

Prevalence and Genotypic Characterization of Hbv In Hiv- Infected Patients From Kwazulu-Natal, South Africa

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Research article

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Abstract

Introduction: The co-infection of HIV with HBV is very common due to shared mode of transmission. HBV/HIV co-infection impact on low HBeAg expression, high HBV replication, causes progressive liver disease, cirrhosis, liver cancer and high mortality. Co-infection may lead to cross-resistance of HBV and HIV drugs due to drug-related immune therapeutic pressure or hepatotoxicity. These challenges necessitate continuous surveillance for HBV among HIV infected individuals to aid patient treatment management. Hence we conducted this study to characterise HBV among HIV infected patients in Durban, KwaZulu-Natal of South Africa.

Methods: Serum was screened for HBsAg using ELISA, followed by DNA extraction from all samples. Genotyping of HBV was done through PCR amplification, Sanger sequencing and phylogenetic analysis.

Results: Of the 50 samples in this study 100% (n = 50/50) were HBsAg positive. HBV/HIV co-infection was 82% (n = 41/50) based on PCR amplification of the HBV partial surface gene and 63% (n = 26/41) of the amplicons were successfully sequenced. Phylogenetic and sequence analyses identified patients nucleotide sequence as genotype A. Mutations prevalence in the HBsAg region was 43% (n = 18/26); including mutations associated with diagnostic failure (K122R and T143S) and 7 vaccines escape mutations (P127T, G145R, S207N, Y200T, E164D, Y206H and L209V). Mutations were determined within the polymerase region of HBV and the amino acid substitutions were identified at 54% (14/26) in different positions within the reverse transcriptase (RT) region

The prevalence of mutations associated with drug resistance was 43% (n = 6/14) within the RT region. Drug resistance mutations was 67% (n = 4/6) for both lamivudine (LMV) and telbivudine (LdT) resistance, 17% (n = 1/6) for entecavir (ETV) and 33% (n = 2/6) for adefovir (ADV) resistance. Mutations causing resistance to lamivudine and telbivudine were M204V, L180M, V163I, and S202K; with S202K also causing resistance to entecavir and adefovir resistance mutation were I253Y and M250I. Multiple drug resistance mutations within a single sample contained L180M, M204V, S202K and M250I mutations.

Conclusion: This study shows the predominance of HBV genotype A in HIV-infected patients and the HBV mutations present in HBV/HIV co-infected individuals. HBV mutations associated with drug resistance suggest the need for continuous HBV screening and use of tenofovir ART regimen among HBV/HIV co-infected individuals.

Introduction

Hepatitis B virus (HBV) is an enveloped virus a prototype of the *Hepadnaviridae* family and the *Orthohepadnavirus* genus is important human pathogens [1]. HBV is estimated to infect more than 300 million people globally and cause of liver disease and liver cancer with approximately 1 million deaths from chronic liver disease and hepatocellular carcinoma (HCC) per year [2]. According to the Baltimore virus classification HBV belongs to the Class VII viruses, it contains a partly inimical relaxed

and circular (RC-dsDNA) double stranded DNA genome consisting of 3200 nucleotide base pairs, it replicate via a ssRNA intermediate [3].

Hepatitis B virus infects cells in the hepatocytes causing liver infection, which may clinical manifest into symptomatic or asymptomatic acute hepatitis characterized by inflammation of liver to fulminant hepatitis. Failure to treat acute HBV hepatitis may proceed to chronic HBV hepatitis which can lead to cirrhosis and HCC [4]. To diagnose HBV infection, disease associated with HBV and to distinguish acute and chronic infections numerous tests such as clinical, biochemical, serology and molecular techniques are conducted. The serological testing diagnose HBV by determining the HBV antibodies and antigens such as antigen (HBsAg); antibody to hepatitis surface antigen (anti-HBs), antibody to hepatitis B core antigen (anti-HBc) and IgM antibody subclass of anti-HBc (IgM anti-HBc).

HBsAg presence may be detected approximately 2 weeks of exposure and is the main clinical marker indicating acute or chronic infection and prevalence as well as endemicity of HBV infection [5]. Molecular techniques diagnose HBV by determining the qualitative and quantitative HBV-DNA. HBV genomes have been classified into ten major genotypes designating A to J based on the whole genome genetic heterogeneity of about 8% [6]. The genetic heterogeneity results from the lack of proofreading mechanism of the viral polymerase during viral DNA replication [6]. HBV sequence heterogeneity is also caused internally by selective immune system pressure and externally by vaccination and antiviral treatment. However, the different HBV genotype distributions differ geographically and that has an impact on the HBV pathogenicity and treatment management. The co-infection of HIV with HBV is very common due to shared mode of blood-born transmission [7].

The common routes of transmission vary geographically depending on the endemicity of HBV. HBV and HIV treatment involves the use of lamivudine (3TC) [8]. However, HIV- infected individuals should not receive 3TC or emtricitabine (FTC) monotherapy for HBV infection because of development of drug resistance with 4 years of single-drug treatment [9]. Resistance to 3TC is characterized by the development of mutation rtm 204 (YMDD mutation). The 3TC should be used only for patients on fully suppressive antiretroviral therapy (ART), this mutation causes rapid uncontrolled HBV DNA replication and that increased death rates associated with HBV infection/ disease.

Several studies have reported HBV prevalence among HIV-infected individuals from Durban in KwaZulu-Natal. However, most studies have focused on the sero-prevalence and studies focusing on the HBV genotype, HBV vaccine escape and drug-resistance are still limited in this area. Hence we conducted this study to determine the prevalence and genotypic characteristics of HBV among HIV-infected individuals in Durban.

Methods

Study population and ethical clearance

This was a cross-sectional study and a total of 50 stored frozen sera samples from HIV-infected individuals in Durban, KwaZulu-Natal cohort were used. The fifty participants were both male and females and they were recruited during the national health laboratory services (NHLS) screening programme in Durban in 2017. All participants were given informed consent and samples were collected and confirmed to be HIV-positive from previous serological tests. The patients were not on ART at that time. Ethical certificate was granted by the North-West University Research Ethics Regulatory Committee (NWU-RERC).

Samples collection processing

A total of 50 stored frozen serum samples from HIV-infected individuals in Durban were donated from NHLS and travelled on ice to the Virology laboratory at the North-West University. Upon arrival the samples were aliquot into numerous 1.5 ml Eppendorf tubes and stored at -80 °C until further use.

Hepatitis B Surface Antigen (HBsAg) Assay

Briefly, 100 µl of undiluted serum samples were used to detect presence of HBsAg using

Monolisa HBsAg ULTRA confirmatory kit following the manufacturer instructions (BioRad, Raymond Poincare, Marnes-la-Coquette, France). This was performed using neutralization by excess of antibodies to anti-HBs (anti-HBs diluent: neutralization reagent) of the HBsAg found in specimens. The presence or absence of HBsAg in the specimens was determined by comparing the rate of fluorescent formation by the test specimens. The O.D. index of specimens was measured at 450 nm and compared to an O.D. cut-off rate mean index calibrator of a negative control HBsAg rate. Samples with an index greater or equal to the cut off rate were considered reactive for HBsAg.

DNA Extraction of HBV

Serum obtained from patients was used to extract HBV DNA using QIAamp DNA Mini kit

(Qiagen, Hilden, Germany) following the manufacturer's instructions. This technique allows the purification of total DNA from contaminants, inhibitors and nucleases from the serum.

PCR Amplification Assay- HBV

A nested PCR amplification of the overlapping surface/ polymerase gene covering nucleotides 256 to 796 from *EcoRI* site as described previously with modifications [10]. Outer sense strand primer, S1 (5' CCT GCT GGT GGC TCC AGT TC-3'), and antisense strand primer, S2Na (5'-CCA CAA TTC KTTGAC ATA CTT TCC A-3') were used. For each sample the following reagent volumes and concentration were prepared: 18.5 µl nuclease free water, 2.5 µl 10x PCR buffer with MgCl₂, 0.5 µl 10 mM dNTP mix, 0.5 µl (50 uM) forward primer S1; 0.5 µl (50 uM) reverse primer S2Na anti-sense primer, 0.125 *Taq* DNA polymerase. A total of 22.5 µl of master mix was aliquot into a 0.5 ml thin-walled PCR tube and 3 µl of DNA template was added. The PCR reaction mixtures (25.5 µl) was subjected to amplification of HBV DNA, carried out

in an automated touch down thermal cycler CFX96 (Bio-Rad, Raymond Poincare, Marnes-la-Coquette, France).

The HBV DNA amplification conditions were 40 cycles involving denaturation at 95 °C for 4 minutes, annealing at 55 °C for 45 seconds, elongation at 72 °C for 1 minute, and final extension at 72 °C for 10 minutes. First round PCR product was used as a template for nested PCR. An aliquot of 3 µl of the first round PCR reaction was subjected to a nested PCR, the master mix volume and concentration were prepared as same for the first round PCR. The nested PCR conditions used were the same as first round PCR protocol. Forward primers S6E (5'-GAGAATTCCGAGGACTGG GGA CCC TG-3) and reverse primer S7B (5'-CGG GAT CCT TAG GGT TTA AAT GTATAC C-3') were used during nested PCR. The negative control consisting of nuclease free water and a positive control were included in the PCR amplification assays.

PCR products verification

PCR products were qualitatively verified using 1% agarose gel (ThermoFischer, Waltham, Massachusetts) stained with 0.15 U/ µl ethidium bromide (Biorad, California, USA). Aliquot of 10 µl PCR product was mixed with 2 µl 10x loading buffer. The mixtures were run on 1% gel along with a molecular weight maker (based on the viral gene of interest) as a band size reference. The agarose gel was run at 100 V for 45 minutes. Gel was placed inside the UV trans illuminator to visualise and image capturing.

Sequencing reaction

Sequencing reaction mix for each sample was prepared as follows: 1.5 µl 5X sequencing buffer, 1.0 µl BigDye terminator, 1.0 µl primer (5 pmol/ µl), 2.0 µl template DNA, DEPC treated water and primer sequences. A total of 10 µl mixture was added into 96-well plate and subjected into sequencing under the cycling condition of 40 cycles involving denaturation at 94 °C for 1 minute, annealing at 50 °C for 5 seconds, elongation at 60 °C for 1 minute, and final extension at 60 °C for 1 minute.

Sequence reaction clean-up

An aliquot of 50 µl of the 1:1 ratio of sodium acetate: ethanol (NaAc:EtOH) was added into samples and centrifuged at 2 000 g for 30 minutes. The well plates were inverted and centrifuged at 150 g for 1 minute. Pre-chilled 70% ethanol was added into the wells, and then centrifuged at 2000 g for 5 minutes. The samples were dried at 65 °C for 5 minutes and loaded into the sequencing machine ABI 3130XL Genetic analyser (Applied Biosystems, Foster City, CA). An aliquot of 10 µl of the Hi-Di formamide was added into the sample for 5 minutes and loaded into a sequencing machine.

Sequencing

The PCR products were sent for direct sequencing at the Inqaba biotechnological industry,

PTY, LTD, Pretoria, South Africa. The amplicons were prepared for direct sequencing using the BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems., Foster City, USA) and

sequencing was done using the ABI 3130XL Genetic analyser (Applied Biosystems, Foster City, CA).

Sequences analysis

Nucleotide sequences of HIV and HBV were edited and contiguous sequences were formed using sequencher v4.5 (Gene Codes Corporation, USA). The chromatograms were edited by removing unwanted and mixed nucleotides character we removed spaces within in the sequences. The contiguous sequences were formed by joining overlapping DNA sequences of a gene. The consensus sequences were compared with complementary genotype sequences from the GenBank using Basic local alignment search tool (BLAST). Representative sequences belonging to distinct genotypes were redeemed from the GenBank to make comparison with study sequences. Multiple sequence alignment of the sequenced nucleotides region was performed with CLUSTALW within the MEGA software package version 7.0.; the aligned nucleotide base sequences were subjected into phylogeny inference on MEGA 7.0. The neighbour-joining method was used to generate dendrograms and the evolutionary relationship was performed using pairwise genetic distance with 1000 bootstraps replicate [11-13]. Frequency estimates of evolutionary divergence between nucleotide sequences were then estimated using the Kimura 2-parameter model [14].

Ethical approval

Ethical certificate was granted by the North-West University Research Ethics Regulatory

Committee (NWU-RERC). Written informed consent was obtained from all study participants prior to their recruitment.

Results

Serology and PCR amplification

The HBsAg was positive in 50/50 patients resulting in a 100% HBV sero-prevalence. Of the 50 HBsAg positive patients, HBV DNA was amplified in 82% (n=41/50) of the HBsAg positive samples. HBV overlapping surface/polymerase region amplicons are shown as 547 bp bands below (Figure 1). PCR amplification could not be obtained for the other 9 (18%) samples.

Sequence Analyses of overlapping Surface/ Polymerase Gene Region

Sequencing of the overlapping surface/polymerase gene region was successful in 63% (n= 26/41) HBV-DNA positive samples. Phylogenetic tree analysis revealed the prevalent genotype A of individual nucleotide sequences Figure 2. The genotype and subgenotypes of sequences were confirmed by depositing all the surface gene nucleotides sequenced into the Genotype2pheno database. The 26 individuals sequence were identified as genotype A and subgenotype A1 based on the results retrieved from the Geno2Pheno database with the percentage of similarity to sub-genotype profile of 96.85% -99.0%.

Amino acids mutations within HBsAg

Within the HBsAg region 18 amino acid mutations were detected from the 26 sequences 69% n= (18/26). Prevalent mutations were found in the “a” epitope, “β”-cell epitope and outside the “a” epitope as shown in (Table 1).

Table 1 Amino acids mutations within the HBsAg region

Amino acid variation	Frequency (%)	Function	Reference
K122R	3	Sub-serotype change (d/y)	[25]
F134L	10	Unclear	[25]
S117N	3	Unclear	
T143S	3	Reflect the genotype variations	[25] [27]
S207N	21	Unknown in genotype A	
Y200T	3	Unknown in genotype A	
G145R	3	Vaccine-escape mutant	[30, 31]
P127T	3	Vaccine-escape mutation Lower reactivity in HBsAg assay	[27]
E164D	8	Vaccine-escape mutation	[34]
L209V	10	Unknown	
Y206H	3	Unknown	
L216 V	13	Unknown	
A194V	13	Unknown	
P70H	13	Overt HBV infections	[25]
T189I	5	Unknown	
P217L	10	Unknown/genotype	
S204R	3		
F129T	3		

Amino acids mutations within Polymerase region

Mutations were determined within the polymerase region of HBV and the amino acid substitutions were identified at 54% (n=14/26) in different positions within the reverse transcriptase (RT) region. The M129L mutation was the most common in this study followed by V163I, I253Y and M250I and other amino acid variations identified included L217R, A233S, Q125E, T128A, V214A, V204I, M204V, L180M, V173L and S202K (Table 2). The prevalence of mutations associated with drug resistance was 43% (n= 6/14) within the RT region (Table 3). Drug resistance mutations included lamivudine (LMV) and telbivudine (LdT) each at 67% (n=4/6), 17% (n=1/6) for entecavir (ETV) and 33% (n=2/6) for adefovir (ADV) resistance. Mutations causing resistance to LMV and LdT were M204V, L180M, V163I, and S202K. S202K mutation also causes resistance to ETV. ADV resistance mutations were I253Y and M250I (Table 3).

Table 2 Distribution of amino acids substitution in reverse transcriptase region of Polymerase of HBV positive patients co-infected with HIV

Amino acids substitutions	Frequency	Function
M129L	24%	Unknown
M204V	3%	Drug resistance associated
L180M	3%	Drug resistance associated
V163I	18%	Drug resistance associated
V173L	3%	Unknown
A223S	7%	Unknown
S202K	3%	Drug resistance associated
Q125E	4%	Unknown
L217R	8%	Unknown
V214A	3%	Susceptible
V204I	3%	Susceptible
I253Y	12%	Drug resistance associated
T128A	3%	Susceptible
M250I	10%	Drug resistance associated

Table 3 Distribution of drug resistant mutations in reverse transcriptase region of Polymerase of HBV positive patients co-infected with HIV

Amino Acids	Frequency (n/7)	<u>Drugs level of susceptibility</u>				
		LMV	LdT	ETV	ADV	TDF
M204V	1	Resistance	Resistance	Susceptible	Susceptible	Susceptible
L180M	1	Resistance	Resistance	Susceptible	Susceptible	Susceptible
M129L	1	Unclear	Unclear	Unclear	Unclear	Unclear
V163I	1	Resistance	Resistance	Susceptible	Susceptible	Susceptible
L180M	1	Resistance	Susceptible	Susceptible	Susceptible	Susceptible
M204V						
S202K	1	Resistance	Resistance	Resistance	Susceptible	Susceptible
M250I	1	Susceptible	Susceptible	Susceptible	Resistance	Susceptible
I253Y	1	Susceptible	Susceptible	Susceptible	Resistance	Susceptible

LMV- lamivudine; LdT- telbivudine; ETV- entecavir; ADV- adefovir; TDF-tenofovir

Discussion

In the current study, the HBsAg sero- prevalence in HIV-infected patients in Durban was 100%. The HBsAg prevalence is higher than that from previous studies in South Africa ranging from 4.8% to 20% [15-17]. The HBsAg sero-prevalence from this cohort is not coherent with published data from Durban, in this region the reported HBsAg among HIV positive people ranges from 2.9% to 10% [18]. In this study HBsAg positive is a marker of active HBV infection in 100% HBsAg positive. However, the HBV DNA of the partial surface gene region was successfully amplified in 82% (41/50) individuals indicating that HBV DNA levels are not always associated with HBsAg levels because HBV DNA replication occur independent of HBsAg synthesis. The HBsAg and HBV DNA results from this study confirm the hyper endemic nature of HBV and HBV-HIV co-infection in South Africa. The phylogenetic analysis of the overlapping surface/polymerase gene covering nucleotides 256 to 796 from the *EcoRI* site of HBV in this study show the predominance of genotype A in the 26 individuals. This phylogenetic result is coherent with previous studies in South Africa [19-21]. The patients sequence were further identified as sub-genotype A1 by Geno2Pheno indicating that HBV significant diversity suggesting an African origin.

The surface antigen serves as an epitope to which the antibody attaches; hence it is used in current recombinant vaccines. The HBsAg contains the alpha (α), beta (β) and T – cell epitopes, the “ α ” epitope is the main region targeted by antibodies and it contains the (β) and T – cell epitopes [22]. The “ α ” epitope is referred to as the major hydrophilic region of HBsAg, which is between the amino acids 124- 150 region [23]. Amino acid substitution within the “ α ” epitope domain of HBsAg may cause structural and functional changes in the S protein [24]. We determined the mutations on the partial surface gene of HBV/HIV co-infected individuals from the Durban cohort. A total of 18 amino acid substitutions were observed within the HBsAg region from 26 sequences. The sequences contained mutations at α , β and T – cell epitopes. There were four mutations (K122R, F134L, T143S and S117N) identified at the “ α ” epitope in the major hydrophilic region (aa79 to aa150) of the surface antigen. The “ β ” epitope contained S207N, Y200T and G145R variants while in the “T” epitope P127T was identified. Mutations identified outside the “ α ” epitope mutations included E164D, L209V, Y206H, A194V, P70H, P217L, T189I, S204R, L216V and F129T. K122R and T143S mutations are reported to be associated with diagnostic failure [25]. Previous studies have failed to detect K122R mutation in HBsAg region using certain ELISA diagnostic tests [26]. We identified seven vaccine escape mutations (P127T, G145R, S207N, Y200T, E164D, Y206H and L209V) identified in the HBsAg. The most common mutation was S207N and has been reported to cause diagnostic escape within anti-HBc positive patients [27]. G145R was detected in 1 patient sequence and this mutation has previously been identified by [28, 29]. G145R mutation is a major vaccine escape mutation in the “ β ”-cell epitope of the “ α ” epitope of HBsAg, because it changes amino acid sequence of the “ α ” epitope structure hence it interferes with the antibody binding causing the HBsAg antigenicity to be reduced [30, 31]. G145R mutation is a classical mutation because it was the first vaccine-escape mutation identified from Italian vaccinated children [32]. Its occurrence has increased in recent years with an increase of HBV endemicity and use of universal immunization [22]. This mutation is crucial in causing failure on the detection of HBV by serological routine assays [25]. G145R has become more dominant with vaccine selection, and it is recommended to be incorporated into future vaccine design [28]. However, the prevalence of G145R and P127T mutants were low in this study, this might be linked to the incorporation of HBV vaccination into South African EPI in 2005 [33].

Another significant vaccine-escape mutation identified from this study was E164D. E164D was detected in 2 individuals; it was reported outside the “ α ” determinants epitopes. The E164D causes substitution change in the overlapping polymerase region creating the V173L mutation which is associated with lamivudine resistance therapy and causes the reduction of anti-HBs [34]. Mutation E164D causes substitution change in the overlapping polymerase region creating the V173L mutation associated with lamivudine resistance therapy [34]. Other vaccine-escape mutations identified in this study were Y206H, L209V, S207N, Y200T and P70H however, there is limited literature on their role in surface protein and HBV infection still needs to be explored.

The HBV polymerase is the target of antiviral agents which are used to suppress the DNA replication of the HBV by blocking the reverse-transcription process. Antiviral agents used against HBV polymerase include the nucleoside/nucleotide analogues such as LMV, adefovir (ADV), TDF, telbivudine (LdT), and entecavir (ETV). In South Africa, lamivudine treatment is still the preferred treatment among the HBV/HIV

co-infected patients, regardless of the introduction of TDF. In addition, to the development of high mutation rate by the HBV error prone, antiviral drugs may also cause the development of drug resistance as a result of selection pressure during long-term use of the antiviral treatment [35]. Mutations causing resistance to LMV were M204V, L180M, V163I, and S202K. Mutations associated with LdT resistance were M204V, L180M, V163I, and S202K; with S202K also being resistance to ETF. A combination of L180M and M204V gives cross-resistance to other nucleosides and reduces sensitivity to ETV but not ADV and may cause vaccine escape mutations in the overlapping S-region and prevent the secretion of HBsAg [36]. ADV resistance mutations were I253Y and M250I.

Multiple drug resistance mutations within a single sample were identified from 1 patient containing L180M, M204V, S202K and M250I mutations. All the mutations associated with drug resistance identified in the polymerase (RT) were identified from genotype A sequences. Other compensatory mutations identified included S202K, Q125E, L217R, V124A, V204I, I253Y and T128A and moreover. The effects on these mutations on the RT functions still need to be explored. There were mutations associated with resistance to tenofovir in this study, hence, we suggest tenofovir-based regimen in treatment of HBV in HBV/HIV co-infected individuals.

In this study, we identified mutations in the HBsAg region which are known to allow escape from neutralizing antibodies. These mutations in the HBsAg region may cause less reactivity with serological assays leading to failure in the detection of HBV by commercial enzymelinked immunosorbent assay (ELISA) assays. We also detected mutations within the polymerase and reverse transcriptase regions which are associated with antiviral drug resistance; hence mutations within this region may lead to treatment failure.

Conclusion

This study shows the predominance of HBV genotype A in HIV-infected patients and the HBV mutations present in HBV/HIV co-infected individuals. HBV mutations associated with drug resistance suggest the need for continuous HBV screening and use of tenofovir ART regimen among HBV/HIV co-infected individuals. In conclusion, this study has added to the limited South African data on HBV genotypes and mutations associated with pathogenesis and drug resistance in HBV/HIV co-infected individuals. We suggest that, the HIV samples be tested for mutations associated with drug resistance to compare the impact of drug resistance in the context of HIV/HVB co-infection.

Declarations

Ethical certificate was granted by the North-West University Research Ethics Regulatory

Committee (NWU-RERC). Written informed consent was obtained from all study participants prior to their recruitment.

Availability of data and material

The data and materials supporting this study finding are available and will be shared per request.

Competing interests

The authors of this study declare no competing interest.

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Authors' contributions

Lorato Modise (LM) designed the study, LM collected samples, LM performed laboratory analyses on samples, LM analysed the data and prepared the manuscript.

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Figures

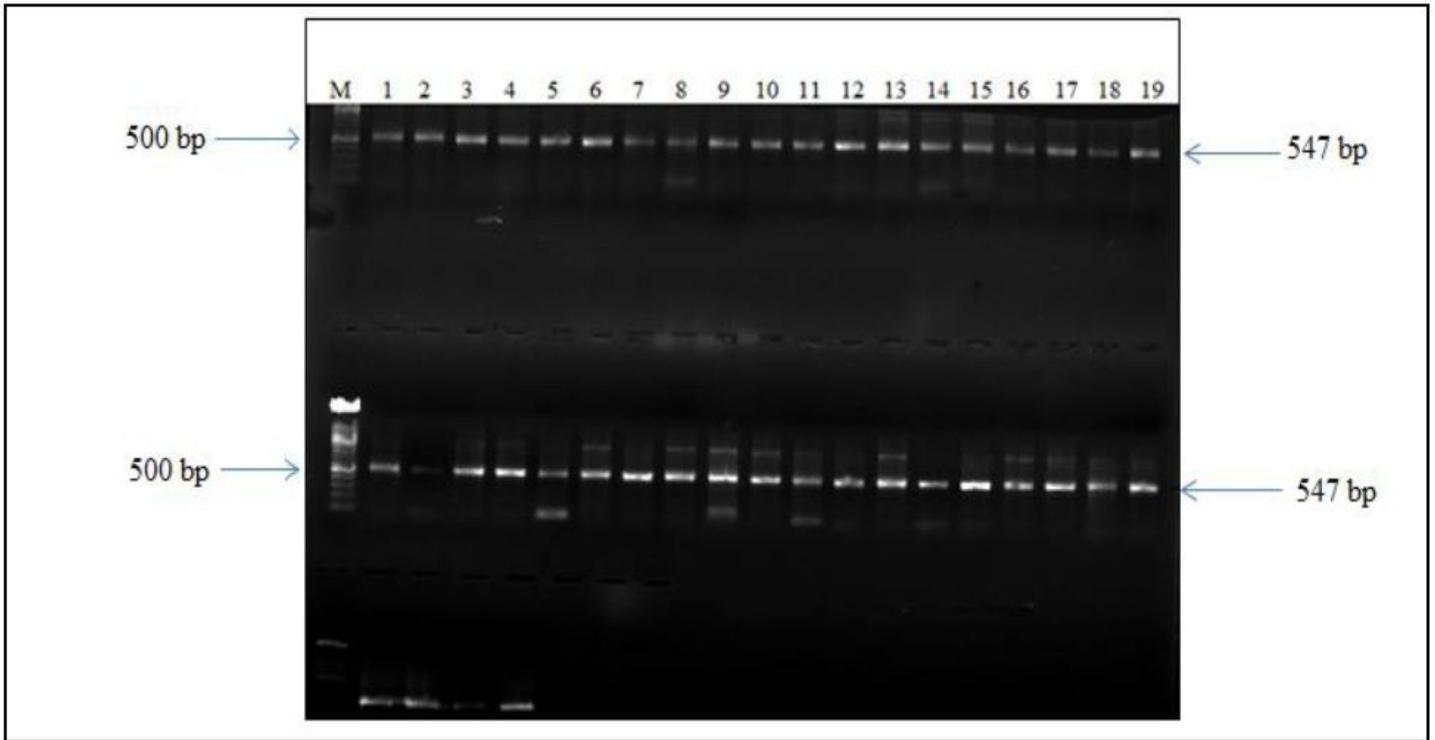


Figure 1

Amplified overlapping surface/polymerase region HBV. Genome position (256 to 796 EcoRI site) shown as 547 bp amplicons on a 1% ethidium bromides-stained agarose gel obtained from the HIV infected KZN cohort. M (first well) = 1000 plus bp molecular weight marker Well 2 (lane 1, lane 2 and lane 3) = positive control Well 3 to well 19 (lane 1, lane 2 and lane 3) = partial surface region amplicons of targeted band size ~ 547 bp

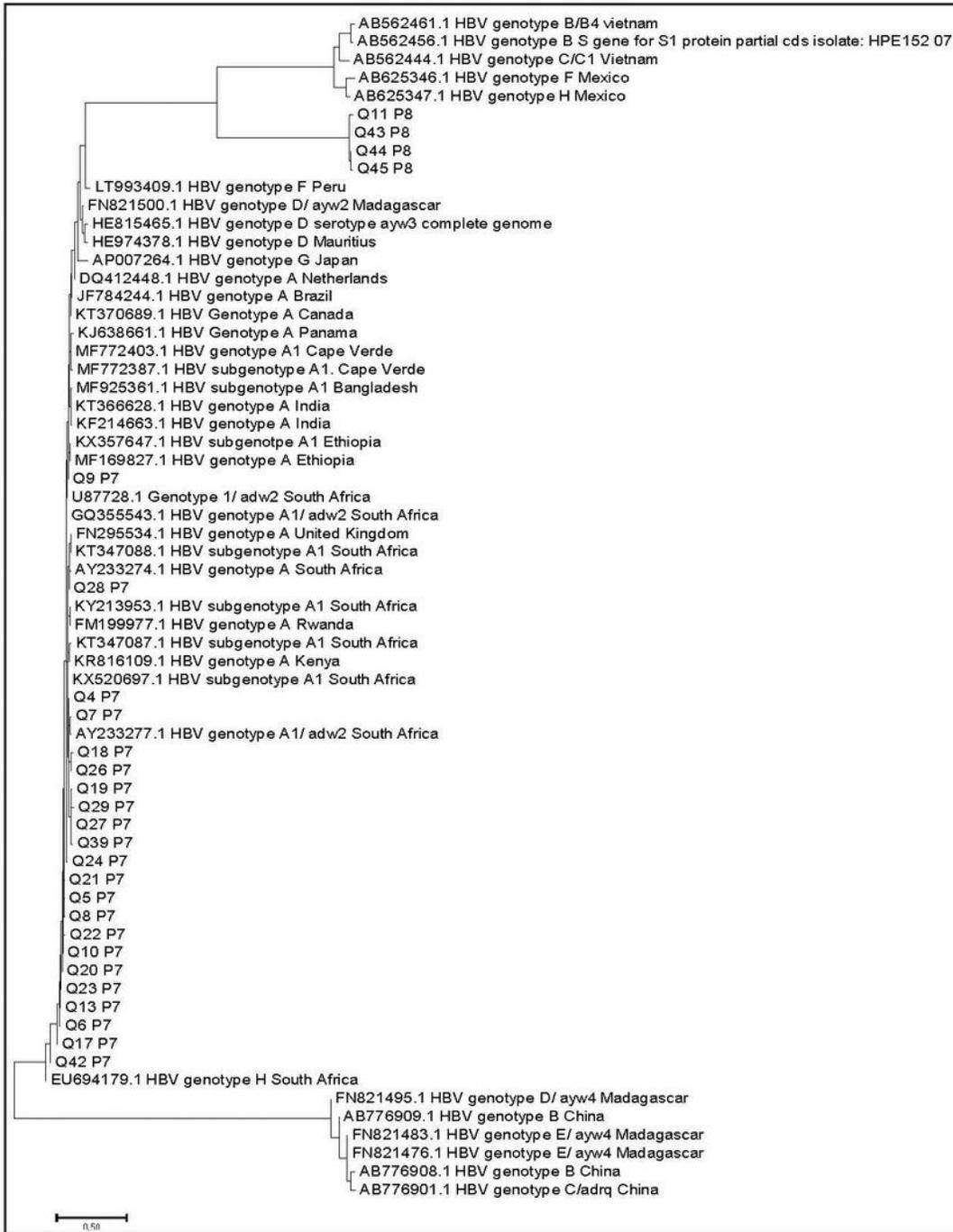


Figure 2.

Figure 2

Phylogenetic tree comparing the S gene sequences of this study. Representative sequences obtained from the GenBank (designated by accession numbers). Study sequences *are represented by letters Q.