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Rapid quantitative detection of *Human immunodeficiency virus type 1* by a reverse transcription-loop-mediated isothermal amplification assay ☆

Yalan Zeng [a,b,1], Xiaoguang Zhang [a,1], Kai Nie [a], Xiong Ding [a,b], Brian Z. Ring [c], Lanying Xu [d], Lei Dai [d], Xiying Li [d], Wei Ren [d], Lei Shi [b,*], Xuejun Ma [a,**]

[a] Key Laboratory of Medical Virology, Ministry of Health, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China
[b] College of Light Industry and Food Sciences, South China University of Technology, Guangzhou, China
[c] Institute of Genomic and Personalized Medicine, School of Life Science and Technology, Huazhong University of Science and Technology, 430074 Wuhan, China
[d] Zhengzhou Center for Disease Control and Prevention, Zhengzhou, China

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ABSTRACT

Accurate and rapid quantitation of *Human immunodeficiency virus type 1* (HIV-1) RNA levels is a critical aspect in estimating the effect of antiviral therapy and establishing therapeutic schedule. Thus, for the first time, a rapid quantitative reverse transcription-loop-mediated isothermal amplification (RT-LAMP) was designed to quantitate HIV-1 RNA. The results showed that the dynamic range was from 2.5×10^2 to 10^7 copies with a coefficient of determination (R^2) of 0.991, and the limit of detection of RT-LAMP by Probit analysis at the 95% detection level was 196 copies. The intra-assay coefficient of variation (CV) ranged from 0.67% to 2.08% at 10^7 copies and 7.25% to 12.97% at 250 copies. The CVs of inter-assay were 2.39% and 13.93% for the high and low copy numbers, respectively. No cross-reaction with *Human immunodeficiency virus type 2* (HIV-2), *Human T lymphotrophic virus type 1* (HTLV-1) and *Hepatitis C virus* (HCV) was observed and a good agreement between the RT-LAMP method and the real-time reverse transcription-polymerase chain reaction (qRT-PCR) test was achieved. This proposed RT-LAMP method could be useful for rapid diagnosis of high risk group and pharmacodynamic assessment of anti-HIV drug, especially in less-equipped laboratories of impoverished areas.

1. Introduction

Human Immunodeficiency Virus type 1 (HIV-1) is the most common and virulent form of HIV retrovirus, and is responsible for attacking human immunocytes and leading to the development of AIDS. According to a recent report by the Chinese Ministry of Health and the WHO, there are 780,000 people infected with HIV in China in 2011. Without an available vaccine or cure, the development of effective HIV-1 diagnostic and detection tools is crucial for the monitoring and control of the virus. A variety of diagnostic assays, such as enzyme-linked immunosorbent assay (ELISA), HIV p24 antigen-based tests (Curtis et al., 2008), and nucleic acid amplification tests (NAAT) (Curtis et al., 2012) have been developed for the detection of HIV. While ELISA is the accepted test for screening, detection in the first months after infection may produce false-negative results. NAAT (nucleic acid amplification tests) is commonly used for detection of early-stage HIV infection, HIV RNA tests can display the positive results about 12 days prior to antibody detection tests (Fiebig et al., 2003).

While assays that test for nucleic acids, such as real-time PCR, are the most reliable technologies for detecting acute infection at present (Curtis et al., 2009), they cannot meet the demands of poverty-stricken areas or field investigation because of their dependence on expensive instruments and reagents. Thus, more cost-effective, rapid, and reliable detection tools are needed for use in epidemic areas or resource-limited places. Additionally these rapid tests can be used for the standard quantitative diagnosis of pathogens in order to quickly estimate curative effect.

A rapid nucleic acid detection method named loop-mediated isothermal amplification (LAMP) has been developed by Notomi et al. (2000). This procedure has been adapted for RNA amplification by employing a reverse transcriptase (RT) for the reverse transcription step (Kurosaki et al., 2007; Manmohan Parida, 2008; Parida et al.,

Abbreviations: HIV-1, *Human immunodeficiency virus type 1*; RT-LAMP, reverse transcription-loop-mediated isothermal amplification; CV, coefficient of variation; HIV-2, *Human immunodeficiency virus type 2*; HTLV-1, *Human T lymphotrophic virus type 1*; HCV, *Hepatitis C virus*; qRT-PCR, real-time reverse transcription-polymerase chain reaction; RT, reverse transcriptase; HAART, Highly Active Anti-Retroviral Therapy; Tt, threshold times; CI, confidence interval.

☆ The views expressed in this article are those of the authors and do not necessarily represent the views of the Chinese Centers for Disease Control and Prevention.

* Correspondence to: L. Shi, College of Light Industry and Food Sciences, South China University of Technology, Wushan Rd 381, Tianhe district, Guangzhou 510641, China.
** Correspondence to: X. Ma, Key Laboratory of Medical Virology, Ministry of Health, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Changbai Rd 155, Changping District, Beijing 102206, China.
E-mail addresses: leishi@scut.edu.cn (L. Shi), maxj@ivdc.chinacdc.cn (X. Ma).

[1] First two authors contributed equally to this study.

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2005; Soliman and El-Matbouli, 2006). RT-LAMP is a one step amplification technique that uses a strand displacement DNA polymerase and a RT to produce 10^9 fold amplification (Inacio et al., 2008; Notomi et al., 2000) from limited RNA sample under isothermal conditions of 60–65 °C (Mori et al., 2006) in 15–60 min (Hosaka et al., 2009). Two inner primers and two outer primers that specifically recognize six separate regions in the target sequence (Curtis et al., 2009) are added to the RT-LAMP reaction. Additionally, two loop primers, first described by Nagamine et al., are used additionally to shorten the reaction time and improve the sensitivity (Manmohan Parida, 2008) and specificity (Nagamine et al., 2002) of the RT-LAMP.

The application of RT-LAMP for qualitative detection of HIV-1 has been demonstrated previously (Curtis et al., 2008, 2009, 2012; Hosaka et al., 2009), but current assays lack the quantitative aspect necessary for the assessment of treatment outcome or the infection course of HIV-infected patients. This study, for the first time, aims to develop a rapid RT-LAMP assay for the quantification of HIV-1 RNA levels for use in the diagnosis of HIV-1 and the guidance of antiviral therapy.

2. Methods

2.1. Generation of HIV-1 subtype B RNA standard and clinical specimen extraction

The international HIV-1 B subtype standard HXB2 [GenBank: K03455], HIV-1 subtypes of CRF_07BC [GenBank: EF368371], C [GenBank: EF122539], CRF01_AE [GenBank: EF122518] and CRF08_BC [GenBank: EF122531], HIV-2 [GenBank: J04542], HTLV-1 [GenBank: AF259264], and HCV [GenBank: AY460204] were obtained from the National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention. Clinical plasma samples from 52 diagnosed HIV-1 infected patients were provided by Zhengzhou Center for Disease Control and Prevention. These HIV-1 infected patients, who were from 11 districts in and around Zhengzhou City, were under Highly Active Anti-Retroviral Therapy (HAART). Nucleic acids from 280 μL of plasma were extracted using QIAamp Viral RNA Mini Kit according to the instruction of the manufacturer and eluted into 30 μL of RNAse-free and DNAse-free water. All aspects of the study were performed in accordance with national ethics regulations and approved by the institutional review boards of the Chinese Center for Disease Control and Prevention (reference number: IVDC2012-001). Participants received written informed consent of the study's purpose and of their right to keep information confidential.

A 1057-bp fragment in HIV-1 gag region was amplified from the HIV-1 B subtype standard using forward primer: 5′-CTTCAGACAGGA ACAGAGGAACT-3′ and reverse primer: 5′-TGTCCTTCCTTTCCACATTTCC AAC-3′. The PCR-amplified products were purified and cloned into pGEM-T Vector (Promega, USA) to construct the plasmids containing DNA inserts. The recombinant plasmids were confirmed by sequencing. The circular plasmids were linearized using restriction enzyme *Spe* I and then transcribed into RNA in vitro using RiboMAX™ Large Scale RNA Production System-T7 (Promega, America). The concentration of purified HIV-1 RNA was determined using spectrophotometry (Eppendorf, Germany) at 260 nm (Aoi et al., 2006). An RNA linearity panel, ranging from 10^7 to 10^3 copies, 500 and 250 copies per 5 μL, was prepared to determine the quantitative standard curve.

2.2. RT-LAMP primer design

HIV-1-specific RT-LAMP primers were designed using the PrimerExplorer V4 software (http://primerexplorer.jp; Eiken Chemical Co., Ltd., Tokyo, Japan) based on the alignment of 281 HIV-1 gag gene sequences from 6 subtypes including CRF08_BC, CRF07_BC, B, C, CRF01_AE and BC. These subtypes account for 98.1% of HIV subtypes found in China and 78.1% in the world (http://www.hiv.lanl.gov/components/sequence/HIV/geo/geo.comp). A 718-bp conserved sequence was identified by comparative sequence analysis and used as a reference for generating the RT-LAMP primers. The RT-LAMP primers, which recognize eight distinct target sites in a conserved region within the p24 gene, consist of the six primers: forward outer (F3), backward outer (B3), forward inner primer (FIP), backward inner primer (BIP), and two loop primers (loop F and loop B). The sequences of the HIV-1 RT-LAMP primers are listed in Table 1.

2.3. RT-LAMP protocol

The RT-LAMP reaction was carried out in a 25 μL reaction mixture with a final concentration of 0.2 μM of each F3 and B3 primers, 1.6 μM of each FIP and BIP primers, 0.8 μM of each loop F and loop B primers, 1× ThermoPol buffer, 1.4 mM dNTPs, 0.1 M betaine, 6 mM $MgSO_4$, 8 U Bst DNA polymerase, 2 U AMV reverse transcriptase, and 5 μL of extracted RNA and RNase-free water. The reaction mixture was incubated at 60 °C for 60 min and then heated at 80 °C for 2 min to terminate the reaction. To monitor for false positives due to reagent contamination, a blank control was included in every run.

For real-time monitoring, the RT-LAMP amplification was performed in a Loopamp real-time turbidimeter (LA-320c; Teramecs, Kyoto, Japan), which records the turbidity in the form of O.D. at 400 nm every 6 s (Manmohan Parida, 2008) and reports positive when the turbidity value is more than 0.1. For the purpose of confirming the amplified products of RT-LAMP, three other testing methods were applied besides the real-time turbidimeter. These were 2% agarose gel electrophoresis stained with 5× Green GoTaq Flexi Buffer and SYBR Green I for visualization of bands, observation of magnesium pyrophosphate precipitate by naked eye, and color change after addition of 120 μM HNB to the reaction tube after amplification.

2.4. Evaluation of the RT-LAMP assay

A standard curve for HIV-1-specific RT-LAMP was constructed by measuring dilutions of transcribed RNA in vitro from 10^7 to 10^3, 500 and 250 copies, with 3 replicates at every dilution on three different runs. Linearity of quantification was established by the relation between the threshold times (Tt) of each dilution and the log of the amount of initial RNA. Limit of detection was determined by detecting serial dilutions (1000, 500, 250, 100, 50, 10, 5, 1 copies, respectively) in 5 replicates on three respective tests. Reproducibility of intra-assay and inter-assay was determined by calculating CV of 10^7 and 250 copies per tube in triplicate in one experiment and among three different runs, respectively. Specificity of the RT-LAMP was corroborated by amplifying blood-borne virus such as HIV-2, HTLV-1, HCV and 5 subtypes of HIV-1. The distribution proportion of these HIV-1 subtypes in China is about 93.6% (http://www.hiv.lanl.gov/components/sequence/HIV/geo/geo.comp).

2.5. Comparison of qRT-PCR and RT-LAMP

All clinical specimens were evaluated using a commercial real-time quantitative fluorescent probing diagnostic RT-PCR kit for *Human*

Table 1
Primers for HIV-1 RT-LAMP.

Primer name	Sequence (5′ to 3′)	Genomic position[a]
F3	GCCCATATCACCTAGAACTT	1224–1243
B3	CTGGATGTAATCTATCCCATTC	1441–1420
FIP	CTGATAATGCTGAAAACATGGGTAT	1318–1294
	-CATGGGTAAAAGTAGTAGAAGAGA	1250–1273
BIP	TTAAACACCATGCTAAACACAGTG	1339–1363
	G-GCAGCTTCTTCATTGATGG	1418–1400
Loop F	TACTTCTGGGCTAAAAGC	1293–1276
Loop B	TCAAGCAGCCATGCAAATGTT	1371–1391

[a] In $HIV\text{-}1_{HXB2}$.