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Molecular Characterization of Hepatitis B Virus Genotypes Circulating Among Individuals in Zamfara State, Northwestern Nigeria

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Abstract

Hepatitis B virus (HBV) is a highly infectious virus that remains one of the most important human pathogens. Recent data indicates that the prevalence of HBV in many Sub-Saharan African countries is among the highest in the world, often exceeding 8% of the population. This study was conducted to carry out molecular characterization of Hepatitis B virus genotypes circulating in Zamfara State, Northwestern Nigeria. Three hundred and ten (310) participants were enrolled in the study. Blood samples were collected by venipuncture, processed, and tested for hepatitis B surface antigen (HBsAg). The positive samples were analyzed using multiplex nested polymerase chain reaction and agarose gel electrophoresis to determine the virus genotypes. Out of the twenty-one HBsAg-positive samples, only 13 have detectable HBV-DNA. The study identified three (3) genotypes with genotype A predominating followed by genotype E with Genotype B having the least occurrence.

Keywords: Hepatitis B virus, Molecular Characterization, Nigeria, Zamfara.

Introduction

Hepatitis B virus (HBV) is a highly infectious virus that remains one of the most important human pathogens **[1]**. 'The virus is usually spread by exposure to infected blood and other blood products' **[2]**. The virus has also been detected in peripheral mononuclear cells, tissues of the pancreas, spleen, kidney, skin, and other body fluids such as saliva, semen, sweat, breast milk, tears urine, and vaginal secretions **[3]**. The infection is highly endemic in Africa, with a significant portion of the population affected by chronic HBV **[4,5]**.

Efforts to control HBV in Africa have made significant strides, particularly with the widespread implementation of vaccination programs aimed at newborns and children. However, challenges persist, including disparities in vaccine coverage, insufficient screening programs, and limited access to antiviral treatments. Nigeria is one of the countries with high incidence, the male to female predilection varies and children are not spared. Medical personnel are at the greatest risk of infection, while commercial sex workers and drivers have also been found to be at significant risk of getting infected **[6]**. Chronic HBV infection can lead to severe liver complications, including cirrhosis and hepatocellular carcinoma (HCC), contributing significantly to the burden of liver disease **[7]**.

Hepatitis B virus has evolved with ten genotypes (A–J) so far identified with distinct geographical distribution **[8]**. This evolution is driven by a high mutation rate which leads to a high degree of genetic variation. 'The dominant HBV genotypes differ by geographic location, transmission dynamics, disease progression, and response to antiviral therapy' **[9]**. The entire genomic sequences within each group diverge from other groups by more than 8% **[10]**. Phylogenetic and phylogeographic evidence suggest that genotype E most likely originated from Nigeria **[11]**. In Africa, one of three genotypes, E, A, and D are predominant depending on the region **[8]**. Genotype A is distributed globally and is the main genotype found in Europe, North America, and India. Genotypes B and C are predominant in East and Southeast Asia. Genotype D is mainly found in the Middle East and Mediterranean countries but has also been reported globally **[12]**. End-stage liver disease and poor response to interferon therapy are commonly observed in chronic HBV infection associated with genotypes C and D. However, it has been observed that horizontal HBV transmission is more common with genotypes A and D.

Hepatitis B virus infection remains a significant health burden affecting millions of individuals worldwide with distinct variations in disease

progression and treatment response linked to viral genotypes **[13]**. The classification of HBV genotypes indicates that these genotypes not only influence viral replication dynamics but also impact disease severity and response to antiviral therapy **[14]**. Despite advancements in understanding HBV genotypes, comprehensive genotype-specific epidemiological data are lacking particularly in regions with overlapping genotype distributions **[15]**. Thus, knowledge gaps persist regarding the specific impact of genotype variability on host immune response and the development of hepatocellular carcinoma **[16]**. Therefore, emerging genotypic variants and their implications for vaccine efficacy and antiviral resistance necessitate continuous surveillance and research efforts **[17]**.

Hepatitis B virus genotyping is important because it provides information on the molecular epidemiology of the virus including phylogenetic and phylogeographic histories **[18]**, it answers questions on the relation between the course of disease and viral strain **[19]**, and it provides insight into the impact of human migration on viral evolution **[20]**. Although HBV infection is widely reported to be endemic in Nigeria **[21,22,23]**, the molecular nature of the virus is poorly understood. Therefore, investigating HBV genotypes will provide critical insights into genotype-specific virulence factors which influence transmission, pathogenesis, disease progression, and response to antiviral therapy facilitating targeted interventions to reduce the burden of HBV in the study area, thus this study is designed to determine the circulating genotypes of Hepatitis B virus in Zamfara State, Northwestern Nigeria.

Materials and Method

Study Area

This study was conducted in Zamfara state northwestern Nigeria. The state is located at 12o 101 N 6o151E with an area of 39,762 km2 and an estimated population of 9,838,160 (2011 census). Until 1996, the area was part of Sokoto State. Zamfara comprises 14 Local Government Areas with three senatorial districts i.e., the Central senatorial district comprising four Local Government Areas (Tsafe, Gusau, Bungudu and Maru), the East senatorial district comprising four Local Government (Kaura, shinkafi, Zurmi, Birnin Magaji) and the West senatorial district comprising six Local Government (Mafara, Maradun, Bakura, Gummi, Bukuyyum and Anka). Most inhabitants of the area are predominantly Hausa whose major source of livelihood is from farming and rearing of domestic animals.

Ethical Approval

Ethical approval to carry out the study was obtained from ethical committees of Federal Medical Center (FMC) Gusau (FMC/2021/985/008/NHREC/TR/19/03/2016), Ahmad Sani Yariman Bakura Specialist Hospital (ASYBSH) Gusau (ASYBSH/SUB/205/VOL.1) and ethical committee of the Zamfara State Ministry of Health (ZSHREC04112022/101). Individual consent was obtained from all the participants. Adequate information was given to all participants for the purpose of the study and confidentiality of all information obtained was assured.

Study Population

This consists of individuals adults, males, females, outpatients, and patients who visited the hospitals during the period of the study, and who willingly gave consent for the study.

Sample Size Determination

The sample size was calculated using a single proportion formula:

$$N=Z^2PQ/D^2$$

Where:

N = Minimum sample size.

Z = Constant, standard normal deviation (1.96 for 95% confidence interval).

P = local prevalence rate of a previous study on [24] Hepatitis B virus among patients = 23.4% = 0.234.

Q = 1-P

D = acceptable margin of error (5% or 0.05).

$$N = \frac{1.96^2 \times 0.234 \times 0.864}{0.05^2}$$

N = 310

Inclusion and Exclusion Criteria

The inclusion criteria used include individuals who attended the hospitals during the period of the study and whose consent was obtained. However, persons who did not give consent to participate, children not older than five years, and persons who were too ill to understand the procedures involved in the study were excluded.

Sample Collections

Four (4) ml of blood samples were aseptically collected from each participant by venipuncture into an appropriately labeled sterile plain tube. This was allowed to clot at room temperature and spun for 5 min at 3000 rpm. The resultant serum was harvested and transferred into well-labeled sterile cryovials. Thereafter, the samples were transported from the sampling hospitals to the Antimicrobial Resistance (AMR) Fleming's laboratory, Veterinary Teaching Hospital of Usmanu Danfodiyo University Sokoto. Subsequently, the sera samples were stored at −20 °C before analysis.

Nucleic Acid Extraction

The extraction was carried out using a Qiagen genomic DNA extraction kit, following the manufacturer's instructions. All samples and reagents were equilibrated to room temperature for a few minutes to thaw prior to the commencement of the procedure. One hundred and fourty (140 µl) of the sera was pipetted into a new Eppendorf tube. Five hundred and sixty (560 µl) of binding buffer was added into the tube. Twenty (20 µl) of proteinase K was added to the mixture. The tube was closed and mixed by vortexing for 15 Seconds and incubated at room temperature for 10 min. The tubes were centrifuged for 5 minutes and 560µl absolute ethanol was added to the mixture. The mixture was then decanted into a DNA binding column, the cap was closed and centrifuged at 8000 rpm for 1 min. The QI-Aamp Mini column was transferred to a clean 2 ml collection tube, and the tube containing the flow-through was discarded. 'Subsequently, 500 µl Buffer AW1 was added to each mixture and centrifuged at 14000 rpm for 1 minute'.

The flow-through was then discarded. 'Five hundred (500 µl) of wash Buffer AW2 was then added and centrifuged at 14,000 rpm for 1 minute'. The flow through discarded centrifuged for 3 minutes at 14,000 rpm to remove excess ethanol from the column. The QIA-amp Mini column was placed in a clean nuclease-free 1.5 ml microcentrifuge tube and the old collection tube containing the filtrate was discarded. About 30 µl elution Buffer AVE equilibrated to room temperature was added directly to the silica membrane of the spin column and incubated at room temperature for 5 min, centrifuged at 14000 rpm for 1 minute. The spin column was discarded and the microtube containing the eluted DNA was caped and stored at -20^0 C until required for subsequent PCR and genotyping.

The Genotyping Procedure

The genotyping of HBV was carried out based on a rapid and specific genotypes system corresponding to six genotypes A through F by nested polymerase chain reactions method using a type-specific primer as described by Shuaibu *et al.* **[1]** with slight modifications in cycling profiles.

Hepatitis B Virus DNA Amplification

Multiplex-nested polymerase chain reaction (PCR) using a type-specific primer was used. Primers were designed based on the conserved nature of the nucleotide sequences in regions of the pre-SI through the S genes. P1B and S1-2 being the universal outer primers and B2 the inner sense (forward) primer with a combination of BA1R, BB1R, and BC1R as anti-sense (reverse) inner primers for genotypes A, B, and C in a multiplex system tagged 'Mix A. For genotypes D, E, and F, anti-sense primers B2R were used in combination with BD1, BE1, and BF1 as sense (forward) primers, also in a multiplex system tagged 'Mix B'.

First Round Polymerase Chain Reaction: Detection of Hepatitis B Virus DNA

The HBV was detected by amplification of pre-SI region through the S genes using universal primers (P_1B) sense primer, (S_{1-2}) antisense primer. The total reaction mixture volume used for the first round of PCR was 20µl. All the premix tubes were labeled with sample Identification numbers. About 2µl of extracted DNA was added to a Master Mix (cocktail of 16µl of deionized water {D.H_2O}, premix of 250µM each of dNTP, 1X PCR buffer,15mM of $Mgcl_2$ and 1U of thermostable Taq polymerase) and 1µl each of P_1B (forward) and S_{1-2} (reverse) outer primers. The PCR was performed using a thermal cycler (PTC-100^{TM} Programmable thermal controller, MJ Research, Inc.), and the reaction conditions were set as initial activation at 94^{O}C for 10 minutes, denaturation at 94^{O}C for 30 seconds, annealing at 53^{O}C for 30 seconds and extension at 72^{O}C for 1minutes. A total cycle of 35 from denaturation to extension was observed. The final extension was set at 72^{O}C for 5 minutes.

Second Round Polymerase Chain Reaction: Hepatitis B Virus Genotyping

This was performed in two different tubes for each sample, one with universal sense primer B2 and type-specific primers for genotypes A, B, and C in 'Mix-A' and the other with universal antisense primer B2R and type-specific primers for genotypes D, E, F in 'Mix B'. Seventeen (17µl) of deionized water (D.H2O) was added into each tube of premix A and B. Two (2µl) of the cocktail primers (containing 0.5µl each of the four primers were added to the mixtures. 'One (1µl) of the first round PCR product was also added into each tube of the premix. The mixture was gently agitated and then centrifuged at 3000rpm for 5 minutes. The PCR condition was set as initial activation at 94ºC for 3 min, followed by 30 cycles of denaturation at 94ºC for 1 minute, annealing at 50ºC for 1 minute, and extension at 72ºC for 1 minute for both "Mix A" and "Mix B", with a final extension at 72ºC for 5 minutes.

Table 1: Sequences of primers for HBV amplification and genotyping

Primer	Sequence (5'-3')	Specificity	Position	Polarity
1st round PCR				
P1	TCACCATATTCTTGGGAACAAGA	Universal	2823-2845	Sense
S1-2	CGAACCACTGAACAAATGGC	Universal	685-704	Antisense
2nd round PCR: Mix A				
B2	GGCTCCAGTTCCGGAACAGT	Type A-E	67-68	Sense
BA1R	CTCGCGGAGATTGACGAGATGT	Type A	113-134	Antisense
BB1R	GGTCCTAGGAATCCTGATGTTG	Type B	165-186	Antisense
BC1R	CAGGTTGGTGAGCTGGAGA	Type C	2979-2996	Antisense

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2nd round PCR: Mix B				
B2R	GGAGGCGGATTTGCTGGCAA	Type D-F	3078-3097	Antisense
BD1	GCCAACAAGGTAGGAGCT	Type D	2979-2996	Sense
BE1	CACCAGAAATCCAGATTGGGACCA	Type E	2955-2978	Sense
BF1	GTTACGGTCCAGGGTTACCA	Type F	3032-3051	Sense

Agarose Gel Electrophoresis

The amplicons were resolved using Agarose electrophoresis. The agarose gel was prepared by weighing 0.5g of Agarose powder in 50 ml 1XTris- Acetate EDTA (TAE) buffer and subjected to heat until the Agarose was completely dissolved and appeared as a clear transparent solution. The molten agar gel was allowed to cool to 50°C and then 2µl of ethidium bromide (0.5µg/ml) dye was added and swirled until the color disappeared. Thereafter, the gel was poured into the gel casting tray held within the gel casting gates and the comb was placed into the slots on the tray. The gel was allowed to solidify for 30 min and then the comb was gently removed. The gel slab along with the running tray was submerged carefully into the electrophoresis tank containing 1X TAE buffer. A total volume of 10µl amplicon was dispensed carefully into the wells of Agarose gel and electrophoresed for 40 minutes at 100V to determine the size of the amplified PCR product. The bands were visualized under the gel documentation system (BioRad Gel Doc-XR, USA) and screenshots were captured. The size of the separated bands (DNA fragments) was compared with the GeneRuler^{TM}100bp+ DNA ladder (MBI Fermentas, Life Sciences, Canada).

Phylogenetic Analysis

Phylogenetic analysis was used to compare the obtained sequence with corresponding sequences. Nucleotide sequences of the 6 genotypes (A-F) were obtained from the GenBank, European Molecular Biology Laboratory, and DNA Data Bank of Japan databases. A phylogenetic tree was constructed by using the neighbor-joining method component of the Molecular Evolutionary Genetics Analysis (MEGA) 3 program on the basis of the nucleotide sequences of the amplified pre-S gene of the HBV genome. Genetic distances were estimated by using the 6-parameter method, and phylogenetic trees were constructed by the neighbor-joining method. The principle of the neighbor-joining method is to find pairs of operational taxonomic units (i.e., neighbors) that minimize the total branch length at each clustering stage of operational taxonomic units starting with a star-like tree. The alignments for the complete genome were edited manually in GeneDoc.

Results

Out of the 21 Hepatitis B surface antigen (HBsAg) positive samples, Hepatitis B viral DNA (HBV-DNA) was detected in 13 samples at 1070bp. The HBV-DNA positive samples show bands representing identified genotypes **(Plate I and Plate II)**.

Plate I: Agarose gel electrophoretogram of HBsAg positive HBV-DNA sample

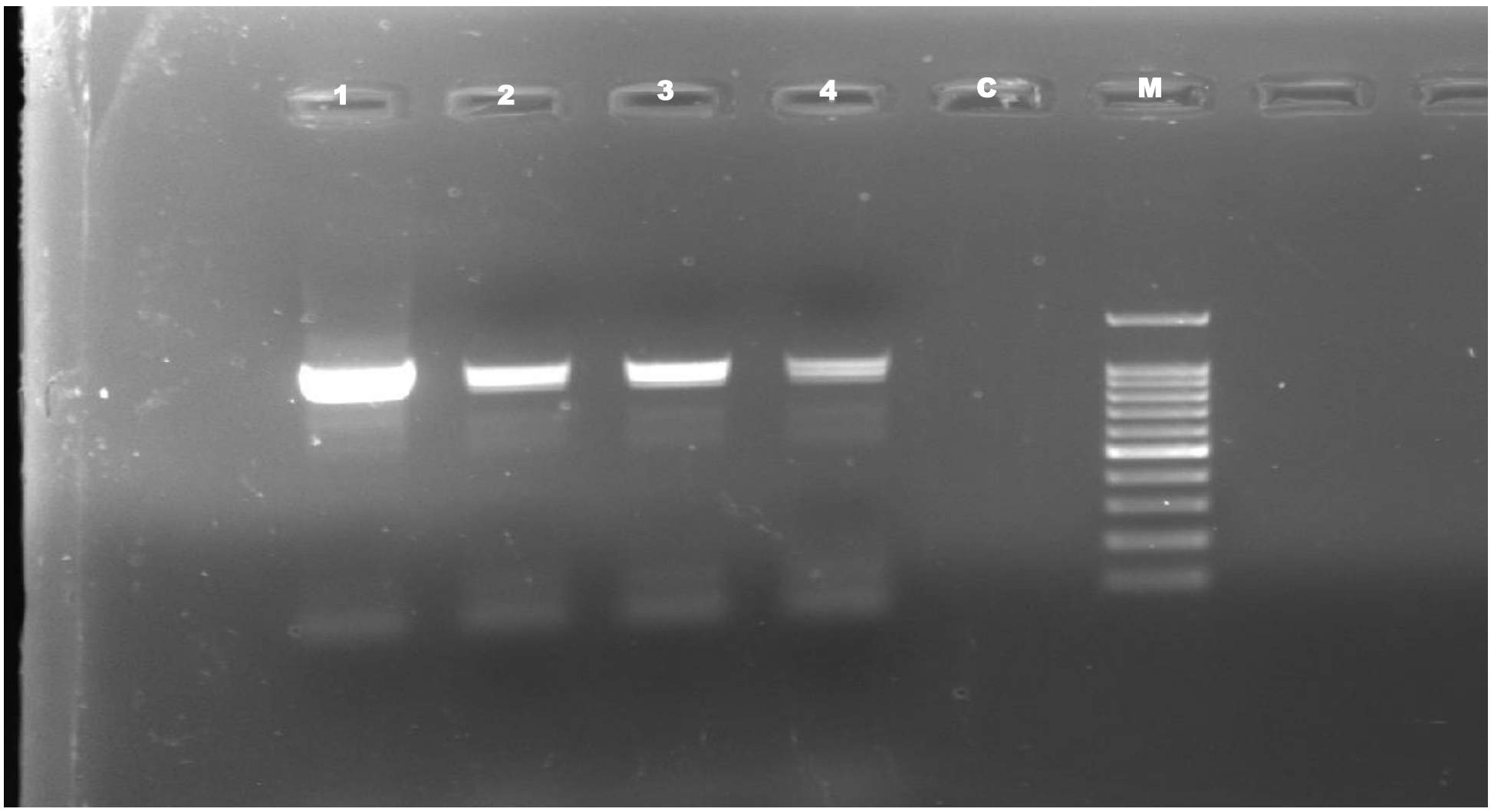
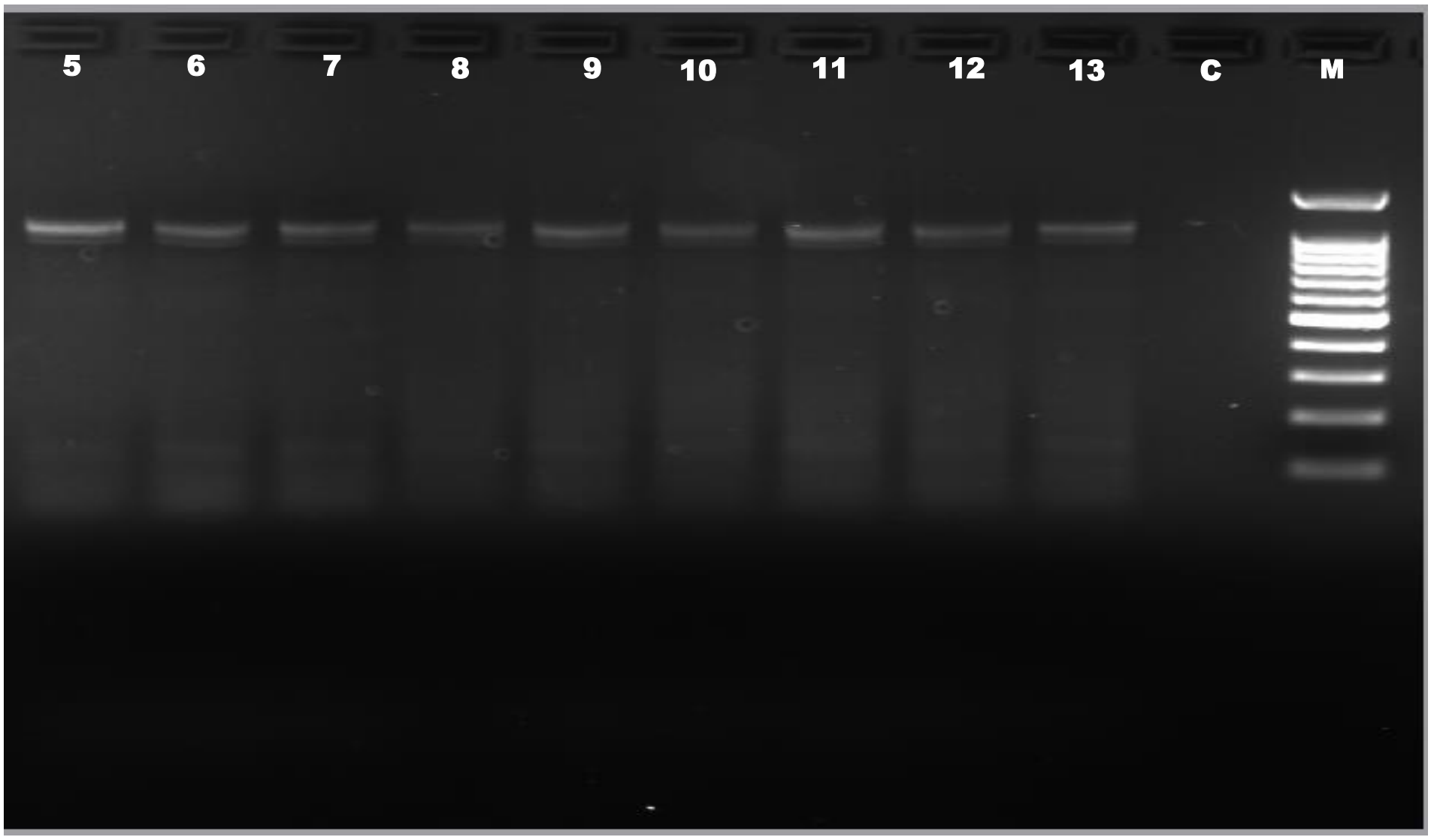


Plate II: Agarose gel electrophoretogram of HBsAg positive HBV-DNA sample



In Mix 'A' of the primers set-up, 7 bands appeared at 68bp **(Figure 3)**, and 2 bands appeared at 281bp **(Figure 4)** corresponding to genotypes A 53.8% (7/13) and B 15.3% (2/13) respectively. In Mix 'B', 4 bands appeared at 167bp corresponding to genotype E. However, eight HBsAg positive samples appeared to be uncategorized with no band indication **Plate I-V**.

Plate III: Showing Agarose gel electrophoretogram HBsAg positive HBV-DNA sample Mix A

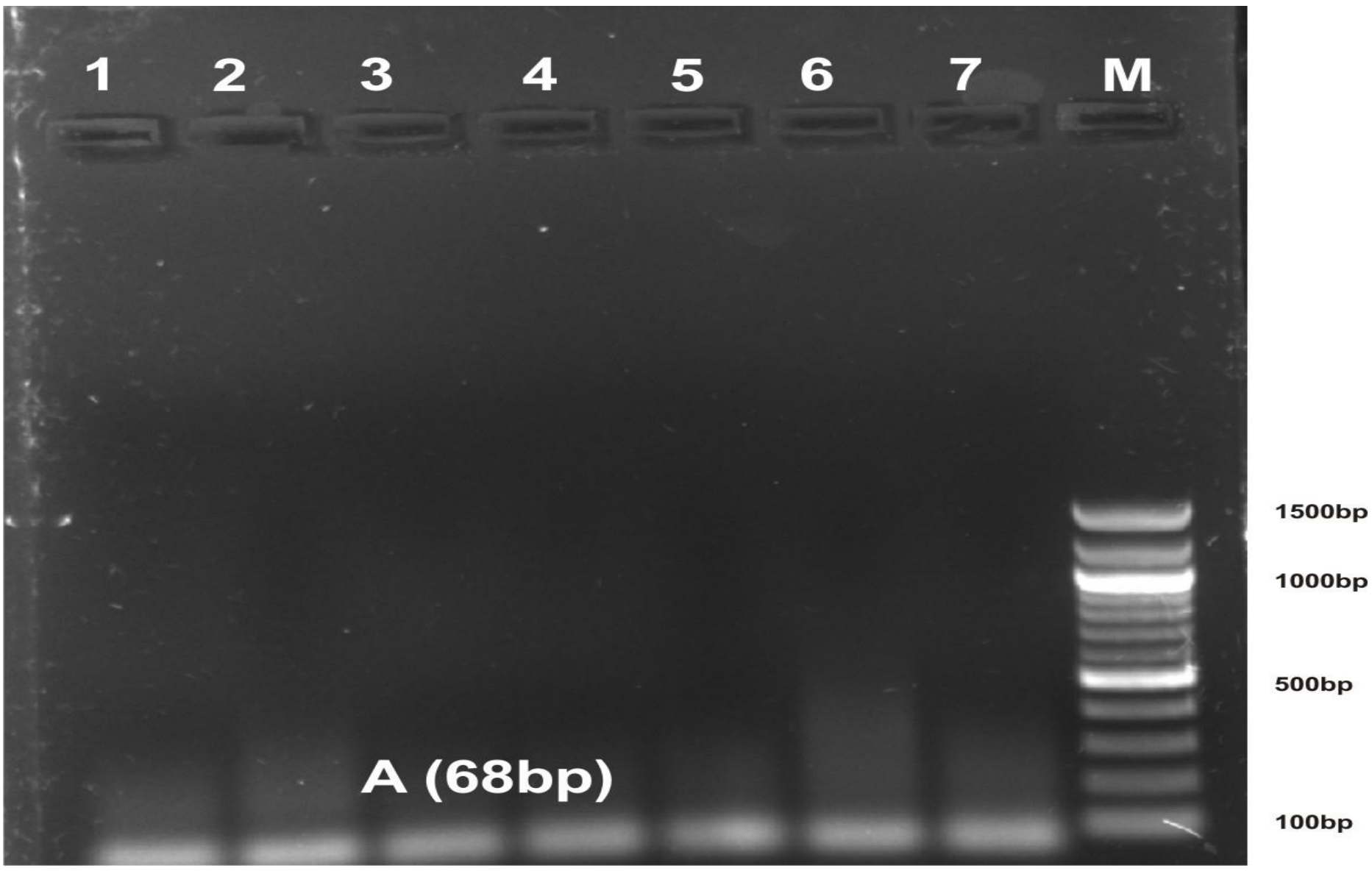


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Plate IV: Showing Agarose gel electrophoretogram HBsAg positive HBV-DNA sample Mix A

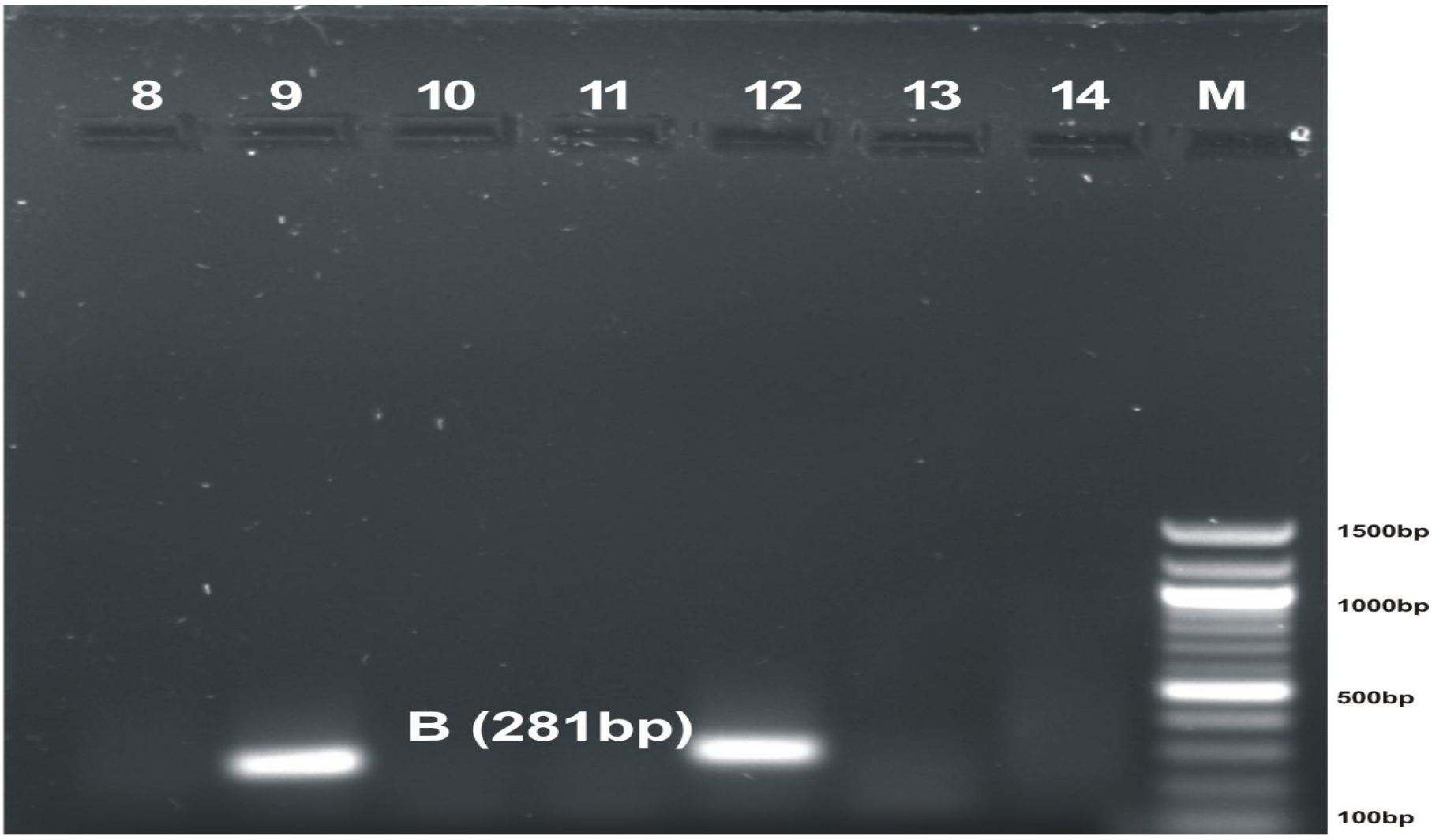
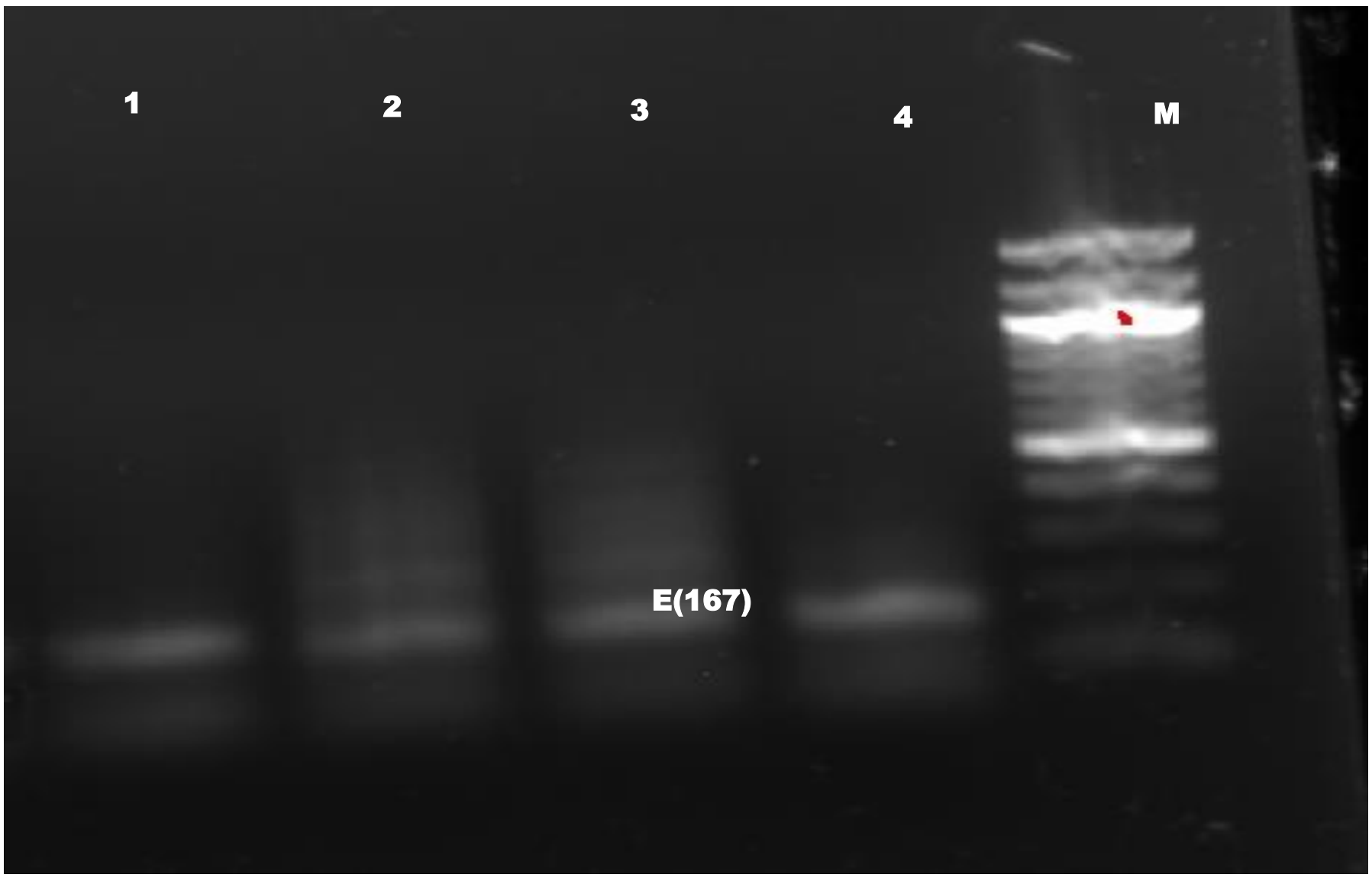


Plate V: Showing Agarose gel electrophoretogram HBsAg positive HBV-DNA sample Mix B



Five representative amplicons from Mix A and Mix B set-ups were subjected to Sanger's sequencing. Sequences obtained corresponded to Genotypes A, B, and E. The obtained sequences were registered and assigned a specific accession number by the GenBank of the National Centre for Biotechnology Information, USA.

Phylogenetic analysis of the complete genome sequences of genotypes A, B, and E was compared to those of 18 HBV genotypes from the GenBank database. 3 genotypes clustered with five other HBV genotypes (accession: LC461604, MH932713, KT365832, HE616583, and MN172189). The nucleotide homology within the cluster ranged from 100% to 99.8%. The nucleotide sequence data reported in this study will appear in the NCBI/GenBank nucleotide sequence databases with the accession numbers PP869431-5.

Table 1: Identity of Hepatitis B virus in Zamfara State, Northwestern Nigeria

Sample code	Accession No.	Base match accession number	% Similarity	E-Value	Query Cover	HBV Type
FMC 31	PP869431	LC461604	100	0.0	100	B
GHM 12	PP869432	MH932713	100	0.0	100	A
FMC 5	PP869433	MH932713	100	0.0	100	A2
GHT 17	PP869434	HE616583	100	0.0	100	E
YSH 2	PP869435	MN172189	100	0.0	100	E

Key

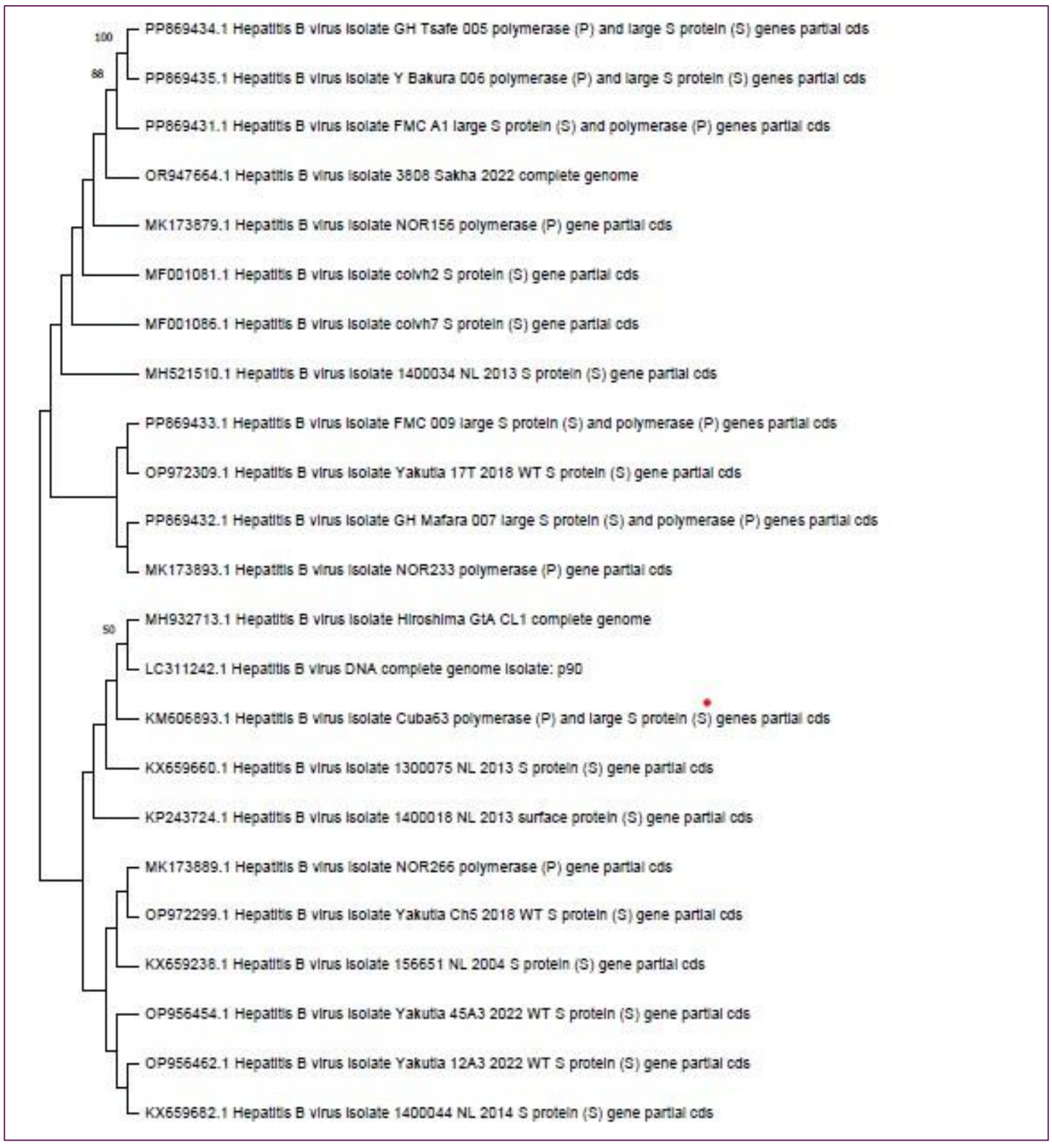
FMC: Federal Medical Centre Gusau.

GHM: General Hospital Talata Mafara

GHT: General Hospital Tsafe

YSH: Yeriman Bakura Specialist Hospital Gusau

Figure I: showing phylogenetic tree constructed by maximum likelihood analysis using the MEGA 6 program



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Discussion

This study was able to detect 13 HBV-DNA in 21 HBsAg-positive samples. This is a clear indication that despite the presence of HBsAg, which is typically the first marker of HBV infection, not all individuals necessarily have detectable levels of HBV-DNA. This low or undetectable level of HBV-DNA could be due to prior treatment or natural clearance **[25]**.

In relation to obtained genotypes, the presence of HBV genotypes A and E in this study was in conformity with the findings of Shuaibu *et al.* **[1]** who reported genotypes A and E among genotypes of this virus that are prevalent in West Africa. However, the discovery of genotype B was incidental as it had not been much reported in West African countries. Genotype B must have been introduced to this area due to the migration of people from rural areas across all the 14 Local Government Areas as a result of insecurity. Moreover, according to Malagnino *et al.* **[26]** global migratory flow of people could alter the normal geographic distribution of HBV genotypes with the appearance of some genotypes in areas where they were not previously identified or detected.

The appearance of an uncategorized band on the gel electrophoresis image represents a recombinant genotype that was not detected by the Mix 'A' and Mix 'B' type-specific primer set-up used in this study as it was capable of detecting only six (6) out of ten (10) so far identified genotypes.

Similarly, the clinical outcome of HBV infection and response to antiviral therapy can be influenced by the HBV genotypes. HBV genotype can influence the likelihood of developing complications such as liver cirrhosis and hepatocellular carcinoma **[27]**. Seroconversion of HBeAb is faster with genotype A and B than with genotype C-infected individuals. Genotype variability might also impact the effectiveness of HBV vaccines, as different genotypes may exhibit varying degrees of antigenic diversity. This study identifies 3 genotypes (A, B, and E) in Zamfara state with genotype A predominating, followed by genotype E with genotype B having the least occurrence. However, genotype B was incidentally found in addition to genotypes A and E which are known to be prevalent in Nigeria and other West African countries.

Conclusion

The study determined the circulating genotypes of the Hepatitis B virus in Zamfara State, North Western Nigeria. It is concluded that despite the presence of HBsAg, which is typically the first marker of HBV infection, not all individuals necessarily have detectable levels of HBV-DNA. This low or undetectable level of HBV-DNA could be due to prior treatment or natural clearance. It is recommended that there is a need for continuous screening and awareness as well as vaccination of the populace to reduce the menace of the infection.

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