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Genotyping and molecular characterization of hepatitis B virus in liver disease patients in Kenya

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ABSTRACT

Hepatitis B virus (HBV) genotypes are important in both the clinical manifestation of disease and treatment response. Although Kenya belongs to the African Region (AFR-E) characterized by high mortality and hyperendemicity of HBV, there is a paucity of HBV genotyping data. The aim of this study was to molecularly characterize the basic core promoter/precore (BCP/PC) and complete surface (S) regions of HBV isolated from 61 HBsAg-positive liver disease patients attending Kenyatta National Hospital in Nairobi. HBsAg, HBeAg and viral loads were determined. HBV DNA was amplified and sequenced from 58/61 patients. In addition to the complete genome of two isolates, the BCP/PC and the complete S regions of 43 and 38 isolates, respectively were sequenced. Following phylogenetic analysis of the S region, 38 isolates clustered with subgenotype A1, whereas two isolates clustered with genotype D, one with subgenotype D1 and another as an outlier of the clade containing subgenotype D6 and the D/E recombinant. When the complete genome of the latter isolate was sequenced it clustered with D6. The majority of isolates belonged to serological subtype *adw*2 and only four to *ayw*2. Three distinct groups of subgenotype A1, distinguished by different amino acid motifs, circulate in Kenya: two in the African cluster and a monophyletic clade in the "Asian" cluster. HBeAg-negativity was a result of G1896A in genotype D isolates, whereas in subgenotype A1, the HBeAg-negativity was a result of mutations in the Kozak region (1809–1812) or precore start codon (1814–1816). Mutations at positions 1762 and 1764 occurred more frequently in HCC patients ($p < 0.05$). In conclusion, subgenotypes A1, D1 and D6 circulate in liver disease patients in Kenya, with A1 predominating.

1. Introduction

Kenya, on the east coast of Africa, has been classified by the World Health Organization (WHO), to belong to the African Region (AFR-E) (Merrill and Hunter, 2011). This region is characterized by high child, and very high adult mortality, and a hyperendemicity of hepatitis B virus (HBV), with a median HBsAg prevalence of 14% (Merrill and Hunter, 2011).

Although in the late 1970s and 1980s, there was a spate of studies on the prevalence of HBV markers in Kenya, more recent studies have been scarce, with the latest studies concentrating on HBV in human immunodeficiency (HIV)-infected individuals. Exposure to HBV, as measured by the presence of at least one of the following markers, HBsAg, anti-HBc and anti-HBs, was found to range from 10% in preschool children to 77% in adults in Nairobi (Bowry, 1983). The frequency of HBsAg-positive individuals in a rural community was found to decline with age, from 30% in children younger than 10 years to 3% in those older than 50 years (Mutuma et al., 2011). An earlier study found a 3% HBsAg prevalence in adult outpatients, and 1.4% in school children (Okoth et al., 1991). HBsAg prevalence of 3.2% was found in a population study, with the antigenemia being higher in males, and demonstrating a certain degree of familial clustering (Bagshawe et al., 1975). In addition to confirming the predisposition of males for HBsAg-positivity, studies

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[1] Spent 3 weeks at the HVDRP in order to carry out experiments for the characterization of HBV isolated from Kenyan liver disease patients.
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in central Kenya showed a higher HBsAg prevalence of 12% (Hyams et al., 1989), and 11.4% in eastern Kenya (Wankya et al., 1979). The difference between the earlier and later studies may reflect the improvement in the sensitivity of the assays used and thus comparisons may not be possible. A survey of seroprevalence of HBV markers in pregnant women found an average HBsAg-positivity of 9.3%, ranging from 3% to 4% central Kenya and some coastal regions to 17.8% in the Rift Valley region (Okoth et al., 2006). Perinatal transmission is rare because of low HBeAg positivity and HBV DNA levels in mothers at birth (Greenfield et al., 1986; Okoth et al., 2006).

Based on an intergroup divergence of greater than 7.5%, HBV has been classified into genotypes A to I, with distinct geographic distribution (Kramvis et al., 2005; Norder et al., 2004; Yu et al., 2010), with genotype J being described in a single individual (Tatematsu et al., 2009). Genotype A prevails in Southern, Eastern and Central Africa, genotype D in Northern Africa and genotype E in Western Africa (Kramvis and Kew, 2007a). Subgenotypes have also been identified within genotypes A and D (Kramvis et al., 2008; Kramvis et al., 2005; Norder et al., 2004).

The genotype distribution of HBV in Kenyan liver disease patients is unknown because the only two genotyping studies undertaken in Kenya, have characterized HBV isolated from blood donors. Using a enzyme linked immunosorbent assay (ELISA), 85% of 59 HBsAg-positive blood donors were infected with genotype A, 12% with genotype D and 3% could not be classified (Usuda et al., 1999). In the second study, where ~300 nucleotide region of the pre-S1 of HBV was amplified and sequenced, genotypes A, D and E were detected in 52 HBsAg-positive blood donors, with genotype A prevailing (Mwangi et al., 2008).

Hepatocellular carcinoma (HCC) is the third most common cancer in Kenyan males, with its highest incidence at the age of 40 years (Mwangi and Gatei, 1993). A strong association of HBV exists with both cirrhosis and HCC, with HBsAg being detected in 25% of cirrhotic livers, and in 30% to 75% of HCC biopsies (Mutuma et al., 2011; Mwangi and Gatei, 1993). Seventy percent of sporadic acute hepatitis cases were as a result of HBV infection (Greenfield et al., 1984). Patients undergoing maintenance dialysis of end stage renal disease had a HBsAg prevalence of 8% (Otedo et al., 2003). The 12.2% HBsAg seroprevalence in acquired immunodeficiency syndrome (AIDS) patients did not differ from groups without AIDS (Ogutu et al., 1990). Approximately 6% of HIV infected adults were coinfected with HBV, with older males being more likely to be coinfected (Harania et al., 2008; Kim et al., 2011), whereas the percentage of HBsAg-positive jaundice patients in the Kisumu district found to be coinfected with HIV was 53% (Otedo, 2004).

Genotypes play a role in both clinical manifestation of HBV infection as well as its treatment management (Kao et al., 2002; Kramvis and Kew, 2005) therefore information about the genotypes circulating in Kenyans, with or without liver disease, is essential. The aim of the present study was to amplify the basic core promoter/precore (BCP/PC) and the complete S regions of HBV isolated from liver disease patients and to carry out the first comprehensive molecular characterization of HBV from Kenya.

2. Materials and methods

2.1. Patients

The cross-sectional study was conducted from July 2009 to March 2010, during which sera and clinical data were collected from 61 consecutive HBsAg-positive patients attending Kenyatta National Hospital, Kenya. The majority of patients, 48.4% were from Nairobi Province, 17.2% Central Province and only 1.6% from North Eastern Province. The serum samples were stored at −80 °C until use. Inclusion criteria included HBsAg-positive patients, with or without liver disease. All patients were negative for antibodies to hepatitis C virus (HCV) and HIV-positive patients were not excluded. The presence of HBeAg was examined after serum collection. The study protocol was approved by the Kenya National Ethical Review Committee in KEMRI, Kenyatta National Hospital (KNH) Ethical Review Committee, and the Human Research Ethics Committee (Medical) of the University of the Witwatersrand. Patients were recruited and serum samples collected in Kenya. After HBsAg, HIV and HCV serological testing, the sera were shipped to South Africa for further testing including: HBeAg, ALT levels, viral loads, amplification and sequencing of HBV.

2.2. Serogical and biochemical investigation

HBsAg and HIV detection were carried out using commercial enzyme immunoassay, Hepanostika ultra II HBsAg and Vironostika HIVAg kits (Netherlands), respectively, according to the manufacturer's instructions. Antibodies to HCV (anti-HCV) were tested using Murex anti-HCV enzyme immunoassay (Abbott, Abbott Park, IL). HBeAg and anti-HBe tests were performed on HBV DNA – positive sera using the Monolisa™ HBeAg-Ab PLUS kit (Bio-Rad, Hercules, CA). Alanine aminotransferase (ALT) levels were determined by an automated bioanalyzer.

The diagnosis of HBV-related liver disease was based on clinical data, laboratory tests, liver biopsy and imaging studies. Patients were classified into four groups:

Group-I (HCC-hepatocellular carcinoma): patients diagnosed by ultrasound scan and elevated serum α-fetoprotein levels ($\geqslant$400 ng/mL) and the presence of a lesion of $\geq$5 cm.

Group-II (CR-cirrhosis): patients with necro-inflammatory damage, fibrosis with nodule formation confirmed by liver biopsy, with ultrasonographic evidence of portal hypertension.

Group-III (CH-chronic hepatitis): patients, with intermittently elevated ALT (1.5 times upper limit of normal)

Group-IV (IC-inactive carrier): patients with persistently normal ALT/AST levels

2.3. Polymerase chain reaction (PCR) for genotyping and molecular characterization

Total HBV DNA was extracted from 200 μl serum using QIAamp DNA Blood Mini kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions. The complete S open reading frame (ORF) was amplified using a nested PCR.

Primers S1F 5′- CAATCGCCGCGTCGCAGAAGATCTCAATC-3′ (2410–2439 from *Eco*RI site) and S1R 5′-TCCAGACCXGCTGCGA GCAAAACA-3′ (1314–1291 from *Eco*RI site) were used for the first round and S2F 5′-AATGTTAGTATTCCTTGGACTCATAAGGTGGG-3′ (2451–2482 from *Eco*RI site) and S2R 5′-AGTTCCGCAGTATG-GATCGGCAGAGGA-3′ (1280–1254 from *Eco*RI site) for the second round PCR, using reaction conditions that were previously reported (Vermeulen et al., 2012). The BCP/PC region of HBV isolates was amplified using a nested PCR. Primers BCP1F 5′-GCATGGAGAC-CACCGTGAAC-3′ (1606–1625 from *Eco*RI site) and BCP1R 5′-GGAAA-GAAGTCCGAGGGCAA-3′ (1974–1955 from *Eco*RI site) were used for the first round and BCP2F 5′-CATAAGAGGACTCTTGGACT-3′ (1653–1672 from *Eco*RI site) and BCP2R 5′-GGCAAAAAACAGAGTAACTC-3′ (1959–1940 from *Eco*RI site) for the second round, using reaction conditions that were previously reported (Bell et al., 2012). The complete genome of representative isolates belonging to subgenotype A1 (sample KM2002) and genotype D (sample KM2044) was amplified using a single amplification method with modifications (Gunther et al., 1995).