



Development and evaluation of a one-step reverse transcription loop-mediated isothermal amplification for detection of *Citrus leaf blotch virus*



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ABSTRACT

Citrus leaf blotch virus (CLBV) is a type member of the genus *Citivirus* belonging to *Betaflexiviridae*. In this study, a reverse transcription loop-mediated isothermal amplification (RT-LAMP) method was developed to detect CLBV; this technology has been widely used in the detection of various plant pathogenic microorganisms and exogenous genes. The sensitivity of the RT-LAMP method was increased 100-fold compared to that of the conventional RT-PCR. In addition, this method was fast, simple and specific; it could provide better technical support for field diagnosis, customs quarantine and the control measures of CLBV. To our knowledge, this is the first report detecting CLBV using RT-LAMP.

CLBV is a single-stranded RNA virus; it is the type species of *Citivirus* in the family *Betaflexiviridae* (King et al., 2011). This virus is rarely reported in China. CLBV-infected citrus is primarily found under natural conditions (Navarro et al., 1984; Guardo et al., 2007). According to a recent survey on viruses infecting kiwifruit, CLBV is the dominant virus species, in kiwi plants infected with CLBV, the leaves showed chlorotic or yellow, and most of the leaves had no obvious disease symptoms (Liu et al., 2019).

At present, there are only three methods that can detect CLBV: RT-PCR (Ruiz-Ruiz et al., 2009; Osman et al., 2015), dot blot hybridization (DBH) and tissue blot hybridization (TPH) (Galipienso et al., 2004). Compared to the above detection methods, RT-LAMP is a nucleic acid amplification reaction method in which reverse transcriptase (M-MLV) is added, reverse transcription and gene amplification are performed at the same temperature (Notomi et al., 2000). A water bath can be used to meet the analysis requirement of LAMP without expensive equipment such as PCR amplifier (Sema et al., 2015), measuring fluorescence with SYBR green or calcein, instead of gel electrophoresis, allows avoiding any chance of contact with ethidium bromide (EB). In this study, one pair of specific primers for the RT-LAMP detection of CLBV was designed, the reaction system and conditions of the RT-LAMP were optimized, these included: the concentration of each component, the reaction temperature and time. In addition, the specificity, sensitivity and reproducibility of the RT-LAMP assay were tested using field samples.

The nucleotide sequence of the coat protein (CP) gene from different CLBV isolates (AJ318061, EU857540, FJ009367, JN936275, NC003877, MF784853 and JQ013955) were obtained from the NCBI. The Primer Explorer online software V5 (<http://primerexplorer.jp/lampv5e/index.html>) with the default settings was used to design the RT-LAMP primers. The sequence alignment was performed using MEGA 6.0 software (Liu et al., 2014) to ensure the primers were designed in the conserved region of the CP gene. The inner (CLBV-FIP and CLBV-BIP) and outer primers (CLBV-F3 and CLBV-B3) were used for the initiation of the RT-LAMP (Supplementary Fig. S9). The primers, CLBV-1F/CLBV-5R, which were designed and published by Chavan et al. (2013) were also used in this study, and the PCR products were sequenced by the Tsingke Company (Beijing, China) to ensure the correct amplification. All primer sequences are shown in Table 1.

Kiwifruit leaf samples infected with CLBV, ASGV (*Apple stem grooving virus*), CMV (*Cucumber mosaic virus*), AcVA (*Actinidia virus A*) and AcVB (*Actinidia virus B*) were stored at -80°C in the State Key Laboratory of Crop Stress Biology, Northwest A&F University. The total RNA was extracted from the leaves of *A. chinensis* using OminiPlant RNA Kit (CWBI, Beijing, China); it was used as the template for the RT-LAMP reaction. The reaction mixture contained 2 μL RNA template, 1.0 $\mu\text{mol L}^{-1}$ of CLBV-FIP and CLBV-BIP, 0.1 $\mu\text{mol L}^{-1}$ of CLBV-F3 and CLBV-B3, 1.2 $\mu\text{mol L}^{-1}$ of dNTPs, 0.6 mol L^{-1} of betaine, 10 U of RNase Inhibitor, 8 U of *Bst* DNA polymerase, 50 U of M-MLV (Promega, Madison, WI, USA), 1 \times Isothermal Amplification Buffer (20 mmol L^{-1}

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Table 1
Specific primers for detection of CLBV by RT-LAMP and RT-PCR.

Primer	Length (nt)	Genome position ^a	Sequence (5'–3')
CLBV-F3	19	806–824	CTCCTGAAAACGGAGGAA
CLBV-B3	18	1012–1029	ACTCGAGGTCACATGTCC
CLBV-FIP	43	891–912	ATCAGCTTCTGTTGGAATTGCT-
(F1c + F2)		844–864	ACTAAATTTGCGGCTTTTAC
CLBV-BIP	43	933–952	GCCTCCAACGAATGAGGAGA-
(B1c + B2)		980–1002	GGAAATTTTGCTCATATATGTGAC
CLBV-1F	22	45–66	AGCCATAGTTGAACCATTCCTC
CLBV-5R	20	450–469	GCAGATCATTACCACATGC

^a Genomic location reference GenBank ID: JN936275.

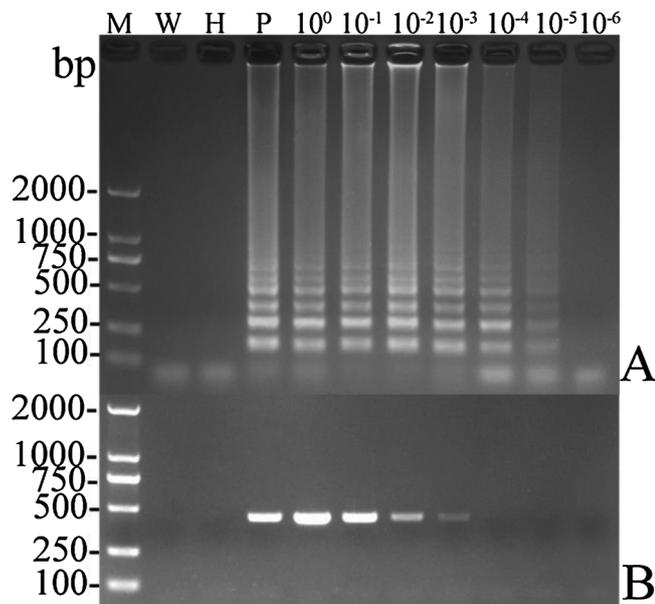


Fig. 1. Comparison of the sensitivity of the RT-LAMP and RT-PCR to detect CLBV. Total RNA extracted from CLBV-infected leaf tissues was diluted in 10-fold serial dilution (10^0 – 10^{-6}) and assayed by RT-LAMP (A) or RT-PCR (B); Lane W, Non-template (water) control; Lane H, Health (Negative) control; Lane P, Positive control; Lane M, DL2000 DNA Ladder.

Tris-HCl, 10 mmol L⁻¹ KCl, 10 mmol L⁻¹ (NH₄)₂SO₄, 0.1% TritonX-100, 2 mmol L⁻¹ MgSO₄. RNA-free water was added to give a total volume of 20 μL; The reaction was incubated at 60 °C for 60 min. For the specific conditions for RT-PCR, please refer to the previously published studies by Chavan et al. (2013).

The reaction conditions including temperature, time and concentrations of each primer, Mg²⁺, *Bst* DNA polymerase, dNTPs and betaine in the RT-LAMP assay were optimized. The reaction temperature was increased from 60 °C to 65 °C by increments of 1 °C. The reaction time was increased from 30 min to 90 min by increments of 15 min. The concentrations of primers CLB-V-FIP/BIP were tested at 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 μmol L⁻¹. The primers CLB-V-F3/B3 were tested at 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 μmol L⁻¹; Mg²⁺ was tested at 0, 1, 2, 3, 4, 5 mmol L⁻¹; the dNTPs were tested at 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 mmol L⁻¹; betaine was tested at 0, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 mol L⁻¹; *Bst* DNA polymerase was tested at 4, 6, 8, 10, 12, 14 U. The RT-LAMP optimization experiment was repeated three times to ensure reliable results.

The optimized RT-LAMP detection contained 1.4 μmol L⁻¹ of CLB-V-FIP/BIP (Supplementary Fig. S1), 0.2 μmol L⁻¹ of CLB-V-F3/B3 (Supplementary Fig. S2), 2 mmol L⁻¹ of Mg²⁺ (Supplementary Fig. S3), 8 U of *Bst* DNA polymerase (Supplementary Fig. S4), 1.4 mmol L⁻¹ of dNTPs (Supplementary Fig. S5) and 0.4 mol L⁻¹ of betaine (Supplementary Fig. S6), with an optimal temperature set at 62 °C for

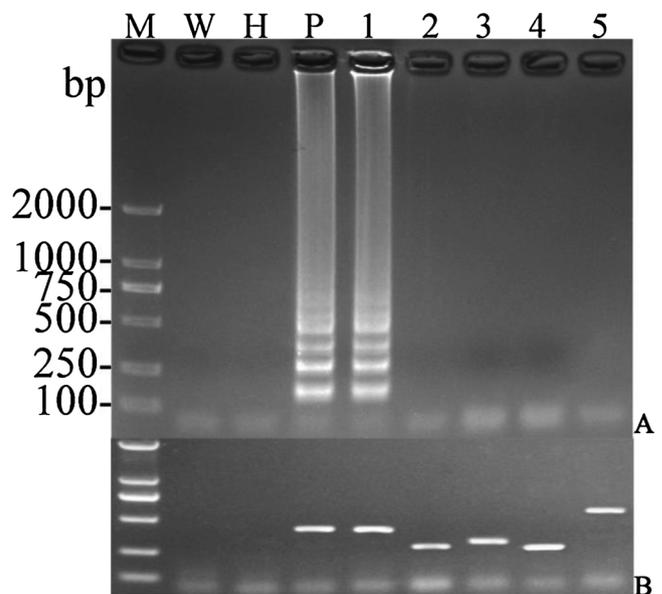


Fig. 2. The specificity of RT-LAMP assay for CLB detection (A) and the specificity of RT-PCR assay for CLB, AcVA, AcVB, CMV and ASGV detection (B). Lanes 1–5 show RNA templates of CLB, ASGV, CMV, AcVA and AcVB, respectively. Lane W, Non-template (water) control; Lane H, Health (Negative) control; Lane P, Positive control; Lane M, DL2000 DNA Ladder.

60 min (Supplementary Figs. S7 and S8).

The detection limits for RT-PCR and RT-LAMP were compared using 10-fold serial dilutions (10^0 – 10^{-6}) of RNA. The amplification product was analyzed using gel electrophoresis. The highest dilution of the RT-LAMP assay was 10^{-5} , and the detection limit of RT-PCR was 10^{-3} . The sensitivity of RT-LAMP increased 100-fold compared to RT-PCR (Fig. 1).

To evaluate the specificity of the RT-LAMP assay, CMV, ASGV, AcVA and AcVB were selected. These viruses either occur widely in nature or belong to the same family as CLB. The methods of RT-PCR for the four viruses have been described in previous studies (Blouin et al., 2013; Hao et al., 2016; Dai et al., 2012). In the RT-LAMP test, no amplification was observed from the samples infected with ASGV, CMV, AcVA and AcVB (Fig. 2-A), but these samples were positive in their respective RT-PCR tests (Fig. 2-B). The results showed that RT-LAMP had high specificity in detecting CLB.

In order to prove the universality of RT-LAMP, a total of 23 different individual plants samples were collected in the major kiwi fruit production regions. Six positive samples were detected by RT-LAMP while five positive samples were detected by RT-PCR (Fig. 3). The results showed that the RT-LAMP method could be easily applied and is more specific to the detection of CLB.

To test the RT-LAMP method more broadly, more than 160 kiwi fruit leaf samples were collected from various cultivars, which covered almost all of the kiwi fruit growing areas in Shaanxi Province, these included: Meixian, Wugong, Yangling, Zhouzhi, Hanzhong and Ankang (Table 2). The results showed that RT-LAMP was sensitive, convenient and efficient.

CLBV can be detected without any expensive equipment and complex experimental techniques by RT-LAMP, for which the sensitivity increased 100-fold compared to that of RT-PCR. In addition, if 0.1 μL of the fluorescent dye SYBR green I was added to the amplification product, the positive sample was observed visually to be green in the absence of electrophoresis, whereas the negative sample was orange (Fig. 4). Such a colour contrast is ideal for the rapid detection of introduced kiwi fruit germplasm and grafted material in local test stations or research institutes.

The one-step RT-LAMP assay for CLB detection is fast and

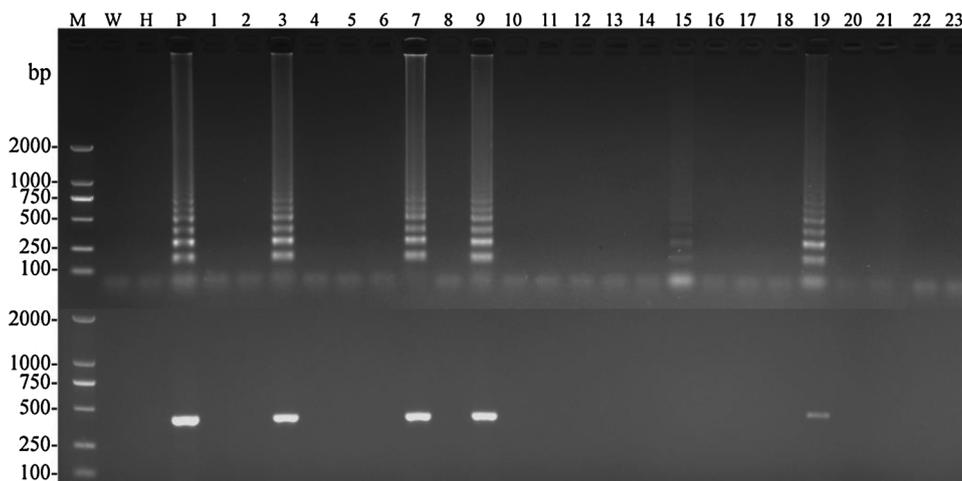


Fig. 3. Field kiwi fruit samples were detected by optimised RT-LAMP (top) and RT-PCR (lower). Lanes 1–23 show kiwi fruit samples; Lane W, Non-template (water) control; Lane H, Health (Negative) control; Lane P, Positive control; Lane M, DL2000 DNA Ladder.

Table 2
The incidence of CLBV in tested kiwi plants growing in Shaanxi, using RT-LAMP and RT-PCR.

Sampling region	Total samples tested (RT-PCR/RT-LAMP/Total)	RT-PCR detection level (%)	RT-LAMP detection level (%)
Meixian	7/7/44	15.9	15.9
Wugong	1/2/20	5.0	10.0
Yangling	1/1/18	5.6	5.6
Zhouzhi	8/9/38	21.1	23.7
Hanzhong	9/10/32	28.1	31.2
Ankang	1/1/15	6.7	6.7
Means	27/30/167	16.2	18.0

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jviromet.2019.05.009>.

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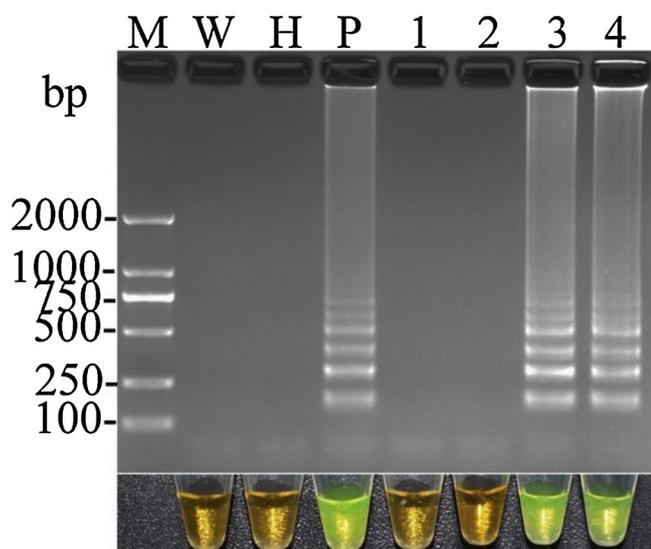


Fig. 4. Visual detection of CLBV. A, detection by agarose gel electrophoresis; B, Amplification products with SYBR green I. Lanes 1–2 were kiwi fruit plant negative for CLBV; Lanes 3–4 were kiwi fruit plant positive for CLBV. Lane W, Non-template (water) control; Lane H, Health(Negative) control; Lane P, Positive control; Lane M, DL2000 DNA Ladder.

accurate. On the basis of its simplicity and convenience, this method can provide technical support for the prevention and control of *A. chinensis* viral disease, and it could play an important role in the inspection and quarantining of CLBV.