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TITLE

Development and clinical validation of loop-mediated isothermal amplification (LAMP) assay to diagnose high HBV DNA levels in resource-limited settings

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ABSTRACT

Objectives

A massive scale-up of testing and treatment is indicated to globally eliminate hepatitis B virus (HBV) infection. However, access to a polymerase chain reaction (PCR), a key test to quantify HBV DNA levels and determine treatment eligibility, is limited in resource-limited countries. We developed and evaluated loop-mediated isothermal amplification (LAMP) assay to diagnose clinically important HBV DNA thresholds defined by WHO (≥20,000 and ≥200,000 IU/mL).

Methods

Pan-genotypic primer sets were designed on conserved HBV gene regions. Accuracy of LAMP to identify highly viraemic patients was evaluated in 400 and 550 HBV-infected people in France and Senegal, respectively.

Results

Our primers successfully detected eight major HBV genotypes/sub-genotypes ($A_{1/2/3}$/B/C/D/E/F) with a detection limit ranging between 40-400 IU/mL. In France, the area under the receiver operating characteristic curve (AUROC), sensitivity and specificity of bead-based extraction and real-time turbidimetric LAMP were 0.95 (95% CI: 0.93-0.97), 91.1% and 86.0%, respectively, to diagnose HBV DNA ≥20,000 IU/mL; and 0.98 (0.97-0.99), 98.0% and 94.6% for ≥200,000 IU/mL. The performance did not vary by viral genotypes. In Senegal, using a field-adapted method (reagent-free boil-and-

spin extraction and inexpensive end-point fluorescence detection), the AUROC, sensitivity and specificity were 0.95 (0.93-0.97), 98.7% and 91.5%, respectively, to diagnose HBV DNA ≥200,000 IU/mL. The assay was not adapted to discriminate low-level viraemia.

Conclusions

We developed a simple, rapid (60 minutes), and inexpensive (US$8/assay) alternative to PCR to diagnose high viremia ≥200,000 IU/mL. HBV-LAMP may contribute to eliminating HBV mother-to-child transmission by identifying high-risk pregnant women eligible for antiviral prophylaxis in resource-limited countries.

KEYWORDS

LAMP; hepatitis B; diagnosis; sensitivity and specificity; Africa

INTRODUCTION

Worldwide, 257 million people are chronically infected with hepatitis B virus (HBV) and 887,000 annually die from cirrhosis or liver cancer [1]. In 2016, WHO adopted a global strategy to eliminate HBV as a public health threat by 2030, and increasing treatment uptake from 8% to 80% in HBV-infected people eligible for antiviral therapy has become a key intervention [2]. Since >95% of HBV-infected people live in low-income and middle-income countries (LMICs) and only 12-25% of infected people are eligible for anti-HBV therapy [3,4], it is crucial to expand diagnostic services to assess treatment eligibility in these countries.

After testing positive for HBV surface antigen (HBsAg), a nucleic acid test (NAT) to quantify serum HBV DNA is required to determine the treatment eligibility. WHO recommends to initiate nucleos(t)ide analogues in HBV-infected patients with high viral load (≥20,000 IU/mL) who have persistently abnormal alanine transaminase (ALT) levels [5]. More recently, to prevent mother-to-child transmission (MTCT), tenofovir is recommended for HBsAg-positive pregnant women with very high viraemia (≥200,000 IU/mL) [6]. However, access to real-time polymerase chain reaction (PCR), a standard NAT to quantify HBV DNA, is severely limited in LMICs [7,8]. PCR is expensive (US$20-130/assay), requires well-equipped laboratories and trained personnel [5,9]. WHO therefore underlines a critical unmet need for simple, low-cost point-of-care tests to evaluate HBV replications [1].

Loop-mediated isothermal amplification (LAMP) assay is a NAT using DNA polymerase with high auto-cycling strand displacement activity and six specially designed primers

[10]. In contrast with PCR requiring a thermal cycler, LAMP can be performed under constant temperature (60-67 °C) using a simple heat block or water bath. Moreover, LAMP has the following characteristics allowing its use as a rapid, reliable and inexpensive point-of-care test in LMICs: high amplification efficiency enabling rapid detection of nucleic acids; generation of a large amount of amplified products allowing the use of a simplified low-cost detector; and high tolerance to well-known PCR inhibitors facilitating the process of DNA extraction [11]. A commercial kit to detect tuberculosis or malaria costs only US$6-8/reaction [12]. LAMP has therefore been put on the WHO List of Essential In Vitro Diagnostics, and recommended to be used in primary care setting to diagnose tuberculosis [13].

We therefore developed a pan-genotypic HBV-LAMP to diagnose high viral loads defined by the WHO guidelines (≥20,000 and ≥200,000 IU/mL) [5,6,14] using two amplification protocols: a semi-quantitative real-time LAMP and a qualitative end-point LAMP. We then conducted clinical validation in France and Senegal.

METHODS

Primer design

Conserved HBV gene regions were selected by multiple sequence alignments of all genome sequences available in GenBank and National Reference Center of Infectious Risks in Blood Transfusion (CNR-RIT) database for eight genotypes (A-H). A set of six degenerated, pan-genotypic primers were designed using LAMP explorer software

(Premier Biosoft). Primer specificity and genotypic coverage were verified *in silico* by BLAST analysis. Twenty-six newly designed candidate primer sets and three from previous publications [15–17] were evaluated.

Real-time LAMP

Analytical validation was performed using real-time turbidimetric LAMP (Loopamp™ LA-500, Eiken Chemical, Japan). This was carried out in a 25 µL reaction volume with 5 µL of the nucleic acid sample, 40 pmol of forward and backward inner primers, 5 pmol of forward and backward outer primers, 20 pmol of loop forward and backward primers, sterile deionized water, a commercial freeze-dried reagent (Loopamp™ DNA Amplification Reagent D, LMP207, Eiken), 30mM KCl and 8 units of Bst 2.0 polymerase (New England Biolabs, USA). Reaction mixture was incubated at a constant temperature (65°C) for 60 minutes. Amplified DNA products were detected in real-time by measuring turbidity generated with a release of magnesium-pyrophosphate.

End-point LAMP

In Senegal, we evaluated end-point HBV-LAMP specifically designed to identify high-risk pregnant women (≥200,000 IU/ml) to prevent MTCT [6,18,19]. The optimal time-to-result threshold obtained from the real-time LAMP to diagnose ≥200,000 IU/ml (*i.e.*, 40 minutes) was applied to stop a reaction using end-point LAMP incubator (HumaLoop M, HUMAN, Germany). The incubator accommodates 16 tubes/run; each run consisted of 40 minutes of isothermal amplification at 65°C, followed by 5 minutes of inactivation of

the enzyme at 80°C. Reaction tubes were read by naked eyes under ultraviolet light in the fluorescence visualization unit of HumaLoop.

Analytical validation

Analytical sensitivity and genotypic coverage were assessed using five replicates of 10-fold serial dilutions of the quantified synthetic DNAs. From GeneCust and NIBSC, we obtained plasmids containing synthetic DNA of the complete HBV genomes representing eight major genotypes/sub-genotypes (A1/A2/A3/B/C/D/E/F) with the following GenBank ID: AY233277/AY738140/AM180624/AB073843/AB111946/AB104712/AB194948/AY090456. For each genotype, an additional 15 replicates were tested at the lowest HBV DNA concentration detected in all five replicates. The limit of detection (LoD) was defined as the lowest concentration that was detected in ≥95% of 20 replicates. Reproducibility (intra-assay and inter-assay variability) was evaluated by testing eight replicates per sample and per experiment by three independent operators using a clinical sample (genotype A3, HBV DNA: 1,200 IU/mL) and reported as a coefficient of variation of time-to-result values. Analytical specificity was evaluated using a panel of genomic extracts of HCV/HIV/cytomegalovirus/parvovirus.

Field-adapted extraction methods

To optimize HBV DNA extraction methods for LMICs, we evaluated efficiency of two simplified low-cost protocols: a magnetic bead-based extraction (Dynabeads SILANE Viral NA, Thermo Fisher Scientific, US) and a reaction-free “boil-and-spin”. The boil-

and-spin, originally described by Nyan [17], was optimized as below; plasma samples were diluted twofold in nuclease-free water, heated at 95°C for 5 minutes and centrifuged at 12000 g for 10 minutes. The supernatant (10 µL) was subsequently used to amplify HBV DNA. Using a serially diluted plasma sample infected with HBV genotype A1, viral DNA recovery following an in-house real-time PCR [20] was compared between three extraction methods: bead-based, boil-and-spin, and a reference spin-column extraction kit (QIAamp Viral DNA Mini Kit, Qiagen, USA).

Clinical validation in France

The real-time HBV-LAMP was retrospectively validated at the CNR-RIT, Paris, using well-characterized plasma samples from deferred blood donors found positive for HIV, HBV, or HCV across France. HBV DNA, HCV RNA, and HIV-1 RNA were detected/quantified using TaqMan-based COBAS system (Roche Diagnostics, France, LoD for HBV DNA: 4.8 IU/mL). HBV DNA-positive samples were genotyped by sequencing a 445-bp region in the S gene [21]. Of those collected in 2004-2018, 400 HBV mono-infected cases and 50 HBV DNA-negative controls (30 HIV mono-infected and 20 negative for HBV and HIV and HCV) were randomly selected. An investigator blinded to the PCR results performed the bead-based extraction and real-time HBV-LAMP.

Clinical validation in Africa

Field-adapted HBV-LAMP was retrospectively validated using 550 sera from HBsAg-positive patients stored at -80 °C at the Institut Pasteur de Dakar, Senegal. Viral loads

were quantified using a real-time PCR (AMPLIX, Biosynex, France, LoD: 26 IU/mL). To ensure good representation of the target conditions (HBV DNA ≥20,000 and ≥200,000 IU/mL), a total of 550 HBsAg-positive sera were randomly selected from four different strata defined by viral loads: undetectable (<26 IU/mL, n=100); low (26-19,999 IU/mL, n=150); high (20,000-199,999 IU/mL, n=150); and very high (≥200,000 IU/ml, n=150). Viral genome extracted by the boil-and-spin method was mixed with the primer set and a commercial lyophilized reagent with calcein (Loopamp™ RNA/DNA Amplification Reagent D, LMP247, Eiken) for both real-time and end-point HBV-LAMP. The study was approved by the Clinical Research Committee at the Institut Pasteur and the National Ethics Committee in Senegal, and reported in accordance with the STARD (Supplementary Table 1) [22].

Data analysis

Receiver operating characteristic (ROC) curve was used to select optimal cutoffs for the real-time LAMP (time-to-result) that maximize the sum of sensitivity and specificity to diagnose the target viral loads. Correlation between LAMP index (60 minutes/time-to-result (minutes)) and HBV DNA level (log IU/ml) was assessed using Pearson's correlation coefficient.

RESULTS

Analytical validation

Reaction conditions of HBV-LAMP were optimized to attain the best amplification efficiency and test reproducibility (data not shown). Of the 26 primer sets designed, the one with the best amplification efficiency was selected (Supplementary Table 2); this set performed better than those previously reported [15–17].

Eight major genotypes/sub-genotypes were all successfully detected by the turbidimetric HBV-LAMP with a LoD ranging from 40 to 400 IU/mL (Table 1). The intra-assay and inter-assay coefficient of variation was 8.51% and 7,94%, respectively. Genomic DNA extracts from HCV, HIV, cytomegalovirus, and parvovirus were not detected by HBV-LAMP. DNA extraction efficiency was comparable between the bead-based and the reference spin-column extraction, but inferior for the boil-and-spin (Supplementary Table 3).

Clinical evaluation in France

Supplementary Table 4 presents characteristics of 400 case samples. Median viral load was 3.3 log IU/mL (IQR: 2.7-4.0). There was broad distribution of HBV genotypes thanks to wide variation in their country of origin: genotype A (n=87), B (66), C (72), D (84), E (76), and F (15). Using the bead-based extraction and the real-time turbidimetric LAMP, a total of 282/400 (71%) HBV DNA-positive samples were detected, with a median time-to-result of 24.5 minutes (IQR: 20.1-30.4). All the samples undetected by the LAMP (n=118) had viral loads below our target condition of 20,000 IU/mL (median: 299 IU/mL, range: 11-18,296). In the samples detected by HBV-LAMP, a correlation

coefficient was r=0.77 (p<0.0001) between HBV-LAMP index and HBV DNA levels (Figure 1A). The correlation coefficient was r=0.88 and r=0.19 in samples with viral loads ≥20,000 IU/mL and <20,000 IU/mL, respectively, demonstrating the linearity of the assay in samples with high viral loads. In each genotype, the linearity was similarly observed for the samples with viral loads ≥20,000 IU/mL (Figure 1B).

Table 2 presents the performance of HBV-LAMP to discriminate between low and high viraemia. The AUROC was 0.95 (95% CI: 0.93-0.97) for ≥20,000 IU/mL and 0.98 (0.97-0.99) for ≥200,000 IU/mL. The optimal cutoff, sensitivity, and specificity were 23.3 minutes, 91.1%, and 86.0% for ≥20,000 IU/mL, and 20.1 minutes, 98.0%, and 94.6% for ≥200,000 IU/mL, respectively. The AUROC was high across the genotypes (Figure 2): A (0.92), B (0.98), C (0.96), D (0.93), E (0.95) and F (0.88) for ≥20,000 IU/mL; and A (0.99), B (0.99), C (0.97), D (0.97), E (0.98) and F (0.96) for ≥200,000 IU/mL. The performance was intermediate to discriminate viral load threshold of ≥2,000 IU/mL (Table 2). None of the 50 samples negative for HBV DNA reacted with HBV-LAMP.

Clinical evaluation in Senegal

In 550 HBsAg-positive samples, median viral load was 4.4 log IU/mL (IQR: 2.3-5.6). The proportion detected by the real-time LAMP was 0% (0/100), 0% (0/150), 40.7% (61/150), and 98.7% (148/150), in undetectable (<26 IU/mL), low viraemia (26-19,999 IU/mL), high viraemia (20,000-199,999 IU/mL), and very high viraemia (≥200,000 IU/mL), respectively. In those detected by the LAMP (n=209), the median time-to-result

was 29.8 minutes (IQR: 25.9-37.2). The correlation coefficient between the HBV-LAMP index and viral loads was r=0.80 ($p<0.0001$) (Figure 1C). The AUROC of the real-time LAMP was 0.85 (95% CI: 0.82-0.87) for ≥20,000 IU/mL and 0.98 (0.97-0.99) for ≥200,000 IU/mL (Table 2). The optimal cutoff, sensitivity, and specificity were 60.0 minutes, 69.7%, and 100% for ≥20,000 IU/mL, and 40.0 minutes, 94.0%, and 93.5% for ≥200,000 IU/mL, respectively. HBV-LAMP was not adapted to diagnose viral loads ≥2,000 IU/mL (Table 2).

Finally, the end-point LAMP was evaluated to diagnose very high viraemia (≥200,000 IU/mL). The proportion detected by the end-point LAMP, stopped at 40 minutes, was 0% (0/100), 0% (0/150), 22.7% (34/150), and 98.7% (148/150), in undetectable (<26 IU/mL), low (26-19,999 IU/mL), high (20,000-199,999 IU/mL), and very high viraemia group (≥200,000 IU/mL), respectively. The sensitivity and specificity of the end-point LAMP were 98.7% and 91.5% to diagnose ≥200,000 IU/mL (Table 2).

DISCUSSION

This is the first study that developed a field-adapted pan-genotypic HBV-LAMP assay, aiming to assist clinicians to diagnose high viral loads indicating anti-HBV treatment eligibility. Irrespective of HBV genotypes, we found a sufficient accuracy of the real-time HBV-LAMP to diagnose clinically important viraemia of ≥20,000 and ≥200,000 IU/mL. Moreover, using the reagent-free boil-and-spin extraction and the low-cost robust

LAMP incubator, we observed high accuracy (sensitivity 98.7%, specificity 91.5%) of the end-point HBV-LAMP to diagnose very high viraemia ≥200,000 IU/mL in Senegal.

Although LAMP has been evaluated to diagnose HBV, its primary focus has been to detect small amounts of HBV DNA rather than to identify highly viraemic patients who need antiviral therapy [15–17,23,24]. Two studies from China assessed LAMP to quantify HBV DNA [15,16]. However, both focused on local viral genotypes (B/C), and we confirmed a lack of reaction of these primers with other genotypes.

In order to optimize the assay for LMICs, we examined two distinct methods for viral genome extraction, and for amplification/detection. Table 3 compares their operational characteristics by referring to WHO-ASSURED criteria for ideal point-of-care tests in LMICs [25]. The bead-based extraction and real-time turbidimetry provides semi-quantitative results allowing to diagnose both WHO's viral load thresholds: ≥20,000 to treat HBV-infected adults and ≥200,000 IU/mL to prevent MTCT [5,6]. As a drawback, the turbidimeter costs US$18,000 and extraction reagents requires a cold chain. In contrast, the boil-and-spin extraction and end-point fluorescence detection only provides qualitative results just to diagnose ≥200,000 IU/mL, however, this is likely to be readily applicable to LMICs as a simple, rapid (60 minutes), and affordable (US$8/reaction) point-of-care test to identify high-risk pregnant women for MTCT. To date, over a hundred end-point LAMP incubators (HumaLoop) have been deployed in LMICs for tuberculosis or malaria program; this only requires a two-day training for local laboratory technicians, no need for regular maintenance, and high throughput (70/day) with 16 modules [26,27]. The risk of contamination is minimal as results can be determined

without opening reaction tubes [28]. In Africa, a prospective study is ongoing to evaluate the feasibility, acceptability, and cost-effectiveness of HBV-LAMP in rural health centers. Moreover, a mobile solar panel for LAMP incubator has been commercialized [12], a potential solution to decentralize the assays in areas with limited electronic supply.

The study has limitations. First, HBV genotypes were not available in samples from Senegal, where the major genotypes are E and A [29,30]. Second, our assay was not adapted to detect low-level viraemia. Our objective was to develop a tool to assist clinicians to evaluate the WHO's treatment eligibility in LMICs, and not to monitor the response to treatment, to diagnose occult HBV infection or to define treatment eligibility in resource-rich countries. Third, HBV-LAMP was evaluated using different extraction methods according to the country. In France, we used bead-based extraction which provided better amplification efficiency (ie, faster time-to-result) than boil-and-spin; the latter does not completely remove inhibitors leading to a slower LAMP reaction. In contrast, boil-and-spin was used in Senegal because this method does not require reagent, resulting in a lower cost and no need for refrigerator.

To achieve the WHO's global elimination goals, it is critical to have a simple, reliable and affordable diagnostic test to evaluate active HBV replication. HBV-LAMP, an attractive alternative to PCR, deserves further field evaluation.

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CONFLICT OF INTEREST

MS is employee of Eiken Chemical. The others declare no conflict of interest.

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TABLES

Table 1. Detection limit of HBV-LAMP for eight reference HBV genotypes

HBV genotype	Real-time turbidimetric LAMP				
	Limit of detection (IU/mL)*	Mean time-to-result (minutes)	Number of replicates	Number detected	% detected
A1	400	27.54	20	19	95
A2	400	26.32	20	19	95
A3	400	24.41	20	20	100
B	400	24.61	20	19	95
C	40	33.54	20	19	95
D	400	27.41	20	20	100
E	400	32.04	20	19	95
F	400	29.13	20	20	100

* The limit of detection was defined as the lowest HBV DNA concentration that was detected in ≥95% of 20 replicates.

Table 2. Performance of HBV-LAMP to diagnose high HBV DNA levels using different extraction/detection methods

	European study (n=400)	African study (n=550)	
	Method 1	**Method 2**	**Method 3**
Extraction method	Bead-based	Boil-and-spin	Boil-and-spin
Detection method	Real-time turbidimetry	Real-time turbidimetry	End-point fluorescence
To diagnose HBV DNA level ≥2 000 IU/mL			
AUROC (95% CI)	0.86 (0.82-0.89)	0.80 (0.77-0.82)	N/A
Optimal cutoff	25.3 minutes	60.0 minutes	
Sensitivity, % (95% CI)	65.5 (58.5-72.1)	59.4 (54.0-64.5)	
Specificity, % (95% CI)	89.5 (84.4-93.4)	100 (98.2-100)	
To diagnose HBV DNA level ≥20 000 IU/mL			
AUROC (95% CI)	0.95 (0.93-0.97)	0.85 (0.82-0.87)	N/A
Optimal cutoff	23.3 minutes	60.0 minutes	
Sensitivity, % (95% CI)	91.1 (82.6-96.4)	69.7 (64.1-74.8)	
Specificity, % (95% CI)	86.0 (81.7-89.6)	100 (98.5-100)	
To diagnose HBV DNA level ≥200 000 IU/mL			
AUROC (95% CI)	0.98 (0.97-0.99)	0.98 (0.97-0.99)	0.95 (0.93-0.97)
Optimal cutoff	20.1 minutes	40.0 minutes	40.0 minutes
Sensitivity, % (95% CI)	98.0 (89.6-100)	94.0 (88.9-97.2)	98.7 (95.3-99.8)
Specificity, % (95% CI)	94.6 (91.6-96.7)	93.5 (90.6-95.7)	91.5 (88.3-94.0)

N/A, not applicable

Table 3. Operational characteristics of two HBV-LAMP methods according to the WHO ASSURED criteria for a point-of-care test in a resource-limited settings [25]

	Bead-based extraction & real-time turbidimetry	**Boil-and-spin extraction & end-point fluorescence**
Affordable by those at risk of infection	(+) Reagent cost: US$ 14 Instrument cost: US$ 18,000	(+++) Reagent cost: US$ 8 Instrument cost: US$ 2,750
Sensitive (few false-negatives)	(+++) High clinical sensitivity to diagnose the two viral load thresholds defined by the WHO (≥20,000 IU/mL to treat chronic hepatitis B & ≥200,000 IU/mL to prevent mother-to-child transmission)	(+) High clinical sensitivity to diagnose only one viral load threshold (≥200,000 IU/mL to prevent mother-to-child transmission)
Specific (few false-positives)	(+++)	(+++)
User-friendly (simple to perform and requiring minimal training)	Field study ongoing	Field study ongoing
Rapid (to enable treatment at first visit) and **R**obust (does not require refrigerated storage)	(+) 95 min (30 min for extraction & 60 min for amplification & 5 min for detection) (-) Reagent for bead-based extraction requires refrigerated storage	(+++) 60 min (15 min for extraction & 40 min for amplification & 5 min for detection) (+++) No cold chain requirement
Equipment-free	(-) Water bath Realtime turbidimetry	(-) Water bath Centrifuge Incubator with UV light
Delivered to those who need it	Field study ongoing	Field study ongoing

FIGURE LEGENDS

Figure 1. Correlation between HBV-LAMP index (60 min / time-to-result min) and HBV DNA levels quantified by PCR

1A. Using bead-based extraction in France (n = 282)*†

1B. Using bead-based extraction in France, by viral genotype (n = 282)*

1C. Using boil-and-spin extraction in Senegal (n = 209)*

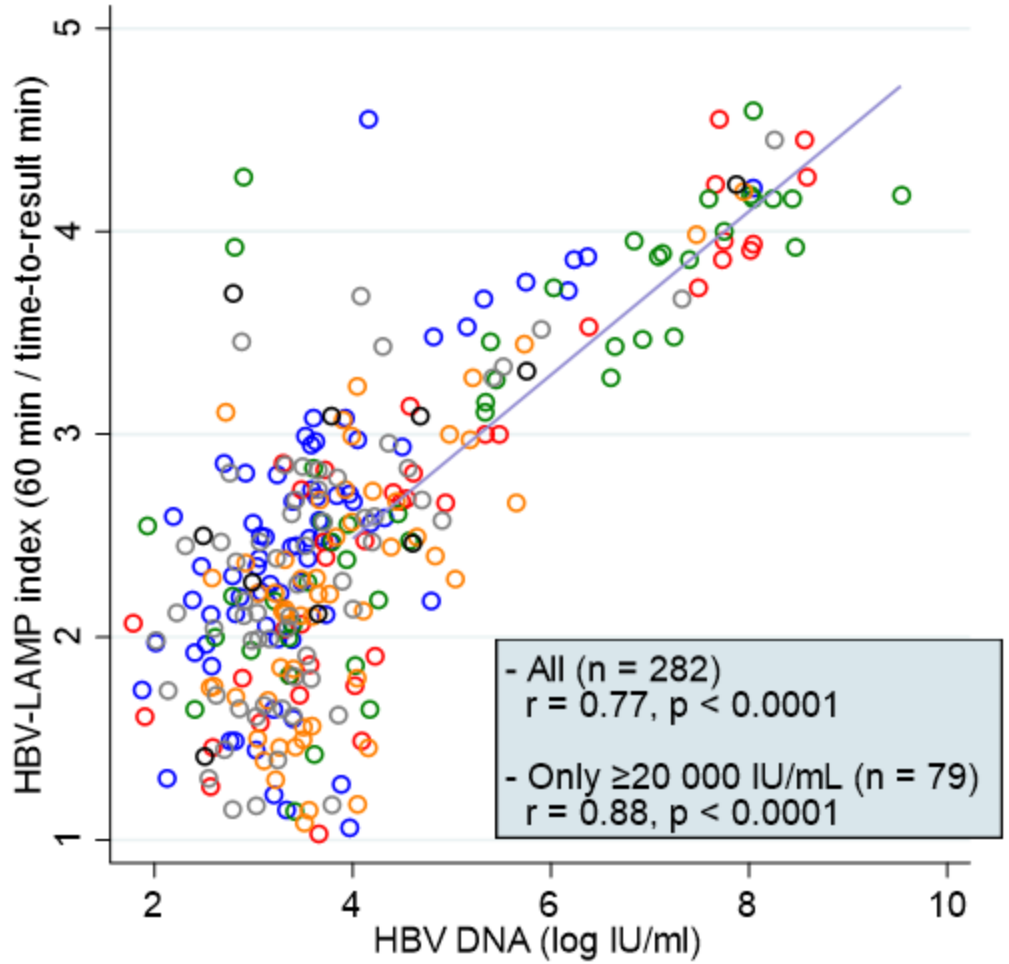
Figure 2. Receiver operating characteristic (ROC) curves to indicate elevated serum HBV DNA levels by different HBV genotypes (n=400)

2A. To diagnose HBV DNA level ≥20,000 IU/ml

2B. To diagnose HBV DNA level ≥200,000 IU/ml

Figure 1. Correlation between HBV-LAMP index (60 min / time-to-result min) and HBV DNA levels quantified by PCR

1A. Using bead-based extraction in France (n = 282)*†

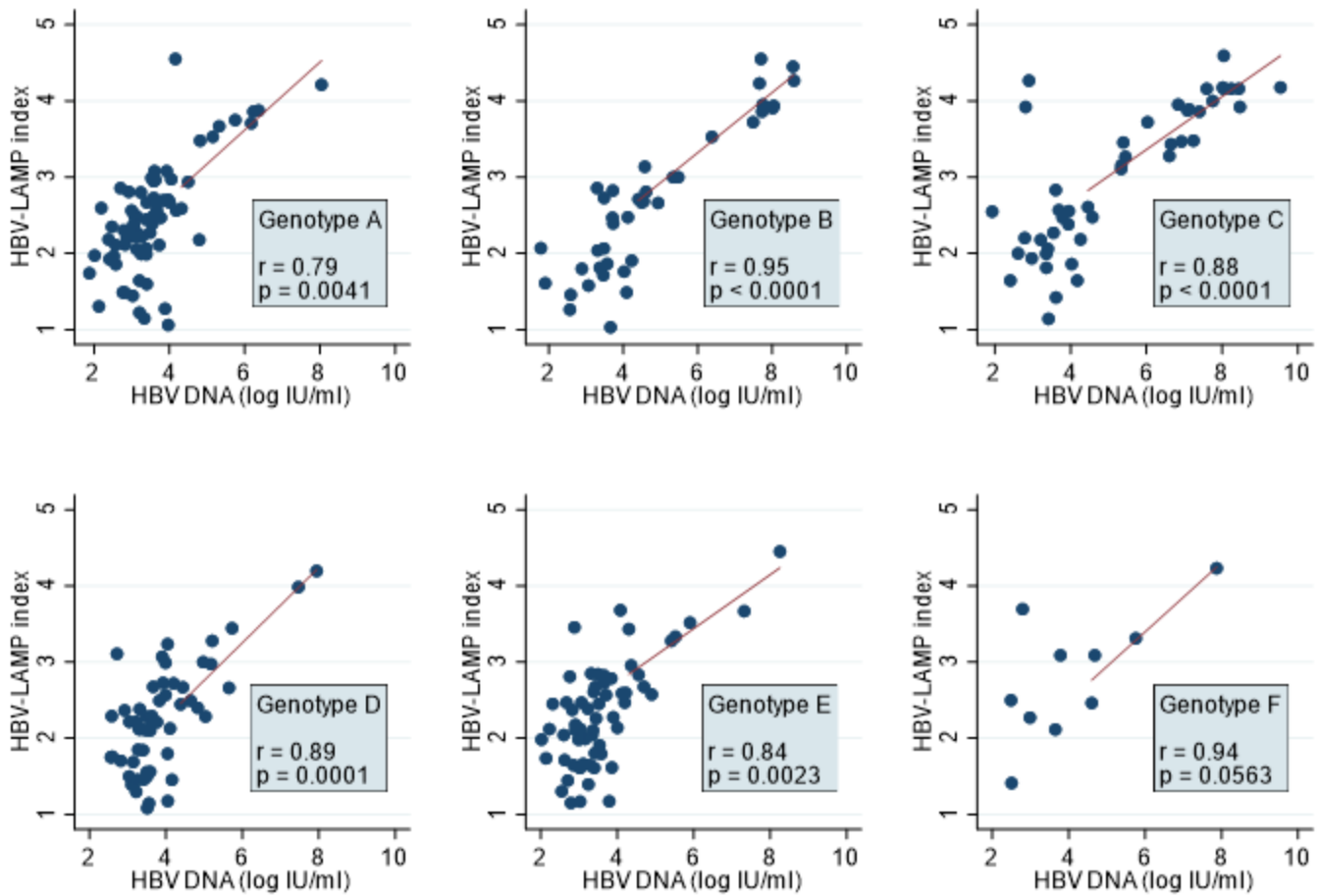


* These analyses included only the samples detected using HBV-LAMP. The regression line was drawn in a range of HBV DNA levels ≥20,000 IU/mL.

† The color of circle indicates HBV genotypes.
Genotype 1: blue
Genotype 2: red
Genotype 3: green
Genotype 4: orange
Genotype 5: gray
Genotype 6: black

Figure 1. Correlation between HBV-LAMP index (60 min / time-to-result min) and HBV DNA levels quantified by PCR

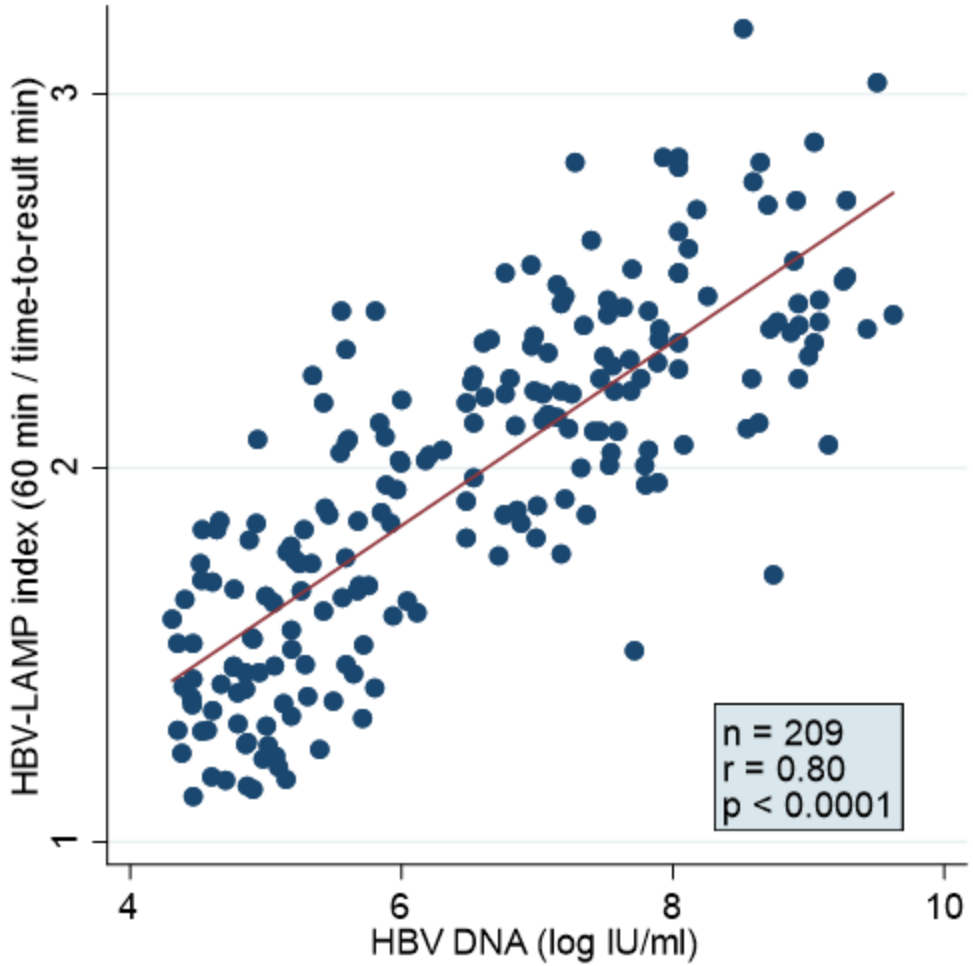
1B. Using bead-based extraction in France, by viral genotype (n = 282)*



* These analyses included only the samples detected using HBV-LAMP. The regression line was drawn in a range of HBV DNA levels ≥20,000 IU/mL.

Figure 1. Correlation between HBV-LAMP index (60 min / time-to-result min) and HBV DNA levels quantified by PCR

1C. Using boil-and-spin extraction in Senegal (n = 209)*



* These analyses included only the samples detected using HBV-LAMP. The regression line was drawn in a range of HBV DNA levels ≥20,000 IU/mL.

Figure 2. Receiver operating characteristic (ROC) curves to indicate elevated serum HBV DNA levels by different HBV genotypes (n=400)

2A. To diagnose HBV DNA level ≥20,000 IU/ml

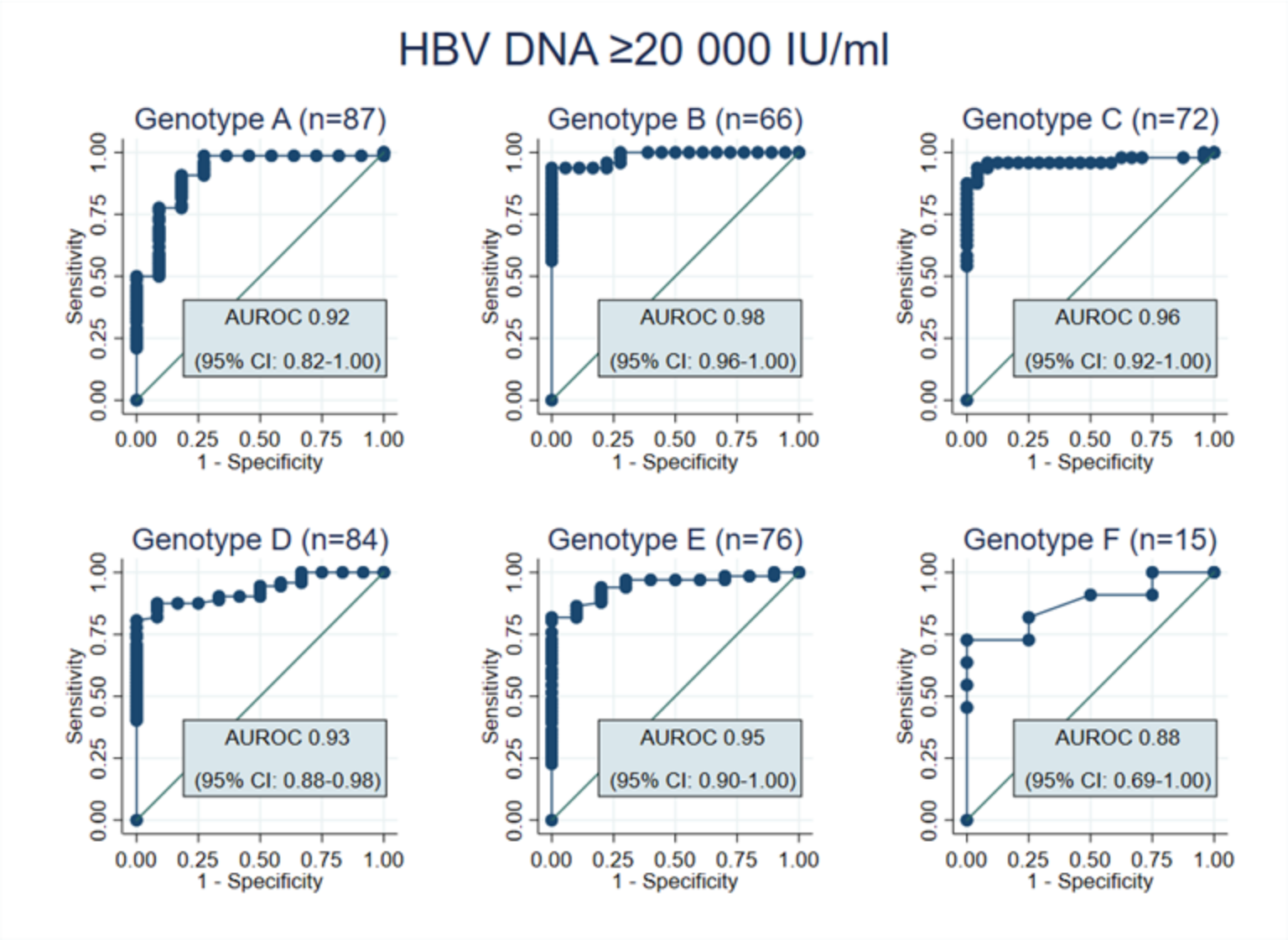
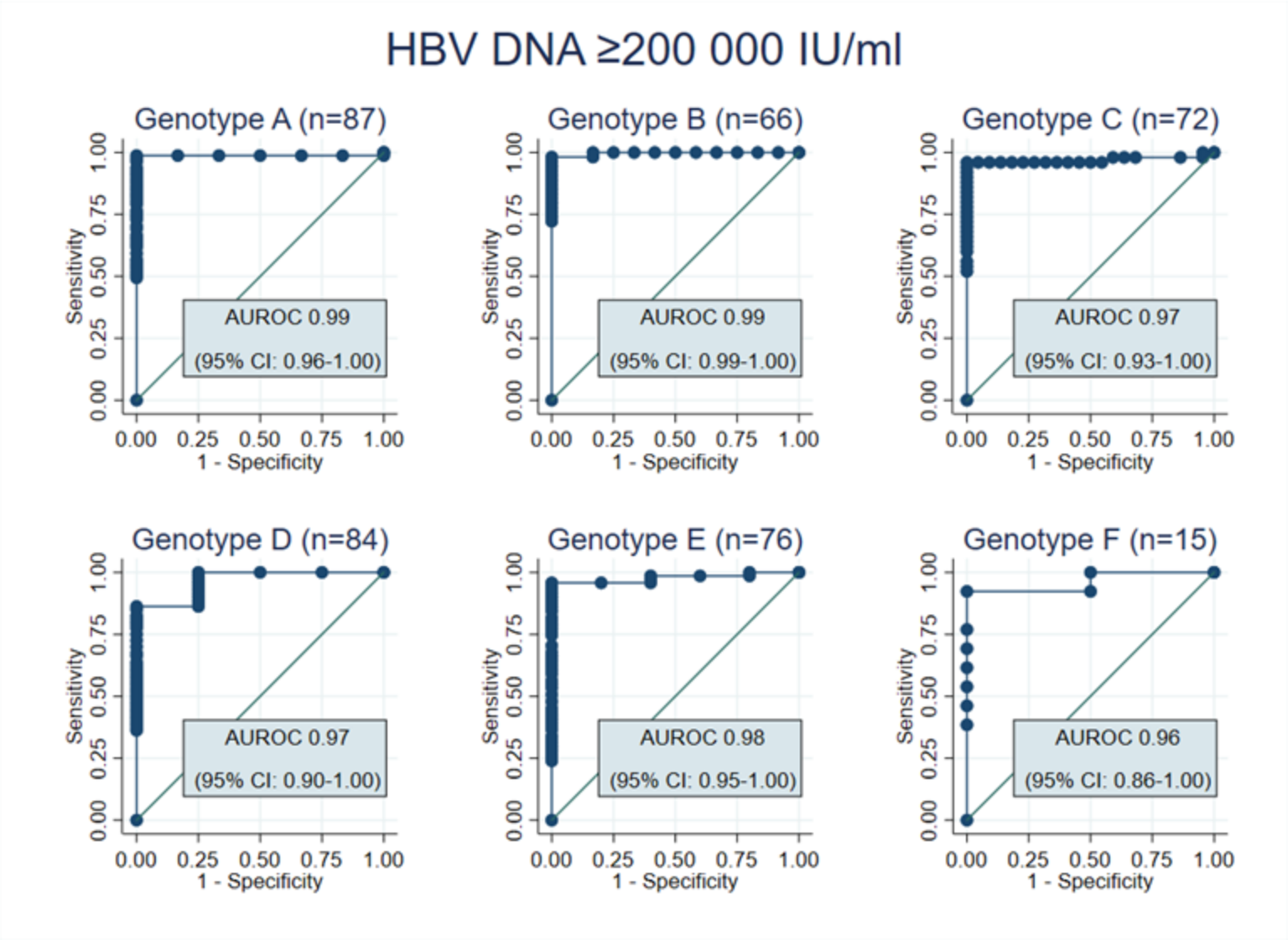


Figure 2. Receiver operating characteristic (ROC) curves to indicate elevated serum HBV DNA levels by different HBV genotypes (n=400)

2B. To diagnose HBV DNA level ≥200,000 IU/ml