Nanoparticle-Based Lateral Flow Biosensors Integrated With Loop-Mediated Isothermal Amplification for the Rapid and Visual Diagnosis of Hepatitis B Virus in Clinical Application

Xu Chen1,2*, Shoshi Wang2†, Yan Tan3†, Junfei Huang4, Xingui Yang4 and Shijun Li4*

1The Second Clinical College, Guizhou University of Traditional Chinese Medicine, Guiyang, China, 2Central Laboratory of the Second Affiliated Hospital, Guizhou University of Traditional Chinese Medicine, Guiyang, China, 3Guizhou Provincial Center for Clinical Laboratory, Guiyang, China, 4Laboratory of Bacterial Infectious Disease of Experimental Centre, Guizhou Provincial Centre for Disease Control and Prevention, Guiyang, China

Hepatitis B virus (HBV) infection remains one of the major public health issues worldwide. Developing a rapid, sensitive, specific, easy-to-operate, and cost-saving approach for the diagnosis of HBV is essential for its therapy and prevention. Here, we first devised a novel approach, termed “loop-mediated isothermal amplification integrated with a nanoparticle-based lateral flow biosensor (LAMP-LFB),” for the detection of HBV in clinical application. The results indicated that a set of LAMP primers based on the S gene were valid for the establishment of HBV-LAMP-LFB. The optimal HBV-LAMP can be carried out at a constant temperature of 65°C for 40 min. The whole detection process, including HBV genomic DNA preparation (~10 min), LAMP (40 min), and LFB reading (within 2 min), can be accomplished within 60 min. The limit of detection of the HBV-LAMP-LFB assay was 7.5 IU per test. The specificity of this assay was one hundred percent, and there was no cross-reactivity with other pathogens. Hence, these results indicated that the HBV-LAMP-LFB assay established in the current study is a sensitive, rapid, specific, visual, simple, and cost-saving method for the screening of HBV agents. More importantly, the HBV-LAMP-LFB has remarkable potential to develop a point-of-care testing in clinical application, especially in resource-scarce regions.

Keywords: hepatitis B virus, loop-mediated isothermal amplification, lateral flow biosensor, rapid of detection, limit of detection

Abbreviations: BC, blank control; CL, control line; DW, distilled water; FAM, 6-carboxy-fluorescein; GZCDC, Guizhou Provincial Center for Disease Control and Prevention; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; mer, monomeric unit; MG, malachite green; NC, negative control; NCBI, National Centre for Biotechnology Information; nt, nucleotide; POCT, point-of-care testing; second GZUTCM, Second Affiliated Hospital, Guizhou University of Traditional Chinese Medicine; TL, test line; WHO, World Health Organization.
INTRODUCTION

Hepatitis B virus (HBV) is a life-threatening pathogen that can cause acute and chronic infection and puts people at high risk of death from cirrhosis and liver cancer (Bollinger et al., 2020; Liaw, 2019). Generally, HBV spreads through blood or blood-related products from HBV-infected patients. Most people do not experience any symptoms when newly infected with HBV. When the disease progresses, some people show jaundice, extreme fatigue, nausea, acute liver failure, hepatitis, liver cirrhosis, and hepatocellular carcinoma (Blackard and Sherman, 2018; Revill et al., 2019). It is hard to detect HBV-infected cases, owing to the absence of hepatitis B surface antigen and a low amount of HBV-DNA in blood at the early stage of HBV infection (Liang, 2009; Tong and Revill, 2016). The problem mainly compounded by the lack of advanced diagnostic methods and medical services for donor screening or routine testing of patients. Hence, developing a rapid, sensitive, specific, easy-to-use assay is critical for addressing the burden of HBV infection.

Traditionally, enzyme-linked immunosorbent assay (ELISA) and real-time quantitative polymerase chain reaction (qPCR) have been widely used to probe HBV based on HBsAg and HBV-DNA (Mallika, 2015; Villar et al., 2015). However, the sensitivity of the ELISA was remarkably low, especially in the early stage of HBV infection (Sara, 2011). In recent decades, nucleic acid amplification technologies (NAATs), including polymerase chain reaction (PCR) and real-time quantitative PCR were considered as good alternative methods for the diagnosis of HBV infection owing to being more sensitive than ELISA (Jain et al., 2018; Coffin et al., 2019). Nevertheless, these methods need special laboratory layout and expensive instruments that may not be readily available in many resource-shortage settings. Moreover, it is time-consuming (approximately 130 min). Herein, developing a cost-effective, rapid, simple, sensitive, and specific assay for identification of HBV is necessary for the diagnosis of blood donors and HBV-infected patients.

To address the shortcomings of these approaches, loop-mediated isothermal amplification (LAMP), a rapid and simple NAAT, has been devised and gradually applied to the diagnosis of many pathogens, such as human papillomavirus, influenza virus, and severe acute respiratory syndrome coronavirus 2 (Zhu et al., 2020; Landaverde et al., 2020; Le Thi et al., 2020), which can robust amplify the target sequence at a fixed temperature (58–69°C) for 30–60 min. The primer set includes a pair of outer primers (F3 and B3), loop primers (LF and LB), and inner primers (FIP and BIP) (Seki et al., 2018). Then, analysis of the LAMP reactions has become another major concern. Conventionally, agarose gel electrophoresis, fluorescent agents, real-time turbidimetry, and colorimetric indicators (MG reagent) have been widely used to read out LAMP products (Romero and Cook, 2018; Kubota and Jenkins, 2015; Notomi et al., 2000). However, these methods require special facilities and reagents. To overcome these drawbacks, the gold nanoparticle-based lateral flow biosensor (LFB) with easy accessibility, good robustness, visualization, low limits of detection, and specificity features has been reported for the detection of nucleic acid and protein molecules in recent years (Quesada-González and Merkoçi, 2015; Huang et al., 2021).

In this study, the loop-mediated isothermal amplification integrated with the gold nanoparticle-based lateral flow biosensor (LAMP-LFB) was devised first for rapid, visual, simple, specific, and sensitive identification of HBV by targeting on the S gene (Nyan et al., 2014). The principle of the HBV-LAMP-LFB is illustrated in Figure 1, and its feasibility was validated with clinical samples.

MATERIALS AND METHODS

Standard Panels and Clinical Samples Preparation

HBV, hepatitis C virus (HCV), and human immunodeficiency virus (HIV) nucleic acid standard substances were obtained from the Chinese Academy of Metrology (Code No. GBW09150, GBW(E)090118, and GBW(E)0900272, respectively). 115 clinical serum specimens were collected from patients with suspected HBV infection at the Second Affiliated Hospital, Guizhou University of Traditional Chinese Medicine (Guiyang, China), during June 2019 to May 2020 and stored at −80°C until use; other non-HBV pathogens used in the current study are shown in Table 1. The genomic DNA templates were extracted using nucleic acid-releasing agents (Sansure Biotech, Changsha, China) in accordance with the manufacturer’s instructions and stored at −20°C before use. The concentrations were measured using NanoDrop ND-2000 (Beijing, China) at A260/280.

MATERIALS AND INSTRUMENTS

Microbial nucleic acid-releasing agents were obtained from Sansure Biotech (Changsha, China). The colorimetric indicator (malachite green, MG), isothermal amplification kits, and gold nanoparticle-based LFB were obtained from HuiDeXin Bio-technique (Tianjin, China). The dye streptavidin-coated gold nanoparticles (streptavidin-GNPs) were obtained from HuiDeXing Biotech Co., Ltd. (Tianjing, China). The size of GNPs is 34.46 ± 3.43 nm, and the extinction coefficient of GNPs is 6.0 × 10⁹ M⁻¹ cm⁻¹ at 506 nm. Biotin-BSA (biotinylated bovine serum albumin) and anti-FAM (rabbit anti-fluorescein antibody) were purchased from Abcam Co., Ltd. (Shanghai, China). The real-time turbidimeter (LA-500) was obtained from Eiken Chemical Co., Ltd. (Japan). The HBV real-time quantification PCR diagnosis kit was obtained from DaAn Gene Co., Ltd. (Guangzhou, China).

Construction of the Gold Nanoparticle-Based Lateral Flow Biosensor

The gold nanoparticle-based LFB (60 mm × 4 mm) used in the current study is devised and elaborated in Figure 2. In brief, the biosensor consists of four sections, including sample pad, conjugate pad, nitrocellulose (NC) membrane, and absorbent
FIGURE 1 | Outline of the HVB-LAMP-LFB workflow. The HVB-LAMP-LFB assay system contains three closely linked steps: DNA extraction (step 1), LAMP (step 2), and gold nanoparticle-based LFB readout (step 4). The whole detection process can be completed within 60 min.

TABLE 1 | Pathogens used in this study.

| No. | Pathogen | Pathogen no. (source of pathogens) | No. of strains | LAMP-LFB result |
|-----|----------|----------------------------------|----------------|-----------------|
| 1   | HBV (standard substance) | The Chinese Academy of Metrology | 1 | P |
| 2   | HBV (clinical samples) | 2nd GZUTCM | 13 | P |
| 3   | HCV (standard substance) | The Chinese Academy of Metrology | 1 | N |
| 4   | HIV (standard substance) | The Chinese Academy of Metrology | 1 | N |
| 5   | H1N1 (nucleic acid samples) | GZCDC | 1 | N |
| 6   | Influenza B | GZCDC | 1 | N |
| 7   | Respiratory syncytial virus type A | GZCDC | 1 | N |
| 5   | Human rhinovirus | GZCDC | 1 | N |
| 7   | Adenoviruses | GZCDC | 1 | N |
| 8   | Mycobacterium tuberculosis | GZCDC | 1 | N |
| 9   | Pseudomonas aeruginosa | 2nd GZUTCM | 1 | N |
| 10  | Mycoplasma pneumoniae | 2nd GZUTCM | 1 | N |
| 11  | Streptococcus pneumoniae | 2nd GZUTCM | 1 | N |
| 12  | Mycoplasma pneumoniae M129/FH | 2nd GZUTCM | 1 | N |
| 13  | Haemophilus influenzae | ATCC49247 | 1 | N |
| 14  | Streptococcus pyogenes | 2nd GZUTCM | 1 | N |
| 15  | Staphylococcus aureus | 2nd GZUTCM | 1 | N |
| 16  | Achromobacter baumannii | 2nd GZUTCM | 1 | N |
| 17  | Leptospira interrogans | GZCDC | 1 | N |
| 18  | Shigella flexneri | 2nd GZUTCM | 1 | N |
| 19  | Bacillus cereus | GZCDC | 1 | N |
| 20  | Bordetella parapertussis | GZCDC | 1 | N |
| 21  | Bordetella pertussis | GZCDC | 1 | N |
| 22  | Enteropathogenic Escherichia coli | GZCDC | 1 | N |
| 23  | Candida glabrata | 2nd GZUTCM | 1 | N |
| 24  | Cryptococcus neoformans | ATCC14035 | 1 | N |
| 25  | Hemophilus parasinfluenzae | GZCDC | 1 | N |

*2nd GZUTCM, The Second Affiliated Hospital, Guizhou University of Traditional Chinese Medicine; GZCDC, Guizhou Provincial Center for Disease Control and Prevention; ATCC, American Type Culture Collection.

*P, Positive; N, Negative.
The detector reagents (dye streptavidin-coated gold nanoparticles (streptavidin-GNPs), 129 nm, 10 mg·ml⁻¹; 100 mM borate, pH 8.5 with 0.05% Tween 20; 0.1% BSA; and 10 mM EDTA) were laminated onto the conjugate pad. Biotin-BSA (4 mg·ml⁻¹; Abcam) and rabbit anti-FAM (0.25 mg·mL⁻¹; Abcam) were coupled onto the NC membrane for the control line (CL) and test line (TL), respectively. Each band was separated by 5 mm. In the end, the assembled biosensor was preserved in a plastic box and conserved with a silica gel desiccant at room temperature.

**Design and Synthesis of Hepatitis B Virus–Loop-Mediated Isothermal Amplification Primers**

Three pairs of HBV-LAMP special primers based on the S gene (GenBank Accession No. AB809557.1) were designed through Primer Explorer V5 and PRIMER PREMIER 5.0 software (Eiken Chemical, Japan). The HBV-LAMP primers were analyzed with the basic local alignment search tool (BLAST) for the primer sets to validate sequence specificity. The position of each primer is shown in Figure 3, and the sequences and modifications are shown in Table 2. All of the oligomers were synthesized and purified by TsingKe Biotech Co., Ltd. (Beijing, China) at an HPLC purification grade.

**The Standard Hepatitis B Virus–Loop-Mediated Isothermal Amplification Reaction**

The HBV-LAMP was carried out in a 25 μL reaction system, including 12.5 μL of 2 × reaction buffer (40 mM Tris-HCl (pH 8.8), 40 mM KCl, 16 mM MgSO₄, 20 mM (NH₄)₂SO₄, 2 M
Detection of Hepatitis B Virus–Loop-Mediated Isothermal Amplification Products

Four monitoring techniques, including 2% agarose gel electrophoresis, colorimetric indicator (MG), real-time turbidimeter, and gold nanoparticle-based LFB, were used for the detection and confirmation of the LAMP products. For positive results, the agarose gel presented ladder-like bands and the color changed from colorless to light green in the reaction system. However, there were no bands in gel electrophoresis and it was colorless in NC and BC. For real-time turbidity measurement, turbidity >0.1 is considered as the positive result. With LFB detection, two visible red lines (CL and TL) appeared simultaneously indicating a positive result, and only the CL was observed for a negative outcome.

Temperature Optimization of the Hepatitis B Virus–Loop-Mediated Isothermal Amplification Assay

Reaction temperature is of vital importance to LAMP. In this study, the reaction temperature was tested in a range from 60 to 67°C with 1°C interval for 60 min. The LAMP products were monitored using real-time turbidity (LA-500). Each temperature was tested independently at least thrice.

Sensitivity of the Hepatitis B Virus–Loop-Mediated Isothermal Amplification–Lateral Flow Biosensor Assay

To identify the limit of detection (LoD) of the HBV-LAMP-LFB assay, HBV nucleic acid standard substances (Chinese Academy of Metrology) were serially diluted 10-fold from 1.0 × 10^4 to 1.0 × 10^-1 IU. The HBV-LAMP operation is as described above, and the LAMP products were analyzed simultaneously with MG and LFB. The sensitivity of HBV-LAMP-LFB was confirmed as the last dilution of each positive test. All examinations were confirmed in triplicate.

Optimization of Amplification Time for the Hepatitis B Virus–Loop-Mediated Isothermal Amplification–Lateral Flow Biosensor Assay

In order to optimize the reaction time for preamplification of the assay, four reaction time periods ranging from 20 to 50 min with 10 min interval were tested at the optimal amplification temperature. The HBV-LAMP products were analyzed with the MG reagent and LFB. Each of the reaction time was tested independently in triplicate.

Analytical Specificity of the Hepatitis B Virus–Loop-Mediated Isothermal Amplification–Lateral Flow Biosensor Assay

The HBV nucleic acid standard substance, 13 HBV-positive clinical samples, and 25 non-HBV pathogens (Table 1) were employed for testing the specificity of HBV-LAMP-LFB. The operation was performed as described above. All of the LAMP amplicons were analyzed through the LFB. Each sample was identified at least three times.

Confirming the Feasibility of the Hepatitis B Virus–Loop-Mediated Isothermal Amplification–Lateral Flow Biosensor Assay Using Clinical Samples

In order to confirm the applicability of HBV-LAMP-LFB assay in clinical setting, 115 serum samples with suspected HBV infection were collected from the Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine. All of the clinical specimens were evaluated simultaneously using qPCR and HBV-LAMP-LFB. The qPCR diagnosis was carried out using a commercial real-time TaqMan PCR kit (DaAn Gene Co., Ltd. China), and the detection was carried out with the Applied Biosystem™ 7,500 Real-Time PCR system (Life Technologies, Singapore). The concentrations of HBV less than 30 IU will be considered as the negative result according to the manufacturer's instructions. The HBV-LAMP-LFB operation is as described above.

**TABLE 2 | The primers used in the present study.**

| Primer name | Sequence and modifications | Length | Gene |
|-------------|----------------------------|--------|------|
| F3          | 5'-ACCAGCAGCGGACATG-3'     | 17 nt  | S    |
| B3          | 5'-GCACAGATTAACTGAGCGAC-3' | 20 nt  |      |
| FIP*        | 5'-FAM-AAGGTTTCTGACAGGAACTG-3' | 45 mer |      |
| BIP         | 5'-CGGACAGGAAAACCTGAGCTTGTAATTC-AGAAACGGACTGAGGC-3' | 41 nt  |      |
| LF*         | 5'-Biotin-AACATAGAAGTTCTTGGAG-3' | 19 nt  |      |
| LB          | 5'-GGCTTTCGCAAGATTTCCA-3'  | 19 nt  |      |

**FIP*, 5'-labeled with FAM when used in the HBV-LAMP-LFB assay; LF*, 5'-labeled with biotin when used in the HBV-LAMP-LFB assay; FAM, 6-carboxy-fluorescein; nt, nucleotide; mer, monomeric unit.**
RESULTS

Overview of the Hepatitis B Virus–Loop-Mediated Isothermal Amplification–Lateral Flow Biosensor Assay System

The principle of HBV-LAMP-LFB assay is illustrated in Figure 1. In brief, the HBV genomic DNA was extracted using nucleic acid–releasing agents within 10 min, and then, preamplification was carried out at a fixed temperature (65°C) for 40 min. We modified the LAMP primers LF and FIP at 5’-end with biotin and FAM, respectively. The HBV-LAMP amplicons were labeled with biotin and FAM; finally, the LAMPs were read out with LFB (Figure 1). For biosensor detection, the dye streptavidin-coated gold nanoparticles (streptavidin-GNPs) were laminated onto the conjugate pad, by adding 1.0 µL of HBV-LAMP products and 100 µL of running buffer (100 mM PBS, pH 7.4 with 1% Tween 20) (Zhu et al., 2021) to the sample pad. The running buffer containing HBV-LAMP products were absorbed, and the detection results were read out visually on the NC membrane (red line) within 2 min. In the positive sample, the biotin/FAM-labeled HBV-LAMP amplicons were captured by anti-FAM at the TL and streptavidin-GNPs were captured by biotin-BSA at the CL. However, in negative results, only the streptavidin-GNPs were captured the biotin-BSA at the CL (Figure 2).

Confirmation and Verification of Hepatitis B Virus–Loop-Mediated Isothermal Amplification Assay

In order to identify the availability of the set of LAMP primers for the HBV assay, the LAMP reactions were performed in the presence and absence of HBV DNA (Chinese Academy of Metrology) as the template at a constant temperature of 64°C for 1 h. Then, the LAMP products were monitored simultaneously by 2% agarose gel electrophoresis, with a colorimetric indicator (MG) and LFB. The results showed that the positive outcomes appeared with the nucleic acid from HBV, but not from HCV, HIV, and BC (Figures 4A–C). Therefore, these results suggested that the set of HBV-LAMP primers for S gene designed here was valid for the development of the HBV-LAMP-LFB assay.

Optimal Reaction Temperature for Hepatitis B Virus–Loop-Mediated Isothermal Amplification Assay

To determine the optimal reaction temperature at the preamplification stage, a HBV-LAMP reaction was carried out at a range from 60 to 67°C (with 1°C interval) with 100 IU of HBV nucleic acid as the template per test. The results were monitored by real-time turbidity (LA-500). The kinetic data showed that the robust reactions of HBV-LAMP were obtained at the reaction temperature range from 65 to 67°C (Figure 5). Hence, 65°C was selected as the optimal amplification temperature for the HBV-LAMP-LFB assay.

Sensitivity of the Hepatitis B Virus–Loop-Mediated Isothermal Amplification–Lateral Flow Biosensor Assay

The sensitivity of the HBV-LAMP-LFB assay was verified with serial dilutions of the HBV DNA standard substance (ranging from $1.0 \times 10^6$ to $1.0 \times 10^{-1}$ IU) as the template, and the LAMP products were visually read out with the MG reagent and LFB,
respectively. As shown in Figures 6A,B, the LoD of the HBV-LAMP assay was 7.5 IU obtained by both detection methods.

Optimal Amplification Time for the Hepatitis B Virus–Loop-Mediated Isothermal Amplification–Lateral Flow Biosensor Assay
To further optimize the reaction time for the HBV-LAMP-LFB assay at the preamplification stage, four reaction time intervals (20, 30, 40, and 50 min) were tested at 65°C, respectively. The LAMP products were identified through MG and LFB methods, respectively. The results confirmed that the limit level of genomic DNA (7.5 IU per reaction) was tested when the reaction lasted 40 and 50 min (Figure 7). Hence, 40 min was recommended as the optimal reaction time for the preamplification stage of the HBV-LAMP assay. Hence, the whole detection procedure, including genomic DNA extraction (~10 min), preamplification (40 min), and LFB reading (~2 min), can be accomplished within 60 min.

Specificity of the Hepatitis B Virus–Loop-Mediated Isothermal Amplification–Lateral Flow Biosensor Assay
For evaluation of HBV-LAMP-LFB assay specificity, the HBV nucleic acid standard substance (Chinese Academy of Metrology), 13 HBV-positive clinical samples, and 25 non-
HBV pathogens (Table 1) were used in the current study. The process of HBV-LAMP-LFB assay was as described above. The results showed that only genomic DNA extracted from HBV samples presented positive results and there was no cross-reactivity with other pathogens (Figure 8). Hence, these results indicated that the HBV-LAMP-LFB assay can accurately differentiate an HBV agent from other microbes.

Evaluation of the Feasibility of the Hepatitis B Virus–Loop-Mediated Isothermal Amplification–Lateral Flow Biosensor Assay With Clinical Samples
To further demonstrate the feasibility of HBV-LAMP-LFB assay as a valuable method for the detection of HBV agents, 115 clinical serum samples were collected from patients and tested simultaneously using the HBV-LAMP-LFB and qPCR assay. Among them, 76 of 115 clinical samples were confirmed as positive results through qPCR (>30 IU), which have also been verified as positive results through the HBV-LAMP-LFB assay. More importantly, 2 of the 39 “negative samples” (approximately 20 IU) were tested as positive outcomes using the HBV-LAMP-LFB assay (Table 3 and Supplementary Table S1). These results suggested that the HBV-LAMP-LFB assay devised here shows more sensitivity in detecting HBV-infected patients, especially for those at the early stage of HBV infection.

DISCUSSION
HBV infection remains one of the main public health issues worldwide (Nguyen et al., 2020; Coffin et al., 2019). It can lead to a wide spectrum of liver diseases, such as acute hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Nelson et al., 2016; Loomba and Liang, 2017). Besides, HBV, HCV, and HIV co-infections become increasingly frequent owing to shared routes of transmission and resulting in serious public health burden, especially in underprivileged regions of the world (Sarmati and Malagnino, 2019; Leoni et al., 2018). Screening and diagnosis of HBV remain a considerable challenge in many resource-limited settings with cost and equipment limitations (Mallika, 2015). In these regions, screening of HBsAg in blood with ELISA is the main approach for detecting HBV. However, this assay often poses a serious threat of misdiagnosis and transmission of HBV with low sensitivity (Coffin et al., 2019). In recent years, NAATs have become the primary tests for the identification of HBV in clinical application (Zhao et al., 2005; Li et al., 2018). Nevertheless, their use in resource-limited regions is not readily affordable, owing to requiring special facilities and trained experts. Here, the novel HBV-LAMP-LFB assay, integrating the gold nanoparticle-based LFB with preamplification, was devised first for the detection of HBV agents. Achieving this assay just requires exceedingly simple facilities, such as heating block and water bath that can hold a fixed reaction temperature of 65°C for 40 min. In addition, the
The qPCR diagnosis was carried out using the commercial real-time TaqMan PCR kit (DaAn Gene Co., Ltd., China). The concentrations of HBV less than 30 IU will be considered as negative results according to the manufacturer’s instructions.

LAMP, as a rapid, sensitive, low equipment–cost assay, was devised first in 2000 by Notomi et al. (2000), and it has been extensively used to detect various pathogens (Dolka et al., 2019; Huang et al., 2017; Kanitkar et al., 2016). The isothermal amplification of the target gene is completed by using a set of four (or six) specific primers spanning six (or eight) distinct regions of the special sequence with Bst DNA polymerase at a fixed reaction temperature (between 58 and 69°C) (Notomi et al., 2000). In the current study, three pairs of HBV-LAMP primers were successfully designed and they specifically identified eight regions of the target gene S (Figure 3). The specificity of the HBV-LAMP-LFB assay was strongly verified using the nucleic acid extracted from HBV clinical samples and other various pathogens. The positive results only appeared in the positive control and HBV clinical samples and there was no cross-reactivity with other pathogens (Figure 8). Apart from its remarkable specificity, the newly devised HBV-LAMP-LFB assay was able to detect the concentration as low as 7.5 IU (Figure 6), which is sufficient for the diagnosis of HBV agents in clinical application. Moreover, 2 of the 39 clinical “negative samples” (HBV concentrations below 30 IU) were tested as positive outcomes with the HBV-LAMP-LFB. The results indicated that our HBV-LAMP-LFB assay has higher sensitivity than that of qPCR (Table 3 and Supplementary Table S1).

In the current study, the gold nanoparticle-based LFB was successfully designed and used to read out the HBV LAMP reactions. Gold nanoparticles are the most appropriate materials for making a biosensor, owing to their high adsorption, high surface-to-volume ratio, good biocompatible, and easy-to-manipulate features (Aldewachi et al., 2017; Fuentes et al., 2016; Quesada-González and Merkoçi, 2015). The LFBs have been powerfully proven and widely applied for rapid and sensitivity detection of disease, drug abuse, and food safety in resource-limited areas (Huang et al., 2019; Jiang et al., 2021; Huang et al., 2021). Here, the LFB can visually read out HBV-LAMP reactions for labeling with biotin-BSA and anti-FAM gold nanoparticle-based LFB provides an easy-to-operate detection platform, which can rapidly and visually read out the HBV-LAMP outcomes. The whole HBV-LAMP-LFB detection process, including genomic DNA extraction (approximately 10 min), preamplification (40 min), and LFB reading (approximately 2 min), can be accomplished within 60 min.

### TABLE 3 | Comparison of qPCR and LAMP-LFB methods to identify HBV in clinical samples.

| Detection method | Clinical samples (n = 115) | Time consumption |
|------------------|---------------------------|------------------|
| qPCR LAMP-LFB    |                           |                  |
| Positive         | 76 (>30 IU)               | 39 (37, <7.5 IU; 2, approximately 20 IU) |
| Negative         | 78                        | 37               |
|                  |                           | Approximately 130 min |
|                  |                           | Within 60 min      |

Note. The qPCR diagnosis was carried out using the commercial real-time TaqMan PCR kit (DaAn Gene Co., Ltd., China). The concentrations of HBV less than 30 IU will be considered as negative results according to the manufacturer’s instructions.
with the Declaration of Helsinki. Before our team obtained the samples/isolates and conducted the study, any personal identifiers of the suspected HBV-infected patients had been removed by the monitoring stations. So, the patient informed consent was waived by the Human Ethics Committee of the Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine.

**AUTHOR CONTRIBUTIONS**

XC and SL conceived and designed the study. XC, SW, and YT participated in primers design. XC, SW, YT, JH, and XY contributed to all the laboratory works. SW, YT, and JH contributed to the data collection. XC, JH, and XY performed statistical analysis. XC wrote the initial draft of the manuscript, and SL revised the manuscript. All the authors read and approved the final manuscript.

**FUNDING**

This work was supported by the Program of Scientific and Technological Project in Guizhou Province (Grant No. Qian Ke He (2020)-4Y184), the Scientific and Technological Project in Guiyang City (Grant No. Zhu Ke He (2020)-10-5), the Program of Scientific and Technological Innovation Team of Guizhou Province (Grant No. Qian Ke He Platform Talent (2018)5606), and the Public Welfare Technology Research Program in Zhejiang Province (Grant No. LGF21H190001). The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

**ACKNOWLEDGMENTS**

The authors would like to express their sincere appreciation to the medical workers in the second GZUTCM and GZCDC for their contribution to the current study.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fbioe.2021.731415/full#supplementary-material

**REFERENCES**

Aldewachi, H., Chalati, T., Woodrooﬁe, M. N., Bricklebank, N., Sharrack, B., and Gardiner, P. (2017). Gold Nanoparticle-Based Colorimetric Biosensors. *Nanoscale* 10 (1), 18–33. doi:10.1039/c7nr06367a

Blackard, J. T., and Sherman, K. E. (2018). Hepatitis B Virus (HBV) Reactivation- The Potential Role of Direct-Acting Agents for Hepatitis C Virus (HCV). *Rev. Med. Virol.* 28 (4), e1984. doi:10.1002/rmv.1984

Bollinger, R. C., Thio, C. L., Sulkowski, M. S., McKenzie-White, J., Thomas, D. L., and Flexner, C. (2020). Addressing the Global burden of Hepatitis B Virus while Developing Long-Acting Injectables for the Prevention and Treatment of HIV. *The Lancet HIV* 7 (6), e443–e448. doi:10.1016/S2352-3018(19)30342-X

Coffin, C. S., Zhou, K., and Terrault, N. A. (2019). New and Old Biomarkers for Diagnosis and Management of Chronic Hepatitis B Virus Infection. *Gastroenterology* 156 (2), 355–368. doi:10.1053/j.gastro.2018.11.037

Dolka, B., Cisek, A. A., and Szelesczuk, P. (2019). The Application of the Loop-Mediated Isothermal Amplification (LAMP) Method for Diagnosing...
