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Title: Development and validation of a new and rapid molecular diagnostic tool based on RT-LAMP for Hepatitis C virus detection at Point-of-Care

Running head: New RT-LAMP system for HCV detection at POC

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Abstract

PURPOSE: Globally, it is estimated that 1.0 million individuals are newly infected by Hepatitis C virus (HCV) every year, and nearly 50 million people live with a chronic infection, according to World Health Organization. To overcome underdiagnosis of HCV infection among hard-to-reach populations, it is essential to develop new rapid and easy-to-use molecular diagnostic systems. In this work, we have developed a pangenotypic diagnostic tool based on Loop-Mediated Isothermal Amplification (LAMP), coupled to a direct sample lysis procedure for molecular detection of HCV at point-of-care (POC).

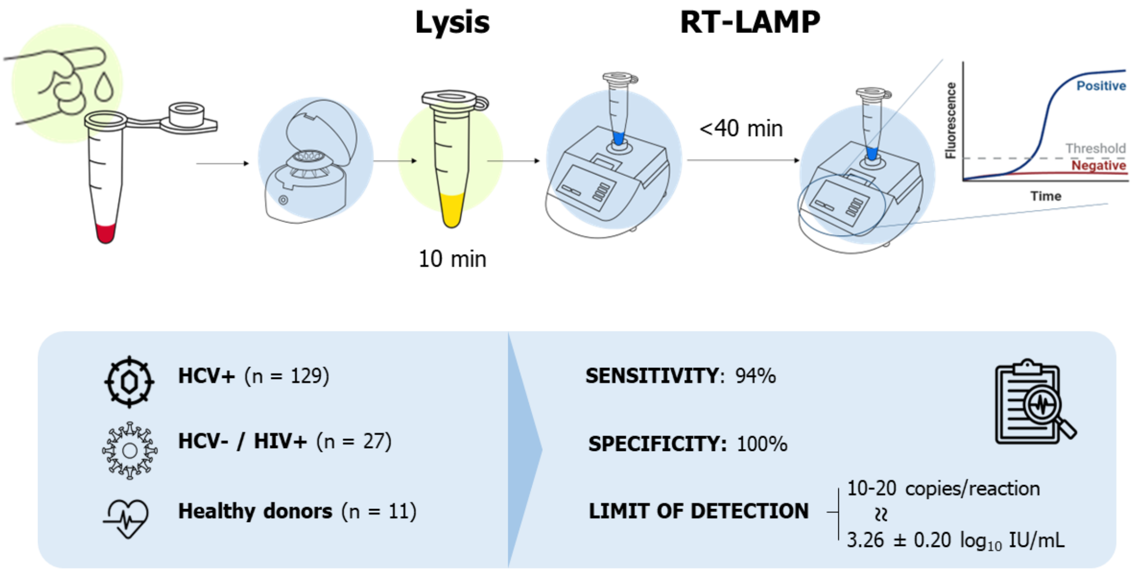
METHODS: Procedure validation was performed using 129 different samples from HCV infected patients (116 serum samples, and 13 fresh blood samples), 27 individuals who tested negative for HCV but positive for HIV, and 11 healthy donors. Serum was collected, lysed for 10 minutes at room temperature, and assayed by RT-LAMP. To achieve this, a set of 9 LAMP-primers was used for the first time. Parallel RT-qPCR assays were conducted for HCV to both validate the procedure and quantify viral loads.

RESULTS: HCV was detected by RT-LAMP in 109/116 HCV positive serum samples, and in 11/13 positive blood samples in less than 40 minutes. Compared to RT-qPCR results, our RT-LAMP procedure showed a sensitivity of 94%, 100% specificity, and a limit of detection of 3.26 $\log_{10}$ IU/mL (10-20 copies per reaction).

CONCLUSIONS: We have developed an accurate system, more affordable than the current available rapid tests for HCV. Since no prior RNA purification step from capillary blood is required, we strongly recommend our RT-LAMP system as a valuable and rapid tool for the molecular detection of HCV at POC.

Key words: HCV, RNA virus, molecular diagnosis, POC, RT-LAMP

Graphical abstract



1. Introduction

Hepatitis C virus (HCV) is a single stranded, positive sense RNA virus, mainly transmitted through blood exchange, that can develop liver cirrhosis, hepatocellular carcinoma, and even death in chronically infected patients [1]. The World Health Organisation (WHO) estimated that 50 million people live with a chronic HCV infection, with around 1.0 million individuals newly infected every year. Risk of death is especially higher in HCV-HIV coinfected individuals, three-fold higher than among HIV-monoinfected people [2].

A total of 8 genotypes and 90 subtypes reflects the high diversity of HCV genome, with different geographical distribution worldwide [3]. Genotype 1 (GT1) is the most prevalent genotype, comprising 66% of cases in high-income countries, followed by genotype 3 (GT3, 18%), genotype 2 (GT2, 12%), and genotypes 4 to 6 in smaller proportions. Prevalence of genotypes 4 (GT4) and 5 (GT5) is higher in low-income countries while genotype 6 (GT6) proportion is greater in East and Southeast Asia [4]. As a bloodborne virus, HCV is recurrently transmitted after exposure of individuals to unsafe injection practices, comprising mostly people who inject drugs (PWID), and those exposed to unsafe health care in low- and middle-income countries

(LMIC). Due to the widespread availability of Direct-acting antivirals (DAAs), the HCV elimination rate has reached up to 90% among HCV-infected people [5].

Consistently, in 2016 the WHO already defined the objective for HCV elimination as a public health problem for 2030 [6]. For this purpose, implementing efficient HCV screening strategies is crucial for reducing underdiagnosis of HCV and expanding the access to DAA-based therapies particularly among hard-to-reach population. The most recurrent rapid tests used for viral diagnosis are based on specific immunoassays for antibody or antigen detection. Despite antibody-tests cannot be used for the determination of an HCV active infection [7], the detection of HCV core antigen (HCV cAg) does show a strong correlation with the presence of HCV RNA. Due to one- to two-day delay in HCV cAg detectability in serum compared to viral RNA, HCV antigen test generally have lower sensitivity than molecular tests: 93.4% for the Abbot ARCHITECT HCV Ag assay, and 93.2% for the Ortho ELISA Ag, with high negative predictive values for viral loads bellow 1000 to 3000 IU/mL [8].

Therefore, in clinical settings, active HCV infection is actually diagnosed through its viral RNA detection [7], in particular by RT-qPCR (Reverse Transcription-real time Polymerase Chain Reaction). However, this technique is more expensive and requires bulky equipment along with highly trained personnel. Besides, it usually implies a time-consuming step of RNA extraction and purification, that also use expensive equipment and reagents. These drawbacks pose notable challenges for the implementation of RT-qPCR as HCV molecular test at POC. Despite of that, several portable and automatized molecular methods have been developed such as the Genedrive HCV ID Kit pre-qualified by WHO (Sysmex Asia Pacific Pte Ltd, Singapur), showing a remarkable analytical sensitivity of 2,362 IU/mL [9]. As well, the Xpert HCV Viral Load Finger-stick (Cepheid, USA) shows both sensitivity and specificity of 100%, capable of detecting its RNA in less than an hour [10]. Its limit of detection (LOD) is 22 or 35 IU/mL for GT1a and GT6e, respectively. These values are very close to RT-qPCR sensitivity levels of the Abbott RealTime HCV Viral Load assay (Abbott Molecular, USA). However, although cheaper than conventional RT-qPCR, costs per test are still higher to be globally implemented as a massive screening method for HCV in decentralized settings and LMIC [11].

As an alternative for molecular HCV detection at POC, several systems based on RT-LAMP (Reverse Transcription loop-mediated isothermal amplification) technology have recently emerged [12-17]. In contrast to RT-PCR, RT-LAMP procedures generally use a set of 4 to 6 specific oligonucleotide primers, such as F3/B3, FIP/BIP, and Loop-FIP/Loop-BIP pairs. Besides, thanks to a unique DNA polymerase with strand displacement activity and the “Loop” primers, nucleic acid amplification occurs continuously at a constant temperature [18], with different molecular weight concatemers of the amplified target sequence as a result. Visualization of LAMP amplification has been conveniently adapted to POC conditions. It includes the use of hydroxynapftol blue (HNB) [14, 16], pH-based colorimetric indicators [15], fluorescent dyes [12, 13], lateral-flow strips [17, 19], or CRISPR/Cas12a coupled biosensors [20, 21]. Nevertheless, the analytical and diagnostic sensitivity and specificity of these systems are usually still far from the gold standard RT-qPCR values. Notably, Nyan and Swinson [13] developed a RT-LAMP-based procedure for HCV RNA detection coupled with fluorescence visualization that showed a sensitivity of only 91.5%. Moreover, end-point addition of dyes can easily lead to cross-contamination and, consequently, to false positive results. Some RT-LAMP systems coupled to CRISPR/Cas12a have achieved sensitivity and specificity rates of 96% and 100%, respectively, such as the assay of Kham-Kjing et al [21]. However, this assay still needs more than an hour to get test results, in addition to the required RNA extraction time. Additionally, a rapid RT-LAMP-based test has been recently developed to detect HCV RNA from plasma samples using microfluidic chips [22]. This test shows an LOD of 500 virions/mL in just 45 minutes reaction. Of note, this procedure has been developed exclusively using spiked plasma samples with JFH1-HCV virions (GT2a).

Except for the HNB-based RT-LAMP system developed by Hongjaisse et al. [16], all the systems mentioned so far are still in development, and most have yet to be validated with HCV-positive samples across the different HCV genotypes. Moreover, their LOD is significantly higher than the threshold recommended by the European Association for the Study of the Liver (EASL) for widespread HCV screening [23]. Specifically, the HNB-based system has an LOD of 5.00 log10 IU/mL, which is 100 times higher.

Therefore, we consider that the development and validation of new, rapid, and portable molecular tests, including sample preparation and viral RNA detection, is still a need. In this work, we have developed a rapid RT-LAMP-based system that detects HCV RNA in less than an hour directly from blood samples obtained by capillary puncture. This system may be very helpful in the WHO goals for elimination of hepatitis C as a public health threat by 2030.

2. Materials and methods

2.1. Sample collection and handling.

Samples and data from patients included in this study were provided by three different hospitals in the Community of Madrid, as the Hospital Universitario la Princesa, the Hospital Universitario La Paz, and the Biobank of the Hospital Universitario Puerta de Hierro Majadahonda (HUPHM)/Instituto de Investigación Sanitaria Puerta de Hierro-Segovia de Arana (IDIPHISA). The serum panel included only patients with chronic HCV infection (positive PCR and antibodies). Samples and data were processed following standard operating procedures with the appropriate approval of the Research Ethics and Scientific Committees in all institutions involved [Instituto de Salud Carlos III (CEI PI 25_2018-v2), Hospital La Paz (PI-3691), Hospital Universitario La Princesa (3917), Universitario Puerta de Hierro Majadahonda (HUPHM)/Instituto de Investigación Sanitaria Puerta de Hierro-Segovia de Arana (IDIPHISA) (PT17/0015/0020 in the Spanish National Biobanks Network)].

A total of 129 samples from people who tested positive for HCV and had not started antiviral treatment (116 serum samples and 13 fresh blood samples; **Supplementary Table S1**), 27 blood samples from individuals who tested negative for HCV but positive for HIV, and 11 healthy controls were analyzed. Serum samples were stored at -80ºC until their use. Regarding fresh blood samples, 10 mL of blood were processed as follows: 50 µL of whole blood were centrifuged for

2 minutes in a minispin centrifuge (Labnet) to obtain its serum fraction immediately before analysis, and 9 mL were centrifuged for 10 minutes at 2500 rpm in gel separator tubes to obtain a larger serum fraction following standard venipuncture methods, as control. The later fraction was also used for RNA extraction and storage at -80ºC. To determine the minimum inhibitory haemolysis level, control blood samples were diluted in PBS and spiked with HCV G2a single stranded DNA (ssDNA).

2.1. Design of oligonucleotide primers

HCV consensus sequences from the most prevalent genotypes and subtypes were obtained from GenBank (**Supplementary Table S2**). HCV reference sequences were aligned by Clustal Omega algorithm [24] to determine the most conserved regions of the HCV genome for primer design. For the amplification of HCV RNA by RT-LAMP, specific primer sets for the simultaneous detection of HCV most prevalent genotypes 1 to 6 were designed using NEB LAMP Primer Design Tool (New England Biolabs Inc., USA) and PrimerExplorerV5 (Eiken Chemical CO., Japan) by targeting HCV 5'UTR region (**Supplementary Table S3**). HCDN primer set was previously described [13]. Oligonucleotide LAMP primers were synthesized by Macrogen (Korea).

2.2. RNA extraction

RNA was extracted and purified from serum samples using the automatized robot Chemagic 360/96 RodHead and Chemagic Viral DNA/RNA 300 Kit H96 purification kit (Chemagen Technology, Perkin Elmer), as recommended by the manufacturer. RNA was eluted in 60 µL elution buffer and stored at -80ºC until its use.

2.3. ssDNA synthesis

Recombinant plasmid pTNCC containing HCV DNA from TN strain [25] was used as a control. Plasmid DNA was purified following alkaline extraction procedures [26]. 5'-UTR region was amplified by conventional PCR using Taq polymerase as recommended by the manufacturer [Maxime PCR PreMix (i-Taq) (Intron Biotechnologies, Korea)]. To obtain ssDNA, an asymmetric

PCR was carried out using Taq polymerase and the forward primer. Amplified fragments were purified with silica membrane columns (NZYTech Spin Columns & Collection Tubes, NZYTech, Portugal).

2.4. Synthesis of *in vitro* transcripts for HCV GT5 and GT6

Synthetic fragments (600 nt length) covering the 5'UTR-Core region of HCV GT5 and GT6 were designed using the online software GeneArt (ThermoFisher). Fragments were synthesized and cloned by the manufacturer in a pUC vector including T7 promoter. *In vitro* transcription was performed with a T7 RNA polymerase (Thermofisher Scientific, USA), as recommended by the manufacturer.

2.5. Real time quantitative RT-PCR amplification

Before sample shipment, HCV viral load was determined with the Cobas® HCV assay on the cobas® 6800 system (Roche Diagnostics, Switzerland), and genotyped with the Abbott RealTime HCV genotype II assay (Abbott Laboratories, USA). To verify and assess the HCV viral load of serum samples upon arrival to our laboratory, purified viral RNA was analyzed by one-step real time RT-qPCR using Hepatitis C virus One-Step RT-PCR Kit (NZYTech, Portugal) as recommended by the manufacturer.

2.6. Conventional RT-PCR

To check viral RNA integrity, amplification of the 5'UTR region of HCV RNA was performed by conventional OneStep RT-PCR using the Maxime RT-PCR PreMix kit (Labotaq, Spain), as recommended by the manufacturer. Purified HCV RNA was amplified with forward primer 5'-GTATGAGTGTCGTACAGCCTC-3' and reverse primer 5'- TCATTCCCATATAGGGGCCAG-3'. The analysis of PCR products was performed by agarose gel electrophoresis in 1% agarose-TAE gels for 10–20 min at 100 V (Mupid-One Electrophoresis System). DNA was visualized under UV light by incubation with RedSafe dye (Intron Biotechnologies, Kyungki-Do, Korea).

2.7. RT-LAMP reaction optimization

Purified HCV RNA or serum lysates were amplified by RT-LAMP. To obtain serum lysates, serum samples were thoroughly mixed in a 1:1 ratio with BLBio lysis buffer and incubated for 10 minutes at room temperature. Purified RNA or serum lysates were directly amplified by RT-LAMP using WarmStart® Fluorescent LAMP/RT-LAMP Kit [with Uracil-DNA Glycosylase (UDG), New England Biolabs]. Reaction was performed as recommended by the manufacturer. Briefly, 12.5µL 2X WarmStart MasterMix was mixed with 5.75µL of a 5X mix of each primer set (5X stock concentration of each primer: F3/B3, 5µM; LF/LB, 10µM, FIP/BIP, 40µM), 0.25µL LAMP Fluorescent Dye, 5µL of lysate or purified RNA, and RNAse free water up to a final reaction volume of 25µL. To reduce amplification times, reaction mix was supplemented with 3% DMSO, 0.05mM DTT, and 0.8U/µL Ribonuclease inhibitor. To enable UDG enzyme activity, the reaction mix was prepared at room temperature (22-25°C) and maintained for 5 minutes during the sample lysis step. After sample addition, RT-LAMP reaction mix was incubated at 45ºC for 10 minutes for reverse transcription, followed by LAMP amplification at 68ºC for up to 50 minutes. Nuclease-free water was used instead of lysate/RNA as non-template control (NTC). Fluorescence signal was recorded in real time along DNA amplification in a QuantStudio™ 5 Real-Time PCR System (ThermoScientific, USA), and analyzed using QuantStudio Design and analysis software v2.6.0.

2.8. Determination of the limit of detection

HCV RNA purified from serum samples of each genotype (1 to 4) were used for this purpose. Viral load was determined by real time RT-qPCR, and subsequently used on the RT-LAMP assay immediately, so that the RNA would not undergo any additional freeze/thaw cycle. RNA was serially diluted to less than 1 copy per reaction, with each concentration was tested in triplicate.

The following equation was used to determine the viral load correspondence in samples subjected to lysis procedure:

$$Viral\ load\ (IU/mL) = \frac{Cp\ x\ D}{4.4\ x\ Vr}$$

Where:

- ***Cp***: RNA copies per RT-LAMP reaction
- ***D:*** dilution factor in lysis buffer (e.g. 1:1 ratio [sample:buffer] used in this study corresponds to dilution factor of 2)
- ***4.4***: equivalence factor of HCV RNA copies per IU [27]
- ***Vr***: sample volume added to RT-LAMP reaction

The correlation between RT-LAMP amplification time and viral load was determined by Spearman's rank correlation coefficient for each genotype (statistical significance at p-value < 0.05). Statistical software R (v 4.3.1) (http://www.r-project.org, RRID:SCR_001905) was used for statistical analysis.

3. Results

3.1. Detection of HCV RNA

HCV RNA purified from a first panel of 20 serum samples including genotypes 1, 2, 3 and 4 was amplified with HCV-LAMP primers sets HCGen or HCDN. RNA from HCV-GT3 samples was not detected with any of these primer sets, so HCGen and HCDN primer sets were further optimized for GT3 detection (HCGen-G3 and HCDN-G3). As depicted in **Fig. 1**, the amplification time was shortened by 27.07 minutes for HCV-GT1 and 23.96 minutes for HCV-GT3 using respective HCDN or HCDN-G3 primer sets, compared to the previous HCGen or HCGen-G3 sets. To enable pangenotypic HCV detection in a single-well reaction, LAMP primers were redesigned by replacing certain nucleosides with degenerate bases (HCDN-Deg set). However, amplification was unexpectedly delayed in 7 minutes for GT3 samples, and GT1, GT2, and GT4 positive samples were no longer detected. Therefore, we determined that detection of these HCV genotypes should be carried out in two independent RT-LAMP reactions using either HCDN or HCDN-G3 primer sets.

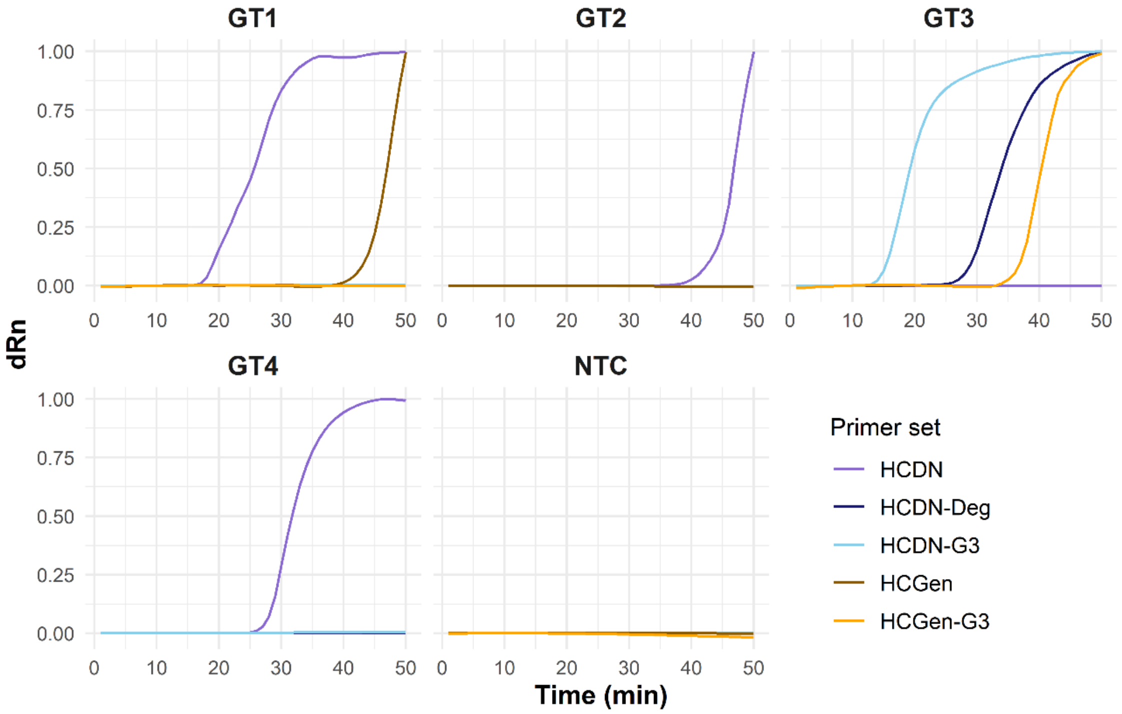


Fig. 1. Representative real time fluorescence RT-LAMP amplification plot comparing primer set yields after 50 minutes incubation with extracted RNA from HCV-GT1, -GT2, -GT3 or -GT4 samples. NTC: non-template control.

3.2. Enhancing HCV detection through RT-LAMP using lysed serum samples and a novel 9-primer set

Conventional pre-processing RNA extraction methods increase both time and expenses of molecular diagnostic tests, that should be preferably avoided at POC. For this purpose, a panel of 20-samples was lysed upon treatment with BLBio buffer before their analysis by RT-LAMP. As shown in **Fig. 2**, amplification was delayed in about 15 min when using lysed samples compared to purified RNA. Real-time fluorescence signal recording reached the plateau-phase after about 40 minutes reaction time. Noteworthy, this treatment had a more pronounced effect on those samples with low viral load. Indeed, HCV GT4 samples (PL14, and PL15), and HCV-GT2 (PL20) were all undetectable. To facilitate sample distinction in overlapped amplification plots, individual plots are available in **Supplementary Fig. S1**.

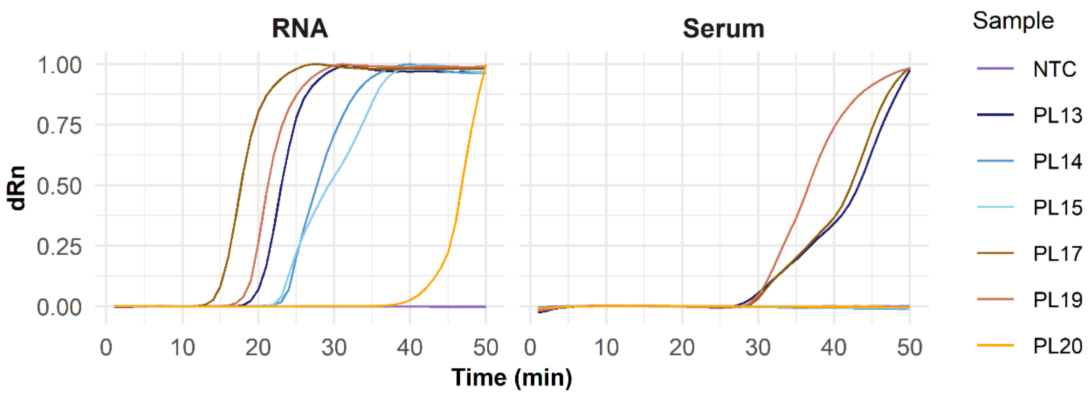


Fig. 2. Representative real time fluorescence RT-LAMP amplification plot using HCDN primer set comparing amplification time with extracted RNA samples (left panel) or lysed serum samples (right panel). NTC: non-template control; samples PL13, PL14, PL15, PL17, PL19 (GT4) and PL20 (GT2).

To mitigate the impact of genotype on LAMP amplification time, HCDN primers were redesigned to create a new set of pangenotypic primers by replacing certain nucleotides with inosine (**Supplementary Table S3**). As shown in **Fig. 3a**, the best LAMP performance was displayed by primer mixes C and E (**Supplementary Table S4**), which efficiently detected GT1, GT2, and GT4 genotypes in lysed samples. However, results showed that amplification time was still delayed for GT2 samples. Thus, to improve their RT-LAMP amplification, specific GT2 primers were included in the mixture (DN2_F3-G2, DN2_FIP-G2, and DN2_LF-G2) together with HCDN primers. Additionally, DN2_LF primer was substituted by DN2_LF_1. Therefore, the new LAMP-primer set for GT1, GT1 and GT4 detection includes a total of nine different primers (HCG124 set, **Supplementary Table S5**). As depicted in **Fig. 3b**, this HCG124 primer set led to a notable decrease of 13.90 minutes in amplification time for HCV GT2, maintaining the yields for GT1 and GT4 unchanged. To facilitate sample distinction, individual amplification plots are available in **Supplementary Fig. S2**.

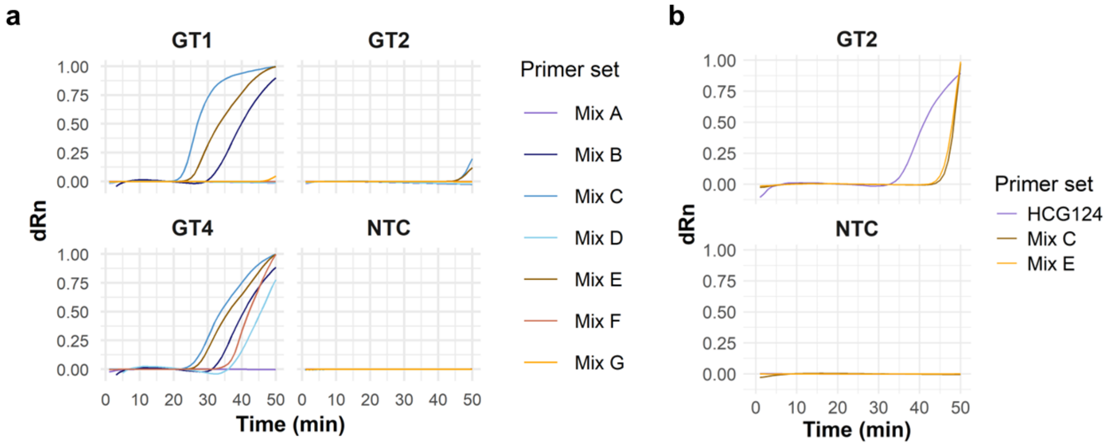


Fig. 3. Representative real time fluorescence RT-LAMP amplification plot comparing: (a) primer combinations for detection of both GT1, GT2 and GT4 from lysed serum samples, using Mix A, B, C, D, E, F, G; (b) primer sets Mix C and E, and new 9-primer set HCG124, for the detection of GT2 serum samples. NTC: non-template control.

Due to the unavailability of serum samples from genotypes 5 and 6, RT-LAMP assay was validated for these genotypes using *in vitro* transcription of their 5' UTR-Core region. Both genotypes were also successfully amplified with the HCG124 LAMP-primer set (**Supplementary Fig. S3**).

3.3. Limit of detection of the HCV RT-LAMP assay

To determine the LOD of the HCV RT-LAMP assay, serum samples with the highest reported viral load were thawed and their purified RNA was quantified by RT-qPCR, then serially diluted 1/10 up to less than 1 copy per RT-LAMP reaction (**Table 1**). Each dilution was tested in triplicates. Results revealed a limit of detection of about 10-20 copies/reaction for GT1, GT2 and GT4, amplified with primer set HCG124, and about 16 copies/reaction for GT3, with HCDN-G3 primer set. RT-LAMP amplification plots are available in the Supplementary Data (**Supplementary Fig. S4**).

Table 1. Limit of detection of the HCV RT-LAMP assay with primer sets HCG124 and HCDN-G3. The amplification time is presented as "mean (standard deviation)". Last quantity detected by RT-LAMP is highlighted in bold.

Genotype	Primer set	Quantity (copies/rxn)	RT-LAMP result	n/N	Amplification time (min)
1	HCG124	5000	Positive	3/3	12.60 (0.07)
1	HCG124	500	Positive	3/3	14.73 (0.86)
1	HCG124	50	Positive	3/3	16.85 (2.19)
1	HCG124	25	Positive	3/3	22.29 (1.44)
1	HCG124	**12.5**	**Positive**	**3/3**	**22.05 (2.93)**
1	HCG124	5	Negative		
1	HCG124	0.5	Negative		
2	HCG124	1300	Positive	3/3	20.73 (0.93)
2	HCG124	650	Positive	3/3	22.16 (0.49)
2	HCG124	320	Positive	3/3	24.54 (1.03)
2	HCG124	160	Positive	3/3	26.08 (1.71)
2	HCG124	40	Positive	3/3	28.06 (2.18)
2	HCG124	**10**	**Positive**	**2/3**	**27.08 (5.26)**
2	HCG124	2.5	Negative		
3	HCDN-G3	1600	Positive	3/3	12.06 (1.30)
3	HCDN-G3	160	Positive	3/3	13.63 (0.64)
3	HCDN-G3	**16**	**Positive**	**2/3**	**22.37 (11.41)**
3	HCDN-G3	1.6	Negative		
3	HCDN-G3	0.16	Negative		
4	HCG124	200	Positive	3/3	19.49 (1.01)
4	HCG124	**20**	**Positive**	**3/3**	**23.77 (4.74)**
4	HCG124	2	Negative		
4	HCG124	0.2	Negative		

Considering the dilution factor of serum in BLBio buffer for its lysis, and an equivalence of 4.4 HCV RNA copies per IU [27], the RT-LAMP assay LOD was 3.26 ± 0.20 $\log_{10}$ IU/mL in blood samples for HCV genotypes 1 to 4.

Regarding RT-LAMP amplification time, GT1 and GT3 RNAs showed lower amplification times compared to GT4 for equivalent viral loads. Moreover, we found that viral load was negatively associated with RT-LAMP amplification time, as determined by their Spearman's correlation coefficient (r_S): GT1, $r_S = -0.91$, $p < 0.001$; GT2, $r_S = -0.83$, $p < 0.001$; GT3, $r_S = -0.79$, $p = 0.019$; GT4, $r_S = -0.29$, $p = 0.573$. However, this association was not significant for GT4.

3.4. Analytical and diagnostic performance of the HCV RT-LAMP system

Clinical validation was performed using the most optimized conditions for HCV detection with a full serum sample panel (116 HCV-positive and 38 HCV-negative samples). A total of 116 serum lysates positive for HCV were analyzed using the HCVG124 or HCDN-G3 primer set, according to their genotype. HCV RNA was detected in 109 out of 116 serum samples (93.97% [IC95% 88.00 – 97.50]), corresponding with a diagnostic sensitivity of 90.28% for GT1 (n = 65/72); 100% for GT2 (n = 6/6); 100% for GT3 (n = 20/20) and 100% for GT4 (n = 18/18).

The diagnostic specificity of the RT-LAMP assay was assessed using 38 HCV-negative serum samples of which 27 tested positive for HIV. The RT-LAMP for HCV demonstrated 100% (IC95% 90.70 – 100) specificity as none of these samples were successfully amplified.

Purified viral RNA from undetected RT-LAMP samples was quantified by RT-qPCR. All those HCV positive samples by RT-qPCR but not detected by RT-LAMP had a viral load under 1,015.00 IU/mL (PLA3a, PLB5a, PLB6b, PLC6b, PLD6b, PLE5b, **Supplementary Table S1**), except sample PLB7a (12,306.93 IU/mL) likely degraded as PCR amplification of its 5'UTR – Core region was unsuccessful (**Supplementary Fig. S5**). However, samples with an intermediate viral load (3.00 – 4.00 $\log_{10}$ IU/mL range) were readily detected.

3.5. Validation of the HCV RT-LAMP assay from blood samples

To determine the potential inhibitory effect on the RT-LAMP reaction by residual haemoglobin in haemolyzed blood samples, an HCV-negative blood sample was mixed with HCV-positive serum (1:1 ratio) and directly lysed in BLBio buffer. Additionally, 1:2 serial dilutions of two HCV-negative blood samples partially haemolyzed were each spiked with 1ng of HCV ssDNA (GT1a). Then, their serum fraction was subjected to lysis in BLBio buffer. Results are summarized in **Table 2** and showed that the haemolysis level directly correlated with the amplification time. Indeed, the greater the degree of sample haemolysis, the more delayed amplification. Despite of that, all the HCV-spiked samples were readily detected by RT-LAMP (**Table 2**).

Table 2. Amplification times, presented as “mean (standard deviation)”, after RT-LAMP reaction using HCG124 primer set. BL and BH, partially haemolyzed HCV-negative blood samples serially diluted (2^n) and spiked with HCV ssDNA; BS, HCV-negative blood sample mixed with HCV-positive serum sample; S, HCV-positive serum sample; PC, HCV ssDNA as positive control; NTC, non-template control.

Sample	Amplification time (min)
BL 2^{-1}	18.46 (1.49)
BL 2^{-2}	14.90 (0.70)
BL 2^{-3}	14.72 (1.20)
BH 2^{-1}	21.48 (0.32)
BH 2^{-2}	18.18 (0.20)
BH 2^{-3}	15.56 (0.19)
BS	29.69 (1.74)
S	33.59 (1.39)
PC	15.54
NTC	UD

To evaluate the diagnostic sensitivity of RT-LAMP on blood samples, 13 HCV-positive fresh blood samples were analyzed. Our protocol for serum separation from 50 µL of whole capillary blood was compared with standard serum obtained from 9 mL venipuncture samples used as control. Once their serum was lysed with BLBio buffer, those samples with unknown genotype were tested in parallel with either HCG124 or HCDN-G3 primer sets. As shown in **Table 3**, 11 out of 13 samples were detected by RT-LAMP assay and all of them tested positive for primer set HCG124, indicative for genotypes GT1, GT2 and GT4. Significantly, two samples remained undetected due to their low viral load of 533 IU/mL and 159 IU/mL. Remarkably, these samples were also not detected in the serum lysates from venipuncture. Of note, all assays included a positive control (HCV sample previously detected by RT-LAMP) and a non-template control to ensure proper viral particle lysis and nucleic acid amplification, while discarding any reaction contamination.

Table 3. Characteristics of the HCV positive fresh blood samples included in this study, indicating: HCV genotype/subtype, when available; viral load reported by the hospital; RT-LAMP amplification time with serum samples from a finger prick volume (50 µL of whole blood centrifuged for 2 min) or venipuncture (9 mL of whole blood in serum separation tubes centrifuged for 10 min). "NA": not available; "UD": undetected.

Sample	Genotype	Viral load (IU/mL)	RT-LAMP amplification time (min)	
			Finger prick	Venipuncture
S01	1a	1,730,000.00	40.10	34.90
S02	1b	860,000.00	29.69	NA
S03	1/2/4	9,410,000.00	34.97	31.65
S04	1b	198,000.00	38.28	42.45
S05	1/2/4	533.00	UD	UD
S06	1/2/4	4,500,000.00	19.76	19.64
S07	1a	1,010,000.00	18.99	24.74
S09	1/2/4	2,150,000.00	27.12	25.70
S10	4	384,000.00	41.82	37.61
S11	1a	975,000.00	17.19	23.94
S12	1a	243,000.00	22.33	26.85
S13	1a	159.00	UD	UD
S14	1a	3,270,000.00	25.14	23.88

4. Discussion

RT-LAMP has emerged as suitable alternative to currently viral molecular detection methods mainly based on qPCR procedures, which are labour-intense, and require expensive equipment as well as specific trained personnel. Amplification of nucleic acids at isothermal conditions reduces equipment requirements, as well as facilitates the development of POC devices that could be easily implemented in LMIC, reducing test costs and facilitating its rapid readout.

In this work, we have developed an RT-LAMP system for molecular detection of HCV-RNA directly from lysed serum samples. The described 10-minute lysis method demonstrates advantages over conventional RNA extraction and purification procedures [28], thus reducing time-consuming sample preparation steps, leading to shorten test duration and lower costs as result. To reduce sample handling, new automated or semiautomated RNA extraction methods have been developed [29, 30]. However, although automated extraction methods reduce contamination risk, they are not adapted for implementation in low-resource settings. Other simple and rapid RNA extraction methods have been studied for HCV, such as silica-based or magnetic-beads-based methods, and sample lysis by boiling in distilled water or in a commercial lysis buffer. Both Hongjaisee S et al [31] and Pauly et al [32] concluded that RNA purification using magnetic beads is the preferred one for HCV RNA extraction from plasma or whole blood samples, respectively. Viral particles lysis in nuclease-free water, commercial buffers, or by heat shock often leads to low RNA purity, which may result in delayed or unsuccessful detection by nucleic acid amplification tests (NAAT) [32, 33]. In the present study, a new homemade lysis buffer named BLBio was tested. Results showed that HCV RNA is readily extracted from serum or blood samples upon 10-minutes lysis at room temperature. Of note, BLBio buffer composition (patent pending) did not inhibit RT-LAMP reaction, making it highly suitable for a rapid RNA analysis on-site. Compared to magnetic beads-based methods, treatment with BLBio buffer takes less hands-on time, since it does not require any additional washing or elution step, thus reducing the risk of contamination, particularly crucial in POC settings.

Considering that 5’-UTR is one of the most conserved regions in the HCV genome together with 3’-UTR [34], it has been largely the target for molecular diagnosis of HCV. In fact, Nyan and Swinson [13] previously described three LAMP-primers sets for detection of HCV genotypes 1 to 6. In this research, our new LAMP-primer set (HCGen) was compared to this previously outlined by Nyan and Swinson (named HCDN). Despite both primer sets targeting the 5’-UTR, HCDN primer set showed higher amplification efficiency but, unexpectedly, it proved incapable to detect either HCV GT2 or GT3 samples in our hands. Moreover, it showed a very low sensitivity for HCV GT4. Of note, these authors used a quantified Armored RNA standard for HCV2ac instead of serum samples. Moreover, due to the high tendency of HCV 5’-UTR to form stable secondary structures [34], it is possible that HCV RNA has a more compacted structure and then less availability in the viral particle than synthetic RNA. Nevertheless, this HCDN primer set could be only validated with HCV GT1 samples.

Despite being the most conserved region in HCV genome, there is not an 100% identity at nucleotide level at the 5’ UTR region among the different HCV genotypes, hardening the design of a pangenotypic set of LAMP-primers. In fact, HCV GT3 genome has considerable nucleotide differences compared to GT1, GT2 and GT4 in this region. Therefore, only a 100% GT3-complementary primer set showed robust RT-LAMP amplification for HCV-GT3 samples. To optimize HCV GT2 and GT4 detection we designed a 9-primer LAMP set, named HCG124. Notably, as far as we are aware, this represents the first time of employing 9 primers for RT-LAMP. The results confirmed that these 3 additional primers not only did not impede HCV detection by RT-LAMP but also enhanced its diagnostic and analytical sensitivity. Since it was only possible to obtain serum samples from genotypes 1 to 4 due to their higher prevalence in Europe, the primer set HCG124 was also validated for GT5 and GT6 by using *in vitro* transcribed fragments. Therefore, our results validated the developed RT-LAMP assay for detection of HCV genotypes 1 to 6. In fact, it demonstrated a diagnostic sensitivity of 93.97%, specificity of 100%, and an analytical sensitivity of 10-20 copies per RT-LAMP reaction, corresponding with a viral load of 3.26 $\log_{10}$ IU/mL on blood. A recent new study has published a novel HCV LAMP-primer set targeting also 5’ UTR region for genotypes 1- 6. The novel universal primers detect up to 10 copies/reaction of synthetic RNA [19]. Validation of this primer set with a clinical sample panel would be of interest for future studies.

It is worth mentioning that, without considering the undetected and partially degraded PLB7a sample, our test parameters would be even better. The remaining undetectable samples showed viral loads under 3.00 $\log_{10}$ IU/mL as demonstrated by RT-qPCR, viral titers that are notably atypical in individuals who have not started any HCV treatment. Besides, it is noteworthy that serum samples had undergone a freeze/thaw cycle before its quantification by RT-qPCR and subsequent RT-LAMP analysis, a fact that could affect the viral RNA stability.

RT-LAMP is not a quantitative methodology but, however, the results evidenced a negative correlation between viral load and RT-LAMP amplification time. The absence of significant association for HCV-GT4 RNA was probably due to the low baseline viral load, resulting in insufficient data to perform a proper statistical analysis. This correlation was not so noticeable in fresh blood samples. This discrepancy can be attributed to the different storage time and conditions that some samples (i.e. S01 and S10) were subjected to during their shipment, which in some cases exceeded 3 hours.

Overall, the test parameters show a global diagnostic test accuracy for HCV detection higher than other studies, where diagnostic sensitivity is under 91.5% [13, 17], specificity does not reach 90% for GT2a and GT3 [15], or the assay LOD is higher than 5.00 $\log_{10}$ IU/mL [16]. With a similar analytical sensitivity, a microfluidic test based on RT-LAMP technology was designed by Sharma et al. [22] for HCV RNA detection, with an LOD of 500 virions/mL. However, this technique has not been validated with HCV-positive samples, but just with plasma samples spiked with JFH1 isolate virions (GT2a). Thus, their results cannot be compared with the present study using whole blood samples from people with HCV. In another recent study, Pauly et al [32] achieved 94% sensitivity and an LOD of 2.8 $\log_{10}$ IU/mL for HCV genotype 1b, using a magnetic bead-based purification method coupled to RT-LAMP detection. However, although this approach shows great analytical and diagnostic performance in an overall reaction time of 50 min, it still needs higher hands-on time compared to our fast and simple lysis procedure. In terms of procedural complexity, the lysis in BLBio buffer would parallel the authors' heat shock strategy, but with a noticeably higher performance compared to their sensitivity of merely 70%. Besides, as the authors state, validation with real whole blood samples and not only spiked blood would be necessary to confirm the test parameters.

Using LAMP-primer sets HCG124 and HCDN-G3 in a two-well reaction set-up, we have detected most prevalent HCV genotypes 1 to 4. In fact, 108 out of 109 HCV-positive serum samples analyzed so far with a viral load above 1,000.00 IU/mL (99.09%), and 11 out of 13 blood samples were detected, but missing only samples under 550 IU/mL. Although this assay does still not reach RT-qPCR sensitivity levels of the *in vitro* diagnostics tests (12 – 30 IU/mL with Abbott Real*Time* HCV assay or 9.2 – 15.3 IU/mL with Cobas® HCV test), its analytical sensitivity accomplishes EASL recommendations of an LOD > 1,000 IU/mL for HCV RNA qualitative detection in LMIC or specific settings in high-income countries [23], where HCV prevalence and underdiagnosis is higher. As well, WHO established that an LOD of 3,000 IU/mL is acceptable for POC diagnostic systems with the aim of improving population access to HCV diagnosis [7]. Even though missing people with HCV viral loads under 1,000 IU/mL is still significant, low viral titters are associated with acute HCV infection, that in 15-45% of patients resolve spontaneously, otherwise rapidly increases their viral loads. For this reason, the developed test in this study would be valuable for its implementation on screening strategies at POC, then diverting to clinic patients who tested positive to confirm diagnosis and start an effective treatment for HCV.

5. Limitations

We should bear in mind some limitations of this study. First, our sample panel only included genotypes 1 to 4, so our RT-LAMP assay could be fully validated for the most worldwide prevalent genotypes 1 to 6 only by using *in vitro* transcripts. Second, the distribution of genotypes 1 to 4 was not homogeneous in our cohort, with a larger representation of GT1 due to its high prevalence in Europe. Further studies in areas with a different HCV genotype distribution should be carried out to increase sample size of HCV genotypes 2 to 6. Thirdly, a single-well reaction would be preferred, due to the reduced sample handling and test costs implied. However, since sensitivity decreased when degenerate bases were added to primer sequences, the HCV RT-LAMP assay proposed in the present study has been designed as a two-well reaction using either HCDN or HCDN-G3 primer sets in independent reactions. Nevertheless, the estimated production cost for an HCV RT-LAMP assay is about 6€ per sample, notably cheaper than current HCV molecular diagnostic tests. Moreover, this procedure only requires minimal and basic laboratory equipment as a mini-spin centrifuge for immediate serum separation, a thermostatic bath, and a portable fluorescent reader for RT-LAMP reaction like those portable systems already described for quantitative PCR analysis of *Mycobacterium tuberculosis* [35] or for Malaria diagnosis by LAMP [36-38]. Even though portable fluorescent readers are becoming increasingly available at lower costs, cheaper POC visualization methods such as colorimetric sensors based on gold-nanoparticles [39] or lateral flow strips should also be considered, which can be coupled to

CRISPR/Cas12a sensing [21], or based in the conventional streptavidin-biotin interaction, that may even allow multiplex diagnosis [19].

Finally, a limited fresh-blood sample panel was available for RT-LAMP validation. This study was performed in Madrid (Spain) where, fortunately, thanks to the highly effective and extensive therapeutic scope of DAAs, the access to individuals with an active HCV infection is too limited. Thus, it becomes a challenge to obtain fresh blood samples from individuals that currently have an HCV infection in Spain. However, there was a strong agreement between the results on fresh blood samples and those obtained using the serum sample panel, as confirmed by those undetected samples, all with a LOD bellow 3.00 $\log_{10}$ IU/mL. This is reasonable considering that the proposed POC design of the RT-LAMP assay includes a serum separation step, which can be easily achieved from a small blood sample using a mini-spin centrifuge. In low resource settings, where active HCV infection is still a problem, capillary punction is the preferred option for sample obtention, since it is a less invasive and safer procedure than venipuncture. In fact, capillary punction easily gets a blood sample of up to 250 µL [40], and enough serum volume for HCV RT-LAMP assay. Indeed, in this work serum was obtained from less than 50 µL of blood and HCV particles were lysed for just 10 min before RT-LAMP assay in less than 40 minutes (reverse transcription for 10 minutes and LAMP amplification time to positivity of about 30 minutes). As demonstrated, no remarkable differences were observed in RT-LAMP amplification time between the serum fraction obtained from a finger prick or standard venipuncture. Thus, the test accuracy as determined with the serum sample panel would also be applied to whole capillary blood.

Moreover, partial sample haemolysis has shown to have a slightly inhibitory effect. Because the intended use of this test is as a rapid test on-site, RT-LAMP reaction would be performed immediately after sampling. However, if this is not possible, serum separation should be preferably performed before 2h after blood sampling.

6. Conclusion

In conclusion, we developed a low-cost, high sensitive, specific, and rapid RT-LAMP assay for HCV detection, delivering results in less than 50 minutes since sample collection. The new system would help to improve HCV screening strategies among hard-to-reach populations at POC, taking a step towards viral hepatitis C elimination.

Abbreviations

DAAs	Direct-acting antivirals
EASL	European Association for the Study of the Liver
HCV	Hepatitis C virus
HCV cAg	HCV core antigen
HNB	Hydroxynapftol blue
LMIC	Low- and middle-income countries
LOD	Limit of detection
NAAT	Nucleic acid amplification tests
POC	Point-of-care
PWID	People who inject drugs
RT-LAMP	Reverse Transcription loop-mediated isothermal amplification
RT-qPCR	Reverse Transcription – real time Polymerase Chain Reaction
ssDNA	Single stranded DNA
WHO	World Health Organisation

DECLARATIONS

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Competing interests

The authors declare no competing interests.

Conflict of interest

The authors declare that they have no conflict of interest.

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Ethics approval statement

This study was reviewed and approved by the Research Ethics and Scientific Committees in all institutions involved [Instituto de Salud Carlos III (CEI PI 25_2018-v2), Hospital La Paz (PI-

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Patient consent statement

The patients/participants provided their written informed consent to participate in this study.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

S.A.L.: Investigation, Validation, Data Curation, Formal Analysis, Writing – Original Draft; C.Y.N: Investigation, Validation, Data Curation; P.C.B.: Investigation, Validation; V.L.A. and C.C.B.: Investigation; L.M.C. and I.G.S.: Resources; V.B. and R.M.: Conceptualization, Supervision and Writing – review & editing. All authors read and approved the final manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2024.10.008.

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