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FULL PAPER

Evaluation of the Dry Format Isothermal Amplification-Based Biosensor for Rice Blast

Rubigilda Paraguison-Alili*, Jeshua Paul P. Antonino, Dan Charlie Joy C. Pangilinan, Aldrin M. Corpuz, Renmar M. Dela Cruz, and Maria Excelsis M. Orden

Central Luzon State University,Science City of Muñoz, Nueva Ecija, Philippines 3119.
*Corresponding author, rpalili@clsu.edu.ph

Seeds and propagating resources of rice plants can also be the primary source of the pathogens that cause various diseases. A modified isothermal amplification-based biosensor was developed to determine the presence of these pathogens. The isothermal amplification-based biosensor was used for rice blast in seeds, storage, and warehouse swabs. The device was improved for dry format Loop-Mediated Isothermal Amplification (LAMP) technique, which provides precise results for detecting pathogens compared with conventional methods. The formulated assay was tested on-station using different varieties of rice and seed storage swabs for repeatability, sensitivity, and specificity analyses. The formulated assay was specific as tested against other related fungi and sensitive and detected even crudely extracted DNA down to 40.8×10^{-6} ng/µL or 40.8 femtograms. The device will be a decision tool for stakeholders to discard rice seeds or provide phytosanitary treatment before storage, seed banking, and quarantine.

Keywords: Biosensor, dry format, isothermal amplification-based, rice blast

INTRODUCTION

Seeds and propagating resources of rice plants can be the primary source of pathogens that cause various diseases. Seedborne plant pathogens can cause significant yield and quality losses and are an overlooked source of pathogen spread and dissemination (du Toit 2004). They can also transmit the pathogen from one place to another and spread to uninfected areas. Hence, it is essential to detect these seedborne pathogens in the quarantined seeds before storage, banking, and even before transporting and sowing to guarantee that no potentially damaging pathogens are introduced in the field through these seeds and other accessories planting.

Among these important seedborne pathogens that infect rice is rice blast caused by *Pyricularia oryzae*. Rice blast is one of the most damaging diseases of rice of its widespread and destructive nature, making yield losses during epidemic years of up to 100% (Dean et al. 2005). A 50% yield loss was attributed to the disease. The fungus may infect any above portion of rice plants, including roots and seeds. The fungus can infect plants in upland and lowland rice production systems at all stages of growth and development. 20,000 spores can be produced from one lesion, and 60,000 conidia can be produced from spikelets. Rice plants are more susceptible to blast infection when soil conditions are drier and are experiencing water stress (Teng et al. 1991).

In contrast, sowing on wet soil allows seed transmission (Vipin et al. 2017). This disease generally causes a yield loss of 10%-20%, but in severe cases, yield loss may reach up to 80% (Koutroubas et al. 2009). Rice blast disease is seedborne and affects 20%–51% of seeds (Amruta et al. 2019; Reddy et al. 2021). The common symptoms of rice blast are found on all parts, including leaf collars, necks, pedicels, panicles, roots, and seeds. However, the most frequent symptoms are the diamond-shaped lesions on the leaves. In addition, seeds can undergo a blanking condition, causing failure of the seeds to fill or causing the entire panicle to fall over as if rotted.

The frequently used seed assays include a visual examination, selective media, seedling grow-out, and serological assays, which, while appropriate for some pathogens, often exhibit inadequate sensitivity, specificity, and accuracy levels. Effective platforms for early warning and fast response are crucial in preventing or mitigating the impact of these pathogenic invasions in rice, especially at the harbors of entry. This study formulated a dry format LAMP assay that can be used outside the laboratory where nucleic acid extraction and the assay are included. The study demonstrates that molecular detection can offer advantages over conventional methodologies and improve the screening of quarantined seeds and management of the most important diseases in rice and other crops. The technique provides evidence of potential tools for screening rice seeds and recommends the feasibility in screening quarantined seeds' SOP. This method holds the promise for detecting these pathogens in countries with resource-limited settings. The formulated assays recommend advancing the comprehensive seed screening approach in countries with limited resources. Its simplicity, fast, specificity, and sensitivity were verified compared to polymerase chain reaction (PCR) quarantine methods.

Table 1. Designed LAMP DNA markers for Pyricularia oryzae

Target Gene		Primer Design
P.ory F3	Outer primers	CCATCCCCTCTCAAATCGGA
P.ory B3		ATGCGTACGGACTAGGTCTA
P.ory FIP	Inner primers	TTAGCGATTGGGCGGTAGGC - AGTTGGCTTTGGGGGTCT
P.ory BIP		AGAAGACAACAGCGCTCATCCC - AGCGTTATCGCATGGAACAA

MATERIALS AND METHODS

Pathogen Detection Procedures

Survey Area and Sample Collection
A small quantity of a seed lot was examined to represent the seed lot. Primary, composite, and finally, working samples were drawn. Samples were submitted to the lab. Sampling with triers was used taken from sacks. Three of the Central Luzon State University's Special Purpose Rice varieties (CLSU Variety 1, CLSU Variety 2, and CLSU Variety 3) with 3 replicates were purposively obtained from the cold storage rooms of the Seed Laboratory, Central Luzon State University, while a total of 9 representative sampling seeds, 15 swab samples were collected from the same storage site. In addition, flag leaves showing symptoms of *Pyricularia oryzae* infection were collected in university rice field sites. Samples were kept in sterile collection bags and stored at 4°C temperature. Samples were carefully transferred to a sterile zip-lock bag, labeled, transported to the laboratory, and stored in the refrigerator before DNA extraction. The date, place of collection, and volume obtained for each sample were recorded.

Microscopy and Morphological Classification
Samples were inoculated in Rose Bengal Agar medium in Petri plates and incubated for 3 days before being subjected to cultural and microscopic characterization to identify the isolates. Similarly, seed representatives were laid and incubated on Potato Dextrose Agar. Collected rice seed samples from suspected infected storage facilities were subjected to culture and isolation to prepare possible pure cultures of the target pathogens. Pure cultures of the target pathogen were incubated until maturity for morphological characterization of their anamorphic or teleomorphic spores (Burge et al. 1977; Gebala and Sandle 2013; Tonon et al. 1997).

Evaluation of the generated molecular markers
All generated sets of primer sets were designed using the Primer Explorer (https://primerexplorer.jp/e/) and were evaluated for their efficiency in the isothermal technique assays targeting the chromosome 1 of the *Pyricularia oryzae,* which controls appressorium formation and other aspects of growth and development (Choi and Dean, 1997) with GenBank Accession No. CP050920.1 (Table 1). The amplification temperature for optimization was performed from 60°C to 65°C, and the amplification reaction time was investigated at 30, 45, 60, and 90 min. Finally, a set of primers showed the LAMP products visualized directly with blue or shades of blue, indicating a positive reaction. Serial 10-fold dilutions were carried out to determine the detection limit of LAMP. The initial concentration was diluted up to the 10^{th} dilution and added as a template per LAMP mixture to construct a minimum detection limit.

Dry format loop-mediated isothermal amplification (LAMP) assay
For dry format LAMP, a 11µl reagent mixture was made correspondingly containing the following: 1.2x reaction buffer, 8 mM of MgSO4, 0.24 M Trehalose, 1.2 M Betaine, 1.2 mM dNTP, 1 µL of the primer mix, 0.36 U of Bst polymerase, 9.1 U of MMLV, and 0.5 µL of color-fluorescent indicator (CFI) with hydroxy naphthol blue (HNB). The reaction mixtures were dried inside a Biosafety cabinet (BioBase, PRC) for 45 to 60 min. 8.5 µL of nuclease-free water was added to the reaction mixture to rehydrate the mixture and approximately 4 µL of template RNA. Reaction mixtures were incubated in a dry bath at 63°C for 60 minutes with added CFI, which varies from violet (negative) to sky blue (positive) because of reduced Mg^{2+} in the presence of amplified DNA.

RESULTS AND DISCUSSION

Dry Format LAMP Assay in Collected Swab and Grain Samples
The optimized dry format LAMP assay conditions were employed for the standard LAMP test on the crude-extracted DNA material obtained from swabs and grain samples collected from a rice grain warehouse. Three of the Central Luzon State University's Special Purpose Rice varieties (CLSU Variety 1, CLSU Variety 2, and CLSU Variety 3) with 3 replicates were purposively obtained from the cold storage rooms of the Seed Laboratory, Central Luzon State University, while a total of 9 representative sampling seeds, 15 swab samples were collected from the same storage site. A set of LAMP-designed primers detected the target pathogen *Pyricularia oryzae* (rice blast). The results were determined by naked eye detection based on colorimetric results using hydroxy naphthol blue (HNB), which varies from violet (negative) to sky blue (positive). As seen in Figure 1, the appearance of blue coloration indicated an amplified gene of *P. oryzae*, suggesting the successful detection of the rice blast disease in all three-grain samples. Furthermore, these results demonstrate the effectivity of the formulated dry format LAMP assay in detecting *P. oryzae* or the rice blast disease in the tested macerated samples obtained from both swab and grain samples.

LAMP technique
G1: grain sample 1; *G2:* grain sample 2; *G3:* grain sample 3; *S1:* swab sample from warehouse walls and floor; *S2:* swab sample from *Shelf A* (pooled swab sample from neighboring shelves A); *S3:* swab sample from *Shelf B* (pooled swab sample from

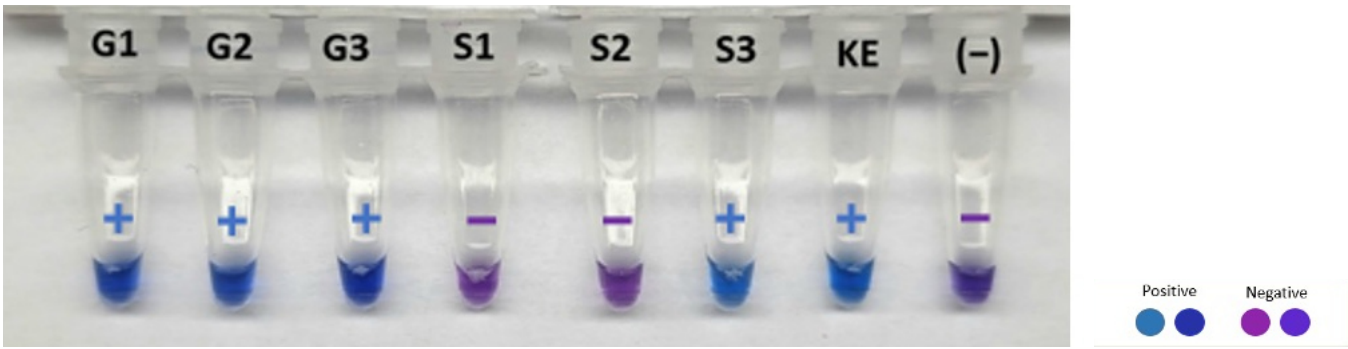

Figure 1. Detection of *P. oryzae* in macerated grain and swab samples using the devised dry format LAMP technique.

G1: grain sample 1; G2: grain sample 2; G3: grain sample 3; S1: swab sample from warehouse walls and floor; S2: swab sample from Shelf A (pooled swab sample from neighboring shelves A); S3: swab sample from Shelf B (pooled swab sample from neighboring shelves B); KE: Kit-extract from rice flag leaves showing symptoms of rice blast infection; (−): Negative Control. Positive: sky blue to indigo coloration; negative: purple to violet.

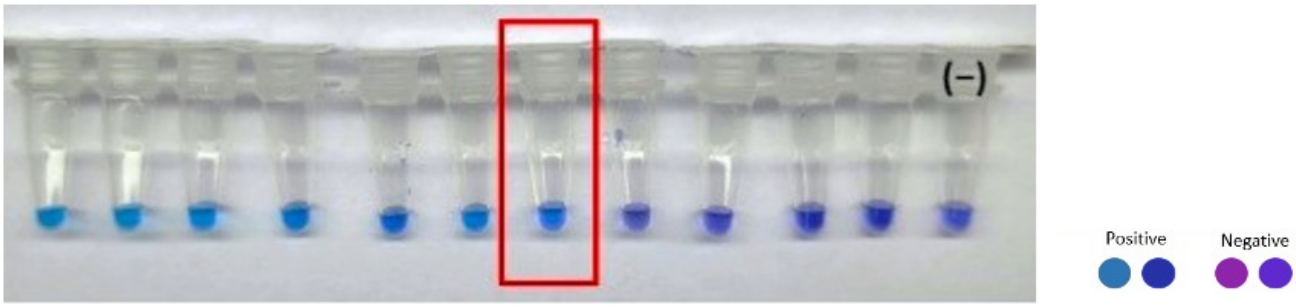

Figure 2. Analytical sensitivity and limit of detection test for the devised dry format LAMP technique in detecting *P. oryzae* (rice blast).

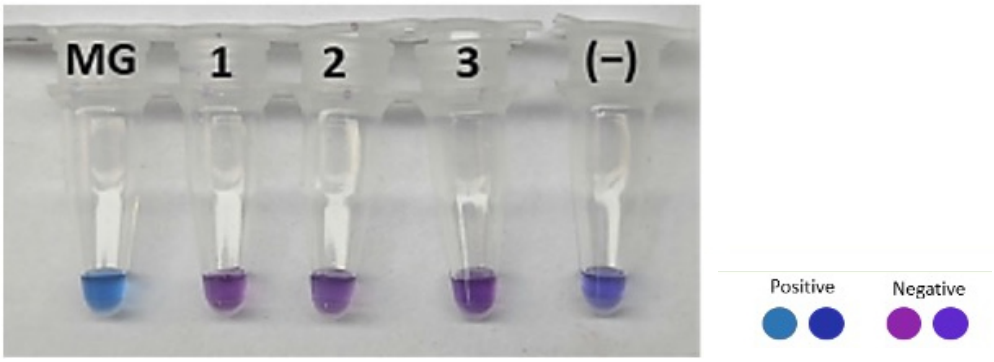

Figure 3. Analytical Specificity test of the devised dry format LAMP technique in detecting *M. oryzae* . 1: *P. oryzae*-positive sample, 2: *Aspergillus sp.* spiked sample; 3: *Pythium sp.* spiked sample; (−): Negative Control

neighboring shelves B); KE: Kit-extract from rice flag leaves showing symptoms of rice blast infection; (−): Negative Control. *Positive*: sky blue to indigo coloration; *negative*: purple to violet.

Analytical Sensitivity and Specificity of the Formulated Technique

For evaluating the analytical sensitivity of the formulated dry format LAMP technique, serial dilutions were prepared from DNA extracts of the target pathogens, using kit-extracted DNA material from the flag leaves showing symptoms of *P. oryzae* infection collected in rice field sites. The DNA was 10-fold diluted with sterile nuclease-free water and tested using the technique. The lowest detectable concentration of DNA was considered to indicate the system's sensitivity.

Based on the results in Figure 2, the detection limit for the *P. oryzae* DNA material was observed at the 10^{-6} dilution, yielding a sensitivity of 40.8×10^{-6} ng/µL based on the initial concentration.

In verifying the specificity of the devised dry format LAMP method, several positive samples from other fungal non-target pathogens were tested alongside the target pathogens. Based on the resulting colorimetric output, only the *P. oryzae*-positive sample indicated a reaction using their respective DNA LAMP primers. In contrast, the rest of their non-target

samples yielded negative results, thus, suggesting specific detection among the treated samples. (Figure 3).

The study aimed to optimize and evaluate the dry format isothermal amplification-based biosensor for rice blast, which can be recommended in seed health procedures, attributable to its high sensitivity, specificity, fast processing results provision, and on-site application. The tool could denote an advantage for seed growers and companies to detect *P. oryzae* in rice seeds before sowing, selling, and importing. Molecular detection techniques of pathogens have been developed as cutting-edge, replacing PCR-based diagnostic methods due to higher cost, besides being a time-consuming technique. LAMP-based assays have been widely reported in phytopathogens because of their high sensitivity, rapidity, and ease of result visualization. However, few molecular detection assays are available for *P. oryzae*, although PCR or qPCR-based assays can be costly and take time to detect. This study points out a necessity for screening rice pathogens to develop an efficient monitoring tool. Moreover, the seed lots supplied to the farmers for farming should also be consistently checked to avoid any unexpected outbreaks and epidemics of the disease.

SUMMARY AND CONCLUSION

A dry format isothermal amplification-based biosensor was devised as a simple and economically designed which can be stored in an ambient isothermal field-based assay for rice blast in rice seeds and seed storage swabs. The assay is a derivative of loop-mediated isothermal amplification (LAMP). The optimized dry format LAMP assay conditions were employed on the crude-extracted DNA material obtained from swabs and grain samples collected from rice grain storage units with a set of designed LAMP primers targeting the *Pyricularia oryzae* (rice blast). The formulated assay was specifically tested against other related fungi and sensitive, which detected DNA down to 40.8×10-6 ng/µL or 40.8 femtograms. These results suggest the effectivity of the dry format LAMP assay in detecting the presence of *Pyricularia oryzae* in rice grains and swabs from a storage facility with competent results. The device can be a decision tool that will provide phytosanitary service before expensive storage, seed banking, and quarantine. As a result, planting seeds infected with pathogens will be avoided.

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