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One-step, rapid, nanoparticle-based biosensor platform for the simultaneous identification of hepatitis B virus and hepatitis C virus in clinical applications

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Abstract

Objectives Viral hepatitis caused by hepatitis B virus (HBV) and hepatitis C virus (HCV) infections remain a major global public health challenge, particularly in low- and middle-income countries. It is crucial to utilize a point-of-care (POC) testing platform that is sensitive, specific, rapid, and user-friendly for screening and diagnosis of the two infections. Here, a novel molecular diagnostic assay, integrating multiplex loop-mediated isothermal amplification with a gold nanoparticle-based lateral flow biosensor (mLAMP-AuNPs-LFB) was developed and applied for one-step, visual, rapid, sensitive, and specific identification of HBV and HCV.

Methods The AuNPs-based LFB was devised and constructed for the simultaneous detection of HBV and HCV. The HBV-LAMP and HCV-LAMP primers were designed against the *S* and 5′-untranslated region (5′-UTR) genes from the major HBV genotypes (B, C, D, B/C recombinant, and C/D recombinant) and HCV subtypes (1b, 2a, 3a, 3b, and 6a) in China, respectively. Our assay conditions, both multiplex-LAMP amplification temperature and time were optimized. The sensitivity and specificity of our assay were tested, and the feasibility of our assay was verified through clinical samples.

Results The AuNPs-based LFB used here was successfully manufactured according to our devise manual. The two unique independent primer pairs were successfully designed based on the *S* and 5′-UTR genes, respectively. The optimal mLAMP-AuNPs-LFB detection process, involving rapid nucleic acid isolation (10 min), mLAMP (63 °C for 35 min), and visual AuNPs-LFB interpretation (less than 2 min), could be completed within 50 min. The HBV&HCV-

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mLAMP-AuNPs-LFB assay can detect the target genes (HBV-*S* and HCV-5′-UTR) with as low as 20 copies of plasmid template per test, and the specificity was 100% for the experimental pathogens.

Conclusions The preliminary results manifested that our mLAMP-AuNPs-LFB assay is a valuable tool and has tremendous potential as a POC testing approach for HBV and HCV identification, especially in undeveloped regions.

Keywords Hepatitis B virus, Hepatitis C virus, Loop-mediated isothermal amplification, Biosensor, Point-of-care platform

Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the two major causes of a wide spectrum of liver diseases, such as acute hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1]. Acute HBV or HCV infection can be either asymptomatic or present with symptomatic acute hepatitis [1, 2], If left untreated, infections that persist for longer than six months are considered chronic, many chronically infected patients have mild liver disease [1, 2]. Other individuals with chronic infection develop active disease, which can progress to cirrhosis and hepatocellular carcinoma [1, 3]. Besides, individuals with dual HBV/HCV infection have an increased risk of cirrhosis and liver cancer [3]. Globally, 295.9 million people are chronically infected with HBV, and approximately 57.8 million individuals are living with chronic HCV infection, the viruses cause approximately 1.1 million deaths each year worldwide [4]. Thus, HBV and HCV infections have been recognized as major global public health concerns, especially in low- and middle-income countries [5]. Both HBV and HCV are bloodborne pathogens that are spread mainly through infected blood exposure, unprotected sexual intercourse, injectable drug use, needlestick injury, and vertical transmission [6, 7]. Most people are asymptomatic when they are newly infected with HBV or HCV, and as the disease progresses, they can become chronic virus carriers [4, 8]. In 2016, the World Health Organization (WHO) launched an ambitious strategy to eliminate viral hepatitis as a public health threat by 2030 [9]. However, the WHO stated that although some achievements had been made, most global targets had been missed by 2022 [10]. The underdiagnosis of HBV and HCV remains a critical barrier to eliminating viral hepatitis as a public health threat, nearly 80% of individuals infected with HBV or HCV remain undiagnosed [4, 5]. Traditional laboratory-based assays for the detection of HBV/HCV nucleic acid markers are well established, but they are often expensive and time-consuming and require extensive facilities and trained technicians. Hence, developing a one-step, specific, sensitive, cost-saving, and easy-to-operate point-of-care (POC) testing platform for HBV and HCV screening is fundamental for the control of further transmission.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification approach that has great promise for point-of-care nucleic acid testing and can robustly amplify target gene at a fixed temperature with *Bacillus stearothermophilus* (*Bst*) DNA polymerase and specific primer sets [11–13]. LAMP primer sets contain six primers (forward outer primer F3, reverse outer primer B3, forward inner primer FIP, reverse inner primer BIP, forward loop primer LF, and reverse loop primer LB) that specifically recognize eight regions of the target gene [13, 14]. The LAMP reaction principle has been previously described [11, 12, 15]. Notably, the LAMP method is an affordable and reliable approach for the identification of several pathogens such as human immunodeficiency virus (HIV), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and monkeypox virus [16–18].

Gold nanoparticle-based lateral flow biosensors (AuNPs-LFBs) have notable advantages as a POC testing tool because of their high sensitivity and specificity, simple test procedure, portability, one-step methodology, prolonged stability, rapid testing ability, naked-eye visual readout, and low cost [19–21]. Thus, AuNPs-LFBs have become an emerging technology in biomedicine for the rapid detection of DNA, RNA, proteins, glucose, and other biomolecules derived from various clinical samples [22, 23].

Here, for the first time,, the multiplex-LAMP integrated with an AuNPs-based LFB platform was successfully developed for one-step, rapid, specific, sensitive, and visual detection of HBV and HCV by targeting their *S* [24, 25] and 5′untranslated region (5'-UTR) [26, 27] genes, respectively[]. The two genes had no homology with other microbial genome sequences according to BLAST searches of the GenBank database. The feasibility of our novel diagnostic approach was validated with serum samples from suspected HBV/HCV infection patients.

Materials and methods

Target gene and clinical sample preparation

The full-length *S* sequences from five dominant HBV genotypes in China (B, C, D, recombinant B/C, and recombinant C/D; GenBank accession Nos. AB014366.1, AB014360.1, AB090268.1, KC774178.1, and AY800249.1) [28], and the HCV 5′-UTR target genes from five dominant subtypes in China (1b, 2a, 3a, 3b, and 6a; GenBank

accession Nos. EU781827.1, HQ639944.1, D17763.1, JQ065709.1, and AY859526.1) [29] were synthetically produced, cloned and inserted into pUC57 plasmids, the concentration of each plasmid was adjusted to 10^8 copies/mL. All the plasmids were applied to positive controls.

Participants at the Second Affiliated Hospital, Guizhou University of Traditional Chinese Medicine provided 73 serum samples for HBV and/or HCV examination between February 2023 and October 2023. The nucleic acids from each of the above samples were isolated using a TIANamp Virus DNA/RNA Fast Kit (TIANGEN BIOTECH Co., Ltd.; Beijing, China). Briefly, 200 µL of each serum sample was treated with 20 µL of protease K, 250 µL of the lysis agent RLC, and 250 µL of isopropanol and incubated at 56 °C for 5 min. Then, the nucleic acids were enriched with a centrifugal RNase-free absorption column. After washing with PWT buffer, the nucleic acids were eluted in 50 µL of RNase-free ddH_2O. The extracted nucleic acids were stored at -80 °C before use.

LAMP primers design and synthesis

The *S* and 5′-UTR sequences were selected as the target genes to detect HBV and HCV, respectively. The *S* genes from HBV genotypes B, C, D, B/C recombinant, and C/D recombinant and the 5′-UTR genes from HCV subtypes 1b, 2a, 3a, 3b, and 6a were aligned by DNASTAR software (Madison, WI, USA), respectively. The conserved sequences were applied to HBV- and HCV-LAMP primer design using Primer Explorer version 5 (http://primerexplorer.jp/e/) and Primer Premier version 5.0 according to the LAMP mechanism. The specificity of primer pair was confirmed through BLAST analysis tool. Table 1 lists the primer sequences and modifications, and Supplementary Figure S1 presents the primer locations. All primers were synthesized and purified via high-performance liquid chromatography grade by TsingKe Biotech Co., Ltd. (Beijing, China).

Preparation of AuNPs-based LFB The AuNPs-based LFB consists of 4 elements: a sample pad, a conjugation pad, a nitrocellulose (NC) membrane, and an absorption pad. Crimson red dye-conjugated streptavidin-coated AuNPs (SA-AuNPs; 40±5 nm, 10 mg/mL; Bangs, Laboratories, Inc., Indiana, USA) were deposited on the conjugation pad. Anti-FAM antibody (0.2 mg/mL, Abcam Co., Ltd.), anti-Dig antibody (0.25 mg/mL, Abcam Co., Ltd.) and biotinylated bovine serum albumin (biotin-BSA, 4 mg/mL; Abcam Co., Ltd.) were immobilized at the NC membrane (detection region) of test line 1 (TL1), test line 2 (TL2) and the control line (CL), respectively, which were separated by 5 mm. All the elements were packaged in a plastic cartridge. Our AuNPs-LFBs used here were manufactured through Tian-Jin HuiDeXin Biotech Co., Ltd. (Tianjin, China) according to our devise manual.

LAMP reaction

The single LAMP reactions for HBV or HCV were performed in a one-step 25 µL reaction volumes. Each reaction contained 12.5 µL of 2×reaction buffer (40 mM Tris-HCl (pH 8.8), 16 mM $MgSO_4$, 2 M betaine, 20 mM $(NH_4)_2SO_4$, 40 mM KCl, and 0.2% Tween-20); 1 µL of *Bst* 2.0 DNA polymerase (8 U); 0.1 µM F3 and B3 (each), 0.2 µM LF or LF* (for AuNPs-LFB only) and LB (each), 0.4 µM FIP or FIP* (for AuNPs-LFB only) and BIP (each); 1 µL of standard plasmid template (5 µL of clinical sample template); 1 µL of AMV reverse transcriptase (10 U) (only for RNA templates); 2 µL of colorimetric indicator

Table 1 The mLAMP-AuNPs-LFB degenerate primers used in this study

Primer name[a]	Sequence and modifications	Length[b]	Gene
HBV-F3	5′-TGTACMAAACCTWCGGACGG-3′	20 nt	*S*
HBV-B3	5′-GTAAYAGMGGYAWAAAGGGA-3′	20 nt	
HBV-FIP	5′-GAGGCCCACTCCCATAGGWAT-GCACYTGTATTCCCATCCCA-3′	41 mer	
HBV-FIP*	5′-FAM-GAGGCCCACTCCCATAGGWAT-GCACYTGTATTCCCATCCCA-3′	41 mer	
HBV-BIP	5′-TGCCATTTGTTCAGTGGTTCGT-TGGCCCCCAATACCACATC-3′	41 mer	
HBV-LF	5′-TTSCGAAAGCCCARGATG-3′	18 nt	
HBV-LF*	5′-Biotin-TTSCGAAAGCCCARGATG-3′	18 nt	
HBV-LB	5′-TGGCTTTCAGTTATATG-3′	17 nt	
HCV-F3	5′-AGTGTCGTRCAGCCTCC-3′	17 nt	5′UTR
HCV-B3	5′-GGCCTTTCGCRACCCAA-3′	17 nt	
HCV-FIP	5′-CDYCCYRGCRATTCCGGTGT-CCTCCCGGGAGAGCCATAG-3′	39 mer	
HCV-FIP*	5′-Digoxin-CDYCCYRGCRATTCCGGTGT-CCTCCCGGGAGAGCCATAG-3′	39 mer	
HCV-BIP	5′-TTGGANMAACCCRCTCWATRCC-CRCTACTCGGCTAGYRRTCT-3′	42 mer	
HCV-LF	5′-TCACCGGTTCCGCAGAC-3′	17 nt	
HCV-LF*	5′-Biotin-TCACCGGTTCCGCAGAC-3′	17 nt	
HCV-LB	5′-GMVATTTGGGCGTGCCCCCG-3′	20 nt	

Note: [a]F3, forward outer primer; B3, backward outer prime; FIP, forward inner primer; BIP, backward inner primer; LF, forward loop primer; LB, backward loop primer. HBV-FIP*, 5′-labeled with FAM, HBV-LF*, 5′-labeled with biotin, HCV-FIP*, 5′-labeled with digoxin, HCV-LF*, 5′-labeled with biotin, when used for the AuNP-LFB assay

[b]nt, nucleotide; mer, monomeric unit

(MG) (for colorimetry only); and double-distilled water (ddH_2O) up to 25 μL. The reactions were performed at 65 °C for 60 min.

The one-step multiplex LAMP reaction was also performed in a 25 μL reaction volumes containing 12.5 μL of 2×reaction buffer; 1 μL of *Bst* 2.0 DNA polymerase (8 U); 0.1 μM *S*-F3 and *S*-B3 (each), 0.2 μM *S*-LF or LF* (for AuNPs-LFB only) and *S*-LB (each), 0.4 μM *S*-FIP or *S*-FIP* (for AuNPs-LFB only) and *S*-BIP (each), 0.25 μM 5′UTR-F3 and 5′UTR-B3 9 (each), 0.5 μM 5′UTR-LF or LF* (for AuNPs-LFB only) and 5′UTR-LB (each), 1.0 μM 5′UTR-FIP or 5′UTR-FIP* (for AuNPs-LFB only) and 5′UTR-BIP (each); 1 μL of each standard plasmid template (5 μL of the clinical sample template); 1 μL of AMV reverse transcriptase (10 U) (only for RNA templates); 2 μL of colorimetric indicator (MG) (for colorimetry only); and ddH_2O up to 25 μL. The reactions were performed at 65 °C for 60 min.

Monitoring methods, including AuNPs-based LFB, real-time turbidity measurements (LA-500, Lumiprobe; Japan), 2% agarose gel electrophoresis, and visual reagent (MG) were utilized for analyzed the LAMP amplification products. For AuNPs-LFB-based evaluation, an HBV-positive result was indicated when both the CL and TL1 of the AuNPs-LFB turned red. An HCV-positive result was indicated when both the CL and TL2 of the AuNPs-LFB turned red. For a negative outcome, only the CL turned red. For real-time turbidity measurements, turbidity > 0.1 indicated a positive outcome. For agarose gel electrophoresis detection, ladder-like bands on the agarose gel manifested a positive result, while no bands indicated a negative outcome. For visual reagent MG analysis, reaction mixtures that turned bright green indicated a successful reaction, while a colorless reaction indicated a negative result.

Optimization of reaction temperature and time for the mLAMP-AuNPs-LFB assay

The optimal amplification temperature for our assay at LAMP reaction stage was test by incubating the HBV-LAMP and HCV-LAMP at 60 to 67 °C (in1°C intervals), and their amplification results were monitored with real-time turbidity. Then, the reaction time for multiplex-LAMP was optimized from 25 to 40 min (at 5 min intervals) under the optimal amplification temperature, the amplification results were analyzed through AuNPs-LFBs. Each assay was conducted in triplicate.

Sensitivity of the mLAMP-AuNPs-LFB assay

Two synthetic plasmids, HBV-*S* and HCV-5′-UTR plasmids, were serially diluted by a factor of ten to provide copy numbers ranging from 2.0×10^4 copies to 1 copy. Each test was performed under optimal reaction conditions, and the results were monitored through visual reagent MG and biosensor AuNPs-LFB. Each test was repeated three times.

Specificity of the mLAMP-AuNPs-LFB assay.

The specificity of our assay was evaluated by assaying various templates, including HBV-*S* plasmids, HCV-5′UTR plasmids, HBV/HCV-positive clinical nucleic acid samples (confirmed by real-time PCR), and other microbe nucleic acids, including HIV, Human papillomavirus, Influenza A virus, Influenza B virus, Epstein-Barr virus, Human enterovirus EV71, Coxsackie virus CAV16, Cytomegalovirus, Herpes zoster virus, *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Klebsiella pneumoniae*, *Cryptococcus neoformans*, and *Mycobacterium leprae* (Table 2), distilled water (DW) was served as the blank control (BC). Each test was performed independently in triplicate on different days under the optimal reaction conditions.

Feasibility of the mLAMP-AuNPs-LFB assay for clinical specimens

Serum samples were collected from 73 patients at the Second Affiliated Hospital Guizhou University of Traditional Chinese Medicine, who were suspected to have viral hepatitis caused by HBV and/HCV. The Genomic nucleic acid templates were rapidly isolated through a TIANamp Virus DNA/RNA Fast Kit (TIANGEN BIOTECH Co., Ltd., Beijing, China) according to the manufacture′s guidelines. We compared our assay to commercially available real-time TaqMan PCR Kit for HBV and HCV (Xi'an Tianlong Technology Co. Ltd., Xi'an, China) on an Applied Biosystems™7500 Real-Time PCR System (Life Technologies; Singapore). The results with HBV > 5 IU (~30 copies) and HCV > 50 IU (~45 copies) were regarded as positive according to the manufacturer's instructions. The Ethics Committee of The Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine approved the lawful collection of these genomic nucleic acid templates (Approval No. KYW2022034). The results of our mLAMP-AuNPs-LFB assay were compared with that of conventional quantitative real-time PCR (qPCR) method.

Results

Schematic of the mechanism of the HBV&HCV-mLAMP-AuNPs-LFB assay

The workflow of HBV&HCV-mLAMP-AuNPs-LFB assay was shown in Fig. 1A (Fig. 1A, **step 1**). For template preparation, nucleic acids were rapidly isolated from serum samples through a DNA/RNA Fast Kit (Fig. 1A, **step 2**). For multiple LAMP amplification, in the mLAMP system, one hapten, FAM, was linked to the HBV *S*-LAMP primer set, and another, digoxin (Dig), was added to the HCV 5′-UTR-LAMP primer set. Hence, the HBV *S*-FIP* and HBV *S*-LF* primers were labeled at the 5′ end with

Table 2 Microbial strains used in this study

No.	Strains/Templates	Source of strain[a]	No. of strains	mLAMP-AuNPs-LFB result[b]	
				HBV	HCV
1	HBV genotype B *S*-plasmid	Tsingke Biotech (Beijing, China)	1	P	N
2	HBV genotype C *S*-plasmid	Tsingke Biotech (Beijing, China)	1	P	N
3	HBV genotype D *S*-plasmid	Tsingke Biotech (Beijing, China)	1	P	N
4	HBV genotype B/C recombinant *S*-plasmid	Tsingke Biotech (Beijing, China)	1	P	N
5	HBV genotype C/D recombinant *S*-plasmid	Tsingke Biotech (Beijing, China)	1	P	N
6	HCV subtype 1b 5′UTR-plasmid	Tsingke Biotech (Beijing, China)	1	N	P
7	HCV subtype 2a 5′UTR-plasmid	Tsingke Biotech (Beijing, China)	1	N	P
8	HCV subtype 3b 5′UTR-plasmid	Tsingke Biotech (Beijing, China)	1	N	P
9	HCV subtype 6a 5′UTR-plasmid	Tsingke Biotech (Beijing, China)	1	N	P
10	HCV subtype 3a 5′UTR-plasmid	Tsingke Biotech (Beijing, China)	1	N	P
11	HBV&HCV plasmids	Tsingke Biotech (Beijing, China)	1	P	P
12	HBV strain (clinical sample)	2nd GZUTCM	1	P	N
13	HCV strain (clinical sample)	2nd GZUTCM	1	N	P
14	HBV &HCV strains (clinical sample)	2nd GZUTCM	1	P	P
15	Human immunodeficiency virus	GZCCL	1	N	N
16	Human papillomavirus	GZCCL	1	N	N
17	Influenza A virus	GZCDC	1	N	N
18	Influenza B virus	GZCDC	1	N	N
19	Epstein-Barr virus	2nd GZUTCM	1	N	N
20	Human enterovirus EV71	GZCDC	1	N	N
21	Coxsackie virus CAV16	GZCDC	1	N	N
22	Cytomegalovirus	GZCCL	1	N	N
23	Herpes zoster virus	2nd GZUTCM	1	N	N
24	*Escherichia coli*	ATCC8739	1	N	N
25	*Staphylococcus aureus*	ATCC25923	1	N	N
26	*Mycobacterium tuberculosis*	GZCDC	1	N	N
27	*Klebsiella pneumoniae*	ATCC700603	1	N	N
28	*Cryptococcus neoformans*	2nd GZUTCM	1	N	N
29	*Mycobacterium leprae*	GZCDC	1	N	N

Note: [a]ATCC, American Type Culture Collection; 2nd GZUTCM, the Second Affiliated Hospital, Guizhou University of Traditional Chinese Medicine; GZCCL, Guizhou Provincial Center for Clinical Laboratory, GZCDC, Guizhou Provincial Center for Disease Control and Prevention

[b]P, Positive; N, Negative

FAM and biotin, and the HCV 5′-UTR-FIP* and HCV 5′-UTR-LF* primers were labeled with Dig and biotin, respectively. The HBV-LAMP products were simultaneously labeled with biotin and FAM, and the HCV-LAMP products were labeled with biotin and Dig during LAMP isothermal amplification process (Fig. 1A, **step 3**). For visual interpretation of the results, the labeled mLAMP products were readout visually through AuNPs-LFB.

The visual interpretation of the HBV&HCV-mLAMP-AuNPs-LFB assay is outlined in Fig. 1B. In brief, aliquots of 1 μL of mLAMP amplicons and 100 μL of running buffer (100 mM PBS with 1% Tween 20 [pH 7.4]) were deposited concurrently onto the sample pad (Fig. 1B). The LAMP amplicons-containing running buffer moves along the biosensor via capillary action, and then rehydrating the dye-coated SA-AuNPs. The FAM/biotin-labeled HBV-LAMP and Dig/biotin-labeled HCV-LAMP products integrate with dye-coated SA-AuNPs at the conjugation pad (Figs. 1B and 2). Finally, the HBV-LAMP amplicons were fixed though anti-FAM antibody at TL1, while the HCV-LAMP amplicons were anchored by the anti-Dig antibody at TL2. Excess dye-coated SA-AuNPs were arrested by BSA at CL (Figs. 1B and 3). The readout of the HBV&HCV-mLAMP products using AuNPs-LFB biosensor was presented in Fig. 1B (**4**). When both CL and TL1 turned red, the sample was regarded as positive for HBV; when both CL and TL2 turned red, the sample was considered positive for HCV; and when only CL turned red, the sample was deemed to negative for both.

Confirmation of the feasibility of the HBV-LAMP and HCV-LAMP primer sets

For confirming the feasibility of the HBV- and HCV-LAMP primer sets in the multiplex-LAMP reaction system, five standard HBV-*S* plasmids (genotypes B, C, D, recombinant B/C, and recombinant C/D), five standard

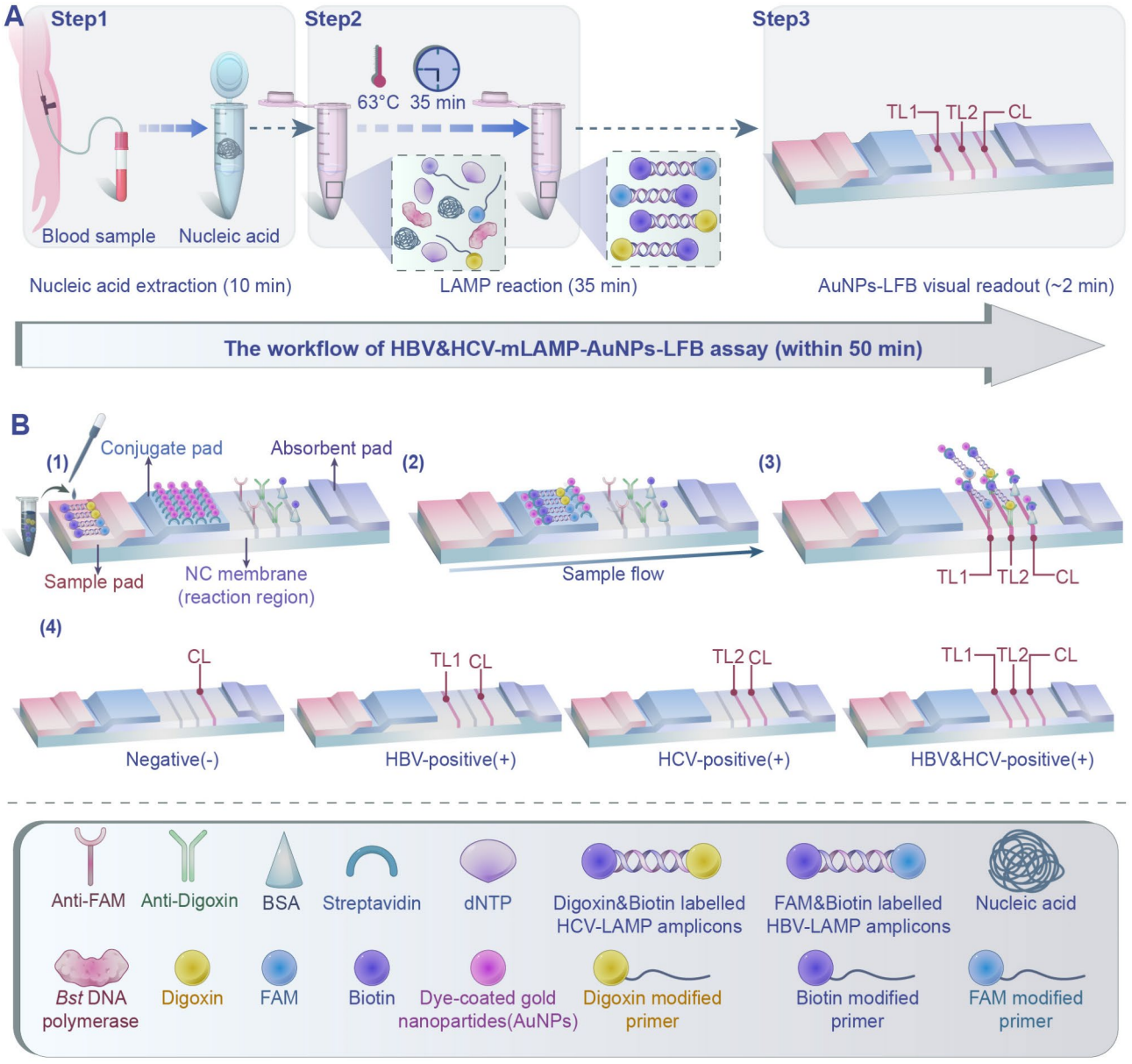

Fig. 1 Schematic diagram of HCV-RT-LAMP-AuNPs-LFB assay's principle: **(A)** Schematic diagram of AuNPs-LFB principles for the analysis of mLAMP products. **1**, mLAMP products (1.0 μL) and running buffer (100 μL) are simultaneously added on the sample pad. **2**, the running buffer containing mLAMP products move forward onto the conjugate pad and NC membrane (reaction region) with capillary action. The crimson dye streptavidin-coated gold nanoparticles (streptavidin-AuNPs) are rehydrated and integrated with FAM/biotin labelled HBV-LAMP products and digoxin/ biotin labelled HCV-LAMP products. **3**, FAM/biotin-labeled HBV-LAMP products are captured by anti-FAM at TL1, digoxin/ biotin labelled HCV-LAMP products are captured by anti-digoxin at TL2, excess dye-coated streptavidin-AuNPs are captured by biotinylated bovine serum albumin (biotin-BSA) at the CL region. **4**, Interpretation of the mLAMP-AuNPs-LFB assay. HBV-positive results are indicated by CL and TL1 bands on the biosensor, HCV-positive results are indicated by CL and TL2 bands on the biosensor, both HBV and HCV positive results are indicated by TL1, TL2, and CL bands on the AuNPs-LFB, negative results are indicated when only the CL band appears on the biosensor.**(B)** Workflow of the mLAMP-AuNPs-LFB assay. the mLAMP-AuNPs-LFB assay's workflow comprises the following closely linked steps: rapid nucleic acid extraction (**step 1**), LAMP reaction (**step 2**), and AuNPs-LFB visual readout (**step 3**). The entire detection process can be completed within 50 min

HCV-5′UTR plasmids (subtypes 1b, 2a, 3b, 6a, and 3a), viral nucleic acid of HIV and human papillomavirus (HPV) were applied to templates for LAMP amplification with a constant temperature of 65 °C for 1 h, whereas distilled water (DW) as a blank control. Agarose gel electrophoresis, colorimetric indicator (MG), and AuNPs-LFB biosensor methods were utilized to readout the LAMP results. The templates from the HBV and/or HCV strand plasmids were amplified and yielded positive results according to each analysis method (Fig. 2A, B, **and**

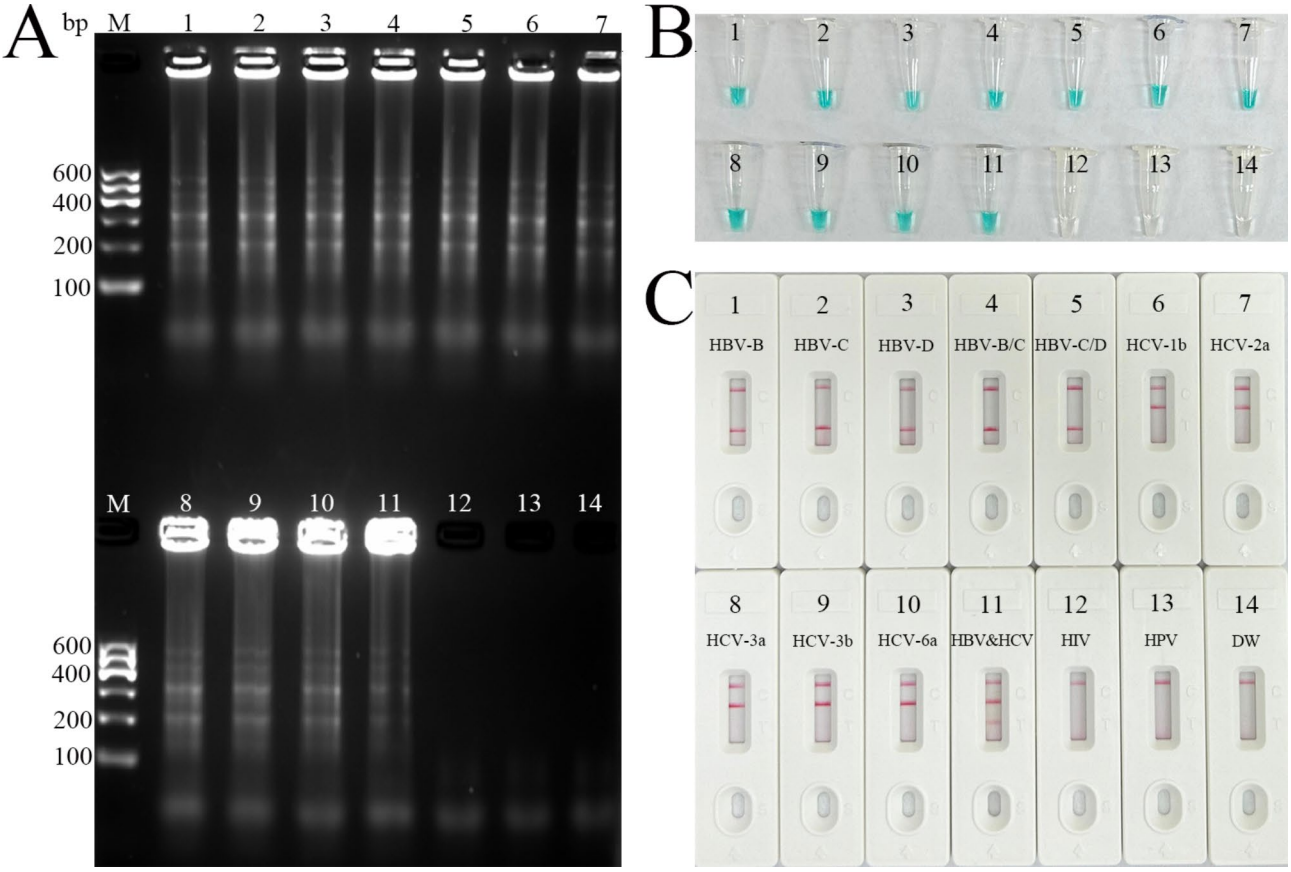

Fig. 2 Confirmation and detection of HBV-, HCV-, and mLAMP product. **(A)** 2% agarose gel electrophoresis, (**B**) visual indicator (MG), (**C**) biosensor platform (AuNPs-LFB). Templates of 1–14 were HBV-B plasmid, HBV-C plasmid, HBV-D plasmid, HBV-B/C plasmid, HBV-C/D plasmid, HCV-1b plasmid, HCV-2a plasmid, HCV-3a plasmid, HCV-3b plasmid, HCV-6a plasmid, HBV-HCV plasmid, human immunodeficiency virus (HIV), human papillomavirus (HPV), and distilled water (DW), respectively. CL: control line, TL1: test line one, TL2: test line two

C). No amplification was detected for the negative controls (HIV and HPV) or the blank control (DW) (Fig. 2A, B, **and C**). In addition, the AuNPs-LFB approach simultaneously detected two targets (HBV and HCV) in a single test (Fig. 4C). These data indicated that our mLAMP-AuNPs-LFB assay utilizing the HBV- and HCV-LAMP primer sets designed in the current study can be applied for the simultaneous identification of HBV and HCV.

Optimization of the reaction temperature for the mLAMP-AuNPs-LFB assay

An optimal reaction temperature is important for the LAMP reaction. In the present study, LAMP reactions were carried out from 60 to 67 °C in 1 °C intervals. The concentration of the HBV-*S*-plasmid or HCV-5′UTR plasmid template used for detection was 1.0×10^4 copies per test. Real-time turbidity measurement was performed to monitor the LAMP products (Fig. 3A **and B**). The data suggested that robust HBV-LAMP reaction appeared at 63 to 65 °C (Fig. 3A) and that HCV-LAMP reaction occurred at 63 to 64 °C (Fig. 3B). Hence, 63 °C was regarded as the most appropriate reaction temperature for HBV&HCV-mLAMP-AuNPs-LFB assay.

Sensitivity of the mLAMP-AuNPs-LFB assay

Serial dilutions of templates (HBV-*S*-plasmid and HCV-5′-UTR-plasmid constructs) ranging from 2.0×10^4 copies to 1 copy were applied to test the sensitivity of our assay. The mLAMP-AuNPs-LFB assay was performed at 63 °C for 1 h, and the LAMP products were analyzed with the AuNPs-LFB platform and MG reagent. The two viruses (HBV and HCV) were identified in a single-tube reaction, and the limit of detection (LoD) was 20 copies per test for both the HBV-LAMP (Fig. 4A **and B**) and HCV-LAMP (Fig. 4C **and D**). The LAMP results obtained using reagent (MG) were consistent with those obtained with AuNPs-LFB. However, the traditional visual detection approach with MG notably cannot discriminate between the two target agents in a signal assay. In addition, the sensitivity of mLAMP-AuNPs-LFB was also in accordance with that of the single HBV- and HCV-LAMP-AuNPs-LFB methods (Fig. 4E **and F**).

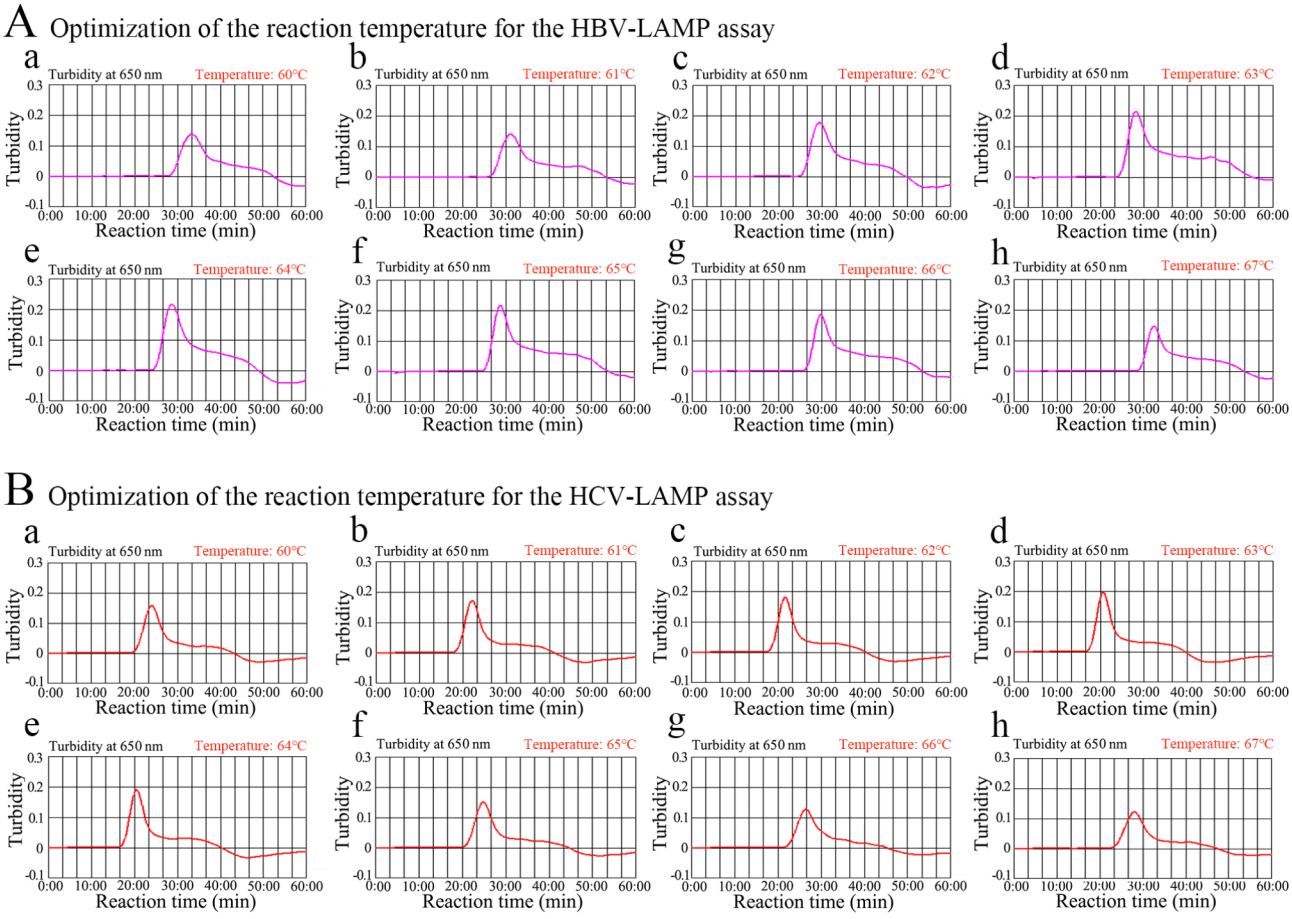

Fig. 3 Temperature optimization for HBV- and HCV-LAMP amplification. LAMP amplifications for HBV (**A**) and HCV (**B**) were monitored using real-time turbidity, and their corresponding amplicon curves were shown as graphs. A turbidity > 0.1 indicated a positive result. Eight kinetic graphs were obtained at different temperatures (60–67 °C in 1 °C increments) with 1×10^4 target gene copies. Graphs **d** (63 °C) to **f** (65 °C) in **A** showed robust amplification. Graphs from **d** (63 °C) to **e** (64 °C) in **B** showed robust amplification

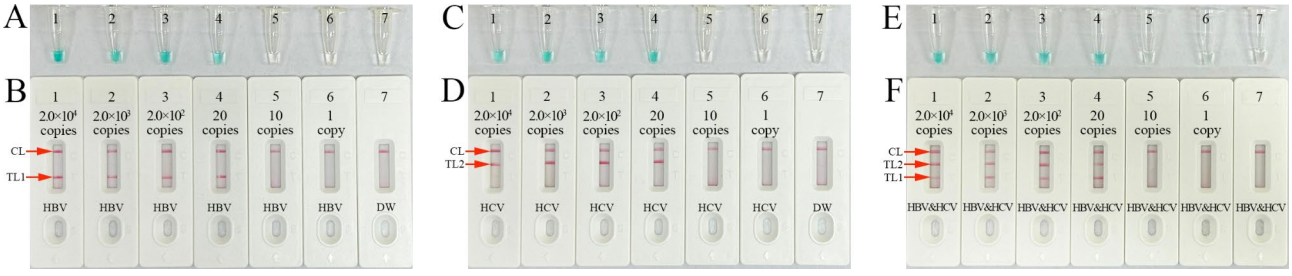

Fig. 4 Sensitivity analysis of the mLAMP-AuNPs-LFB assay with serial nucleic acid template dilutions. Serial dilutions (2.0×10^4 copies, 2.0×10^3 copies, 2.0×10^2 copies, 20 copies, 10 copies, and 1 copy) of HBV-*S* and HCV-5′UTR plasmids were used as templates, and DW was used as the negative control. Results were simultaneously analyzed by visual reagent MG and the AuNPs-LFB. (**A**, **B**): A sensitivity analysis of the HBV LAMP assay indicated its LoD was 20 copies per reaction. (**C**, **D**): A sensitivity analysis of the HBV LAMP assay indicated its LoD was 20 copies per reaction. (**E**, **F**): A sensitivity analysis of the mLAMP assay for HBV and HCV indicated its LoD was 20 copies of the nucleic acid template per reaction. CL: control line, TL1: test line one, TL2: test line two

Optimization of reaction time for the mLAMP-AuNPs-LFB assay

The optimal reaction time for the mLAMP-AuNPs-LFB assay at the multiple-LAMP reaction stage was tested across the range from 25 to 45 min (with 5-min intervals) (Fig. 5A-D). All tests were carried out under the optimal amplification temperature, and the results were analyzed using AuNPs-LFB biosensor. Both plasmid templates (HBV-*S*-plasmid and HCV-5′UTR-plasmid) at the LoD level (20 copies) resulted in positive outcomes after 35 min of incubation (Fig. 5C). Therefore, the entire diagnostic procedure for the mLAMP-AuNPs-LFB assay can

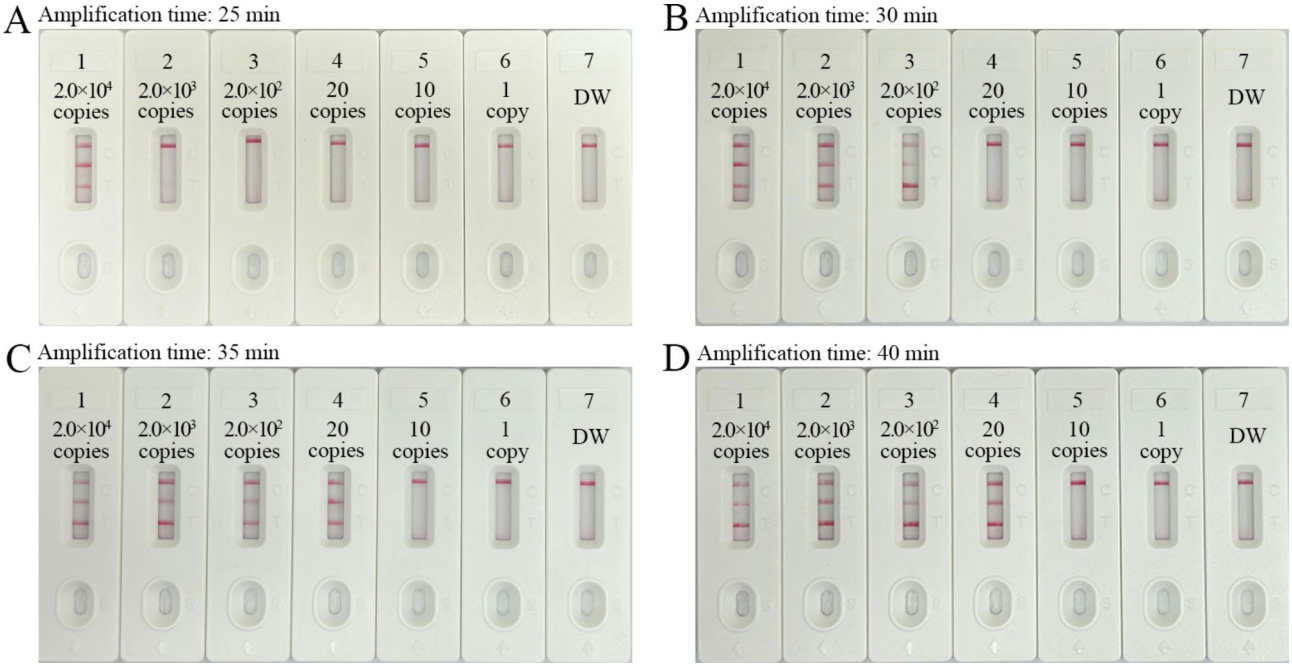

Fig. 5 Amplification time optimization for the mLAMP-AuNPs-LFB assay. Four LAMP reaction times were evaluated at 63 ℃: 25 min (**A**), 30 min (**B**), 35 min (**C**), and 40 min (**D**). biosensor 1–7 represent nucleic acid template levels 2.0×10^4 copies, 2.0×10^3 copies, 2.0×10^2 copies, 20 copies, 10 copies, and 1 copy and negative control (DW), respectively. Results were analyzed using AuNPs-LFBs. The optimal LoD occurred with a 35 min reaction time (**C**). CL, control line; TL1, test line one; TL2, test line two

be finished within 50 min, with rapid nucleic acid preparation (10 min), multiple-LAMP reaction (35 min), and result readout through the visual AuNPs-LFB method (<2 min).

Specificity of the mLAMP-AuNPs-LFB assay

Our assay's specificity was assessed through the use of HBV-*S*-plasmids (genotypes B, C, D, recombinant B/C, and recombinant C/D), HCV-5′UTR-plasmids (subtypes 1b, 2a, 3a, 3b, and 6a), positive HBV and/or HCV clinical samples (confirmed by qPCR), and 15 other microbes (Table 2). All the tests were carried out under the optimal reaction conditions (63 °C for 35 min), and the results were readout through the AuNPs-LFB biosensor. In our study, the positive results observed for all positive controls and negative results for all negative and blank controls (Fig. 6). Hence, the data showed that our assay has no cross-reactions with other pathogens, and presented 100% specificity. In addition, each test of this study was repeated three times at differently days, and showed good reproducibility.

Application of the mLAMP-AuNPs-LFB assay for clinical samples

To confirm the feasibility of our assay for clinical samples, 73 serum samples from patients with suspected HBV and/or HCV infection were analyzed initially using conventional quantitative real-time PCR (qPCR). Among them, 21 samples were confirmed by qPCR to be HBV-positive, 13 were HCV-positive, 7 were from patients coinfected with HBV and HCV, and 32 were tested as negative. The remaining nucleic acids from patients after qPCR diagnosis were tested through our mLAMP-AuNPs-LFB assay. The results of our novel mLAMP-AuNPs-LFB assay were consistent with those of qPCR approach (Table 3), and the details were showed in Supplementary Material Table S1. the data revealed that our proposed mLAMP-AuNPs-LFB assay can function as a useful POC testing approach for detecting HBV and HCV in clinical practice.

Discussion

Here, for the first time, mLAMP isothermal amplification, which was combined with readily available AuNPs-LFB visual readout platform, was successfully devised for rapid, visual, specific, sensitive, and user-friendly simultaneous diagnosis of HBV and HCV infection. The applicability of our assay was verified using clinical specimens from patients with suspected HBV and/or HCV infection.

In recent decades, specific and sensitive PCR-related molecular techniques have revolutionized the diagnosis of acute cases of HBV and HCV infection [25, 30]. However, these methods typically require a significant investment in infrastructure, thermal cyclers, and training. This approach is not feasible for low- and middle-income

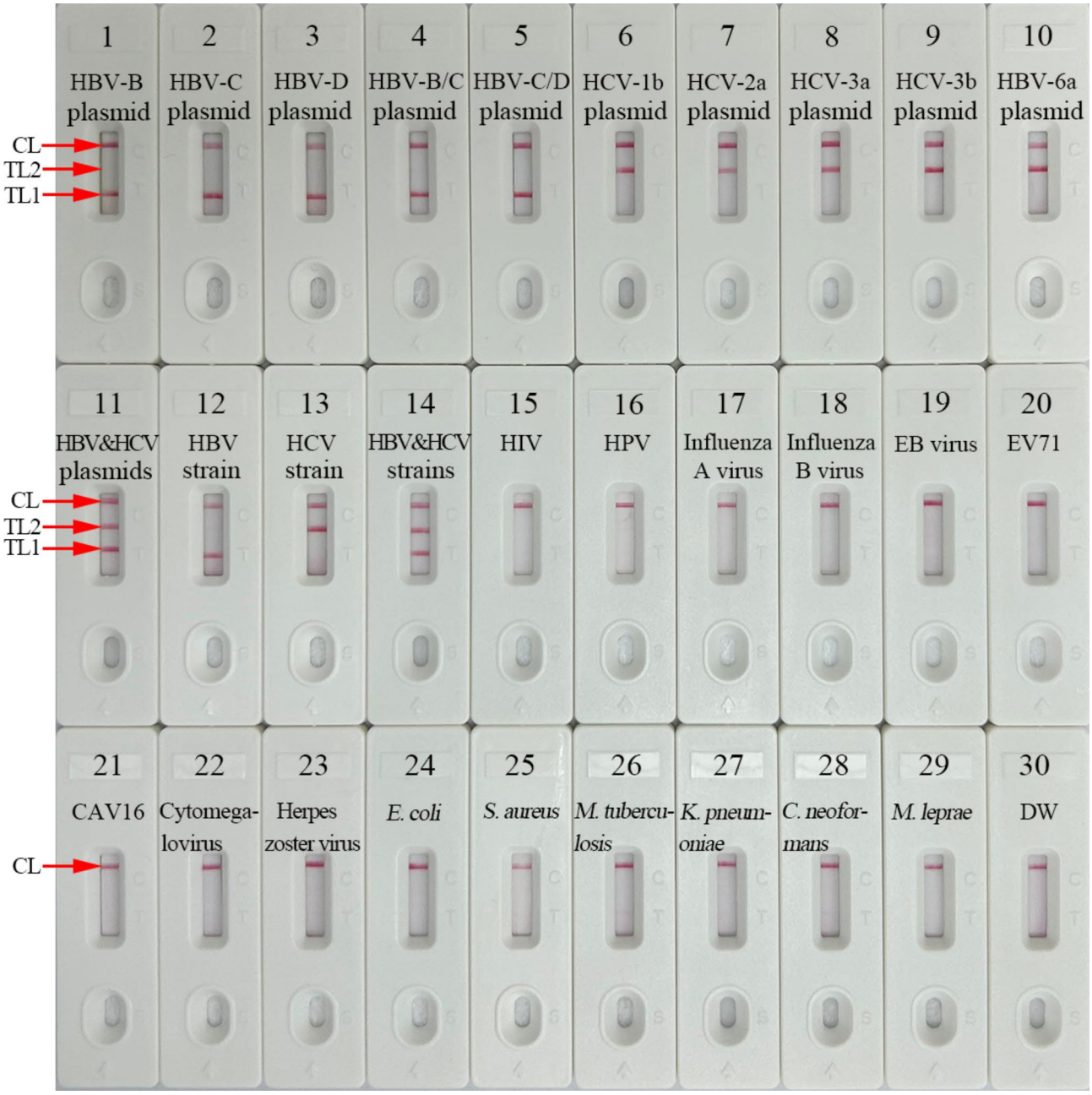

Fig. 6 Specificity analysis of mLAMP-AuNPs-LFB assay with different strains. The mLAMP reactions were performed using different nucleic acid templates, and each of the amplification product was analyzed through visual AuNPs-LFBs. Biosensor 1–30, HBV genotype B plasmid, HBV genotype C plasmid, HBV genotype D plasmid, HBV genotype B/C plasmid, HBV genotype C/D plasmid, HCV subtype 1b plasmid, HCV subtype 2a plasmid, HCV subtype 3b plasmid, HCV subtype 6a plasmid, HCV subtype 3a plasmid, HBV&HCV plasmids, HBV strain (clinical sample), HCV strain (clinical sample), HBV &HCV strains (clinical sample), Human immunodeficiency virus (HIV), Human papillomavirus (HPV), Influenza A virus, Influenza B virus, Epstein-Barr virus, Human enterovirus EV71, Coxsackie virus CAV16, Cytomegalovirus, Herpes zoster virus, *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Klebsiella pneumoniae*, *Cryptococcus neoformans*, *Mycobacterium leprae*, distilled water (DW), respectively. CL, control line; TL1, test line one; TL2, test line two

settings. In the present study, the mLAMP-AuNPs-LFB assay was developed on the basis of the isothermal LAMP technique for simultaneously amplifying two target genes (HBV-*S* and HCV-5′UTR) within a short time (35 min) at a constant temperature (63 °C), which can be carried out using simple equipment, such as a water bath and metal bath. Then, the mLAMP amplicons were rapidly and visually analyzed with AuNPs-LFBs. In our assay, the crude nucleic acids were sufficient for the LAMP reaction owing to the *Bst* DNA polymerase used is less readily inhibited than the *Taq* DNA polymerase used in traditional PCR method [31, 32]. Therefore, our

Table 3 Comparing HBV & HCV levels in clinical samples using our mLAMP-AuNPs-LFB assay and qPCR method

Detection method	Clinical samples ($n=73$)			
	Positive			Negative
	HBV	HCV	HBV & HCV	
qPCR	21	13	7	32
mLAMP-AuNPs-LFB	21	13	7	32

molecular assay is rapid and affordable, and the whole assay process, including rapid nucleic acid extraction (~ 10 min), LAMP (35 min), and AuNPs-LFB visual interpretation (less than 2 min), can be finished within 50 min.

.In the current study the optimal HBV&HCV-mLAMP reaction conditions were determined to be 63 °C for 35 min (Figs. 3 and 5). The specificity of our assay was tested for several HBV genotypes (B, C, D, B/C recombinant, and C/D recombinant), HCV subtypes (1b, 2a, 3b, 6a and 3a), and other pathogens. As expected, our assay presented 100% specificity for both HBV and HCV, and had no cross-reaction with other pathogens (Tables 2**and** Fig. 6). Besides, the sensitivity of our assay was 20 copies per test (Fig. 4), which is sufficient for clinical diagnostic purpose. The WHO International Standard Substances Hepatitis B Virus and Hepatitis C Virus for nucleic acid amplification techniques can better define the sensitivity of the test and compare with other similar assays. However, it is difficult to obtain all WHO International Standard Substances used in this study, including five major hepatitis B virus genotypes (B, C, D, recombinant B/C, and recombinant C/D), and five dominant subtypes hepatitis C virus (1b, 2a, 3a, 3b, and 6a) predominant in China. Fortunately, synthetic plasmids containing target sequences can applied to positive controls and verify the sensitivity of nucleic acid detection technologies [33–35]. Here, the full-length *S* sequences from five dominant HBV genotypes and the HCV 5′-UTR target genes from five dominant subtypes in China were synthetically produced, cloned and inserted into pUC57 plasmids, respectively. The synthetic plasmids were used as positive controls and test the sensitivity of HBV&HCV-mLAMP-AuNPs-LFB assay. To confirm the feasibility of our assay for clinical application, 73 serum samples from suspected HBV- and/or HCV-infected patients were assayed simultaneously with our mLAMP-AuNPs-LFB assay and the traditional real-time PCR technique. The data indicated that our novel approach have ability to detect HBV and HCV in clinical practice. We previously used single LAMP to detect HBV and HCV with an LoD as low as 7.5 IU and 20 copies per test, respectively [36, 37]. Hongjaisee et al. used the LAMP approach and rapidly and visually identified HCV at 10–100 ng per reaction [38]. However, these methods were inadequate for identifying and distinguishing the two important pathogens in a single test. Here, we applied for the first time a one-step multiplex LAMP reaction for simultaneously detecting and distinguishing HBV and HCV.

For visual and rapid interpretation, a novel AuNPs-based biosensor diagnostic platform was used in this study. AuNPs-LFBs are paper-based biosensors that have tremendous potential for POC testing because they are typically user friendly, have high sensitivity and specificity, and have a low cost [39, 40]. The use of AuNPs in the development of our biosensor improved its sensitivity and performance owing to their unique physicochemical characteristics, such as good biocompatibility, chemical stability, low toxicity, and high surface-to-volume ratio [21]. These features facilitate the synthesis and modification of AuNPs with various chemical functional groups for conjugation with biomolecules (DNA probes, antigens, and antibodies), which are vital for the development of biosensors [41, 42]. In this study, the colorimetric indicator MG, real-time turbidity, and agarose gel electrophoresis were also used to detect multiplex LAMP amplicons. Nevertheless, all of them are insufficient for identifying simultaneously two target genes in a one-step reaction. In addition, real-time turbidity measurements and agarose gel electrophoresis require expensive instruments and are not suitable for use as a POC diagnostic platform. Our AuNPs-LFB platform is user-friendly and cost-saving (approximately US$2.0 per test), the approximate total cost of each assay, including nucleic acid rapid extraction (US $0.5), LAMP (US $1.5), and AuNPs-LFB detection (US $2.0), was US $4.0, which is cheaper than that of traditional real-time PCR testing (approximately $7.0 USD) [43]. More importantly, the AuNPs-LFB biosensor can simultaneously identify HBV and HCV in a one-step reaction.

In addition, our assay also has several limitations. First, the HBV-LAMP primers and HCV-LAMP primers identified only the HBV genotypes and HCV subtypes, respectively, dominant in China. Further refinement of the HBV-LAMP and HCV-LAMP primers is needed because HBV and HCV have high genetic heterogeneity. Second, in our assay system, the LAMP amplification tube had to open for AuNPs-LFB interpretation, which increases the risk of cross contamination. For greater suitability for clinical application and to avoid aerosol contamination, it is necessary to refine the HBV&HCV-mLAMP-AuNPs-LFB diagnostic system to eliminate the need for opening the LAMP reaction tube.

Conclusions

by combining multiple LAMP isothermal amplification methods with AuNPs-LFB, a novel mLAMP-AuNPs-LFB assay was successfully developed for the rapid, user-friendly, highly sensitive and specific, and cost-saving detection of HBV and HCV in clinical applications. The whole diagnostic procedure can be finished within

50 min without the need for specific instruments. Our assay presented 100% specificity and had a 20-copy LoD. Hence, our mLAMP-AuNPs-LFB assay is a valuable tool for rapid, specific, sensitive, visual, and user-friendly identification of HBV and HCV and has tremendous potential as a POC testing approach for screening for HBV and HCV infection.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-024-03610-z.

Supplementary Material 1

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Author contributions
Xu Chen: Writing—original draft, review & editing, funding acquisition, investigation, methodology, resources. Yuanfang Shi: Writing—original draft, data curation, methodology, validation. Qi Zhao: Writing—original draft, methodology, resources, validation. Yu Wang: Writing—original draft, data curation, resources. Xinggui Yang: Writing—original draft, methodology, resources, validation. Yan Tan: Writing—original draft, data curation, investigation, resources. Yi Wang: Writing—review & editing, project administration, software. Shilei Dong: Writing—review & editing, resources, supervision, visualization. Zhenghua Xiao: Writing—review & editing, funding acquisition, project administration, supervision.

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Data availability
The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of The Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine (Approval No. KYW2022034), and complied with the Declaration of Helsinki. Before our team obtained clinical samples/isolates and conducted this research, any personal patient identifiers were removed. Patient informed consent was waived by the Ethics Committee of The Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine.

Consent for publication
Not Applicable.

Competing interests
The authors declare no competing interests.
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