

*Full Length Research Paper*

# Hepatitis B virus genotypic patterns in Sudan reflects population movements

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**Hepatitis B virus (HBV) genotypes have important impacts on the natural course of infection, prognosis and treatment outcomes. HBV genotypes A, D and E are predominant in various parts of Africa. Sudan occupies a central position in Africa with waves of migration from surrounding territories and continuous influx of different HBV genotypes. This study aimed to determine HBV genotypic patterns in Sudan as a reflection of population movements. In a prospective, cross-sectional, analytical study and following scientific and ethics approvals, five hundred and thirty five HBV-ELISA/PCR confirmed individuals were consented for participation. DNA was extracted using the guanidine technique. HBV strains were genotyped using type-specific primers in a nested and multiplex PCR technique. Five hundred sera were reactive in ELISA tests and had HBV DNA traces. Thirty five individuals were ELISA HBs Ag non-reactive, but showed HBV DNA traces [Occult hepatitis]. Samples were typed as: genotype A [85%], genotype A+E [10%], genotype D [3%] while recombination of A, D and E was seen in 2%. In conclusion, HBV genotypes in Sudan are a mixture of genotypes A, D and E separately or in recombination reflecting previous and ongoing migration waves from surrounding territories. Sequencing studies are needed for further HBV genotypes confirmation.**

**Key words:** HBV infection, Genotypic patterns, recombinant genotypes, Sudan.

**Abbreviations:**

bp	base pair
HBe Ab	Hepatitis B e Antibody
HBe Ag	Hepatitis B e Antigen
HBs Ag	Hepatitis B surface Antigen
HBV	Hepatitis B virus

**INTRODUCTION**

Hepatitis B virus (HBV) infection is the most widespread viral infection in humans world-wide with more than 350 million chronic carriers. HBV causes a spectrum of liver diseases ranging from asymptomatic state to liver cirrhosis and hepato-cellular carcinoma. Approximately 600,000 deaths occur worldwide annually due to chronic HBV-associated liver disease [Sitnik et al. 2004; WHO

2008; Sunbul et al. 2014; Rahman et al. 2016]. Genotypically, HBV genomes have been classified according to their genetic variability (>8 % for whole genome) into ten major genotypes designated A to J. The genetic diversity occurs due to the replication error of the virus during its multiplication. In addition, recombination between genotypes generates novel variants that further

contribute to the genetic diversity of HBV [Tran et al. 1991; Georgi-Geisberger et al. 1992; Simmonds and Midgley 2005]. Recombination between A and D, B and C are well documented [Bowyer and Sim 2000; Morozov et al. 2000; Yuasa et al. 2000; Sugauchi et al. 2002]. Genotype H is closely related to genotype F and is thought to perhaps have split off from it within the “New World”. Genotype I was described as a novel genotype since the diversity in its complete genome sequence is only 7% from that of its closest neighbor, genotype C. Genotype J is thought to be phylogenetically positioned between human and primate HBV variants being close to strains which had been previously found in Orang-utans and gibbons [Kurbanov et al. 2008; Tatematsu et al. 2009; Panduro et al. 2013]. It has been well documented that HBV genotype A is widely prevalent in East and South Africa where it infects the vast majority (> 90%) of patients. In northern Africa and the Mediterranean Basin, genotype D predominates in up to 80% of patients, while genotype E is prevalent in West and Central Africa [Hasegawa et al. 2006; Ayed et al. 2007]. Although genotype A has been found on every continent, its genetic diversity is higher in Africa (4% over the complete genome) than in the rest of the world (3%). Therefore, it has been suggested that genotype A has emerged in Africa after a long evolution and has been introduced to other continents [Hannoun et al., 2007]. However, despite the high genetic diversity of HBV genotype A, it is very rare in West Africa. On the other hand, genotype E has been found only in Africa, with some rare exceptions on other continents in persons with an African link. In Africa, genotype E is found almost exclusively throughout a wide belt from Senegal in the west to the Central African Republic in the east and Namibia in the south [Vray et al. 2006; Bekondi et al. 2007; Kramvis and Kew 2007]. The markedly low genetic diversity of HBV genotype E compared to HBV genotype A suggests its short natural history relatively and its recent introduction into resident population of this belt. However, the recent presence of HBV genotype E, its high prevalence/extensive geographic distribution in Africa and the long natural history of HBV genotype A are irreconcilable [Mudlers et al. 2004; Andernach et al. 2009]. Genotype G is the least common of the genotypes where the pre-core and core regions are aberrant with a 36-nucleotide insertion within the core gene making it the longest of the HBV genotypes, probably affecting HBe Ag production. The error rate of HBV is high (approximately  $>2 \times 10^4$  base substitutions/site/year) leading to high natural mutation rates [Magnius and Norder 1995; Li 2003; Buti et al. 2005; Yu et al. 2010; Dao et al. 2011; Croagh et al. 2015]. The genetic variability of HBV may come about through natural mutation or by recombination. Recombination leads to the development of divergent strains of HBV and has been described between numerous different genotypes [A and E; G and A]. Recent evidence suggests that the core gene may be a preferred site for recombination as noted in West African

patients with A/E recombinant strains of HBV. In G and A genotypes recombination it has been claimed that co-infection with genotype A leads to enhanced viral replication [Morozov et al. 2000; Wang et al. 2005; Kramvis et al. 2007; Garmini et al. 2009; Dao et al. 2011; Sakamoto et al. 2013; Croagh et al. 2015]. It has been reported that different HBV genotypes have different sensitivities to antiviral therapies like pegylated interferon and lamivudine. Genotype B shows a better virological response to Adefovir dipivoxil therapy compared to genotype C [Wen et al. 2014].

Sudan, a vast country that occupies a central position in Africa that has been a scene for waves of migration from surrounding countries. These waves are due to natural disasters as well as man-made wars. HBV epidemiology is very varied among different parts and populations of the country [Mudawi 2008; Gadour and Abdullah 2011; Mahgoub et al. 2011; Nail et al. 2013; Sunbul et al. 2014; Abuelgasim and Baraka et al. 2015]. This study aimed to determine the different patterns of HBV genotypes hypothesizing that those different genotypes reflect population movement into Sudan.

## MATERIALS AND METHODS

### Study type and Ethical consideration

This prospective, cross-sectional and analytical study was approved by the Ethics and Scientific Committees of the Institute of Endemic Disease, University of Khartoum, Sudan [1/2015; 2/2015; 9/2016 & 1/2017]. Informed consent was obtained from all participating volunteers.

### Study subjects

Five hundred and thirty five consented HBV infected volunteers were enrolled. Volunteers were recruited from hepatology clinics, virology laboratories and blood donor centers in Greater Khartoum, Central Sudan and Red Sea and Kassala States in Eastern Sudan. Sera samples were tested by ELISA tests. All samples were subjected to molecular testing for viral DNA.

### DNA Extraction

HBV DNA was extracted from 200  $\mu$ l of volunteers' sera using a modified Guanidine method [Pramanick et al. 1976]. Briefly: 200  $\mu$ l of lysis buffer were added to the sera and the mixture incubated in a water-bath at 70  $^{\circ}$ C for two hours. Equal volumes of chloroform were added and the upper layer was removed and the process was repeated three times. One ml of ice-cold ethanol was added to precipitate the DNA. The resulting pellet was allowed to dry and was re-suspended in RNase-free water. The DNA concentration was measured and the DNA was stored at  $-20^{\circ}$  C until analyzed.

## Determination of HBV Genomes

The presence of HBV genomes was determined using nested and multiplex PCR with HBV genotype-specific primers as described previously [Naito et al. 2001]. Briefly, the first PCR run was performed using 2 universal outer primers: sense primer [P1] (5'-TCACCATATTCTTGGAACAAGA-3') and anti-sense primer [S1-2] (5'-CGAACCACTGAACAAATGGC-3'). In the second PCR run, a primer [B2] was used as the inner primer (sense) (5'-GGCTCMAGTTCMGGAACAGT-3') with a mixture [mix A] to detect genotypes A, B and C: consisting of anti-sense primers: **BA1R** (genotype A specific, 68 bp) (5'-CTCGCGGAGATTGACGAGATGT-3'), **BB1R** (genotype B specific, 281bp) (5'-CAGGTTGGTGAGTGACTGGAGA-3') and **BC1R** (genotype C specific, 122bp) (5'-GGTCCTAGGAATCCTGATGTTG-3'). **B2R** was used as the inner primer (antisense) (5'-GGAGGCGGATYTGCTGGCAA-3') with a mixture [mix B] for detection of genotypes D, E and F. Mix B consisted of sense primers **BD1** (genotype D specific, 119 bp) (5'-GCCAACAAGGTAGGAGCT-3'), **BE1** (genotype E specific, 167 bp) (5'-CACCAGAAATCCAGATTGGGACCA-3') and **BF1** (genotype F specific, 97bp) (5'-GYTACGGTCCAGGGTTACCA-3') (*Pharmacia Biotech*, Uppsala, Sweden).

The first PCR reaction mix volume was 40 µl: 50 ng of each outer primer, a 200 mM concentration of each of the four deoxynucleotides, 1 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, Conn.) and 1X PCR buffer containing 1.5 mM MgCl<sub>2</sub>. ESCO thermocycler (ESCO PCR machine, Singapore) was programmed as follows: Incubation for 10 minutes at 95°C, followed by 40 cycles consisting of 94°C for 20 seconds, 55°C for 20 seconds and 72°C for 1 minutes. Two second-round PCRs were performed for each sample, with the common universal sense primer (B2) and mix A [Genotypes types

A, B, C] and the common universal antisense primer (B<sub>2</sub>R) and mix B [D, E, F]. One µl of the first PCR product was added to two tubes containing the second sets of each of the inner primer pairs, deoxynucleotides, AmpliTaq Gold DNA polymerase and PCR buffer, as in the first reaction. The 40 amplification cycles were: preheating at 95°C for 10 minutes, 20 cycles of amplification at 94°C for 20 seconds, 58°C for 20 seconds and 72°C for 30 seconds and additional 20 cycles of 94°C for 20 seconds, 60°C for 20 seconds and 72°C for 30 seconds. HBV Genotypes were determined when the two second-round PCR products from each sample were separately electrophoresed on a 3% agarose gel (Jungwon-gu, Seongnam-si, Seoul, Korea), stained with ethidium bromide and evaluated under UV light. The sizes of PCR products were estimated when compared to the migration pattern of a 50-bp DNA ladder (Jungwon-gu, Seongnam-si, Seoul, Korea).

## RESULTS

Sera samples from five hundred and thirty five HBV-infected individuals [one hundred and eighty six volunteers from eastern Sudan (n= 186/535; 35%) and three hundred and forty nine (349/535; 65%) were from Khartoum, Central Sudan]. Five hundred were reactive in ELISA. Thirty five individuals were ELISA HBs Ag non reactive but had HBV DNA traces in their sera [Occult hepatitis].

The majority (454/535; 85%) of sera were typed as genotype A. Recombinant genotype A/E were seen in 10% of volunteers (54/535), while genotype D was seen in a minority (3%, 15/535). In about 2% (12/535) of samples showed multiple recombination of A, D & E. One sample had genotype A and genotype E with an additional band [size 310 bp] which was not previously reported with set 2 primers. It is currently being investigated (Figure 1 & Figure 2).

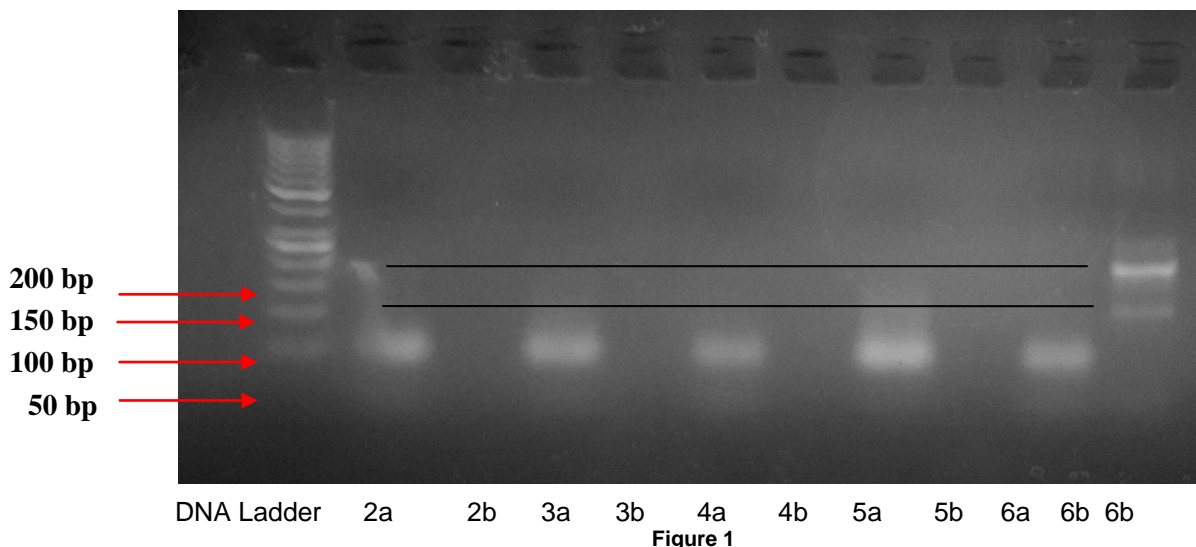
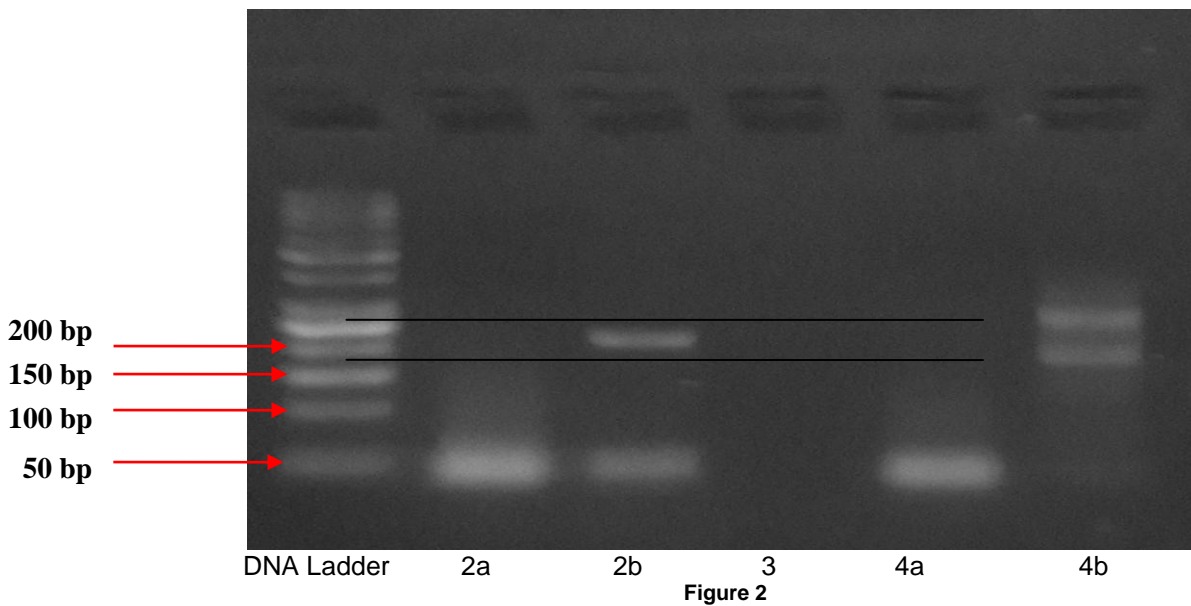


Figure 1



## DISCUSSION

Geographically, Sudan occupies a central position in Africa with high HBV sero-prevalence rates (~8%) and a markedly high exposure rates that can reach 40% in some areas [Mudawi 2008; Gadour and Abdullah 2011; Mahgoub et al. 2011; Nail et al. 2013; Sunbul et al. 2014; Abuelgasim and Baraka et al. 2015]. Various reports claim that different genotypes are associated with severe liver disease [genotype B and D] or hepatocellular carcinoma [genotype A] compared to others. In addition, response to anti-viral therapy is very variable between various drugs and HBV genotypes. Certain subtypes have been reported to be associated with a higher risk of anti-viral resistance compared to others [Kato et al. 2002]. In this study, we attempted to determine the distribution of genotypes in Sudan, hoping that this will point to routes of population migration into Sudan. Genotype A, E and D are circulating in Sudanese individuals with chronic HBV infections, this most likely reflects pouring of genotypes E and D from surrounding areas and its recombination with Genotype A. With the continuous influx of populations from the surrounding areas the patterns of HBV genotypes will continue to change. Changing epidemiology of Hepatitis B and migration has been reported previously by other investigators. Genotype E was always detected in combination with genotypes A and D in our cohort in agreement with previous reports. Recombination between different HBV genotypes has been reported from different parts of the world [Simmonds and Midgley 2005; Wang et al. 2009; Chu et al. 2012].

In conclusion, HBV genotype patterns in Sudan are a mixture of genotypes from surrounding countries (D, E) with recombination with resident genotypes (A) reflecting ancient and recent population movements.

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## Author's contributions

EAGK conceived the study idea. All authors participated in the laboratory work and data analysis, all authors participated in manuscript preparation and approval.

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**Competing interests:** The authors declare no competing interests.

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