Rapid point-of-care test for hepatitis B core-related antigen to diagnose high viral load in resource-limited settings

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Rapid Point-of-Care Test for Hepatitis B Core-Related Antigen to Diagnose High Viral Load in Resource-Limited Settings

Global elimination of hepatitis B virus (HBV) requires scale-up of testing and treatment services in low-income and middle-income countries (LMICs). Following the screening for hepatitis B surface antigen (HBsAg), quantification of serum HBV DNA is invariably required to identify a subset of HBV-infected individuals who should immediately start antiviral therapy. Anti-HBV treatment is recommended for patients with chronic HBV infection (CHB) who have high HBV DNA levels ($\geq$2000 or $\geq$20,000 IU/mL) in the presence of liver fibrosis or inflamation.[1–3] To prevent mother-to-child transmission, antiviral prophylaxis is recommended for HBV-infected pregnant women with high viremia ($\geq$200,000 IU/mL).[1,2,4] Despite its central role in the clinical management of CHB, >95% of HBV-infected people live in LMICs where HBV DNA quantification is not easily accessible.

Hepatitis B core-related antigen (HBcrAg) may be an effective alternative to quantifying HBV DNA in treatment-naive CHB patients because of its close correlation with intrahepatic covalently closed circular DNA levels[5] and serum HBV DNA levels.[6] Indeed, maternal HBcrAg levels during pregnancy accurately predicted mother-to-child transmission events despite infant immunization.[7] However, its measurement requires chemiluminescence enzyme immunoassay (CLEIA), which remains unavailable in decentralized settings in LMICs. We, therefore, developed a rapid diagnostic test based on immunochromatography enabling to detect HBcrAg (HBcrAg-RDT) at the point of care and performed the analytical/clinical validation.

HBcrAg-RDT is a lateral flow assay using anti-HBcrAg monoclonal antibodies. A test kit contains a single-use disposable plastic cassette, pretreatment solution, neutralizing solution, squeeze tube, and applicator tip (Supplementary Figure 1). No special equipment is required. First, 50 μL of the sample was mixed with 150 μL of pretreatment solution containing acid, detergents, and reducing agent. After leaving for 10 minutes at room temperature, 50 μL of neutralizing solution, containing buffer, detergent, and base, was added. Subsequently, 20 μL of the pretreated sample was applied to the sample window of the cassette. On the sample pad, HBcrAg in the pretreated sample was bound to primary antibodies conjugated with alkaline phosphatase (ALP). These antigen-antibody complexes then migrated to the interpretation window where they were captured by secondary anti-HBcrAg antibodies immobilized on the test line, forming sandwich immune complexes. Meanwhile, free ALP-labeled antibodies further migrated to the control line, where they were captured by anti-ALP antibodies immobilized on this line. Whenever ALP was captured on the test line or control line, a blue color appeared with a chromogenic substrate that had migrated with a developing solution precontained in the cassette.

Analytical validation was performed at Fujirebio Hachioji Laboratory, Japan, using 3 types of reference materials (serum, plasma, and whole blood) in which HBcrAg levels were quantified by a reference CLEIA (LUMIPULSE G1200, Fujirebio, Tokyo, Japan). Using serially diluted concentrations of HBcrAg-CLEIA, the limit of detection was defined as the lowest concentration detected in $\geq$95% of 20 replicates. Interoperator and intraoperator reproducibility were evaluated by 3 operators over 1 day and by a single operator over 3 days, respectively, using 3 types of sera: high-positive (5.0 $\log_{10}$ U/mL), low-positive (4.0 $\log_{10}$ U/mL), and negative. Analytical specificity was evaluated by spiking low-positive sera (4.2 $\log_{10}$ U/mL) and negative control with potentially interfering substances (Interference Check A plus/RF plus, Sysmex, Kobe, Japan). Operating temperature was evaluated at 18°C, 20°C, 25°C, 30°C, 37°C, and 39°C using low-positive and negative sera. Stability of HBcrAg after sample pretreatment was assessed by leaving pretreated sera, plasma, and whole blood with 2 different HBcrAg-CLEIA levels (low-positive [4.2 $\log_{10}$ U/mL] or negative) at a constant temperature of 4°C or 25°C for 0, 1, 3, and 7 days. Effects of freeze/thaw cycles were evaluated using 5 freshly collected positive sera (range, 3.6–6.9 $\log_{10}$ U/mL) at 4 different time points: before freezing, and after 1, 2, and 3 freeze/thaw cycles (-60°C).

We previously reported the performance of HBcrAg-CLEIA in 284 treatment-naive CHB patients from the PROLIFICA cohort in The Gambia, West Africa.[8] Using the exact same cohort, we evaluated this time the performance of HBcrAg-RDT to diagnose 3 clinically

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important HBV DNA thresholds (≥2000, ≥20,000, and ≥200,000 IU/mL) and 3 corresponding HBcrAg levels (≥3.6, ≥4.5, and ≥5.3 $\log_{10}$ U/mL).[6] Additionally, we assessed 75 HBsAg-negative Gambians (hepatitis C virus–infected, 12%; HIV-infected, 7%) as HBV-negative control (13 with hepatocellular carcinoma, 24 with cirrhosis, 38 without liver disease). Hepatitis B e antigen (HBeAg) and HBV DNA were tested locally by enzyme immunoassay (EIA; ETI-EBK Plus, DiaSorin, Saluggia, Italy) and in-house real-time polymerase chain reaction (limit of detection, 50 IU/mL), respectively. Stored sera (-80°C) were sent to Toshiba General Hospital, Japan. HBcrAg-CLEIA and HBcrAg-RDT were tested by staff masked to the clinical and virologic data.

We also evaluated the accuracy of simplified HBV DNA–free algorithms using HBcrAg-RDT to identify HBV-infected individuals eligible for antiviral therapy. As a reference method, we referred to each of the eligibility criteria, established by the American Association for the Study of Liver Diseases (AASLD), European Association for the Study of the Liver (EASL), and Asian Pacific Association for the Study of the Liver (APASL) guidelines. All of these considered HBV DNA levels, HBeAg, alanine transaminase (ALT), and liver histopathology or FibroScan.[1–3] We developed 3 simplified algorithms. Model 1 was the same as the reference criteria (AASLD, EASL, and APASL) except that HBV DNA was replaced with HBcrAg-RDT (positive HBcrAg-RDT indicating viremia of ≥2000 or ≥20,000 IU/mL) and histopathology was replaced with FibroScan. Model 2 consisted only of HBcrAg-RDT and ALT, a simple scoring system similar to the TREAT-B, composed of HBeAg and ALT.[9] The total score was obtained by adding HBcrAg-RDT score, negative (±0) or positive (+1), and ALT score, <20 IU/L (±0), 20–39 (+1), 40–79 (+2), or ≥80 (+3). The total score ≥2 indicated the eligibility.[9] Model 3 only used HBcrAg-RDT: positive indicated eligible and negative indicated ineligible. Statistics were performed using Stata version 16.0 (Stata Corporation, College Station, TX). The study was approved by the Gambian Government/MRC Joint Ethics Committee.

Using HBcrAg-CLEIA as a reference, the limit of detection of HBcrAg-RDT was 4.3 $\log_{10}$ U/mL for serum and plasma and 4.9 $\log_{10}$ U/mL for whole blood (Supplementary Table 1). Complete agreement was observed for both the interoperator and intraoperator reproducibility. HBcrAg-RDT results were not affected by the addition of hemolysis (470 mg/dL), lipemia (1630 mg/dL), unconjugated bilirubin (19.9 mg/dL), conjugated bilirubin (20.1 mg/dL), or rheumatoid factor (50 mg/dL). The operating temperature was confirmed to be at least 18°C–39°C. HBcrAg stability in pretreated serum, plasma, and whole blood was preserved for at least 7 days at both storage temperatures (4°C and 25°C). HBcrAg-RDT results in the 5 freshly collected sera remained unchanged after up to 3 freeze/thaw cycles.

In 75 HBsAg-negative Gambians, none tested positive for HBcrAg-RDT, giving a specificity of 100% (95% confidence interval, 95.2–100) in the HBsAg-negative population. As reported in our previous article,[8] in 284 HBsAg-positive Gambians (median age, 36 years [interquartile range, 30–45]; male sex, 66%; genotype A/E, 16/84%; HBeAg-positive, 13%), the distribution of HBV DNA levels was undetectable (42%), 50–1999 IU/mL (35%), 2000–199,999 IU/mL (11%), and ≥200,000 IU/mL (12%). The proportions eligible for antiviral therapy were 21% (AASLD), 20% (EASL), and 22% (APASL). HBcrAg-CLEIA was detected in 53%. HBcrAg-RDT was positive in 23%. The sensitivity and specificity of HBcrAg-RDT to diagnose clinically important HBcrAg-CLEIA levels were 73.3% and 100% for ≥3.6 $\log_{10}$ U/mL, 95.2% and 97.3% for ≥4.5 $\log_{10}$ U/mL, and 100% and 92.8% for ≥5.3 $\log_{10}$ U/mL, respectively. The sensitivity and specificity of HBcrAg-RDT to diagnose clinically important HBV DNA levels were 72.7% and 91.7% for ≥2000 IU/mL, 86.7% and 88.7% for ≥20,000 IU/mL, and 91.4% and 86.3% for ≥200,000 IU/mL, respectively (Table 1). The sensitivity of HBcrAg-RDT was higher than HBeAg-EIA and comparable with HBcrAg-CLEIA (Table 1). In women of reproductive age (n = 67), the sensitivity and specificity of HBcrAg-RDT to indicate viral loads ≥200,000 IU/mL were 100% and 87.5%, respectively. The performance of HBcrAg-RDT did not vary across the viral genotypes.

For the simplified algorithms using HBcrAg-RDT to indicate treatment eligibility (Table 1), the sensitivity and specificity of Model 1 (HBcrAg-RDT/ALT/FibroScan) were 96.6% and 83.2% for EASL, 93.2% and 86.7% for AASLD, and 90.5% and 96.8% for APASL, respectively. Model 2 (HBcrAg-RDT/ALT) performed less well than Model 1, but was comparable with TREAT-B (HBeAg-EIA/ALT).

We developed a novel point-of-care test detecting high HBcrAg levels and high viremia in serum, plasma, or whole blood. Its low production cost (US$<5), simple specimen preparation, no requirement for equipment/cold chains, operating temperature (39°C), and rapid turnaround time (45 minutes) all favor its use at the point of care in LMICs. The World Health Organization recently recommends HBeAg testing as an alternative to HBV DNA to determine eligibility for peripartum antiviral prophylaxis.[4] This recommendation was based on a meta-analysis of HBeAg performance showing pooled sensitivity of 88.2% and specificity of 92.6% for indicating viral loads ≥200,000 IU/mL.[10] These estimates, however, were mostly based on studies using laboratory-based immunoassays (EIA/CLEIA). In studies using RDTs, the pooled sensitivity was only 70.1%.[10] Compared with these, the performance of HBcrAg-RDT observed in Gambian women of childbearing age (sensitivity 100%, specificity 87.5%) is highly promising. Our study also suggested the usefulness of several HBV

Table 1. Sensitivity and Specificity of 3 Antigen Tests (HBcrAg-RDT, HBcrAg-CLEIA, and HBeAg-EIA) to Discriminate Clinically Important HBV DNA Levels and 4 Simplified Algorithms (Models 1–3 based on HBcrAg-RDT and TREAT-B) to Select Patients Eligible for Antiviral Therapy in HBsAg-Positive Gambian Patients

	All patients (n = 284)		Subgroup analyses: Women of reproductive age (n = 67)		Genotype A (n = 38)		Genotype E (n = 198)	
	Sen	Spe	Sen	Spe	Sen	Spe	Sen	Spe
To diagnose clinically important high HBV DNA levels								
Viral load ≥2000 IU/mL								
HBcrAg-RDT	72.7%	91.7%	87.5%	93.2%	72.7%	88.9%	74.1%	91.0%
HBcrAg-CLEIA[a]	83.3%	83.9%	87.5%	81.4%	90.9%	70.4%	83.3%	84.7%
HBeAg-EIA	47.7%	97.6%	50.0%	96.5%	50.0%	96.3%	48.1%	97.2%
Viral load ≥20,000 IU/mL								
HBcrAg-RDT	86.7%	88.7%	100%	90.3%	87.5%	86.7%	88.9%	87.0%
HBcrAg-CLEIA[b]	88.9%	90.4%	100%	91.9%	87.5%	86.7%	91.7%	89.5%
HBeAg-EIA	61.4%	96.0%	60.0%	95.0%	71.4%	96.7%	61.1%	95.0%
Viral load ≥200,000 IU/mL								
HBcrAg-RDT	91.4%	86.3%	100%	87.5%	83.3%	81.3%	93.1%	84.6%
HBcrAg-CLEIA[c]	91.4%	93.2%	100%	93.8%	83.3%	84.4%	93.1%	92.9%
HBeAg-EIA	70.6%	94.9%	66.7%	93.5%	100%	96.9%	65.5%	93.4%
To select HBV-infected patients eligible for antiviral therapy								
EASL 2017								
Model 1 (HBcrAg-RDT, ALT, FS)[d]	96.6%	83.2%	100%	88.3%	90.9%	92.6%	97.7%	84.5%
Model 2 (HBcrAg-RDT, ALT)[e]	87.3%	78.6%	83.3%	91.5%	80.0%	77.8%	87.8%	80.3%
Model 3 (HBcrAg-RDT only)	72.4%	89.4%	85.7%	91.7%	54.5%	81.5%	81.4%	88.4%
TREAT-B (HBeAg-EIA, ALT)[e]	81.5%	82.4%	83.3%	89.5%	66.7%	81.5%	85.4%	83.3%
AASLD 2018								
Model 1 (HBcrAg-RDT, ALT, FS)[d]	93.2%	86.7%	100%	95.0%	84.6%	100%	95.2%	87.8%
Model 2 (HBcrAg-RDT, ALT)[e]	87.5%	79.0%	83.3%	91.5%	83.3%	84.0%	87.5%	79.7%
Model 3 (HBcrAg-RDT only)	69.5%	88.9%	85.7%	91.7%	61.5%	88.0%	76.2%	86.5%
TREAT-B (HBeAg-EIA, ALT)[e]	81.8%	82.8%	83.3%	89.5%	72.7%	88.0%	85.0%	82.8%
APASL 2015								
Model 1 (HBcrAg-RDT, ALT, FS)[d]	90.5%	96.8%	100%	100%	62.5%	96.7%	93.3%	96.7%
Model 2 (HBcrAg-RDT, ALT)[e]	98.4%	83.6%	100%	90.2%	87.5%	75.9%	100%	84.7%
Model 3 (HBcrAg-RDT only)	63.5%	88.2%	100%	90.3%	62.5%	80.0%	77.8%	88.2%
TREAT-B (HBeAg-EIA, ALT)[e]	96.6%	87.9%	100%	88.1%	85.7%	82.8%	97.7%	87.8%

AASLD, American Association for the Study of Liver Diseases; ALT, alanine aminotransferase; APASL, Asian Pacific Association for the Study of the Liver; EASL, European Association for the Study of the Liver; FS, FibroScan; HBcrAg-CLEIA, hepatitis B core-related antigen by chemiluminescence enzyme immunoassay; HBcrAg-RDT, hepatitis B core-related antigen by rapid diagnostic test; HBeAg-EIA, hepatitis B e antigen by enzyme immunoassay; HBV, hepatitis B virus; Sen, sensitivity; Spe, specificity; TREAT-B, Treatment Eligibility in Africa for HBV.

[a]Cutoff level of 3.6 $\log_{10}$ U/mL was applied.

[b]Cutoff level of 4.5 $\log_{10}$ U/mL was applied.

[c]Cutoff level of 5.3 $\log_{10}$ U/mL was applied.

[d]Significant fibrosis (Metavir ≥F2) and cirrhosis (F4) were defined as liver stiffness ≥7.9 and ≥9.5 kPa, respectively (Lemoine M, et al. Gut 2016).

[e]Model 2, and TREAT-B, applied the cutoff of ≥2 points to indicate treatment eligibility.

DNA–free algorithms with HBcrAg-RDT to determine treatment eligibility. Importantly, Model 2 (HBcrAg-RDT/ALT), which performs as well as TREAT-B (HBeAg-EIA/ALT), may provide additional benefits by enabling clinical management at primary care facilities without laboratory-based immunoassays.

As limitations, HBcrAg-RDT may be useful for initiating antiviral therapy, but not for monitoring treatment response. HBcrAg-RDT was only validated using stored sera from a single cohort. Further evaluation is warranted in other cohorts using different sample. In conclusion, HBcrAg-RDT is a simple, affordable, and reliable tool to identify highly viremic patients in LMICs.

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Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Clinical Gastroenterology and Hepatology* at www.cghjournal.org, and at https://doi.org/10.1016/j.cgh.2022.05.026.

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Conflicts of interest
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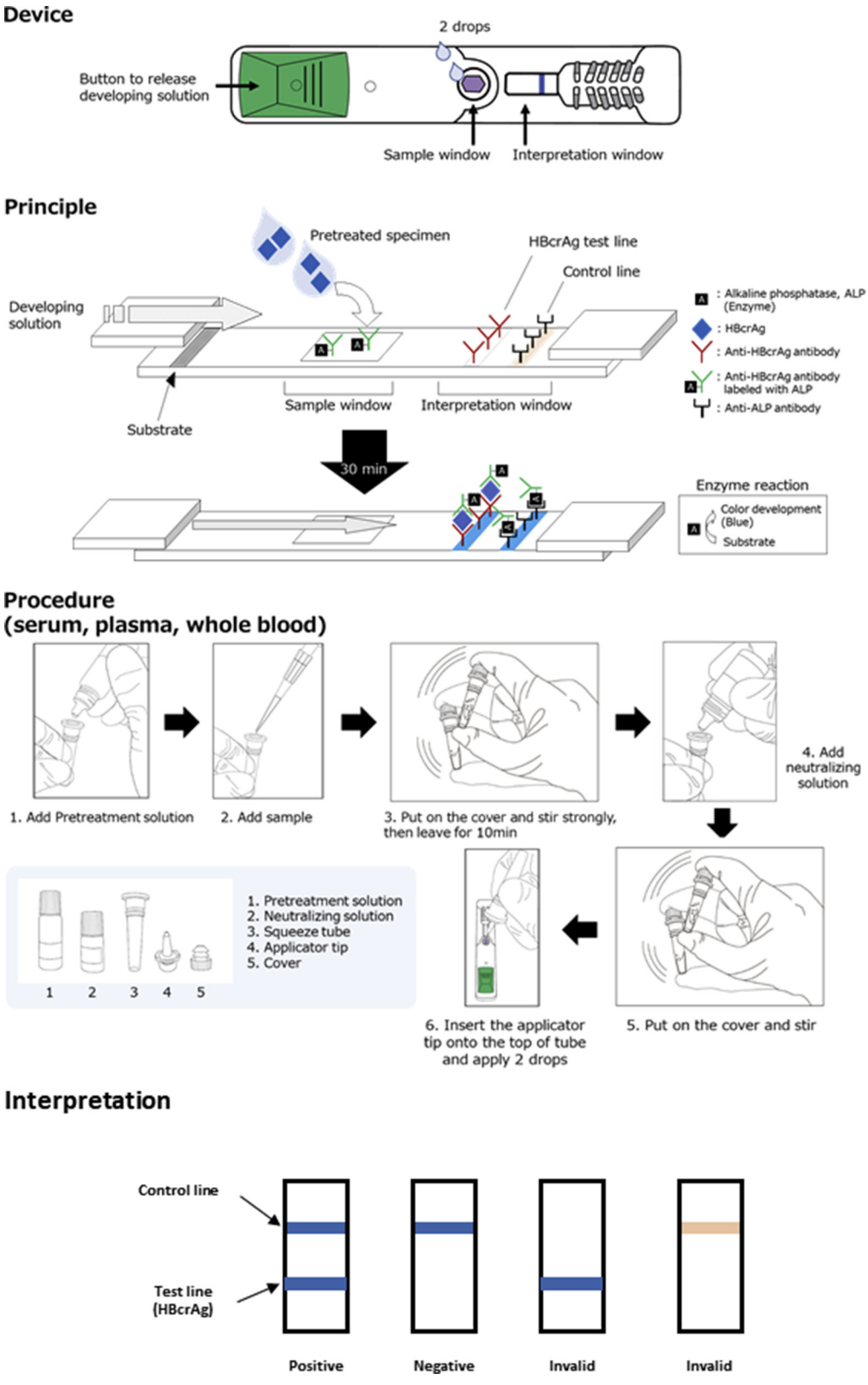

Supplementary Figure 1. Device, principle, procedure, and interpretation.

Supplementary Table 1. Detection Limit of HBcrAg-RDT Using Serum, Plasma, and Whole Blood

HBcrAg levels (defined by the reference CLEIA)	Detected by HBcrAg-RDT, %		
	Serum	Plasma	Whole blood
5.2 $\log_{10}$ U/mL			100
4.9 $\log_{10}$ U/mL			100[a]
4.6 $\log_{10}$ U/mL	100	100	40
4.3 $\log_{10}$ U/mL	95[a]	100[a]	0
4.0 $\log_{10}$ U/mL	25	20	
3.7 $\log_{10}$ U/mL	0	0	

CLEIA, chemiluminescence enzyme immunoassay; HBcrAg-RDT, hepatitis B core-related antigen by rapid diagnostic test.
[a]The limit of detection was defined as the lowest concentration that was detected in $\geq$95% of 20 replicates (4.3 $\log_{10}$ U/mL for serum and plasma and 4.9 $\log_{10}$ U/mL for whole blood).