EVALUATION OF THREE HEPATITIS B VIRUS DETECTION PROTOCOLS IN BLOOD DONORS

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ABSTRACT

Hepatitis B virus (HBV) DNA diagnosis plays a critical role in the transmission of HBV infections. Accurate protocols in HBV detection will reduce the spread of the virus from blood donors. Therefore, this research compared the HBV detecting potentials of three different HBV protocols in the virus detection. Blood from 200 donors at the University college blood bank Ibadan were sampled and tested for HBV using the ABON™ HBsAg Hepatitis B Surface Antigen Tests (strip test), Monolisa TM HBsAg ULTRA (ELISA test) and Huruian biology HBV detection kit (qPCR test). HBV positive samples were genotyped by sequencing to ascertain genotype identity. Results showed that of the 200 samples tested, both ABON™ HBsAg Hepatitis B Surface Antigen Tests and Monolisa TM HBsAg ULTRA (for ELISA) did not record any positive sample. However, Huruian biology HBV detection kit via qPCR recorded a total of twenty-three positive samples. Of the twenty-three samples, twenty were of genotype E while three were of genotype B. The results from this study showed that qPCR is more accurate in HBV virus detection than ELISA technique and also HBV genotype E is the most common genotype isolated in Nigeria.

Keywords: Hepatitis B virus, detection, strip, qPCR, ELISA

INTRODUCTION

Human hepatitis is an inflammation caused by hepatitis B virus (HBV), which infects the liver.
complications, are frequently experienced [Yuen et. al. 2003]. Patients with HBV exhibit severe jaundice and extremely high ALT levels (a part of liver function tests) as indicators of their acute disease [Villeneuve, 2005].

If infected blood is not detected during screening, HBV continues to be a serious blood-borne infection with serious consequences for blood recipients. Blood transfusion services are a crucial component of the healthcare system, a necessary life-saving procedure and one of the essential therapies listed by the World Health Organization. Transfusion-transmitted infections (TTIs) are still a significant health issue in the majority of developing nations due to underfunded facilities and a staffing deficit [Ifeanyi et. al., 2018]. Infections with blood-borne viruses are particularly common in the field of blood transfusion medicine. Occasionally blood donors transmit an infectious agent without displaying any symptom [Dhingra and Kitchen, 2014], and there is a 1% possibility of contracting its diseases during blood transfusion [Erhabor et. al., 2014]. It is illegal to provide contaminated blood to people who need blood transfusions. As a result, blood donors are tested for viral TTIs before giving blood. Enzyme-linked immunosorbent assays (ELISA), enzyme immunoassays (EIA), polymerase chain reactions (PCR) and immunochromatographic tests are some of the techniques used to diagnose viral infections [Hayder et. al., 2012]. ABON™ HBsAg Hepatitis B Surface Antigen Rapid Test is a rapid test for the qualitative detection of Hepatitis B Surface Antigen (HBsAg) in whole blood, serum or plasma. This kit is intended to identify the presence of HBsAg by interpreting the strip's color development visually. The presence of colored band signifies a positive outcome, while its absence signals a negative result [Osei-Boakye et. al., 2023]. This kit is generally used as a point-of-care diagnosis for HBV detection. The Monolisa TM HBsAg ULTRA assay is a qualitative one-step enzyme immunoassay that employs monoclonal and polyclonal antibodies that have been chosen for their capacity to bind to the various HBsAg subtypes now recognized by the WHO and the majority of variant HBV strains. It is based on the sandwich-type principle [Dembele et. al., 2020].

The development of PCR-based techniques has greatly increased the sensitivity of HBV DNA detection, and their commercialization (e.g., Roche Diagnostic Systems and HBV Monitor) has resulted in broad adoption of the technology [Mendy et. al., 2006]. More recently, the invention of real-time PCR technology has enlarged the range over which such levels may be precisely quantified and significantly improved the simplicity with which HBV DNA levels can be monitored [Yeh et. al., 2004; Mendy et. al., 2006]. Contrary to classical PCR, real-time PCR is frequently employed for the quantitative detection of HBV DNA due to its
improved accuracy, larger linear range, and reproducibility [Allice et. al., 2007].

A crucial step in tracking the spread of the virus is genotyping the hepatitis B virus, and different parts of the world have reported different HBV transmission patterns [Abubakar et. al., 2016; Forbi et. al., 2011]. Several studies have connected the HBV genotype to variations in disease development, pathogenicity, and therapeutic response [Tong and Zhang 2016; Revill et. al., 2016; Lin et. al., 2017]. This highlights the value of HBV genotyping as a diagnostic and epidemiological marker [Lin et. al., 2017]. Currently, HBV genotypes A through J have been reported, with genotype E (HBV/E) being the most common and in circulation in Nigeria. However there have also been reports of other genotypes in Nigeria, such as A, B, and C [Ahmad et. al., 2019]. Two crucial factors that every test methodology must take into account are rapid and sensitivity. Yet precision is crucially important. As a result, the goal of this study is to compare the capacities of three alternative HBV detection techniques for genotyping HBV-positive samples and detecting the virus in blood donors.

MATERIALS AND METHODS

Study Area

Blood samples from Donors at the Blood Bank in University College Hospital, Ibadan were used for this research. Sample analysis was carried out at the Molecular Laboratory in the Biorepository Unit at the College of Medicine, University College Hospital, Ibadan, and the Universal Laboratory, University College Hospital, Ibadan. The Hospital is situated at Ibadan North Local Government area longitude 7.3569°N and latitude 3.8743°E. It is bordered to the East by Ibadan North East Local Government and to the West by Ibadan North West Local Government Oyo State, Nigeria.

Study Subjects

A total number of 200 healthy prospective blood donors of age group 20-60 were recruited for this study. A well-structured and validated questionnaire was issued to obtain demographic information from consenting subjects (blood donors) for the study, both verbal and written consent in the form of signatures were also obtained from all participating donors.

Ethical Clearance

The Oyo State Ministry of Health's ethics committee reviewed, authorized, and provided authorization for the study's implementation with ethical number AD 13/479/442754. All study participants were educated on the purpose of conducting the research. Questioners were administered and each individual gave written informed consent prior to participation.

Sample and Sampling Technique

Ten millilitres of blood were collected from the antecubital vein from the participating individuals. Prior to blood collection, surface was sterilized with povidone-iodine and blood
collected was transferred to EDTA bottle. Blood samples were then centrifuged at 2000g for 10 minutes to separate the serum from cells. Two milliliters of serum were then transferred into different EDTA bottles and used each for a rapid screening test, surface ELISA test and Molecular qPCR test respectively. All samples were stored in a -80 °C Revco freezer till analysis.

**Rapid test using the ABON rapid screening test**

The ABON™ HBsAg Hepatitis B Surface Antigen Tests (ABON Biopharm Co. Ltd. Hangzhou, China) kit was used as a rapid test in HBV detection. The HBV rapid test was performed according to the manufacturer’s instruction and the results were interpreted by trained HBV counsellors. The ABON™ HBsAg Hepatitis B Surface Antigen Tests kit has been designed to detect the HBsAg through visual interpretation of colour development in the strip.

**Hepatitis B surface antigen detection using ELISA using Monolisa TM HBsAg ULTRA**

The Monolisa TM HBsAg ULTRA was used as an ELISA test kit in HBV surface antigen detection according to the manufacturer’s manual. Monolisa TM HBsAg ULTRA is based on the principle of sandwich ELISA using monoclonal antibodies and polyclonal antibodies selected for their ability to bind themselves to the various subtypes of HBsAg. The colour intensity of the solution was read spectrophotometrically at 450nm filter [Amini et. al., 2017].

**qPCR**

**DNA extraction and amplification**

Using a DaAn Gene Extraction Kit (deCODE genetics), 200µl of serum samples were used to extract the hepatitis B virus DNA. The extracted DNA was stored at -20°C prior to amplification. Using the Huruian biology HBV detection [PCR fluorescence] kit (Guangdong Huayin Medicine Science Co., LTD) in accordance with the manufacturer's instructions, the HBV in the samples was quantified using qPCR. The kit was run with a profile consisting of an initial 94°C denaturation for 2 minutes, followed by 40 cycles consisting of 94°C denaturation for 5 seconds, annealing, and extension at 60°C for 30 seconds including plate reading. The reaction was terminated at 80°C for 10 minutes.

**HBV Genotyping**

**Primer design**

Four HBV sequences for each genotype were collected from the Genebank database on the NCBI website in order to design appropriate primers for the HBV, which is universal to all genotypes (National Center for Biotechnology Information). By visiting the NCBI website (http://www.ncbi.nlm.nih.gov/) choosing Nucleotide from the drop-down menu, and then typing HBV (with a specific genotype) in the search box, the HBV sequence for each genotype was searched. Four reference
sequences for each HBV genotype were chosen once related sequences surfaced on the page. These sequences were uploaded to a bio edit program, clustered to highlight conserved sections that were peculiar to each genotype, and then used in the design of primers. Accessing the https://www.idtdna.com/PrimerQuest/Home/Ind ex webpage and pasting the sequence into the sequence entry box, several PCR primers were created. A perfect match between the primers is essential since it will improve primer annealing during PCR. Primers must anneal to these conserved HBV genotype sequence areas in order to do this. The optimal primer was then chosen and synthesized at Inqaba in South Africa after each pair of primers had been tested for specificity to be sensitive to only HBV and also for the ability to cut across all genotypes.

Polymerase Chain Reaction

The PCR was carried out in a 50 µl total reaction volume consisting of 10ul of 5x GoTaq colorless reaction, 3 µl of 25mM MgCl₂, 1 µl of 10mM dNTPs mix, and 1 µl of 10 pmol of each primer for The GeneAmp 9700 PCR System Thermocycler (Applied Biosystem Inc., USA) was used to conduct the PCR. The PCR conditions included an initial cycle of initial denaturation at 94°C for 5 min, followed by 40 cycles of each cycle consisting of 30 secs of denaturation at 94 °C, 30 secs of primer annealing at 50 °C, 30 secs of primer annealing at 94 °C, 30 secs of primer annealing at 50 °C, 30 secs of primer annealing at 94 °C, 30 secs of primer annealing at 50 °C, 30 secs of extension at 72°C, and a final extension for 7 min at 72°C.

Gel Electrophoresis

The integrity of the amplified 340 bp gene fragment was examined on a 1.5 % Agarose gel to ascertain successful amplification. 1.5 % agarose gel was made by mixing 1.5grams agarose powder in an already prepared one strength of Tris-acetate-EDTA buffer (1XTAE buffer) the suspension was microwaved to a boil point then the molten agarose was dyed with 5 µl of 0.5 g/ml ethidium bromide after being allowed to cool to 60°C (which absorbs invisible UV light and transmits the energy as visible orange light). The casting tray's slots were fitted with combs, and molten agarose was then poured into the combs. The wells were created by giving the agarose gel 20 minutes to solidify. The gel tank was filled with the 1XTAE buffer to just barely cover the agarose gel. After loading the 100bp DNA ladder into well 1 of each PCR product, two microliters (2 µl) of 10X blue gel loading dye were added to the 4 µl of each PCR product to make it easier to load the samples into the wells and monitor the gel's development. The gel was electrophoresed at 120V for 45 minutes before being imaged under UV trans-illumination. By comparing the mobility of a 100 bp molecular weight ladder with experimental samples run alongside it in the gel, the sizes of the PCR products were calculated.

Amplicon purification

The PCR reagents were eliminated from the amplicon using ethanol purification protocol. Briefly, in a new, sterile 1.5-l Eppendorf tube,
each 40µl PCR amplified product received 7.6 l of Sodium acetate 3M and 240 µl of 95% ethanol. The mixtures were properly mixed by vortexing, and the tubes were then kept at -20°C for at least 30 minutes. The pellet was washed by adding 150 µl of 70% ethanol and mixing, then centrifuged for 15 min at 7500g and 4°C after removing the supernatant and then inverting the tube to trash dry. After drying, the pellet was suspended with 10 µl of sterile distilled water and stored at -20°C. The purified amplicon was quantified using a nanodrop of model 2000 from Thermo Scientific and tested on a 1.5% Agarose gel run at 110V for about an hour as before to confirm the presence of the purified product.

**Sequencing**

The Big Dye terminator v3.1 cycle sequencing kit was used for sequencing the amplified fragments on an Applied Biosystems Genetic Analyzer 3130xl sequencer According to the manufacturer’s instruction the manufacturer's instructions. All genetic analyses were conducted using MEGA 6 and Bio-Edit software, and the altered sequence was blasted on the NCBI website to determine genotypic identity

**RESULTS**

**HBV Diagnosis**

The performance of each of the three HBV protocols as a tool in HBV detection was evaluated in this study (Table 1). Of the 200 blood donors tested, results indicated that both the ABON rapid test kit and the Monolisa TM HBsAg ULTRA test kit both did not record positive result in any of the samples. With the cycle threshold (CT) ≤ 38 as positive for the presence of HBV, the real-time Polymerase Chain Reaction (qPCR) on the other hand, was able to establish the presence of the Hepatitis B virus in 23 samples and recording the remaining 177 samples as negative. Table 2 shows that out of the 23 samples, five samples recorded very low CT with CT ≤ 30 recording very high viral load positives. The highly infected 5 samples were BB144, BB145, BB143, BB141 and BB132 having a CT value of 17.77 and 18.8 22.65 25.03 and 25.73 respectively. While the others moderately infected BB143 (30.38), BB144 (30.85), BB145 (32.13), BB147 (32.69), BB154 (33.02), BB158 (33.03), BB167 (33.05), BB182 (33.29), BBO184 (33.48), BB0185 (33.63), BB0187 (33.74), BB0188 (33.79), BB0189 (34.08), BB0191 (34.28), BB0192 (34.54) and BB0193 (34.87) and BB0194 and BB0197 were the less infected with CT values of 35.04 and 35.58 respectively. The moderately infected ones (17 samples) recorded CT > 30 but CT < 38 recording low viral load positives. Sample BB144 recorded the lowest CT value as CT = 17.77 therefore the most affected individual.

**HBV genotyping**

**Sequence Alignment Using the BLAST method**

The BLAST (Basic Local Alignment Search Tool) was used to calculate the statistical
significance of the matches, presented as the E value. The blast made use of approximately 99%-100% of each sequence [query cover] and identity was each between 98.81%-100% [Table 2]. NCBI Blast result revealed that all sequences were predominated Hepatitis B with 20 of the sequences of genotype E (BB091 BB094 BB132 BB134 BB141 BB143 BB144 BB145 BB147 BB154 BB158 BB167 BB182 BBO184 BB0185 BB0187 BB0188 BB0189 BB0193 and BB0197) and the remaining 3 were of genotypes B (BB0194, BB0192 and BB0191). No other genotype was recorded besides Hepatitis B virus genotype E and Hepatitis B virus genotype (Table 2).

Phylogenetic analysis showing the relationship between isolated HBV strains and HBV reference genotypes generated from the NCBI data base

The Phylogenetic tree was computed using the sample sequences and established sequences from the NCBI data base representing HBV genotype A – J. the well-rooted tree clustered with two main branches with BB0194, BB0192 and BB0191 clustering with HBV genotype B showing a close relationship with HBV genotype B. while the rest samples clustered with HBV genotype E showing a close relationship with HBV genotype E (Figure 2).

DISCUSSION

Hepatitis B is an extremely contagious virus that can cause a long-lasting, insidious infection. In endemic areas, hepatitis B infections are common and continue to be a serious blood-borne virus with serious consequences for blood recipients. If infected blood is missed during screening, the infection spreads more widely. To this end, a rapid and easy to use HBV detection protocol is very important but an accurate testing protocol which has the ability of detecting HBV even at minute viral load is of high value. This study compares the detection of hepatitis B using the ABON rapid screening test kit, Monolisa TM HBsAg ULTRA ELISA test kit and Huruian biology HBV PCR test kit. These kits were not only different but uses different technologies for the determination HBV values.

This study confirmed that although the ABON strip and the ELISA were rapid and cheaper, the qPCR technique was more reliable and sensitive as it gave 23 different cases of positive results compared to the other techniques. The most popular method for checking for this infection is the hepatitis B surface antigen (HBsAg). It is the first detectable viral antigen to manifest itself following infection. Kurdi et. al. [2014] noted that this antigen might not be present in early infection and might go undetected later in the infection because it is being eliminated by the host. The findings by Kurdi et. al. [2014] further support the potential use of the PCR approach for the accurate and reliable detection and
diagnosis of hepatitis virus infection when compared to ELISA.

Nucleic acid Protocols for HBV genotyping have been developed and employed in modern science. Such protocols include nested PCR [Ahmad et. al., 2019; Ayodele et. al., 2019; Umego et. al., 2022] and genotyping by sequencing [Oladeinde et. al., 2018; Chu et.al. 2003]. Although, Protocols for nested PCR are relatively cheap and easy to handle [Bartholomeusz and Schaefer 2004], genotyping by sequencing can be preferred due to the fact that sequencing just doesn’t give the genotype, but it also reveals strain diversity and sequence information [Umego et. al., 2022]. On these bases, we selected sequencing protocol to genotype the positive HBV isolates. It also helps to understand the relationships and potential transmission pathways of different HBV genotypes within a study population.

This study was able to detect and genotype the infecting HBV in 11.5% (23/200) of the collected samples. Of the 23 positive cases, three were identified to be HBV genotype B while the remaining twenty were HBV genotype E with no mixed infection. HBV genotype E is being reported to be restricted to West Africa including Nigeria where it is predominant. Genotype E is predominant in sub-Saharan Africa, and has been discovered in Central African Republic, Senegal, Namibia, and in East Africa [Hübschen et. al., 2008]. Odemuyiwa et. al., [2001] also reported that HBV genotype E is most predominant in Nigeria recording 100% genotype E. In a research conducted in Nigeria on 20 chronic and acute hepatitis patients. Genotype E was also predominant in this study conducted by Valente et. al., [2010] accounting for 66.7% of the successfully genotyped samples obtained in Port Harcourt, Nigeria. The occurrence of predominant genotype E in this study is in line with others studies carried out which examines the frequency of the different HBV genotypes occurrence in Nigeria [Ayodele et. al., 2019].

Other HBV genotypes, however, have been reported to be identified in Nigeria these are HBV genotypes B, A, C and D [Ahmad et. al., 2019]. Ayodele et. al., [2019] also reported that of the 15 HBV positive cases 10 were genotype E while 5 were Genotype B. Lim et. al., [2020] stated that the genotypes of HBV amongst a subset of infected young people in Central Nigeria and reported that a total of 29 cases [8.3%] were both HBsAg and HBeAg positive. Of these, 4 (13.8%) were genotype A (HBV/A), 10 (34.5%) genotype B (HBV/B) and 13 (44.8%) genotype E (HBV/E); suggesting that although genotype E is predominant, genotype B is also a common genotype in Nigeria. This was also in line with this study. Although we did not record HBV genotype A, C and D in this study, HBV genotype B was also detected in three different cases.

CONCLUSION
This study found that a significant percentage of individuals who tested negative for Hepatitis B virus [HBV] using the HBsAg marker was positive for the virus when qPCR technique was used to detect the viral DNA. This highlights the limitations of the current screening procedure and the need for additional measures to ensure the safety of transfused blood. The study also identified two genotypes of HBV present in the study population, with Genotype E being the most common. Overall, the results of this study emphasize the importance of implementing thorough screening procedures to protect the health of both donors and recipients.

CONFLICTS OF INTEREST: None to Declare

REFERENCE


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https://doi.org/10.1016/j.jhep.2004.06.031

Table 1: performance characteristics of Rapid test (ABON) ELISA (Monolisa) and qPCR (Huruian).

<table>
<thead>
<tr>
<th>Protocol tested</th>
<th>Rapid test /ABON/</th>
<th>ELISA /Monolisa/</th>
<th>qPCR /Huruian/</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positives detected</td>
<td>0</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Negatives detected</td>
<td>200</td>
<td>200</td>
<td>177</td>
</tr>
</tbody>
</table>

Table 2: qPCR analysis showing the positive HBV samples and their relative Ct values and genotype.

<table>
<thead>
<tr>
<th>S/N</th>
<th>sample ID</th>
<th>CT value</th>
<th>Status</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BB091</td>
<td>34.87</td>
<td>POSITIVE</td>
<td>Genotype E</td>
</tr>
<tr>
<td>2</td>
<td>BB094</td>
<td>33.48</td>
<td>POSITIVE</td>
<td>Genotype E</td>
</tr>
<tr>
<td>3</td>
<td>BB132</td>
<td>25.73</td>
<td>POSITIVE</td>
<td>Genotype E</td>
</tr>
<tr>
<td>4</td>
<td>BB134</td>
<td>33.63</td>
<td>POSITIVE</td>
<td>Genotype E</td>
</tr>
<tr>
<td>5</td>
<td>BB141</td>
<td>25.03</td>
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<td>Genotype E</td>
</tr>
<tr>
<td>6</td>
<td>BB143</td>
<td>22.65</td>
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<td>7</td>
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<td>8</td>
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<td>Genotype B</td>
</tr>
<tr>
<td>23</td>
<td>BB0197</td>
<td>32.13</td>
<td>POSITIVE</td>
<td>Genotype E</td>
</tr>
</tbody>
</table>
Plate 1: Gel picture showing the positive amplification of the HBV using HBV universal primers. Key amplification of approximately 330bp indicates positive amplification.
**Fig 2.** Phylogenetic analysis showing the relationship between isolated HBV strains and HBV reference genotypes generated from the NCBI data base.