Development of a LAMP assay for the detection of phytoplasma causing Cassava Witches' Broom disease

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ABSTRACT

Cassava Witches' Broom Disease (CWB) is caused by the infection of a phytoplasma. The manifestation of this disease has become serious recently in Vietnam, Thailand and other cassava-growing countries in the Southeast Asia. Although PCR provides a gold standard in diagnostics, it is time-consuming and requires heavy investment in terms of equipment as well as technical skills. To provide a cheaper and handier alternative, we developed an assay based on Loop-mediated isothermal amplification (LAMP) that allows specific detection of phytoplasma from field-collected samples. Three primer sets were designed and tested, of which 2 sets aimed to detect phytoplasma 16S rRNA, the other set was to detect cassava actin. A dual assay for phytoplasma 16S rRNA and cassava actin was optimized at conditions of 63°C for 60 minutes. A positive reaction can be visualized by either agarose-gel electrophoresis, color change of hydroxynaphtol blue (HNB) or appearance of precipitation. The assay was shown to be able to distinguish between healthy and diseased cassava samples collected from the field in single step. Further development of this KIT is underway toward on-site application of the assay.

MATERIALS & METHODS

Healthy and CWB cassava were collected in Dong Nai province (Vietnam). Primers for LAMP were designed by the PrimerExplorer V4 software at the website http://www.primerexplorer.jp.

LAMP assay was carried out in a 20-µL reaction mixture, including: 1.75 µM FIP, 1.75 µM BIP, 0.25 µM F3, 0.25 µM B3, 3µl 1.5 mM each dNTP (NEB, USA), 4.8 µl 25 mM $MgSO_4$ (Thermo Scientific, USA), 8U Bst 2.0 DNA polymerase (NEB, USA), 100 mM HNB as the indicator and 0.5 µl DNA template in the isothermal amplification buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween® 20, pH 8.8 at 25°C) (NEB, USA).

The reaction was conducted at 63°C in 60 minutes. LAMP products were confirmed by color change of HNB from violet to blue, agarose electrophoresis or turbidity appearance.

RESULTS

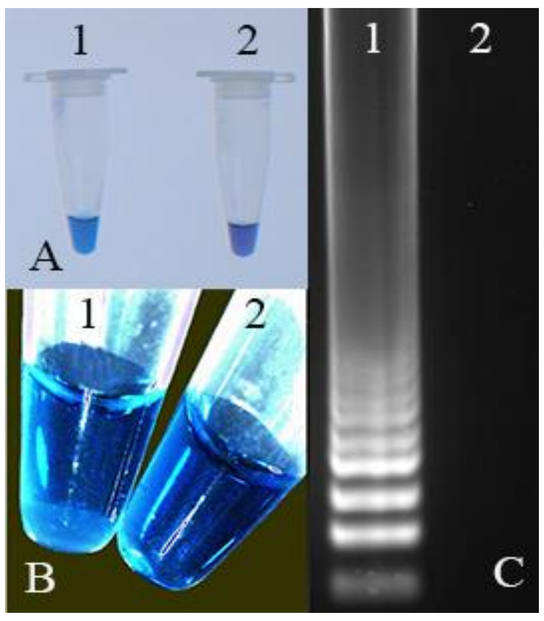

Fig 1. Visualization of the LAMP assay based on (A) HNB change; (B) turbidity appearance and (C) separation on 2% agarose gel. 1: phytoplasma DNA. 2: no DNA template

Table 1. List of primer sets for LAMP assay

Primers	Sequences	Target gene
1-FIP	5'- T GTT CCT CCA TAT ATT TAC GCA TCT AGA GTA AGA TAG AGG CAA GTG -3'	16S rDNA
1-BIP	5'- G ACG CTG AGG CAC GAA AGA GTA CTC ATC GTT TAC GGC -3'	
1-F3	5'- CAT TGT GAT GCT ATA AAA ACT GT -3'	
1-B3	5'- CAA CAC TGG TTT TAC CCA AC -3'	
2-FIP	5'- C ACC ACC TGT GCA ACT GAT AAG GTC TTG ACA TGC TTC TGC -3'	
2-BIP	5'- G GTT AAG TCC CGC AAC GAG CTT GCT AAA GTC CCC ACC AT -3'	
2-F3	5'- AGG TAC CCG AAA AAC CTC ACC -3'	
2-B3	5'- TCC CCA CCT TCC TCC AAT T -3'	
Act-FIP	5'-GCT TCT CCT TCA TGT CAC GGA CTG ATG AAG ATC CTC ACT GAG A-3'	Actin
Act-BIP	5'-TGA ACA GGA ACT TGA GAC TGC CCA TCA GGA AGC TCA TAG TTC TT-3'	
Act-F3	5'-GCT CTT CCA CAT GCC ATT-3'	
Act-B3	5'-CTT CTG GAC AAC GGA ATC TT-3'	

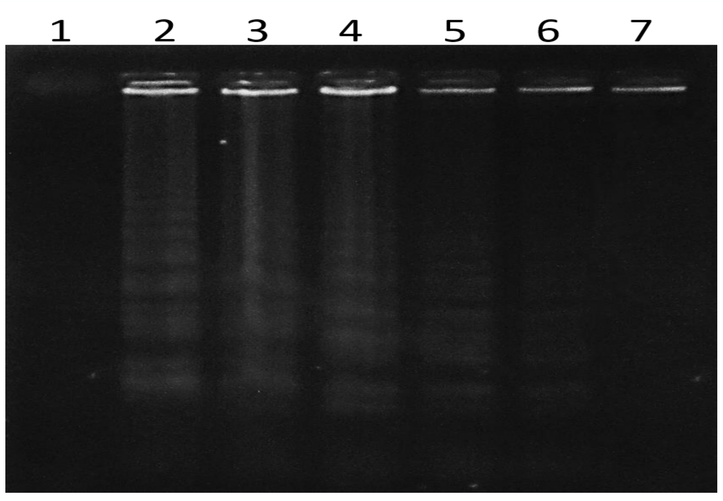

Fig 2. Specificity of LAMP assays to detect phytoplasma DNA in the mixture with serial dilution of cassava DNA. 1: No DNA template; 2-7: Mix of 10^{-6} ng phytoplasma DNA and series of 250; 500; 750; 1000, 1250 and 1500 ng healthy cassava genomic DNA of KM 94 variety

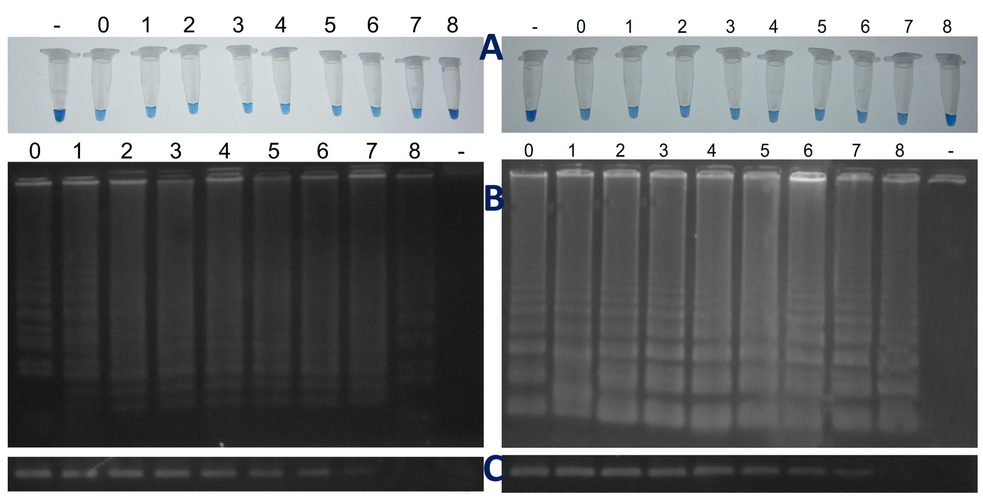

Fig 3. Sensitivity of LAMP assay with primer set A (left) and primer set B (right). A. HNB color change; B. Electrophoresis on 2% agarose gel; C: By PCR with F3/B3 primers; M: 1000 bp DNA ladder; Lane 0: 100 ng plasmid DNA template; Lane 1-8: 10^1-10^8 DNA dilutions from preparations from 100 ng plasmid DNA; (-): negative control

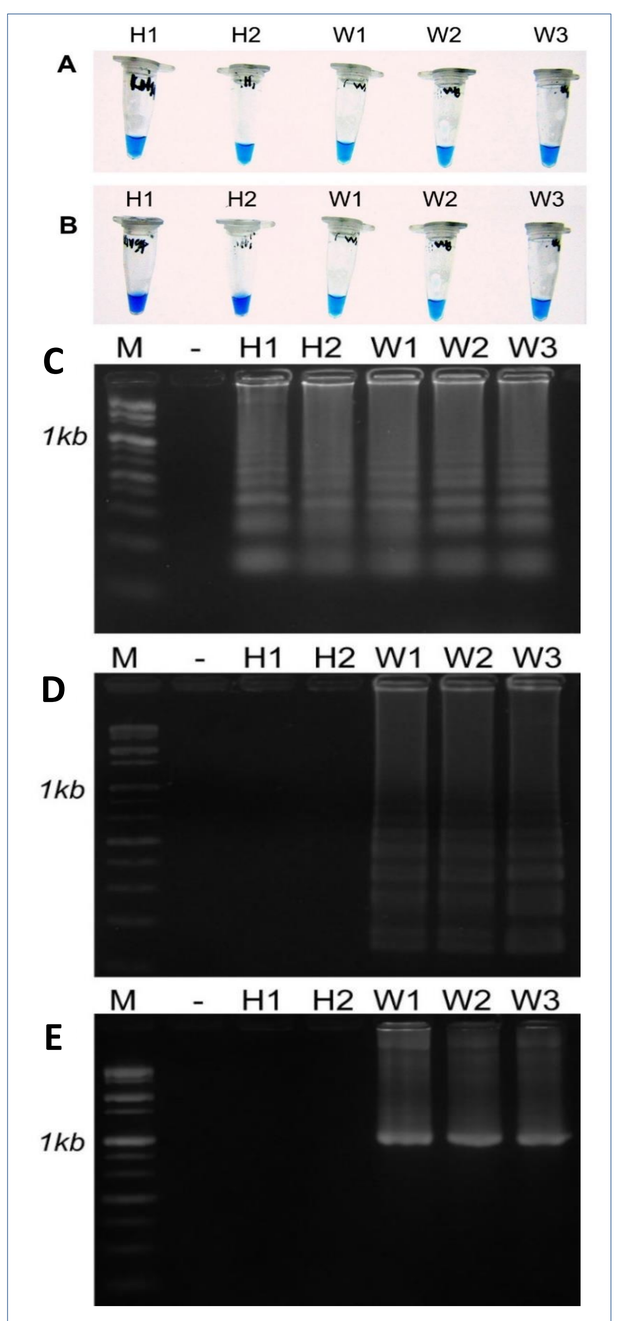

Fig 4. LAMP assay on field-collected cassava with HNB indicator (A, B), visualize on agarose gel (C, D) and Nested-PCR (E). A, C: Actin primers; B, D: 16S rDNA phytoplasma primers. H1, H2: healthy samples; W1, W2, W3: CWB infected samples; M: 1kbp DNA ladder; (-): Negative control;

CONCLUSIONS

1. The LAMP assay to detect phytoplasma causing CWB were established and with two specificity primer sets.
2. The sensitivity of the assays was about 10^{-6} ng target DNA and is better than the conventional PCR for the same samples.
3. The assay is less time-consuming than the nested-PCR to detect phytoplasma or new pathogens of plant diseases.

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