ Artist

☐ Trader ☐ Teacher ☐ Other …….

Other data :

Date of the last donation:……..

Have you ever been transfused? ☐ No ☐ Yes (Date:…….)

Have you ever undergone a surgery? ☐ No ☐ Yes (Date:…….)

Do you have more than one sex partner? ☐ No ☐ Yes (Date:…….)

Have you ever had jaundice in the past? ☐ No ☐ Yes

Weight: ……kg

Blood pressure : ……mmHg

Hematocrit/ Hemoglobin : …….

Appendix 2: Questionnaire administered to blood donors in Kinshasa (French version)

Titre de l'étude : « Génotypes du virus de l'hépatite B chez les donneurs de sang bénévoles à Kinshasa, DRC ».

Date de prélèvement :…….. Nom du site :……

Numéro du donneur :…… Nombre de dons : ……..

Données sociodémographiques

Adresse (y compris le district):…… Date de naissance: …….

Sexe : ☐ M ☐ F

Etat civil : ☐ Marié /cohabitation ☐ Célibataire ☐ Divorcé (e)/Séparé(e)

☐ Veuf (ve) ☐ Autre :……

Profession : ☐ Elève – Etudiant ☐ Ménagère

☐ Commerçant ☐ Enseignant

☐ Artiste ☐ Autre …….

Autres données

Date du dernier don:……..

Avez-vous déjà été transfusé? ☐ Non ☐ Oui (Date:…….)

Avez-vous déjà été opéré? ☐ Non ☐ Oui (Date:…….)

Combien de partenaires sexuels avez-vous?

Avez-vous déjà souffert de jaunisse dans le passé ? ☐ Non ☐ Oui

Poids : ……kg

Pression artérielle : ……mm Hg

Hematocrite/ Hemoglobine : …….

Appendix 3: HBV strains used for phylogenetic analysis.

HBV strain (Isolate name)	Country	Collection year	Genotype /Subge-notype	Source	Reference
DRC0300441	DRC	-	A/A3	Human	Mulders *et al.,* 2004
CAE151	Cameroon	-	A/A3	Human	Mulders *et al.,* 2004
LDA_57-Zaire-A	Angola	2007	A/A3	Human	Valente *et al*., 2010
A5_24074	Nigeria	2004	A/A5	Human	Andernach *et al.*, 2009
179	South Africa	-	D/D3	Human	Kimbi *et al.*, 2004
9210699	Gambia	-	D/A4	Human	Hannoun *et al*., 2005
ik3346	Gambia	-	D/A3	Human	Hannoun *et al.,* 2005
CMR983	Cameroon	1994	A/A3	Human	Kurbanov *et al*., 2005
BV-188	Belgium	2006	A/A6	Human	Pourkarim *et al.,* 2010
BV-56	Belgium	1998	A/A6	Human	Pourkarim *et al.,* 2010
BV-201	Belgium	2001	A/A6	Human	Pourkarim *et al.,* 2010
380	Congo	-	A/A1	Human	Hannoun *et al*., 2005
9414346	Tanzania	-	A/A1	Human	Hannoun *et al*., 2005
LUX92-25059DRC	DRC	1992	A/A1	Human	Mulders *et al*., 2004
LDA 296-Zaire-A	Angola	-	A/A1	Human	Valente *et al.,* 2010
45088	Rwanda	-	A/A1	Human	Hubschen *et al*., 2009
KE20421	Kenya	2012	A/A1	Human	Kwange *et al*., 2013
79	South Africa	-	A/A2	Human	Owiredu *et al*., 2001
HBV-N10	China	-	A/A2	Human	Wu *et al*., 2007
USG916	USA	-	G/-	Human	Kato *et al.*, 2002
0350-03	Namibia	-	E/-	Human	Kramvis *et al.*, 2005
Benin269	Benin	-	E/-	Human	Fujiwara *et al.*
B11	DRC	-	E/-	Human	Hass *et al*., 2005

B3	DRC	-	E/-	Human	Hass *et al*., 2005
CMR936	Cameroon	1994	E/-	Human	Kurbanov *et al*., 2005
C-1858	East Asian	-	C/-	Human	Alestig *et al*., 2001
07D07HCC.	Japan	-	B/-	Human	Takahashi *et al.,* 1998
HBV-BL592	Bolivia	-	F/-	Human	Huy *et al.,* 2006
2928Nic	Nicaragua	-	H/-	Human	Arauz-Ruiz *et al.,* 2002
HBV (AB048702)	Australia	-	D/D4	Human	Sugauchi *et al.*, 2001
PYW310	PNG*	-	D/D4	Human	Okamoto *et al.,* 1988
s02022151	Netherlands	2006	A/A5	Human	Niesters *et al.*, unpubl.
Z35716	Poland	-	D/D2	Human	Pluciennicza k, unpubl.
JYW796	Japan	-	D/D5	Human	Okamoto *et al.*, 1988
B12	Tunisia	2005	D/D1	Human	Meldal *et al*., 2009
Irish-13	Ireland	-	D/D7	Human	Laoi *et al*., 2008
A44	Tunisia	2006	D/D7	Human	Meldal *et al*., 2009
CAR175	CRA*	2007	D/D7	Human	Bekondi *et al.,* unpubl.

PNG* stands for Papua New Guinea, CRA* for Central African Republic and unpubl. for unpublished.

Appendix 4: Ethical approval certificate

REPUBLIQUE DEMOCRATIQUE DU CONGO
Ministère de l'Enseignement Supérieur, Universitaire et Recherche Scientifique
Université de Kinshasa
ECOLE DE SANTE PUBLIQUE
COMITE D'ETHIQUE

No d'Approbation: ESP/CE/027/2015

Kinshasa, le 25 février 2015

Au Dr Maindo Pati Moloko Patience
Investigateur Principal
Faculté de Médecine
Université de Kinshasa
République Démocratique du Congo

Concerne : Décision du Comité d'éthique portant sur l'étude intitulée : «*Génotypes du virus de l'hépatite B et phénotypes érythrocytaires des donneurs de sang à Kinshasa, République Démocratiques du Congo*».

Docteur,

Le Comité d'Ethique de l'Ecole de Santé Publique de l'Université de Kinshasa a bien reçu le protocole dont le titre est repris en marge.

Après examen du protocole selon les normes d'éthique nationales sur les études impliquant les êtres humains, le Comité a donné un avis favorable à cette recherche et autorise sa mise en œuvre pour la période allant du 25 février 2015 au 24 février 2016.

Veuillez agréer, Docteur, l'expression de notre considération distinguée.

Prof. Félicien MUNDAY
Secrétaire du Comité d'éthique de l'Ecole de Santé Publique
et Président du Comité National d'Ethique de la Santé de la RD Congo

ESP UNIKIN COMITE D'ETHIQUE

Appendix 5 : Informed consent for participating in the study (English version)

Study title: "Genotypes of hepatite B virus among voluntary blood donors in Kinshasa, DRC"

My name is Dr Patience MAINDO Patience. I am performing a study on the genotypes of hepatitis B virus in blood donors in Kinshasa, DRC. This study has been initiated in order to gather data that will help to understand the epidemiology of hepatitis B in our country. And these data will also be useful for the improvement of care of blood donors in DRC.

You will be interviewed for 15 minutes and then 10 ml of your blood will be drawn.

Confidentiality about information you will give us is ensured, as well as anonymity, by using your ID instead of your name.

We need your consent for participating in the present study.

For any further question and information, please contact this phone number:

(+243) 810393596

Do you agree to participate in the present study? ☐YES ☐ NO

Date : ……/……/…..

Name et signature of blood donor: ……

Appendix 6 : Informed consent for participating in the study (French version)

Titre de l'étude : « Génotypes du virus de l'hépatite B chez les donneurs de sang bénévoles à Kinshasa, DRC ».

Mon nom est Dr Patience MAINDO Patience. Je mène une étude sur les génotypes du virus de l'hépatite B des donneurs de sang à Kinshasa, RDC. Cette étude a été initiée dans le but de recueillir des données qui permettront l'amélioration de la prise en charge des donneurs de sang en RDC.

Vous serez chacun interviewé pendant environ un quart d'heure et 10 ml de votre sang seront ensuite prélevés. Nous assurerons la confidentialité de toutes les informations que vous nous fournirez et votre anonymat en utilisant un code en lieu et place de votre nom.

Nous avons besoin de votre consentement pour votre participation à cette étude.

Si vous avez des questions après cette interview, vous pouvez me contacter au (+243) 810393596 à n'importe quel moment.

Donnez-vous votre adhésion? ☐ Oui ☐ Non

Fait à Kinshasa, le ……/……/…..

Nom et signature du donneur de sang:……