Evaluation of A Reverse Transcriptase (Rt) Loop Mediated Isothermal Amplification Assay for Detection of Hepatitis C Genotypes 1-4 Viruses Under Limited Logistical Conditions

Abstract

The loop-amplification mediated isothermal amplification (LAMP) presents characteristics that overcome the limitations associated with the current nucleic acid based technologies (NATs), hindering their use in the low resource settings, hence overreliance on serological assays which miss hepatitis C virus (HCV) during the long seroconversion period. The LAMP assay would be ideal for early detection of HCV in routine diagnostics and blood transfusion setups in such settings, drastically reducing transmission related transfusion. This study validated and tested a reverse transcriptase-LAMP for HCV detection under limited logistical conditions in a low resource setting.

Under stringent laboratory conditions, analytical sensitivity and reproducibility were performed using a panel of HCV positive plasma of genotypes 1a, 1b, mixed 1a/1b, 2b, 3a and 4. Cell culture supernatants of HIV-1 B and plasma samples for Hepatitis B virus were used for specificity testing. Upto 227 plasma including 70 (40 RNA positive and 30 negative) from German patients and 157 (43 RNA positive and 114 negative) from Kenyan patients were tested. Kenyan samples were obtained from 121 sero-positive plasma screened from 1121 participants of various cohorts in Kenya.

Although LAMP detected upto 102 IU/mL for genotypes 1a,1b and 2b, a lower detection threshold was established at 103 IU/mL. Overall sensitivity was 94% (PPV 98%) and specificity was 98% (NPV 96%) for RT-LAMP. Sub optimal detection was noted for genotypes 2b, 3a and 4. Sequence analysis revealed mismatches affecting stringency of primer binding at the F1, B1 and the LB primer targets.

RT-LAMP shows potential for early HCV diagnosis and screening in low resource settings. Its robustness is however genotype dependent and can be enhanced by designing primers targeting circulating and suspected genotypes. More studies should be done on the possibility of designing multiplex RT-LAMP primers to capture a wide variety of genotypes. The assay remains simple, rapid, and cost effective for nucleic acid detection and is ideal for use in the limited resource settings.

Keywords: RT-LAMP; Hepatitis C virus (HCV); Kenya; Limited logistical condition

Abbreviations: LAMP: Loop-Amplification Mediated Isothermal Amplification; NATs: Nucleic Acid Based Technologies; HCV: Hepatitis C Virus; HNB: Hydroxynaphthol Blue; IDUs: Injecting Drug Users; cDNA: Complementary DNA; dNTP: Desoxynucleotide Triphosphates; DRC: Democratic Republic of Congo

Introduction

Hepatitis C Virus remains a major public health concern in many parts of the world due to its long sero-conversion period presenting it as a high risk in transfusion related transmission. The transfusion related transmission still remains a concern in many developing countries and resource related settings [1] which are highly dependent on serological tests for diagnosis and routine screening of blood meant for transfusion. Although 15-20% of the infected clear the virus spontaneously, 80-85% of those infected are thought to progress to chronic liver infections characterized by liver cirrhosis and hepatocellular carcinoma [2]. Genetic diversity of HCV has been thought to influence the treatment outcomes [3] and six genotypes denoted as genotypes 1-6 are currently known [4]. The geographical distribution of these genotypes show that genotypes 1 - 3 circulate mainly in the United States of America (USA) and Europe, genotypes 4 and 5 are found in some parts of Africa mainly in Egypt and the Democratic Republic of Congo (DRC) for genotype 4 and South Africa for genotype 5.
with genotype 6 so far reported only in Hongkong [4]. Genotypes specifically associated with Africa are determined as genotype 1a, 2b and 4, all of which have been reported in Kenya [5,6].

The long window period associated with the HCV virus is a major reason for its poor diagnosis in many laboratories in the developing world and the low resource settings. Many laboratories in the latter regions mainly rely on detection of antigen or antibodies to the virus [7-9]. A number of these serological assays have even been designed as combination assays intended to reduce the window periods [10,11]. However, some studies have demonstrated the inefficiency of the same combination assays to detect two proteins with a similar degree of sensitivity in a single run [12] as claimed by the manufacturers. The gap occasioned by the long seroconversion period leaves only the Nucleic Acid based Tests (NATs) as reliable tools for HCV diagnosis early within the window period. The limitations of the conventional and widely used nucleic acid based technique (NATs) of high costs, long time to results, sophistication, need for specialization among others have been well elucidated [13]. Since its development about 15 years ago [14], loop-mediated isothermal amplification (LAMP) has presented characteristics that are ideal as an alternative for use in the limited resource settings. The characteristics of simplicity, cost-effectiveness and rapidity have attracted the assay for evaluation of LAMP for detection of a number of pathogenic microorganisms. The LAMP assay uses a set of primers targeting six arbitrarily designated regions F1, B1, FIP, BIP, LF and LB under isothermal conditions (normally a temperature between 60-65°C). The inclusion of *Bacillus stearothermophilus* (Bst) DNA Polymerase with strand displacement capability ensures loop formation which become the targets of amplification resulting in the generation of high amounts of magnesium pyrophosphate (Mg$_{2+}$P$_{3-}$) deposits. These Mg$_{2+}$P$_{3-}$ deposits then become the targets of detection either visually by use of a color detection dye, or by turbidimetric reading or detection in real time by use of a turbidimeter [15].

With addition of a reverse transcriptase enzyme RT-LAMP has been evaluated for a number of viruses including for Hepatitis C virus detection [16-18]. However, these studies have been carried out under stringent laboratory conditions with clinical samples mainly from the Asian continent. No study has so far been reported on the efficacy of HCV RT-LAMP under limited logistical conditions in sub-Saharan Africa. This study therefore aimed at evaluating the RT-LAMP in detecting HCV under stringent in Europe (Germany) and comparing the results with those performed under limited logistical conditions in sub-Saharan Africa (Kenya).

### Materials

**Comparative assays**

Abbott m2000rt system (Abbott Laboratories, North Chicago, IL, USA) served as a comparative assay for samples analyzed in Germany, whereas multiplex RT-PCR was the only available comparative assay for Kenyan samples tested in Kenya. The Abbott m2000rt system is a commercially available assay used to detect and measure the amount of HCV nucleic acid particles in real time using fluorescent labeled oligonucleotide probes. The fluorescent signals are always proportional to the log of the virus RNA measured in International Units per milliliter (IU/mL).

**Nucleic acid extraction and LAMP amplification**

**Reagents/equipment:** Roche MagNAPure (Roche Diagnostics, Mannheim, Germany) and High Pure Viral Nucleic Acid (cat. no. 1185874401; Roche Diagnostics, Unterhaching, Germany) kits were used for extraction of viral nucleic acid from the plasma samples. The MAST Isoplex™ RNA Amplification kit (Mast Diagnostica, Reinfeld, Germany) was used for LAMP assay amplification. The RNA amplification kit contained 8 U/µL Bst polymerase enzyme, 20 U/µL reverse transcriptase enzyme (Roche Transcripter reverse transcriptase), water, detection dyes (V13 indicator dye for real time turbidimetric detection and hydroxynaphthol blue (HNB) for visual color detection) and an already constituted 2×reaction mix (RM). The reaction mix contained dNTPs (2.8 mM), KCl (20 mM), (NH$_4$)$_2$SO$_4$ (20 mM), Tris buffer (40 mM, pH 8.8), betaine (1600 mM) and MgSO$_4$ (16 mM). For real time turbidimetric readings the Loop Amp real-time turbidimeter (LA-200m; Teramecs, Kyoto, Japan) was used.

**Reaction primers**

All the primers used targeted the 5’ UTR conserved gene (Accession number: Q418245). Whereas the LAMP primers used had previously been described [16], in-house primers with a few modifications to include wobble bases were generated and used during the RT-PCR amplification. These primers for RT PCR were designated as outer primers; HCVN 01- GCC GAC ACT CCA CCA TRR A (forward), HCVN 02 – GTG CAC GGT CTA CTA GAC C (reverse), HCVN 08 -TAC TCA CCG GGT CCG CAG A (Reverse). Inner primers HCVN 03 – CAC TCC CCT GTG AGG AAC T – 3’ (forward), HCVN 04 - CCC GGG GCA CTC GCA AGG A (Reverse) with HCVN 02 and HCVN 08 being used during reverse transcription to generate HCV cDNA. All primers were synthesized and supplied by Ella Biotech (Ella Biotech GmbH, Munich - Germany).

**Hepatitis C samples**

**Validation panel:** An in-house sample panel of known HCV positive plasma were obtained from the Max von Pettenkofer Institute in Munich-Germany. The plasma samples with HCV viral loads V1529272 (1.4 × 10$^6$ IU/mL), V1529317 (2.3 × 10$^6$ IU/mL), V15103800 (6.1 × 10$^5$ IU/mL), V1529319 (5.5 × 10$^5$ IU/mL), V0743586 (1.6 × 10$^6$ IU/mL) representing genotypes 1a, 1b, mixed 1a/1b, 2b, 3a and 4 respectively were used during the validation testing of the RT-LAMP.

**Clinical samples from patients:** A total of 227 plasma samples including 70 (40 RNA positive and 30 negative) from German patients and 157 (43 RNA positive and 114 negative) from Kenyan patients were tested. The Kenyan RNA positive plasma were part of the 121 sero-positive samples screened from a total of 1121 persons of various cohorts including injecting drug users (IDUs), female sex workers, blood donors and patients attending various outpatient clinics in Nairobi Kenya.
Ethical clearance: For Kenyan samples, ethical clearance was obtained from the Scientific and Ethical Review Unit (SERU) of the Kenya Medical Research Institute (KEMRI) in Nairobi (protocol number KEMRI/SERU/CVR/008/3179), while the German samples were used with permission from the routine diagnostic laboratory of the Max von Pettenkofer-Institute (MVP) in Munich.

Methods

Generation of quantitation panel for correlation analysis and test samples

Total nucleic acid was extracted from plasma sample panels of genotypes 1a, 1b, mixed 1a/1b, 2b, 3a and 4 using an automated system, Roche MagNApure (Roche Diagnostics, Mannheim, Germany), according to manufacturer’s instructions. Tenfold dilutions were made from the extracted materials and used to constitute quantitation panels for correlation analysis. The dilutions were further maintained as positive control samples during the testing of clinical samples. Negative controls included known HIV-1 genotype B (MVP 899-87) cell culture supernatant, ultra-pure water (H$_2$O), 0.9% sodium chloride (NaCl) and poly (A) from High Pure Viral Nucleic Acid.

Processing of samples by Abbott Real Time

The sample panel as well as clinical samples tested at the Max von Pettenkofer institute were first amplified and quantified by Abbott Real Time assay (http://www.abbottmolecular.com/static/cms/workspace/pdfs/US/51-602146R6.pdf as described by the manufacturer (Abbott Laboratories, North Chicago, IL, USA). The starting sample volume for automatic nucleic acid extraction was 1 mL and where the appropriate volume could not be attained, samples were filled up with 0.9% NaCl to 1 mL and then the material processed.

Testing samples by RT-PCR

Reverse transcription: Purified RNA extracts were first reverse transcribed to generate complementary DNA (cDNA) using reverse primers HCVN 02 and HCVN 08. Briefly 10 µM of each reverse primer, 4 µL of 5x buffer (100 µM Tris-HCL, 250 µM KCL, pH 8.4), 2 µL DTT (0.1 M), 2 µL desoxynucleotide triphosphates (dNTP) (5 mM) and 40U reverse transcriptase enzyme, 0.5 µL of Bst polymerase enzyme, 0.5 µL reverse transcriptase, 1 µL of the detection dye were added and topped up with water for a volume of 20 µL. A total of 5 µL of the RNA template was added to the 20 µL master mix so as to make a total reaction volume of 25 µL and the reaction mix incubated at 63°C for 60 min in a LoopAmp real-time turbidimeter (LA-200 m; Teramecs, Kyoto, Japan). The threshold value (Abs) equivalent of an optical density above 0.1 by the turbidimeter indicated a positive detection.

Nested PCR

The PCR reaction was performed in a total volume of 50 µL (for first PCR amplification) containing 31.7 µL PCR water, 5 µL reaction buffer, 2 µL of dNTP, 3µL of each of the primers HCVN 01 and HCVN 02, 0.3 µL of taq Polymerase and 5 µL of cDNA to amplify a 296-bp fragment. In the second round of PCR 3 µL of first round PCR product was added to 47 µL of the PCR mixture similar to the first but with the inner primers HCVN 03 and HCVN 04 for 30 cycles in order to amplify a 253-bp fragment as visualized on a 2% agarose gel.

Processing of samples for testing by RT-LAMP assay

Extraction of RNA from plasma: Viral RNA was extracted using High Pure Viral Nucleic Acid Kit (cat. no. 11858874001; Roche Diagnostics, Untersching, Germany) according to the manufacturer’s instructions. In this method 200µL of each sample were used as the starting volume for the extraction process and the RNA was eluted in 50µL of elution buffer supplied in the extraction kit (https://cssportal.roche.com/LFR PublicDocs/rras/11858874001_en_16.pdf).

RT-LAMP amplification: The master mix for RT-LAMP reaction was composed of 40 pmol for each inner primer (F3/B3), 5 pmol for each outer primer (F3/B3), 25 pmol for each loop primer (LF/LB) primers. Further 12.5 µL of the reaction mix, 1µL of Bst polymerase enzyme, 0.5 µL reverse transcriptase, 1 µL of the detection dye were added and topped up with water for a volume of 20 µL. A total of 5 µL of the RNA template was added to the 20 µL master mix so as to make a total reaction volume of 25 µL and the reaction mix incubated at 63°C for 60 min in a LoopAmp real-time turbidimeter (LA-200 m; Teramecs, Kyoto, Japan). The threshold value (Abs) equivalent of an optical density above 0.1 by the turbidimeter indicated a positive detection.

Nucleic acid sequencing

The process of sequencing involved 3 phases which were performed according to the manufacturer’s protocol using a Beckman Coulter CEQ 8800 system (Agencourt Bioscience Corporation, Beverly, MA, United States). This sequencing method is based on use of dideoxynucleotides [19]. The sequence results generated were edited and finally aligned using a Biodit software [20] (Biosciences-Carlsbad, CA, USA) and blasted in the HCV database to identify the genotypes.

Testing analytical parameters for RT-LAMP

Analytical sensitivity and the lower limit of detection (LOD): Analytical sensitivity is the minimum number of copies in a sample which can be measured accurately by an assay [21]. It is currently recommended that lower limit of detection be determined as the end point dilution at which 50% of the tested samples are positive [22].

The tenfold dilution from the validation panels were made ranging from 10$^6$ IU/mL to 10$^3$ IU/mL and each tested ten times and results recorded as positive or negative for each viral load concentration by the LAMP assay and the percentage of positive detections calculated.

Analytical specificity: Analytical specificity was tested using RNA templates HIV-1, Hepatitis B virus DNA with virus concentrations between 10$^4$ to 10$^6$ copies/mL.

Precision

Inter-assay precision was evaluated by testing the concentrations of the panel on 3 different days and measuring the time to detection (Tt) values using a real time turbidimeter. The coefficient of variation (CV) was thus determined based on the
Analytical sensitivity of RT-LAMP assay showed a lower detection threshold at a viral load of 10^3 IU/mL for genotype 1a, 1b and 2b where the rate of detection was above 50% (Figure 2). Viral loads of 10^4 were detected at about 40% rate with the assay detecting down to 51 IU/mL albeit only at 20% detection rate for genotype 1a and 1b. The sensitivity for genotypes 3a and 4 were relatively low with a potential lower limit of detection only for samples above 10^4 IU/mL. Detection rates of samples below 10^3 IU/mL were less than 30% or none for genotypes 3a and 4. The LAMP assay did not detect all the 30 HCV negative samples, including those that had been determined positive for HIV and Hepatitis B infections.

Reproducibility: Reproducibility of the assay was analyzed by testing the precision based on the time taken to threshold detection using a real time turbidimeter. A high precision was observed for all samples tested for samples above 10^3 IU/mL for all the genotypes (although genotype 1b showed a higher variation at viral load 10^5 IU/mL) tested except for genotype 3a which recorded a poor precision for viral loads of 10^3 and 10^4 IU/mL (Table 1) where variations above 10% were observed.

Clinical validation

A total of 227 plasma samples were tested on RT-LAMP assay where the overall sensitivity of 94% (PPV = 97.5%) and specificity of 98.6% (NPV = 96.6%) were determined (Table 2). On the various groups of samples, a lower sensitivity was observed for German samples mainly as a result of the 5 samples (4 for genotype 3a and 1 for genotype 1b) which could not be detected. For Kenyan samples, a reduced positive predictive value was due to 2 samples that were determined as false positives by RT-LAMP. It was also noted that although all Kenyan were detected, genotype 2b and 4 were detected late after 40 minutes as opposed to the genotype 1a which were detected in less than 35 minutes.

Sub optimal detection of genotype 3a and 4

A parallel shift was however shown with genotype 3a and genotype 4 indicating a sub-optimal amplification for the genotypes, a fact which could be potentially true for genotype 2b which also showed a late detection threshold time for all the sample dilution panels (Figure 3). It is thus not clear from the results whether the false negative results recorded here as a result of non-binding of the primers to the viral RNA or the need for a longer run time for LAMP. Sequence analysis of the various genotypes showed mismatches at the F1 the B1 and the LF primer target sites mainly for genotypes 2b, 3a and 4 (Figure 4).

Discussion

Effective diagnosis of HCV within the sero-conversion period remains a major challenge in transfusion medicine and especially in the low resource countries where serological assays remain the major diagnostic systems for use. The long amount of time, sophistication and the high costs associated with the Nucleic Acid based Techniques (NATs) hinder their use in routine diagnosis and screening of blood in these low resource settings. This study reveals HCV RT-LAMP as a reliable alternative to the available NATs for diagnosis of hepatitis C virus in plasma
and compares the performance of the same assay platform for selected genotypes. Although a study [17] has evaluated LAMP on genotypes 1-6, a number of studies published [16,18] have dwelt mainly on genotypes circulating in Asian countries and the Arab world. This study presents the success of RT-LAMP assay under limited logistical conditions and further shows the differences in amplification of LAMP on a selected genotypes 1a, 1b, mixed 1a/1b, 2b, 3a and 4. The results obtained by the clinical samples both of Europe and sub-Saharan Africa and the success in their detection is a clear demonstration of robustness of HCV RT-LAMP under both favorable (stringent) and limited logistical conditions. Studies have shown that HCV RNA become detectable in sera between 7 – 14 days after exposure, before the detection of aminotransferase and the antibodies - all coming between 4 to 10 weeks [23]. The sensitivity by RT-LAMP in this study therefore demonstrate that its use show a potential to reduce the window period for HCV detection to only a maximum of 14 days, similar to any available NAT. In fact some studies have even shown that HCV RT-LAMP is more sensitive compared to nested RT PCR and of similar sensitivity to real – time RT PCR [18]. Of the 121 seropositive Kenyan samples, only 43 were detected by HCV nested RT-PCR whereas 45 were detected by RT-LAMP (2 samples noted as false positive), an indication that the samples not detected may have been as a result of no or low level of HCV RNA normally as a result of spontaneous clearance of the viruses. It can however not be concluded whether the 2 aforementioned samples noted as false positive by RT- LAMP were truly false positive, since other studies have reported a higher sensitivity by RT- LAMP compared to nested RT PCR [18]. Failure to test the Kenyan samples with both real time (q) PCR (since real time RTPCR was not available for testing the Kenyan samples during the course of this study) as well as nested PCR, especially for the two false positive samples was therefore a major limitation of this study.

Table 1: Inter-Assay Precision test for LAMP assay on a panel of known HCV viral loads.

| Genotype       | Log_{10} Viral Load (IU/mL) | Time to Threshold Detection in Minutes | CV   |
|----------------|----------------------------|----------------------------------------|------|
|                |                            | Test D1 | Test D2 | Test D3 | Mean detection time |
| Gen 1a         | 6                          | 24.12   | 24.18   | 25.11   | 24.28 | 0.02 |
|                | 5                          | 29.36   | 31.54   | 30.54   | 30.29 | 0.04 |
|                | 4                          | 34.24   | 34.06   | 36.24   | 34.51 | 0.03 |
|                | 3                          | 39.06   | 44.15   | 44.14   | 42.27 | 0.07 |
|                | 2                          | 42      | 48.13   | 47.34   | 45.49 | 0.07 |
|                | 1                          | -       | -       | -       | -     | -   |
| Gen 1b         | 6                          | 23.42   | 23.24   | 23.06   | 23.14 | 0.01 |
|                | 5                          | 26.36   | 26.54   | 24.54   | 25.49 | 0.04 |
|                | 4                          | 28.33   | 28.06   | 29      | 28.28 | 0.02 |
|                | 3                          | 46.18   | 37.54   | 31.48   | 38.24 | 0.19 |
|                | 2                          | 60      | 48.02   | 56.06   | 54.41 | 0.11 |
|                | 1                          | -       | -       | -       | -     | -   |
| Gen 1a/1b      | 6                          | N/A     | N/A     | N/A     | N/A   | *** |
|                | 5                          | 27.36   | 26.12   | 27.01   | 26.5  | 0.02 |
|                | 4                          | 31.08   | 30.12   | 30.08   | 30.26 | 0.02 |
|                | 3                          | 35.12   | 34.54   | 37.16   | 35.37 | 0.04 |
|                | 2                          | 47.13   | 51.34   | 52.28   | 52.15 | 0.05 |
|                | 1                          | -       | 58.23   | -       | -     | -   |
| Gen 2b         | 6                          | N/A     | N/A     | N/A     | N/A   | *** |
|                | 5                          | 33.52   | 34.48   | 34.59   | 34.12 | 0.02 |
|                | 4                          | 34.14   | 37.11   | 40.13   | 37.08 | 0.08 |
|                | 3                          | 47.02   | 55.36   | 53.3    | 51.53 | 0.08 |
|                | 2                          | 59.56   | -       | -       | -     | -   |
|                | 1                          | -       | -       | -       | -     | -   |
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Table 2: Sensitivity and specificity analysis of RT-LAMP assay on various groups of samples (n = 227).

| Sample Group | Number of Samples | Parameter Tested |
|--------------|-------------------|------------------|
|              |                   | Sensitivity (n/N) (%) | Specificity (n/N) (%) | PPV (%) | NPV (%) |
| Overall      | n = 227           | (78/83) (94%)        | (142/144) (98.6%)     | 97.5     | 96.6     |
| German       | n = 70            | (35/40) (87.5%)      | 100                   | 100      | 100      |
| Kenyan       | n = 157           | (43/43) (100)        | (112/114) (98.2)      | 95.6     | 100      |

PPV: Positive predictive value
NPV: Negative predictive value

Figure 2: Detection rate of LAMP on selected genotypes. A higher detection rate is shown among genotypes 1a, 1b and 2b with a lower limit of detection potentially shown at viral loads within $10^3$ IU/mL. There is a potential suboptimal detection of LAMP for genotypes 3a and 4.
The lower detection threshold established at approximately $10^3$ IU/mL in this study corresponds to other studies [17] which have demonstrated a detection rate of 80% for samples of 50 IU/rxn (approximately 2200 IU/mL) with 10 IU/rxn (400 IU/mL) only detected at 40% rate. The findings of the aforementioned study as well as the results of this study are also not significantly different from the lower limit detection of 660 IU/mL determined some further studies [18]. The lower detection limits determined are
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Amplification Assay for Detection of Hepatitis C Genotypes 1-4 Viruses Under Limited Logistical Conditions. J Hum Virol Retrovirol 5(2): 00145. DOI:

In this study, the development of a rapid and cost-effective assay for detecting hepatitis C (HCV) genotypes 1-4 under limited logistical conditions is reported. The assay utilizes a reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) protocol. Although mismatches in primer sequences were identified, further analyses confirmed primer specificity for genotypes 2a, 2b, 3a, and 4a, suggesting a high level of specificity. The assay was designed to be simple and easy to perform, requiring no specialized equipment, and could be used in remote areas with limited resources.

**Conclusion**

In conclusion, the rapidity, sensitivity, and specificity of the RT-LAMP assay make it a suitable tool for detecting HCV genotypes 1-4 in resource-limited settings. The assay's simplicity and cost-effectiveness make it ideal for use in low-resource countries where traditional laboratory infrastructure is lacking.

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