

Abstract

Hepatitis B virus (HBV), the prototype member of the family *Hepadnaviridae*, is hepatotropic and replicates by reverse transcription. HBV is responsible for the chronic infection of more than 240 million people worldwide, of which 65 million reside in Africa. The nine HBV genotypes (A to I) identified to date, are geographically distributed and exhibit different clinical manifestations and treatment responses. The term occult HBV infection (OBI) refers to a HBV infection in which HBV surface antigen (HBsAg) cannot be detected by conventional serological assays as has been defined by the Taormina expert panel. . HBV and human immune deficiency virus (HIV) are both endemic in many parts of the world and share common transmission routes. Worldwide, 10% of those infected with HIV are also chronically infected with HBV. HIV co-infection has been shown to be a risk factor for the development of OBI in individuals infected with HBV.

The aim of this study was to characterize, at the molecular level, HBV from mono-infected and HBV/HIV co-infected individuals in Sudan

The objectives of this study were the systematic and comparative analysis of HBV genotype D sequences, available in the public databases; the molecular characterization of HBV from mono-infected Sudanese liver disease patients and from HBV/HIV co-infected Sudanese patients; and the development and testing of bioinformatics tools to explore HBV sequence data generated using ultradeep pyrosequencing (UDPS) and comparison of UDPS results with those obtained from cloning based sequencing (CBS).

All available complete genomes of genotype D of HBV from the GenBank database were analyzed. The intra-group divergence of the subgenotypes ranged from 0.8% $\pm$ 0.5 for subgenotype D6 to 3.0% $\pm$ 0.3 for subgenotype D8. Phylogenetic analysis of genotype D

showed separation into six distinct clusters (subgenotypes D1, D2, D3/D6, D4, D5 and D7/D8), with good bootstrap support. The mean intergroup divergence between subgenotype D3 and subgenotype D6 was 2.6%, falling below the accepted threshold of 4% required to define a subgenotype. This suggests that subgenotypes D3 and D6 are the same subgenotype because they also share signature amino acids. Furthermore, subgenotype D8 is a genotype D/E recombinant, which clusters with subgenotype D7. This analysis provided an update on the classification of the subgenotypes of genotype D of HBV.

Although HBsAg seroprevalence in Sudan, a central-African country, is greater than 8%, the only sequencing data for HBV, available prior to our study, was from asymptomatic blood donors, where genotype E predominates, followed by genotype D and subgenotype A2. Ninety-nine HBV-positive liver disease patients were enrolled in our study, including: 15 with hepatocellular carcinoma (HCC), 42 with cirrhosis, 30 asymptomatic carriers, 7 with acute hepatitis and 5 with chronic hepatitis. The surface and basic core promoter/precore (BCP/PC) regions, and the complete genome of HBV were sequenced. Eighty-two percent of the samples from HBV mono-infected liver disease patients were genotyped. Fifty-nine percent were infected with genotype D (74% D1, 10% D2, 3% D3 and 13% D6), 30% with genotype E, 8.5% with genotype A and 2.5% with a genotype D/E recombinant. Patients infected with genotype E had a higher frequency of HBeAg-positivity (29.2%) and higher viral loads compared to patients infected with genotype D. BCP/PC region mutations, including the G1896A mutation, seen in 37% of the HBeAg-negative individuals, could account for the HBeAg-negativity.

A total of 358 Sudanese HIV-positive patients were enrolled. HBsAg was detected in 11.7% of the participants, indicating chronic HBV infection. HBV DNA was detected in 26.8% of the participants: 11.7% were HBsAg positive (overt infection) and the remaining 15.1% were

HBsAg-negative (OBI). Fifty serum samples from the HBV/HIV DNA-positive co-infected participants were selected for genomic analysis of HBV. Of these, the HBV genotype of 37 was determined. The genotype distribution of HBV isolates from the HBV/HIV co-infected participants did not differ significantly from those from the HBV mono-infected patients: genotype D (46%), E (21.6%), A (18.9%) and a D/E recombinant (13.5%). Compared to the HBV isolates from mono-infected liver disease patients, the frequency of the D/E recombinant and genotype A was higher in HBV/HIV co-infected patients, as was the intra-group divergence of genotype E. No difference in BCP/PC mutations affecting HBeAg expression at the transcriptional and translational levels between genotype D and E was observed. The following mutations could account for the HBsAg-negativity: sM133T, sE164G, sV168G and sS174N. No primary drug resistance mutations were found.

Two online bioinformatics tools, the “Deep Threshold Tool (DDT)” and the “Rosetta Tool”, were built to analyze data generated from UDPS and CBS of the BCP/PC region of four Sudanese serum samples, infected with either genotype D or E of HBV, from HBeAg-positive and HBeAg negative patients. A total of 10952 reads were generated by UDPS on the 454 GS Junior platform. The Threshold was calculated using DDT based on probability of error of 0.5%. In total, 39 unique mutations were identified by UDPS, of which 25 were non-synonymous. The ratio of nucleotide substitutions between isolates from HBeAg-negative and HBeAg-positive patients was 3.5:1. From the sequences analyzed, compared to genotype E isolates, genotype D isolates showed greater variation in the X, BCP/PC/C regions. Only 18 of the 39 positions identified by UDPS were detected by CBS.

Using the specific criteria, that have been suggested previously, to define genotypes/subgenotypes of HBV, we determined that genotype D has six and not eight subgenotypes. The importance of HBV genotypes in clinical consequences of infection and

response to antiviral treatment has led us to characterize HBV genotypes circulating in Sudan. HBV mono-infected patients and HBV/HIV co-infected individuals, were mainly infected with genotype D or E. HBV mono-infected patients, infected with genotype E, had higher HBeAg-positivity and higher viral loads than those infected with genotype D. The ratio of genotype A to non- A, as well as the genotype E intra-group divergence were higher in HBV/HIV co-infected individuals compared to HBV mono-infected individuals. OBI was found in 15.1% HBV/HIV co-infected patients and its clinical relevance remains to be determined. In order to overcome the limitations of Sanger sequencing, which include its high cost and inability to detect minor populations in quasispecies, next generation sequencing techniques have been developed. It was demonstrated that correct analysis of UDPS data required appropriate curation of read data, in order to clean the data and eliminate artefacts and that the appropriate consensus (reference) sequence should be used in order to identify variants correctly. CBS detected fewer than 50% of the substitutions detected by UDPS. This new technology may allow the detection of minor variants between the different genotypes of HBV and provide biomarkers for the prediction of clinical manifestation of HBV and response to antiviral therapy.