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Development of an updated loop-mediated isothermal amplification (LAMP) assay for the rapid detection of *Ralstonia syzygii* subsp. *celebesensis* (*Rsc*), the causative agent of banana blood disease

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Abstract: Efficient and accurate detection of *Ralstonia syzygii* subsp. *celebesensis* (*Rsc*), the pathogen responsible for banana blood disease, is vital for controlling its detrimental effects on banana cultivation. This study developed and assessed a loop-mediated isothermal amplification (LAMP), which detected as few as 10 cells per reaction, significantly surpassing conventional PCR, which required a minimum of 10^3 cells per reaction. The LAMP assay utilizes real-time fluorescence-based Ct values and Tm analysis, ensuring reliable detection by confirming target specificity. The assay effectively identified *Rsc* in *Rsc*-spiked banana sucker samples and naturally infected field specimens using a simple, purification-free boiling DNA extraction method, making it highly adaptable for field diagnostics. By integrating real-time fluorescence detection with Tm validation, this study presents an advanced, field-deployable molecular diagnostic tool with superior sensitivity and specificity, addressing critical limitations of existing *Rsc* detection methods and enhancing early disease surveillance in banana cultivation.

Keywords: banana blood disease; DNA fingerprinting; LAMP assay; *Ralstonia syzygii* subsp. *celebesensis*; repetitive-PCR

INTRODUCTION

Banana blood disease (BBD) is a destructive vascular wilt disease caused by *Ralstonia syzygii* subsp. *celebesensis* (*Rsc*) [1]. This bacterial pathogen poses a significant threat to banana cultivation in Southeast Asia, where bananas are a vital staple crop and an important source of livelihood. The prevalence and rapid spread of this disease remain significant challenges, with the potential to cause up to a 100% reduction in banana production [2]. BBD is characterized by reddish-brown discoloration of vascular tissues, necrosis, and oozing of bacterial exudates, ultimately leading to plant wilting and death [3,4]. The disease spreads rapidly through contaminated planting materials, tools, and insect vectors, necessitating effective and timely detection methods to mitigate its impact [5]. Early and accurate detection of *Rsc* is essential for effective disease management and containment strategies.

Several diagnostic techniques have been employed to detect BBD. Culture-based methods, while reliable, are time-consuming and require specialized laboratory facilities for pathogen isolation and identification. Molecular methods, such as polymerase chain reaction (PCR), have improved detection accuracy by offering higher sensitivity and specificity[6,7]. Rincón-Flórez et al. [6] developed a real-time PCR assay for BBD and evaluated existing assays based on conventional PCR. The assays effectively detected DNA extracted from pure cultures of *Rsc* and, to a lesser extent, from symptomatic plant tissues. However, the reliance of PCR on thermocyclers, skilled personnel, and controlled laboratory environments [8] limits its applicability, particularly in

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field settings. Immunological approaches, such as lateral flow immunoassays (LFIA), provide rapid detection but have a higher detection threshold, which may limit early disease identification. Talib et al. [9] developed an LFIA strip for *Rsc* detection, demonstrating its potential for field application. However, they suggested further improvements in signal intensity to enhance detection reliability. While these molecular and immunological assays offer significant advancements, their dependence on sophisticated laboratory equipment or endpoint detection methods compromises their sensitivity, specificity, and practicality in field conditions. These drawbacks underscore the need for rapid, cost-effective, and field-adaptable detection methods, particularly in resource-constrained banana-growing regions.

The loop-mediated isothermal amplification (LAMP) technique offers a promising solution to many limitations associated with conventional diagnostic methods. LAMP is an innovative and highly efficient gene amplification technique recognized for its simplicity and speed in diagnosing microbial diseases. Amplification is typically completed within an hour [10,11]. Unlike PCR, LAMP operates under isothermal conditions, eliminating the need for complex thermocycling equipment. This specific and sensitive molecular method provides faster results, greater tolerance to inhibitors commonly found in plant tissue extracts, and simplicity that makes it well-suited for field diagnostics [10]. Its portability and robustness allow an on-site application that allows for real-time decision-making in disease control. In recent years, LAMP has been successfully applied to detect various plant pathogens, showcasing its potential to revolutionize agricultural disease diagnostics [12-15]. A LAMP assay with carbon particle flocculation, achieving high sensitivity (0.5 pg DNA detection limit) was developed [16]. However, this approach requires additional reagents and handling steps for visualization, adding complexity to field applications. Furthermore, endpoint LAMP assays, such as flocculation-based methods, rely on visual interpretation, which may lead to subjective errors and reduced sensitivity. Immunological assays also have limited sensitivity and require antibody development, making them less adaptable for broad application.

In developing a LAMP assay for detecting plant pathogens, repetitive sequence-based PCR (rep-PCR) is a crucial preliminary tool. Rep-PCR aids in identifying unique genetic markers within the pathogen's genome and is essential for designing specific LAMP primers. This method amplifies DNA segments located between repetitive extragenic palindromic sequences (REPs), enterobacterial repetitive intergenic consensus (ERIC) sequences, or BOX elements [17,18]. These repetitive elements, commonly found in bacterial genomes, are non-coding and dispersed at variable intervals [19,20]. By targeting these sequences, rep-PCR generates unique DNA fingerprint patterns specific to individual strains, enabling differentiation of closely related isolates [21,22]. Consequently, integrating rep-PCR with LAMP provides a targeted, sensitive, and reliable diagnostic method, underscoring the value of combining molecular tools to enhance specificity and accuracy in pathogen detection assays. Beyond its role in assay development, rep-PCR provides reproducible, high-resolution genetic profiles while being cost-effective and technically straightforward [18,23,24].

To address the limitations of current diagnostic methods, this study presents a novel LAMP assay utilizing primers designed from a rep-PCR fingerprinted sequence, ensuring improved specificity for *Rsc* detection. Unlike previous LAMP approaches that utilized colorimetric or particle-based endpoint detection, our assay integrates a portable LAMP machine that provides real-time fluorescence-based Ct values and Tm analysis. This approach offers three distinct advantages over prior methods: higher sensitivity, with the assay detecting as few as 10 cells per reaction compared to 10^3 cells per reaction for PCR; greater specificity and reliability, as Tm analysis confirms true-positive detection and distinguishes specific from non-specific amplification, thereby reducing false positives; and improved field adaptability, with a simple, purification-free boiling DNA extraction method enabling on-site detection without requiring advanced laboratory infrastructure.

This study aims to validate a specific, sensitive, field-deployable LAMP assay for rapid *Rsc* detection. The assay is compared to conventional PCR, assessing specificity, sensitivity, and applicability in controlled and field settings. By integrating real-time fluorescence detection and Tm validation, this study advances molecular diagnostics for *Rsc*, providing a practical and scalable solution for early disease surveillance and management in banana-growing regions.

MATERIALS AND METHODS

Ethics statement

This study did not involve human participants, animals, or genetically modified organisms requiring ethical approval. All samples were collected and handled following institutional and national guidelines, with appropriate permissions and biosafety measures.

Microorganisms and genomic DNA preparation

Rsc and *R. solanacearum* isolates, other bacterial species, and fungal isolates listed in Table 1 were cultured for genomic DNA extraction. *Rsc* and *R. solanacearum* isolates were grown on triphenyl tetrazolium chloride (TZC) agar medium [25] at 28°C for 48 h. Other bacterial isolates were cultured on a nutrient agar (NA) medium at 28°C for 48 h, and fungal isolates were cultured on potato dextrose agar (PDA) medium at 30°C for 7 days. Colonies from these cultures were subsequently used for genomic DNA extraction. Genomic DNA from bacterial isolates was extracted using the GF-1 Bacterial DNA Extraction Kit (Vivantis, Malaysia), and genomic DNA from fungal isolates was extracted using the DNeasy Plant Mini Kit (QIAGEN, USA), following the manufacturer's protocols. The quality and concentration of the extracted DNA were determined using a Thermo Scientific™ NanoDrop™ Lite spectrophotometer. The extracted genomic DNA was stored under appropriate conditions and used in subsequent analyses.

Table 1. List of microorganisms used in this study and specificity test results of the developed LAMP assay versus conventional PCR

Microorganism	Isolate	Host/Source	Assay	
			PCR	LAMP
Ralstonia syzygii subsp. *celebesensis* (*Rsc*)	MY4101	*Musa* sp.	+	+
Rsc	MY2101	*Musa* sp.	+	+
Rsc	RM130	*Musa* sp.	+	+
Rsc	RM26	*Musa* sp.	+	+
Rsc	KP33-2	*Musa* sp.	+	+
Rsc	YH2101	*Musa* sp.	+	+
Rsc	KB 1101	*Musa* sp.	+	+
Rsc	KB35-1	*Musa* sp.	+	+
Rsc	BS1401	*Musa* sp.	+	+
Rsc	TT2501	*Musa* sp.	+	+
Rsc	BT2401	*Musa* sp.	+	+
Rsc	BT4101	*Musa* sp.	+	+
Ralstonia solanacearum	RS832	*Solanum lycopersicum*	-	-
R. solanacearum	RS1350	*Zingiber officinale*	-	-
R. solanacearum	RS-SK04	*Capsicum annuum*	-	-
R. solanacearum	RS-SK05	*Capsicum annuum*	-	-
Enterobacter sp.	No.7	Soil	-	-
Enterobacter sp.	No.21	Soil	-	-
Acinetobacter sp.	No.12	Soil	-	-
Acinetobacter sp.	No.25	Soil	-	-
Klebsiella sp.	No.6	Soil	-	-
Klebsiella sp.	No.16	Soil	-	-
Pseudomonas sp.	No.29	Soil	-	-
Bacillus sp.	JK74	Soil	-	-
Bacillus sp.	JK106	Soil	-	-
Bacillus sp.	YH3-2 B2	Soil	-	-
Pectobacterium sp.	ER01	*Brassica rapa* subsp. *pekinensis*	-	-
Fusarium oxysporum f.sp. *cubense* (*Foc*)	KPS1-3	*Musa* sp.	-	-
Foc	PBR1-5	*Musa* sp.	-	-
Trichoderma sp.	TPK01	Soil	-	-

Selection of unique genetic regions through rep-PCR DNA fingerprinting

Rep-PCR DNA fingerprinting was employed to identify unique genetic regions in *Rsc* isolates, such as MY4101 and BT4101, distinguishing them from other microorganisms. The analyzed microorganisms included *R. solanacearum* isolates RS832, RS1350, and RS1481; *Bacillus* sp. isolates YH3-2 B2, JK74, and JK106; *Klebsiella* sp. isolate No. 6; *Enterobacter* sp. isolate No. 7; *Acinetobacter* sp. isolate No. 12; *Pseudomonas* sp. isolate No. 29; and *Fusarium oxysporum* f. sp. *cubense* isolate KPS1-63. Genomic DNA extracted from these samples was subjected to rep-PCR to generate DNA fingerprints using the primers ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') and ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') [26]. The PCR reaction mixture contained 50 ng of genomic DNA, 0.5 μM of each primer, 2 mM dNTPs,

1.25×buffer A, 1.25 mM $MgCl_2$, and 2 U of Taq DNA polymerase (Vivantis, Malaysia). PCR amplification was performed under the following thermocycling conditions: an initial denaturation step at 95°C for 7 min, followed by 30 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. The reactions were performed using a PCRmax thermocycler (PCRmax, UK). PCR products were analyzed by electrophoresis on a 1.2% agarose gel prepared in 1×TBE buffer (90 mM Tris/borate/EDTA, pH 8.0). Electrophoresis was conducted at 100 V for 90 min. The resulting DNA patterns were visualized under UV light using a Gel Documentation System (UVITEC, Cambridge, UK). The sizes of the DNA bands were determined and analyzed using UVITec-1D software. DNA bands unique to the *Rsc* strains, including MY4101 and BT4101, as distinguished from those of other microorganisms, were identified and carefully excised from the gel using a sterile scalpel. The excised bands were purified using the MEGAquick-spin™ Plus Total Fragment DNA Purification Kit (iNtRON Biotechnology, Korea). The purity and concentration of the purified DNA were measured using a Thermo Scientific™ NanoDrop™ Lite spectrophotometer. The purified DNA was subsequently used for cloning and sequencing.

DNA cloning and sequencing

The purified PCR product was ligated into the pGEM-T Easy cloning vector using T4 DNA ligase (Promega, USA) at an insert-to-vector molar ratio of 3:1. The ligation reaction was incubated at 4°C for 1 h. The ligation mixture was then transformed into *Escherichia coli* JM109 competent cells. Transformed cells were plated on Luria-Bertani agar (LBA) plates supplemented with 100 μg/mL ampicillin, 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG), and 40 μL of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). Plates were incubated overnight at 37°C, and white colonies, indicative of successful transformation, were selected for further analysis. A single white colony was used as a template for PCR amplification using M13 universal primers (M13F_43: 5'-AGGGTTTTCCCAGTCACGACGTT-3' and M13R: 5'-CAGGAAACAGCTATGAC-3'). The PCR reaction mixture contained 0.5 μM of each primer, 2 mM dNTPs, 1.25×Buffer A, 1.5 mM $MgCl_2$, and 2 U of Taq DNA polymerase (Vivantis, Malaysia). Amplification was performed under the following thermocycling conditions: an initial denaturation at 95°C for 5 min; 30 cycles at 95°C for 45 s, 55°C for 45 s, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. The amplified product was purified using the MEGAquick-spin™ Plus Total Fragment DNA Purification Kit (iNtRON Biotechnology, Korea). Purified DNA was sequenced using Sanger sequencing (BIONICS, Korea).

LAMP primer design

The nucleotide sequence of the *Rsc* target region was trimmed and assembled using the Gap4 program. The processed sequence was then used for designing LAMP primers using the LAMP Primer Designer Tool version 1.4.2 (New England Biolabs, https://lamp.neb.com/). The primer design parameters were configured to automatically assign values based on the GC content of the target sequence. All primers were synthesized by Macrogen, Inc. (South Korea).

LAMP assay reaction and conditions

The LAMP assay was performed to amplify the genomic DNA of *Rsc* using the WarmStart® LAMP Kit (DNA & RNA) (New England Biolabs Inc., UK). Each reaction was conducted in a 12.5 μL volume, containing 1×WarmStart LAMP Kit Master Mix, 0.2 μM of each outer primer (F3 and B3), 1.6 μM of each inner primer (FIP and BIP), 0.4 μM of each loop primer (F-Loop and B-Loop), 0.25 μL of LAMP fluorescent dye, and 1 μL of genomic DNA (25 ng). The optimized reaction conditions included an incubation at 67°C for 30 min, followed by a gradual temperature increase (ramp rate: 0.05°C/s) from 80°C and 98°C to determine the annealing temperature of the LAMP amplicon (anneal derivative analysis). The assay was performed using the Genie® III instrument (OptiGene, UK), which provided real-time monitoring and graphical visualization of the amplification process.

Specificity and sensitivity test of the LAMP primers

The specificity of the designed LAMP primers for detecting *Rsc* was evaluated using genomic DNA from various microorganisms, including *Rsc*, *R. solanacearum*, *Bacillus* sp., *Klebsiella* sp., *Enterobacter* sp., *Pseudomonas* sp., *Acinetobacter* sp., *Pectobacterium* sp., *Fusarium oxysporum* f. sp. *cubense* (*Foc*), and *Trichoderma* sp. The LAMP reactions were performed

under the optimized conditions described earlier. The sensitivity of the LAMP primers was assessed using 10-fold serial dilutions of genomic DNA from *Rsc* isolate MY4101, ranging from 25 ng/μL to 2.5×10^{-5} ng/μL. Additionally, a bacterial cell suspension of the same isolate was prepared through 10-fold serial dilutions (10^5 to 0 cells/μL) in sterile distilled water to evaluate the direct detection capability. DNA extraction was performed by boiling the bacterial suspensions for 10 min, following the method described [27].

The performance of the developed LAMP assay was compared to conventional PCR using the published primer set 121F (5'-CGTATTGGATGCCGTATTGGA-3') and 121R (5'-AAGTTCATTGGTGCCGAATCA-3') [6]. The PCR reaction mixture contained 0.4 μM of each primer, 2 mM dNTPs, 1.25× Buffer A, 1.5 mM $MgCl_2$, 2 U of *Taq* DNA polymerase (Vivantis, Malaysia), and 1 μL of genomic DNA. Thermocycling conditions consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. PCR products were analyzed by electrophoresis on a 1.2% agarose gel to evaluate the amplification results. The relative specificity and sensitivity of the LAMP assay were calculated as described [8].

Detection of *Rsc* in spiked banana sucker samples

To evaluate the detection threshold of the developed LAMP assay for *Rsc* in banana suckers using a simple DNA extraction method, healthy banana suckers (10 g) were homogenized in 10 mL of sterile water. A 1-mL aliquot of the resulting homogenate was spiked with 10-fold serial dilutions of the *Rsc* isolate MY4101 bacterial suspension, ranging from 10^8 to 10^2 cells/mL. DNA was extracted by boiling the spiked samples for 10 min. A 1-μL aliquot of the boiled extract was analyzed using both the LAMP assay and the conventional PCR method, following the protocols described previously.

Detection of *Rsc* in naturally infected samples

The applicability of the LAMP assay for detecting *Rsc* in naturally infected banana samples was evaluated. A total of 10 symptomless banana sucker samples were collected from natural fields in Yala Province, Thailand, where *Rsc* infection was previously identified. For assay validation, two additional banana sucker samples were included as controls: one *Rsc*-free sample derived from *in vitro* propagation (negative control) and one *Rsc*-inoculated sample (positive control). Genomic DNA was extracted from 100 mg of each sample using the cetyltrimethylammonium bromide (CTAB) method. The extracted DNA was analyzed using the LAMP assay under the previously optimized conditions. The results were compared with those obtained using the conventional PCR method.

RESULTS

Unique genetic regions derived from rep-PCR DNA fingerprinting

Rep-PCR analysis using the primers ERIC2 and ERIC1R generated distinct DNA fingerprints, differentiating *Rsc* from other examined microorganisms. The *Rsc* isolates MY4101 and BT4101 exhibited identical DNA fingerprint patterns. A unique 1300-bp DNA band observed exclusively in the *Rsc* isolates was selected for sequencing. This sequence was subsequently used to design LAMP primers (Fig. 1).

LAMP primers: specificity and sensitivity

After trimming low-quality sequences, a specific 1017-bp amplicon identified in *Rsc* through rep-PCR was used for designing LAMP primers, and the sequence was submitted to NCBI under a GenBank accession number

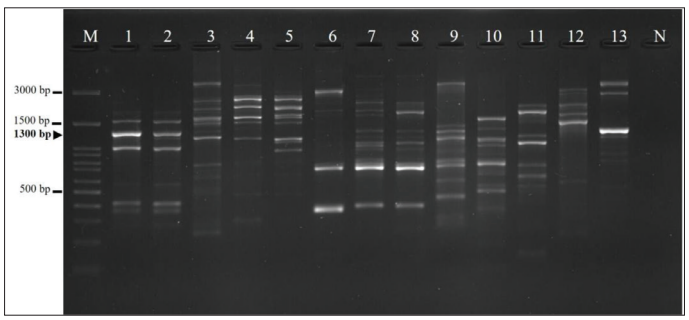

Fig. 1. DNA fingerprint patterns generated using ERIC primers, visualized by 1.2% agarose gel electrophoresis. M: 1000 bp ladder; N: Negative control. Lanes 1-2: *Ralstonia syzygii* subsp. *celebesensis* isolates MY4101 and BT4101. Lanes 3-5: *R. solanacearum* isolates RS832, RS1350, and RS1481; lanes 6-8: *Bacillus* sp. isolates YH3-2 B2, JK74, and JK106; lane 9: *Klebsiella* sp. isolate No. 6; lane 10: *Enterobacter* sp. isolate No. 7; lane 11: *Acinetobacter* sp. isolate No. 12; lane 12: *Pseudomonas* sp. isolate No. 29; lane 13: *Fusarium oxysporum* f.sp. *cubense* isolate KPS1-63.

PV252063. The primer sequences, referred to as the EA2 LAMP primer set, are detailed in Supplementary Table S1. This set includes the outer primers (EA2F3 and EA2B3), the inner primers (EA2FIP, composed of F2 and F1c, and EA2BIP, composed of B2 and B1c), and the loop primers (EA2LF and EA2LB).

The specificity of the designed LAMP primer set was evaluated using genomic DNA from *Rsc* isolates and non-target microorganisms. The results are presented in Table 1 and Fig. 2. The LAMP assay successfully detected all *Rsc* isolates (MY4101, MY2101, RM130, RM26, KP33-2, YH2101, KB1101, KB35-1, BS1401, TT2501, BT2401, and BT4101), showing positive amplification for each. In contrast, the LAMP assay did not produce amplification for any of the non-target microorganisms, including *Ralstonia solanacearum* (RS832, RS1350, RS-SK04, RS-SK05), *Enterobacter* sp. (No.7, No.21), *Acinetobacter* sp. (No.12, No.25), *Klebsiella* sp. (No.6, No.16), *Pseudomonas* sp. (No.29), *Bacillus* sp. (JK74, JK106, YH3-2 B2), *Pectobacterium* sp. (ER01), *Fusarium oxysporum* f.sp. *cubense* (KPS1-3, PBR1-5), and *Trichoderma* sp. (TPK01).

PCR demonstrated identical results, detecting all *Rsc* isolates while showing no amplification for the non-target microorganisms. The specificity and sensitivity of the LAMP assay were calculated to be 100%, indicating that the designed LAMP primer set is specific and sensitive for detecting *Rsc*. The sensitivity of the LAMP assay and conventional PCR was evaluated using 10-fold serial dilutions of genomic DNA from *Rsc* isolate MY4101, ranging from 25 ng/μL to 2.5×10^{-5} ng/μL. The LAMP assay detected genomic DNA concentrations as low as 2.5×10^{-3} ng/μL, with amplification times increasing as the DNA concentration decreased. At 25 ng/μL, the amplification signal was observed at 11.35 min, while at 2.5×10^{-3} ng/μL, the amplification time increased to 18.30 min (Fig. 3A and Supplementary Table S2). The melting temperature (Tm) of the amplified products ranged from 91.87°C to 92°C (Fig. 3B and Supplementary Table S2.). The similarity or identity of the Tm values indicates a consistent GC content of the amplified products, confirming the accuracy of amplification and supporting positive results. No amplification signals were observed for DNA concentrations below 2.5×10^{-3} ng/μL (2.5×10^{-4}, 2.5×10^{-5}, 2.5×10^{-6} ng/μL). In contrast, conventional PCR demonstrated lower sensitivity, with a detection limit of 2.5×10^{-1} ng/μL. PCR yielded positive results for DNA concentrations ranging from 25 ng/μL to 2.5×10^{-1} ng/μL but failed to amplify DNA at concentrations of 2.5×10^{-2} ng/μL and lower (Fig. 3C).

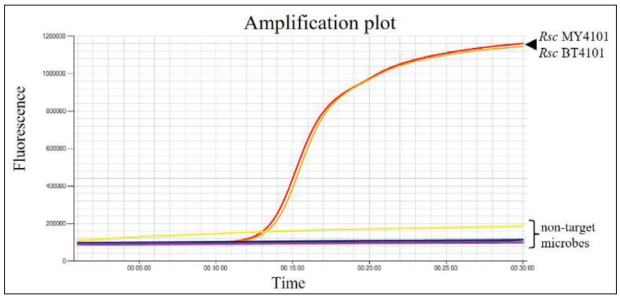

Fig. 2. Specificity test of the designed LAMP primer set. Target samples: DNA of *Ralstonia syzygii* subsp. *celebesensis (Rsc)* isolate MY4101 and BT4101, and non-target microorganisms: *Ralstonia solanacearum* isolates RS832 and RS-SK05, *Pseudomonas* sp. isolate No. 29, *Bacillus* sp. isolates JK74, *and Fusarium oxysporum* f.sp. *cubense* isolate PBR1-5.

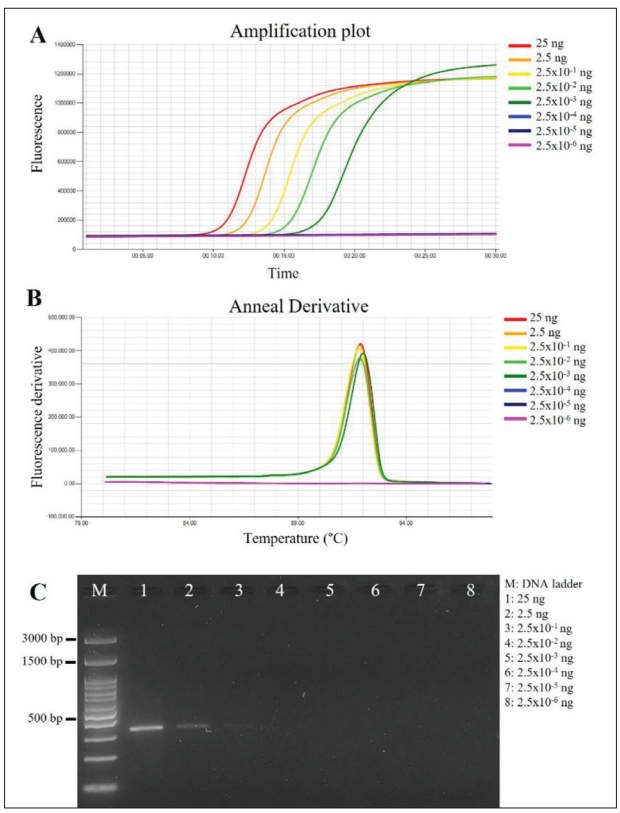

Fig. 3. Sensitivity analysis of the developed LAMP assay for detecting *Ralstonia syzygii* subsp. *celebesensis*. **A** – Amplification signal of the LAMP assay. **B** – Melting temperature of the LAMP products. **C** – Sensitivity analysis using PCR.

The sensitivity of the LAMP and conventional PCR assays in detecting *Rsc* was evaluated using bacterial cell suspensions prepared in 10-fold serial dilutions. DNA was extracted using a simple boiling method. The LAMP assay detected bacterial concentrations as low as 10 cells/reaction. Positive amplification signals were observed at higher concentrations, with 10^5 cells/reaction at 10 min, 10^4 cells/reaction at 11.05 min, 10^3 cells/reaction at 13 min, and 10^2 cells/reaction at 16 min. At 10 cells/reaction, the amplification signal appeared at 18 min (Fig. 4A and Supplementary Table S3). The melting temperature (Tm) of the amplified products ranged between 91.70°C and 92°C (Fig. 4B and Supplementary Table S3.), validating the consistency and accuracy of the LAMP assay. No amplification signals were observed at 1 cell/reaction and 0 cell/reaction. In contrast, conventional PCR demonstrated a detection limit of 10^3 cells/reaction, producing positive results for 10^5, 10^4, and 10^3 cells/reaction but failing to detect lower concentrations (10^2 cells/reaction and below) (Fig. 4C). These results demonstrate that the LAMP assay has a higher sensitivity than conventional PCR, detecting bacterial concentrations as low as 10 cells/reaction, whereas PCR had a detection limit of 10^3 cells/reaction.

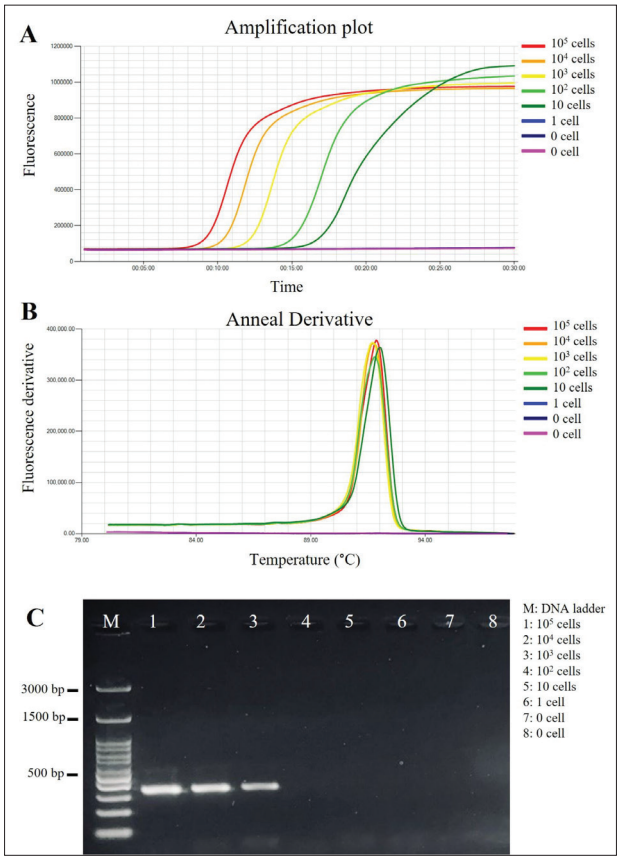

Fig. 4. Sensitivity analysis of the developed LAMP assay for detecting *Ralstonia syzygii* subsp. *celebesensis* from cell suspension and prepared genomic DNA by boiling method. **A** – Amplification signal of the LAMP assay. **B** – Melting temperature of the LAMP products. **C** – Sensitivity analysis using PCRassay.

Detection of *Rsc* in spiked banana sucker samples

The detection threshold of the LAMP assay and conventional PCR for *Rsc* in spiked banana sucker samples was evaluated using a simple boiling DNA extraction method. The LAMP assay detected *Rsc* at concentrations as low as 10 cells/reaction. Positive amplification signals (Fig. 5A and Supplementary Table S4.) were observed at higher concentrations, including 10^5 cells/reaction (10 min), 10^4 cells/reaction (11 min), 10^3 cells/reaction (12.82 min), 10^2 cells/reaction (14 min), and 10 cells/reaction (14.05 min). The melting temperature (Tm) of the amplified products remained consistent (Fig. 5B and Supplementary Table S4), ranging from 91.70°C to 91.90°C, confirming the accuracy of the amplification. No amplification signals were observed at 1 cell/reaction and 0 cell/reaction.

In contrast, conventional PCR failed to detect *Rsc* at all tested concentrations, including the highest concentration of 10^5 cells/reaction, yielding negative results throughout the dilution series. These results demonstrate that the LAMP assay can detect *Rsc* in spiked banana

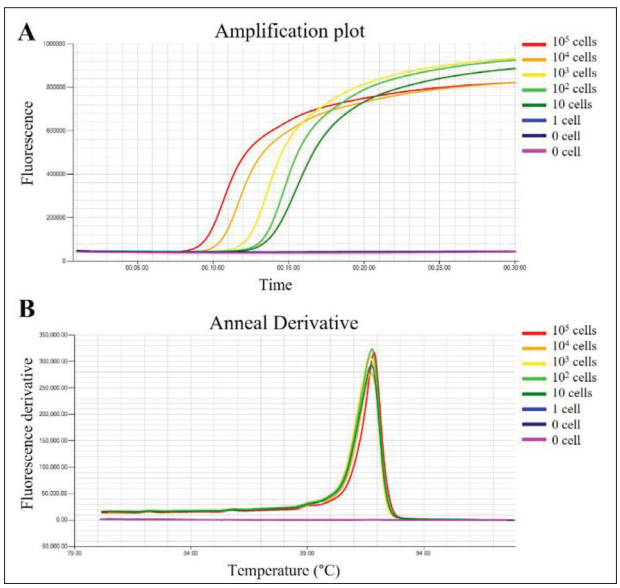

Fig. 5. Sensitivity analysis of the developed LAMP assay for detecting *Ralstonia syzygii* subsp. *celebesensis* in spiked banana sucker samples. **A** – Amplification signals of the LAMP assay. **B** – Melting temperature of the LAMP products.

sucker samples with a detection threshold of 10 cells/reaction; conventional PCR showed no amplification even at higher bacterial concentrations. The LAMP assay's ability to perform well with DNA extracted using a simple boiling method reflects its robustness, sensitivity, and suitability for field-based diagnostics.

Detection of *Rsc* in naturally infected samples

The LAMP assay and conventional PCR were evaluated for the detection of *Rsc* in banana sucker samples, including a positive control (*Rsc*-inoculated sucker), a negative control (*Rsc*-free sucker from *in vitro* propagation), and 10 samples collected from an infected field. Both assays correctly identified the negative control (Sucker 1) as *Rsc*-free and the positive control (Sucker 2) as *Rsc*-positive. Among the 10 field samples, conventional PCR detected *Rsc* in 1 sample (Sucker 7), while the LAMP assay detected *Rsc* in 2 samples (Suckers 3 and 7) (Table 2 and Supplementary Table S5). These results demonstrate that the LAMP assay can detect *Rsc* in naturally infected samples and exhibits slightly higher sensitivity than PCR under these conditions.

Table 2. Detection of *Ralstonia syzygii* subsp. *celebesensis* (*Rsc*) in naturally infected banana sucker samples using LAMP and PCR assays

Sucker No.	Sample Description	PCR Result	LAMP Result
1	*Rsc*-free sucker (negative control)	-	-
2	*Rsc*-inoculated sucker (positive control)	+	+
3	Field sample	+	+
4	Field sample	-	-
5	Field sample	-	-
6	Field sample	-	-
7	Field sample	-	+
8	Field sample	-	-
9	Field sample	-	-
10	Field sample	-	-
11	Field sample	-	-
12	Field sample	-	-

DISCUSSION

The application of rep-PCR as a preliminary tool revealed its utility in developing molecular diagnostic assays. By targeting repetitive extragenic sequences unique to *Rsc*, the rep-PCR-based analysis provided high-resolution genetic fingerprints, enabling the generation of species- and strain-specific genomic fingerprint patterns of the pathogen [28]. These genetic markers served as robust targets for LAMP primer design, ensuring assay specificity while minimizing cross-reactivity with non-pathogenic or closely related bacterial species [21,22]. The integration of molecular techniques accentuates the synergistic potential of combining rep-PCR with LAMP for pathogen detection and differentiation. The LAMP assay developed in this study demonstrated high specificity and sensitivity in detecting *Rsc* from both pure cultures and symptomatic plant tissues [10]. The incorporation of rep-PCR-derived genetic markers ensured the selection of unique and reliable targets for primer design, enhancing assay precision. Amplification was completed within one hour under isothermal conditions, eliminating the need for complex thermocyclers and reducing the technical expertise required for operation [10, 11]. The portability and robustness of the LAMP assay make it highly suitable for on-site diagnostics [29-31]. This capability is crucial for the timely detection of BBD, which enables the rapid implementation of control measures to prevent the spread of this devastating disease [30].

This study further demonstrates the efficacy of the LAMP assay for the specific and sensitive detection of *Rsc*, outperforming conventional PCR in several aspects. The specificity of the designed LAMP primers, validated across a range of microorganisms, guarantees the assay's reliability for field diagnostics, as no cross-reactivity with non-target organisms was observed. The LAMP assay's detection limit of 10 cells/reaction represents a significant advancement in sensitivity compared to the conventional PCR limit of 10^3 cells/reaction. This heightened sensitivity is particularly advantageous in scenarios where pathogen populations are low, such as in the early stages of infection or latent infections. Furthermore, the ability of the LAMP assay to detect DNA at concentrations as low as 2.5×10^{-3} ng/μL underscores its effectiveness in detecting minimal pathogen presence.

The simplicity of the LAMP assay design is another key advantage. Operating under isothermal conditions not only reduces equipment costs but also increases its tolerance to inhibitors commonly present in plant tissue extracts [32]. These attributes make the LAMP assay ideal for use in field environments where laboratory infrastructure is limited [33]. Furthermore, its rapid detection capability supports real-time decision-making, allowing farmers and agricultural practitioners to implement immediate interventions to curtail disease outbreaks, such as the removal of infected plants and disinfection of tools [30].

Compared to other molecular techniques, LAMP offers practical advantages, including the ability to produce a clear color change that is easily observed by the naked eye [34]. The results can be detected using simple colorimetric changes or turbidity, eliminating the need for expensive fluorescence detection systems. This versatility further enhances its efficacy in low-resource settings, aligning with the urgent need for accessible diagnostic tools in banana cultivation regions affected by BBD.

In this study, the capacity of the LAMP assay was further confirmed in field-relevant settings. In spiked banana sucker samples, the LAMP assay consistently detected *Rsc* as low as 10 cells/reaction using a simple boiling DNA extraction method; PCR failed to detect the pathogen even at higher concentrations. This highlights the practical value of the LAMP assay for rapid, low-resource diagnostic applications [34-38]. Similarly, in infected samples, the LAMP assay identified *Rsc* in more samples than PCR, demonstrating its higher sensitivity and potential for uncovering low-level infections in field conditions. The consistent melting temperatures of the LAMP products (91°C-92°C) across experiments validate the reliability and accuracy of the assay, as uniform Tm values indicate correct and specific amplification. The rapid amplification times observed with higher pathogen concentrations further position the LAMP assay as suitable for time-sensitive diagnostics.

The rapid and accurate detection of *Rsc* using the LAMP assay offers a transformative approach to managing BBD in banana cultivation. Early detection is critical for preventing dissemination of the pathogen, which can occur through contaminated planting materials, tools, and insect vectors. Deploying LAMP assays in banana-growing regions could enable proactive monitoring of disease prevalence and targeted application of control measures, reducing economic losses and safeguarding banana production. Furthermore, portable and affordable diagnostic tools like LAMP align with global efforts to promote sustainable agriculture. By reducing reliance on centralized laboratory facilities and expensive diagnostics, LAMP empowers smallholder farmers and enhances disease management in regions where bananas are a vital staple crop [34].

This study underscores the effectiveness of LAMP in detecting *Rsc*, the pathogen responsible for BBD. However, further research is essential to validate its performance under diverse field conditions. Extending LAMP application to other banana pathogens could enhance its role in integrated disease management strategies. Additionally, user-friendly LAMP kits with pre-assembled reagents and straightforward visual readouts could significantly boost adoption rates among farmers and agricultural professionals. These findings reveal LAMP's potential as a practical, cost-effective, and field-adaptable diagnostic tool, addressing the urgent need for accessible detection methods, particularly in resource-constrained banana-growing regions.

CONCLUSIONS

The developed LAMP assay represents a robust, sensitive, and specific diagnostic tool for detecting *Rsc*, with significant advantages over conventional PCR. Its speed, portability, and cost-effectiveness make it indispensable for agricultural disease diagnostics, particularly in resource-constrained settings. Implementation of this assay in field diagnostics could greatly enhance the management of banana blood disease by enabling early and accurate pathogen detection, offering a practical solution to one of the most pressing challenges in banana cultivation.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. LAMP primer sequences designed in this study

Primer name	Sequence (5'-3')
EA2F3	GGACCAGTACGAGGTCAGAC
EA2B3	CCATCGCGACCATGCCA
EA2FIP	ACGCAGCACTGCCAGAGCCGTCAGGCTGGTACCGTCA
EA2BIP	GATCGCATTGAGCCTGCAGGACACCTGATCGATCGACTGTC
EA2LF	AGCAGCGCCAACGTGATG
EA2LB	GATTCGGCGTTTGCTGTGC

Supplementary Table S2. Sensitivity analysis of the developed LAMP assay for detecting *Ralstonia syzygii* subsp. *celebesensis*

DNA (ng)/LAMP reaction	Ct	Tm	Detection Result
25	11.35	91.90	+
2.5	13.17	91.97	+
2.5x10^-1	14.55	92.00	+
2.5x10^-2	16.08	91.87	+
2.5x10^-3	18.30	91.93	+
2.5x10^-4	-	-	-
2.5x10^-5	-	-	-
2.5x10^-6	-	-	-

Amplification signals of the LAMP assay are represented by Ct values, and Tm refers to the melting temperature of the LAMP products. The detection result is determined based on Ct and Tm values, where "+" indicates a positive result and "-" indicates a negative result

Supplementary Table S3. Sensitivity analysis of the developed LAMP assay for detecting *Ralstonia syzygii* subsp. *celebesensis* (*Rsc*) from the cell suspension and prepared genomic DNA by the boiling method

Rsc cell/LAMP reaction	Ct	Tm	Detection Result
10^5	10.00	91.80	+
10^4	11.05	91.70	+
10^3	13.00	91.70	+
10^2	16.00	91.80	+
10	18.00	92.00	+
1	-	-	-
0	-	-	-

Amplification signals of the LAMP assay are represented by Ct values, Tm refers to the melting temperature of the LAMP products. Detection is determined based on Ct and Tm values where "+" indicates a positive result and "-" indicates a negative result

Supplementary Table S4. Sensitivity analysis of the developed LAMP assay for detecting *Ralstonia syzygii* subsp. *celebesensis* (*Rsc*) in spiked banana sucker samples

Rsc cell/LAMP reaction	Ct	Tm	Detection Result
10^5	10.00	91.90	+
10^4	11.00	91.70	+
10^3	12.82	91.70	+
10^2	14.00	91.80	+
10	14.50	91.70	+
1	-	-	-
0	-	-	-

Amplification signals of the LAMP assay are represented by Ct values, Tm refers to the melting temperature of the LAMP products. Detection is determined based on Ct and Tm values where "+" indicates a positive result and "-" indicates a negative result

Supplementary Table S5. Detection of *Ralstonia syzygii* subsp. *celebesensis* (*Rsc*) in naturally infected banana sucker samples using LAMP assays

Sucker no.	Sample Description	Ct	Tm	Detection Result
1	*Rsc*-free sucker (negative control)	-	-	-
2	*Rsc*-inoculated sucker (positive control)	18.00	91.90	+
3	Field sample	17.00	91.96	+
4	Field sample	-	-	-
5	Field sample	-	-	-
6	Field sample	-	-	-
7	Field sample	19.45	92.00	+
8	Field sample	-	-	-
9	Field sample	-	-	-
10	Field sample	-	-	-
11	Field sample	-	-	-
12	Field sample	-	-	-

Amplification signals of the LAMP assay are represented by Ct values, Tm refers to the melting temperature of the LAMP products. Detection is determined based on Ct and Tm values, where "+" indicates a positive result and "-" indicates a negative result

SUPPLEMENTARY MATERIAL

RESEARCH DATASET

The raw data underlying this article is available as an online supplementary research dataset: https://www.serbiosoc.org.rs/NewUploads/Uploads/Kaennuea%20et%20al_Research%20Dataset.pdf