Application of loop-mediated isothermal amplification method (LAMP) for direct detection of the bacterial wilt pathogen *Ralstonia solanacearum* in soil

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Introduction

DNA-based detection methods can provide improved specificity and sensitivity compared to serological methods. However, these methods remain impractical for most field applications due to difficulties in concentrating the organism from the environment and ensuring that enough DNA is available for detection. The objective of this research was to develop a rapid detection method for *Ralstonia solanacearum* in ginger soil, since this pathogen has been shown to dramatically affect yields of both edible and ornamental ginger in Hawaii. Isothermal DNA amplification techniques, such as Loop-mediated isothermal AMPlification (LAMP), might be suitable for rapid field detection because of its ability to amplify DNA with high specificity, efficiency, and speed without thermal cycling.

Figure 1. Bacteria wilt of edible ginger plant caused by *Ralstonia solanacearum*

Objectives

For the detection of *Ralstonia solanacearum* in soil, we set out to develop

- Sample isolation technique from drainage water samples by using filtration columns
- Gene-based discrimination at species level
- Isothermal method for DNA replication and detection
- Comparison of isothermal DNA replication technology to traditional detection methods such as PCR and ELISA

Principle of LAMP

LAMP is a novel method that amplifies target DNA sequences with high specificity, efficiency, and speed without the need for thermocycling. The principle is based on synthesis by a unique strand displacing DNA polymerase. Reaction specificity depends on unique sequences of four specially designed primers that hybridize with a total of six distinct sequences on the target DNA.

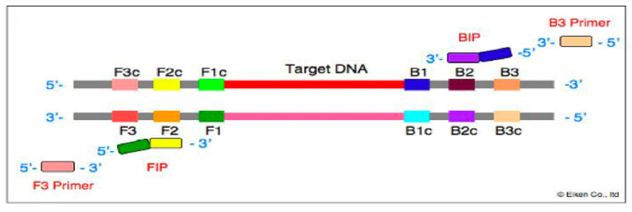


Figure 2. Design of primers

Four unique primer sequences are designed based on the regions of the target gene illustrated in Figure 2. Proper primer design is critical for LAMP reaction. The primer design can be facilitated by using the PrimerExplore (http://primerexplorer.jp/e/) to optimize sequence compositions, GC contents, and secondary structure.

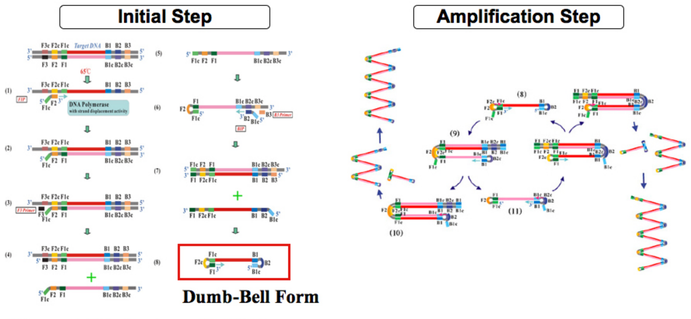


Figure 3. Mechanism of LAMP

The reaction is initiated by inner primer (either FIP or BIP) hybridization to its respective priming site (F2c or B2c) on the target DNA. The outer primer (F3 or B3) secondarily hybridizes to its priming site (F3c or B3c) on the target DNA and initiates synthesis of new complementary sequence that displaces DNA sequences already extended from the inner primer. The result is a DNA sequence which can form stem-loop structures at both ends. This autopriming "dumb-bell" structure is the starting material for LAMP auto-cycling amplification. The amplification products are stem-loop DNAs with several inverted repeats of the target, exhibiting cauliflower-like structure with multiple loops.

LAMP Primer and Process Design

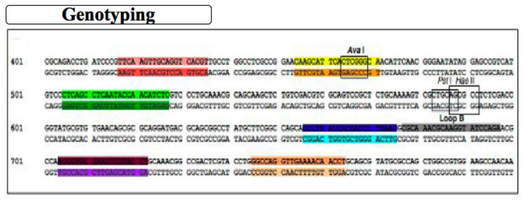


Figure 4. Gene sequence coding for flagellar subunit *fliC* in *Ralstonia solanacearum*

Target gene sequence for flagellar subunit *fliC* of *Ralstonia solanacearum* strain GMI1000. The different colors correspond to the different primer sequences of the LAMP primer set shown Figure 2. Restriction sites of enzymes *Ava I*, *Hae II*, and *Pst I*, which were used to confirm the LAMP products, are shown in rectangular boxes. LAMP reactions were conducted at 65°C for 60 minutes with various primer combination.

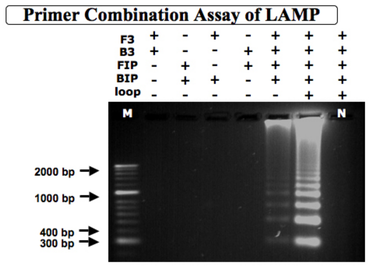


Figure 5. Primer combination assay and acceleration of reaction by loop primer

LAMP products were observed using different primer combinations. Lane 1 (M) has a size marker (BIOLINE), and Lane 8 (N) is a control with no template DNA. All of 4 primers, F3, B3, FIP, and BIP are required for successful LAMP reaction (Lane 6). The LAMP reaction was accelerated by adding a loop primer (Lane 7). 1 µl (out of total 25µl of the reaction solutions) were applied to 2% agarose gel electrophoresis and stained with ethidium bromide.

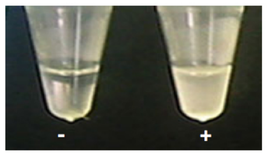


Figure 6. Rapid Visual Assessment

Tubes showing LAMP reaction products with no template DNA (-), and with template DNA (+). One of the characteristics of LAMP is its ability to rapidly synthesize an large amount of DNA. Accordingly, a large amount of by-product, pyrophosphate ion, is produced yielding a white precipitate of magnesium pyrophosphate.

Process of producing magnesium pyrophosphate

$$(DNA)_{n-1} + dNTP \rightarrow (DNA)_n + P_2O_7^{4-} \quad [1]$$

$$P_2O_7^{4-} + 2Mg^{2+} \rightarrow Mg_2P_2O_7 \quad [2]$$

Analysis of LAMP Primer Selectivity

Species	Strain	No. of strains	Race	Host plants (no. of strains)	LAMP
R. solanacearum		4	1	Tomato (2), squash (1), peanut (1)	+
		6	2	Banana (5), heliconia (1)	+
		5	3	Potato (2), geranium (2), *S.phurjea* (1)	+
		4	4	Ginger (4)	+
		5	BDB	Banana (5)	-
R. eutropha	H16	1	---	N/A	-
Erwinia carotovora subsp. carotovora	CC26	1	---	Potato	-
Enterobater	A 5150	1	---	N/A	-

Table 1. A summary of strains of *Ralstonia solanacearum*

Selectivity of LAMP reactions conducted with purified genomic DNA from different strains of *R. solanacearum* (races 1, 2, 3, 4, and *R. solanacearum* BDB) and other soil-borne bacteria strains (*Erwinia carotovora subsp. carotovora* strain CC26, *Enterobacter* strain A 5150, and *Ralstonia eutropha* strain H16). With the exception of all 5 blood-disease strains, all strains tested that belong to the species complex of *R. solanacearum*, including the Race 3 strains, gave an amplification product with a ladder-like pattern typical of LAMP, whereas LAMP amplification products were not observed following LAMP reaction with other soil-borne bacteria strains.

Direct Optical Detection Limit

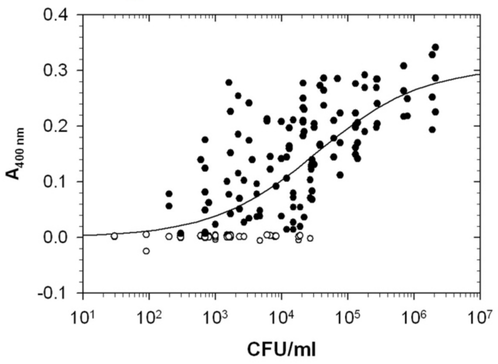
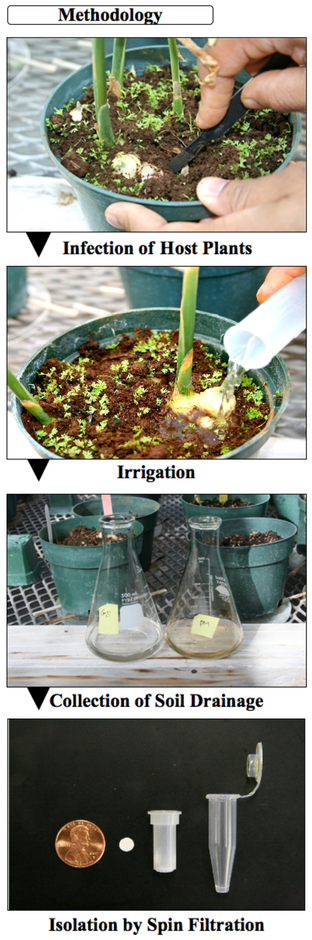


Figure 8. Absorbance of LAMP reaction mixture at 400 nm vs *R. Solanacearum* population of collected drainage water samples.

Solid circles indicate samples classified as "positive" for *R. Solanacearum*, and open circles indicate samples classified as negative based on optical absorbance measurements. The line indicates the best fit sigmoidal curve ($R^2 = 0.54$; Std. Error = 0.066 absorbance units; detection limit = 10^5 CFU/ml).

Application of LAMP for Bacterial Detection in Soil

To demonstrate the application of LAMP in soil, edible ginger plants (*Zingiber officinale*) were infected with *Ralstonia solanacearum* strain A4515, and effluent water samples were collected from daily irrigation water. A simple filtration technique was applied to concentrate bacteria, followed by a species-specific LAMP, PCR, and ELISA, the results of which were compared to traditional plate count.



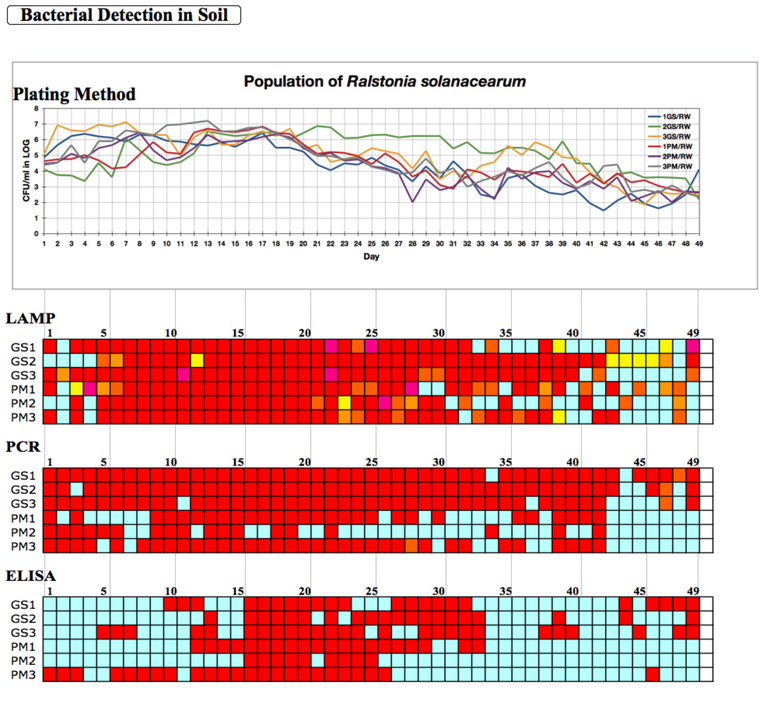


Figure 7. *Ralstonia solanacearum* Diagnostic Results by Method

Viable plate counts compared to LAMP, PCR, and ELISA classifications of drainage water collected from potted ginger samples. Following infection by rhizome wounding (RW) with *Ralstonia solancearum*, samples were collected daily from ginger plants in three separate pots of soil from a ginger farm on Hawai'i (GS) and three separate pots of potting media (PM). Classifications for LAMP, PCR, and ELISA from three replicates of each sample show light blue for no detection, yellow,orange, or red respectively for single, double, and triple positives.

Conclusions

- **The LAMP method with the *fliC* primer set is species specific to *Ralstonia solanacearum*.**
- **LAMP is a rapid, specific, and inexpensive amplification method, and can amplify the target DNA within 60 min under isothermal conditions (65 °C).**
- **The by-product of LAMP reaction, a white precipitate of magnesium pyrophosphate, allows simple visual assessment.**
- **Statistical analysis of the turbidity data showed that direct optical detection limit is 10^5 CFU/ml in soil samples**
- **The detection limit of the direct LAMP assay was between 10^3 – 10^5 CFU/ml, which is the same as PCR and more sensitive than ELISA**

Future work

- **Development of automated diagnostic system for *Ralstonia solanacearum* based on the LAMP reaction.**
- **Redesign primer set to be selective to sub-species level.**

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