Validation d'une nouvelle approche de PCR quantitative (qPCR) : outil de dépistage de l'hépatite B chez des femmes enceintes à haut risque de transmission du virus de l'hépatite B aux nouveau-nés au Mali

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Résumé
Introduction : Au niveau mondial, l'infection par le virus de l'hépatite B (VHB) chez les personnes à risque est un problème majeur de santé publique. La transmission mère-enfant (TME) est importante en Afrique puisque sur 70% des cas d'hépatite B soit 4,5 millions d'enfants infectés ont moins de cinq ans, probablement en raison d'une transmission verticale pendant la grossesse, l'accouchement ou l'allaitement. La prise en charge de l'infection par VHB est une préoccupation majeure, notamment la disponibilité de la charge virale à coût abordable dans un pays a ressources limitées comme le Mali. Cette étude visait à développer et à valider une méthode de détection et de quantification de l'ADN du VHB par la qPCR chez les femmes enceintes, une population à risque de transmettre le virus aux nouveau-nés. Méthodologie : Nous avons recruté 74 femmes enceintes avec un AgHBs positif au Centre de santé de référence de la commune III (CSRéf CIII) de Bamako, Mali dans cette étude. Leur charge virale a été prédéterminée par une technique de référence dans un laboratoire local. Nous avons conçu des sondes et des amorces spécifiques pour le gène PreC du VHB, ce qui a permis la détection et la quantification par qPCR. Nous avons adapté cette nouvelle PCR en temps réel sur différents machines pour HBV DNA quantification. Résultats : Neuf sur neuf (9/9) échantillons avec une charge virale (CV) entre 10000-100000 Unités Internationales/millilitre (UI/ml) et 4/4 avec une CV > 100 000UI/ml ont été détectés et quantifiés. Parmi les cinquante-cinq (55) échantillons avec un CV de 12-10000 UI/ml, 38/55 échantillons avec une CV > à 10000UI/ml ont été détectés et 17/55 échantillons entre 12-1000 n'ont pas été détectés. 6/6 échantillons négatifs n'ont pas été détectés par notre nouvelle qPCR. Conclusion : Ce nouveau protocole qPCR a démontré une détection et une identification efficaces des cas d'échantillons à CV élevées à plus de 1000 UI/ml chez les femmes enceintes. Il pourrait être étendu à d'autres populations à haut risque telles que les personnes immunodéprimées. Mots-clés : Détection ADN, Femmes enceintes, Virus de l'hépatite B, qPCR, Mali.

Abstract
Introduction: Worldwide, hepatitis B virus (HBV) infection in people at risk is a major public health problem. Mother-to-child transmission (MTCT) is high in Africa, where 70% of hepatitis B cases, or 4.5 million infected children, are under the age of five, probably due to vertical transmission during pregnancy, childbirth or breastfeeding. Management of HBV infection is a major concern, particularly the availability of affordable viral load in a resource-constrained country like Mali. The aim of this study was to develop and validate a method for the detection and quantification of HBV DNA by qPCR in pregnant women, a population at risk of transmitting the virus to newborns. Methods: We enrolled 74 pregnant women with positive AgHBs in this study. Their viral loads were previously determined at the Reference Centre Lab. We designed specific probes and primers for the HBV PreC gene, for detection and quantification by qPCR. We adapted this new real-time PCR on different machines for HBV DNA quantification. Results: Nine out of nine (9/9) samples with a viral load (VL) between 10000-100000 International Units/millilitre (IU/ml) and 4/4 with a VL > 100,000 IU/ml were detected and quantified. Of the fifty-five (55) samples with a CV of 12-10000 IU/ml, 38/55 samples with a CV > 1000 IU/ml were detected and 17/55 samples between 12-1000 were not detected. 6/6 negative samples were not detected by our new qPCR. Conclusion: This new qPCR protocol has demonstrated effective detection and identification of high CV samples over 1000 IU/ml in pregnant women. It could be extended to other high-risk populations such as immunocompromised individuals. Keywords: DNA detection, Pregnancy, viral load, Hepatitis B virus, qPCR, Mali.
Introduction
Hepatitis B virus (HBV) infection remains one of the leading causes of morbidity and mortality worldwide, with a high prevalence of cases in sub-Saharan Africa. In 2019, the World Health Organization (1) estimated that 300 million people were living with chronic hepatitis B and more than 800,000 deaths. The chronic phase of HBV infection can lead to cirrhosis and/or hepatocellular carcinoma (2). The serological status of HBV infection and the control of viral replication by antiretroviral (ARV) treatment are essential to control of this endemic. In addition, routine screening of pregnant women for HBV infection and antiviral prophylaxis for those in need are additional methods of preventing mother-to-child transmission (MTCT) (3). Furthermore, access to hepatitis B vaccination for adults and infants is essential for achieving the WHO goal of eliminating HBV by 2030. This is because the risk of developing chronic HBV infection or liver disease is high when infection occurs in the perinatal period rather than later through horizontal transmission (4). Infants remain 80-90% infected for the first years of their life then their infection becomes chronic(5). The gold standard screening and diagnosis method of HBV relies mainly on immunodetection of HBV surface antigen (HBsAg) in Africa (6). While in most high-income countries the screening is reinforced by testing for the presence of antibodies (Ab) directed against certain viral proteins including anti-Hbc or AbHbc (core protein), which helps in identifying current or past infection. Anti-HBs or HBsAg (surface protein) is a sign of cured infection or immunization by vaccination (6). Those methods are not able to detect the occult hepatitis B infection defined by the presence of HBV deoxyribonucleic acid (DNA) in clinical samples without a detectable HBsAg (7). Those molecular techniques are highly sensitive, specific and capable of detecting most circulating genotypes. The detection of HBV DNA indicates an active viral replication in infected persons. The real-time polymerase chain reaction (PCR) method uses a fluorescent probe containing a reporter dye, allowing efficient detection of amplified products in real time. Real-time quantitative PCR (qPCR) is a powerful diagnostic tool, generating reliable and reproducible results with reduced risk of contamination. It allows the identification and the approximation of the DNA load of the targeted pathogens (8). HBV DNA quantification is crucial for assessing the phase of viral infection as well as for monitoring antiviral therapy in patients with chronic hepatitis B (9). The risk of mother to child transmission is higher with a high viral load in pregnant women and support the important to quantify HBV DNA viral load in this population (10,11). However, hepatitis B viral load quantification remains a privilege in low-middle income countries in general due to her high cost of commercials kits and limited access to alternative tool to quantify HBV DNA. In this study, we aimed to develop and validate a qPCR-based detection and quantification of HBV DNA in person with high viral load risk like in pregnant women.

Material and Methods
Samples and clinical controls
We collected 74 clinical samples from hepatitis B surface antigen (HBsAg) positive pregnant women. We first assessed the viral load (VL) for participants at the Medical Laboratory using the "HBV-Real Time-Quant-DX" platform. This platform allows real-time amplification for the quantitative detection of HBV DNA with ready-to-use lyophilised reagents. We used AccuSpan™ HBV DNA Linearity Panel (genotype E) PHD802 (2410-0162 / Batch #10439611) as our positive and negative DNA controls containing nine (9) serial dilutions (from the highest positive to the lowest positive DNA as well as a negative sample) perform all experiments of the assay development and to generate a standard curve. We tested other HBV genotypes. Controls were simultaneously amplified by qPCR and served as internal controls to demonstrate that the detection and amplification were properly operating for each clinical sample.

Primers and probes selection
We selected pairs of primers targeting HBV genome from the literature search based on geographical strains as described elsewhere (12). The selected HBV primer sequences were first blasted using NCBI (National Center for Biotechnology Information) to verify specificity. We selected complete genome sequences from all genotypes from the GenBank nucleotide database. All selected sequences and the sequences of selected primers set were aligned using the MAFFT version 7 online alignment tool to ensure the primers are from a conserved region of HBV. HBV variants such as drug-induced Pol mutations, base polymorphisms, vaccine escape mutants at the S and pre-S genes, mutations resulting in premature stop codons, and recombination prone regions were specifically all checked. The primers’ sequences with highly conserved regions of HBV (PreC and preS genes) for genotype E (prevalent strains from West Africa) were targeted for the qPCR assays. Those primers were tested in PCR end point and four primers sets (set 1, set 2, set 3 and set 4) showed specific amplification of the HBV targets. The primer set 3 was final selected to continue the experiments (5'- GGACCCCCTGCTCGTGTATTACA-3' and 5'-GAGAGAAAGTCCACCCMCGAGTCTTAGA-3') covered the pre-C region is from a well-conserved region of the HBV genome were selected for qPCR assay. Although taken from the literature, they have been adapted to the most prevalent HBV genotype in Mali (genotype E) by changing two nucleotides. These primers have been aligned and analysed for melting temperature (Tm), self-complementarity and secondary structure using the online calculator Primers3 Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The probe (5HEX/TCACCTCCTGCACTA/3MGBEC) used was labelled at the 5’ end with the reporter dye (R) VIC or HEX depending on the qPCR machine used and the 3’ end by quencher (Q).
HBV DNA extraction
The QIAmp DNA mini kit (QIAGEN GmbH, QIAGEN Strasse1, 40724 Hilden, Germany) (REF 51306; LOT 166035662) was used to isolate the HBV DNA from 200 µL of plasma and recover in 100 µL of elution solution (AE) according to the manufacturer's instructions. The extraction principle of this kit relates to affinity chromatography on a silica column, by binding the DNA specifically to the silica gel membrane and by removing the PCR inhibitors, such as divalent cations and proteins, followed by two washing steps, making it possible to obtain pure DNA.

PCR for primers testing
We performed conventional PCR on a known hepatitis virus B positive sample for primer sets validation. We carried this PCR out in a volume of 25 µL total (20 µL master mix and 5 µL DNA) FastVirus 4X (Thermo Fisher Scientific Balcus UAB V.A. Graciumo 8 LT-02241 Vilnius, Lithuania) and ABI Universal master mix amplification kit, ABmix (Life Technologies LTD, Woolston, Warrington WA1 4SR UK) according to the manufacturer's instructions. The PCR amplification conditions were applied according to used kit protocol, the initial denaturation of the DNA strands for 95°C for 20 seconds and finally 40 cycles of 95°C for 15 seconds then 55°C for one minute for annealing and extension. We viewed amplified products under ultraviolet using Enduro GDS Touch II transilluminator after electrophoresis on 2% agarose gel for 25 minutes at 140 volts.

HBV real-time PCR conditions
The development of this quantitative qPCR was carried out using the ABI Universal master mix amplification kit, ABmix. The PCR reaction contained 12.5 µL of ABmix, 10 µM of primers each and 0.25 nM of probe, nuclease-free water adjusted for a final volume of 25 µL and 5 µL DNA or molecular grade water as a negative control. A positive sample for HBV of known viral load (3.128E8) was used as a positive control and for obtaining a calibration curve by making serial dilutions of 10 and tested in duplicate. The amplification program adopted was an incubation at 50°C for two minutes and polymerase activation at 95°C for ten minutes, a two-step amplification at 95°C for 15 seconds for denaturation and 60°C for one minute for annealing and extension for 40 times or cycles. We performed qPCR experiments on Applied Biosystem 7500 FAST and the LightCycler 480 II (LC480) instruments. The detection channel of the used probe was VIC for the 7500ABI FAST instrument and the HEX reference fluorophore of the probe for the LightCycler 480 II instrument. We used a housekeeping gene RNase P as an internal control of the samples to make it possible to highlight the presence or absence of HBV DNA in the samples using specific primer/probe set.

Linearity of the quantification of the technique
To establish the linearity of the quantification of HBV DNA by our qPCR, the "Linearity Panel DNA HBV" was used to validate the experiment. Serial dilutions of 10, from a strongly positive clinical sample (3.128E8) were also prepared to be used as standard in future experiments. These HBV standards made it possible to obtain concentrations of 10², 10³, 10⁴, 10⁵, 10⁶, 10⁷, 10⁸, 10⁹, and one copy/ml and one negative. Each concentration was tested in duplicate in several different experiments of quantification of viral load with our real-time PCR.

Data analysis
Results from standards provided Cycle threshold (Ct) and quantity of DNA samples obtained from AB 7500FAST instrument and converted into copies per milliliter (UI/mL). With the values of the standards in the experiments, the limit of detection (LoD) for the qPCR test was established as well as to quantify the unknown concentrations of the clinical samples. The coefficients of correlation (r) were calculated using the Pearson correlation.

According to the consensual values of the literature for a slope (between -3.6 to -3.2) and for R² (close to 1) made it possible to validate each experiment (13,14). The sensitivity and specificity of this developed qPCR technique compared to the reference commercial technique (HBV RealTM Quant Dx) were evaluated from data entered in Excel. (9)

The sensitivity, specificity, positive and negative predictive values of the new test were determined using OpenEpi online software, version 3.

Ethical approval
This study was approved by the ethics committee of the University of Sciences, Techniques, and Technologies of Bamako (USTTB), Mali under the number Nº2021/175 / EC/USTTB.

Results
PCR for primers’ testing
The effective detection of the HBV DNA by selected primer pairs was carried out by conventional PCR using two samples (sample S1 and sample S2) known positive (Figure 1). The samples called S1 and S2 showed a specific amplification at the expected PCR product size of approximately 180 base pairs (bp), which was specific to the targeted HBV PreC/C gene.

![Image](180bp.png)

Fig1.: Revelation of amplification products by conventional PCR
S: Sample SET: Primer set M: 100bp ladder NC: Negative Control
S1: 100 0000 UI/mL and S2: 76 160 000 UI/mL
The Set 3 primer-probe was chosen for real-time PCR experiment to quantify HBV DNA in the clinical samples.
Using ABI7500 Fast software, a plot of real-time PCR amplification of HBV obtained from serial dilutions in duplquat ranging from a nominal concentration of 3.128E^8 to 3.128 UI/mL and unknown samples (Figure 2a).

**Fig 2a:** Sample amplification curve by qPCR with set 3 primers on the ABI 7500 FAST

A real-time PCR standard curve of HBV showing linearity over a dynamic range. This standard curve was generated from the amplification plot displayed in Figure 2a. Least squares regression was calculated from plots of measured Ct (y-axis) versus DNA detection over a range of 100-10E^8 UI/mL (x-axis) tested in PCR wells in duplicate per dilution. The correlation coefficient was 0.99 and the slope of the line was -3.37. Our study showed an inversely proportional relation between the detection threshold cycle (Ct) and the number of copies (UI/mL as shown in Figure 2b above). The highest viral load (312800000 UI/mL) was detected before the 18th cycle while the lowest load (31.28) was only detected around the 39th cycle. The limit of detection of our qPCR technique was 100 UI/mL. A typical amplification curve, standard curve plot, and linear regression analysis of these data are shown in figures 2a, and 2b. Regression analysis yielded a correlation coefficient of 0.96 and a Y-intercept value of 52.46. The slope value of -3.37 closely approximates the theoretical maximum amplification efficiency of 97.92% validating our experiment. Two (02) replicates experiments were used to calculate the inter- and intra-test precision and the standard curve.

**HBV real-time PCR evaluation**
Seventy-four (74) HBsAg positive samples from a cohort of pregnant women were used to perform the clinical evaluation experiments of the validated qPCR assay in this study.

The reference method detected 68/74 samples and 6 had an undetectable viral load.

Our in-house real-time PCR showed that 51/74 (68.9%) detected positive samples as well as the reference method (HBV-Real Time-Quant-DX).

The sensitivity, specificity, positive and negative predictive values of the new test were determined using OpenEpi online software, version 3. (Table I).

**Table I:**

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>95% [CI]</td>
<td>95% [CI]</td>
<td>95% [CI]</td>
<td>95% [CI]</td>
</tr>
<tr>
<td>New qPCR</td>
<td>75 (63.56 - 83.77)</td>
<td>100 (60.97 - 100)</td>
<td>100 (93 - 100)</td>
<td>26.09 (12.55 - 46.47)</td>
</tr>
</tbody>
</table>

Our in-house real-time PCR showed sensitivity and specificity of 75% and 100% respectively.

**Evaluation of testing methods**
Among fifty-five (55) samples with VL < 10000UI/mL according to the reference qPCR method, 38 of them with VL at ≥1000 were detected by the new qPCR, all the samples with a VL ≥10000UI/mL were detected. Six undetectable VL by reference method were also undetectable by the newly developed qPCR (Table II).

**Table II:**

<table>
<thead>
<tr>
<th>Viral load</th>
<th>Techniques Used</th>
<th>Reference method</th>
<th>New qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undetectable</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>&lt; 12-10000 UI/mL</td>
<td>55</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>[10000-1000000] UI/mL</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>&gt;1000000 UI/mL</td>
<td>4</td>
<td>4</td>
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</table>

**Reference method:** HBV-Real Time-Quant-DX
The undetectable samples by new qPCR method included 12 samples with viral load between 12 and 150
Discussion

The study established a new qPCR that detected HBV clinical samples successfully. The quantification was more accurate in samples with a high VL compared to samples with low VL. This is particularly important due to the fact that people with a higher viral load can easily transmit the virus to other (e.g. in pregnant women during pregnancy or at birth) (15). This protocol can easily detect the high level of viral load notably for pregnant women in the context of developing countries where the implementation of HBV vaccine birth dose needs to be improved and HBV vertical transmission is important.

Out of the four designed primer sets, the set 3 primers were selected for the experiments of this new qPCR assay (Figure 1).

Those primers and probe target a conserved region called HBV PreC which gene codes for capsid proteins precursor to antigen HBe (HBeAg), an indicator of active HBV infection was. Indeed, during HBV infection the HBeAg marker appeared and can be measured to assess the active replication of the HBV infection (5). However, the presence of an HBeAg mutated variant can limit this marker to monitor the active HBV replication in some patients. The HBV DNA quantification or viral load is the best marker to assess the viral replication. (16,17).

Out of 68 samples detected by a reference method, 51 were positive by our qPCR method, showing a 75% concordance with the reference test (Table 1). Hundred percent (100%) of sample with high viral load (>10,000 UI/mL) were detected by the new qPCR, showing that this new developed qPCR is very sensitive to quantify the high VL samples. This result is a significant and can be very helpful in managing HBV infected pregnant women at risk to transmit the virus to their new-born. The risk of mother-to-child transmission of HBV infection is higher when the maternal viral load is high (>1 000 000UI/mL)(1). HBV vaccination at birth is strongly recommended to reduce the risk of infection development in new-born (18,19). However, a study conducted by Cheung in 2019, places particular emphasis on the level of viral DNA to predict the risk of immune-prophylaxis failure in highly viremic carriers who would be at risk of preterm delivery (19). Although this new developed quantitative qPCR showed good results, 17 samples tested by the reference method UI/mL could not be amplified. However, a sample with a VL at 1000 was amplified at later cycle(39th) by the new developed qPCR which might probably signify that low VL samples will be quantify if the cycles numbers of the new developed PCR is increased. None of the HIV mono-infected and negative for HBV samples were detected by our qPCR assay, confirming the great specificity and no cross-reactions of this Assay. This new developed qPCR test has some limitations because since a total of 25% samples of low viral load by reference method were not amplified. This might probably change when the number of cycles will be more than the ones we used (40 cycles) in this study. However, it is able to screen samples with high level of viral load like in pregnant women to start a prophylaxis treatment. A study from pregnant women showed that is essential to perform the HBV DNA quantification before the 22nd week of gestation (19) for an effective management of HBV infection in this population. The authors highlight the importance of alternative technique of qPCR tests on HBV quantification compared to commercial technique in developing countries supporting this study (18).

Benefit of technique

This in-house qPCR assay is accurate to screen high level of viral load and can be performed in open access using several PCR machines which reduce its cost comparing to commercial techniques that are sometimes restricted to some specific equipments.

The implementation of this in-house qPCR could be made available in equipped laboratories with a real-time instrument and may assist to monitor HBV infection progression and the treatment among peoples living with virus.

Conclusion

We developed a new real-time PCR assay suitable to screen and monitor the high VL levels in peoples living with HBV. This qPCR test allows for the efficient detection of at-risk individuals with very high VL, including pregnant women, in order to offer them antiviral prophylaxis and thus limit the risk of mother-to-child transmission. It is sufficiently specific (100%), accurate, reproducible and of acceptable sensitivity (75%) to be use in low-income countries endemic context and it’s could apply to targets other HBV infected populations.

Conflict of interest: none

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