



Establishment and application of the Recombinase-Aided Amplification-Lateral Flow Dipstick detection method for *Pantoea ananatis* on rice

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Abstract

Pantoea ananatis is a major pathogen that causes the new bacterial blight in rice, and its symptoms very similar to rice bacterial blight. Therefore, there is a dire need for an accurate and rapid method for detecting *P. ananatis*. In this study, an early and rapid visual detection method for *P. ananatis* was established. Using GyrB gene as the target sequence, an innovative recombinase-aided amplification detection system integrated with a lateral flow dipstick (RAA-LFD) was constructed. The optimized RAA-LFD detection method can be initiated at body temperature and does not rely on precise instruments. It does not require DNA extraction and can be used directly with plant tissue fluids. The results can be visualized after 10 minutes of amplification. The specificity and sensitivity tests showed that the RAA-LFD method could detect *P. ananatis*, whereas other common plant pathogens were not detected, and its detection sensitivity for *P. ananatis* DNA reached 100 copies/ μ L. The detection of diseased tissues indicated that this method could accurately detect *P. ananatis* in artificially inoculated rice tissues in the early stages of infection before symptoms. The RAA-LFD detection system established in this study is simple and fast, with visual results, excellent specificity, and high sensitivity. It is semi-quantitative and should be used for the early detection and rapid field diagnosis of new leaf blight, which provides technical support for the early warning and real-time detection of field samples.

Keywords *Pantoea ananatis* · Recombinase-aided amplification · Lateral flow dipstick · Point of Care Test

Pantoea ananatis is a gram-negative bacterium which can be isolated from soil, water, plants, animals, human, etc. As a plant pathogenic bacteria, *P. ananatis* has many hosts, including pineapple, onion, eucalyptus, oyster mushroom, corn, wheat, rice, etc. The infections in different hosts perform various symptoms, such as rot of the stem, fruit and bulb, leaf spot disease and blight. It was reported that *P. ananatis* caused rice leaf blight in India, Benin, Togo and Russia. In 2020, *P. ananatis* was identified as the pathogen of a new rice bacterial blight. The blight has an incidence of 45%-60% in China. After the *P. ananatis* soaks the rice, water-soaked linear light brown lesions occur at the upper

part of the leaves. Then the lesions spread down to the leaf margins, and the infected leaves turn light brown, eventually resulting in blade curling and wilting (Yu et al. 2022a, b).

The symptoms very similar to rice bacterial blight. From 2020 to 2021, this blight broke out in Zhejiang, Anhui, and other provinces in the middle and lower reaches of the Yangtze River in China, exhibiting an annually increasing trend. Yongyou, Zhongzheyou, Zhongzao, and other major varieties were all susceptible to this blight, whereas Thiamethoxam, Zinc-thiazole, Bismethiazol, and other pesticides that are highly efficient for the preventive control of bacterial blight exerted insignificant effects on it (Yu et al. 2022a, b), which severely affected the safe production of rices and the sustainable development of this industry. Normally, early infected rices seedlings do not show symptoms; however, early diagnosis of blight is particularly important to avoid uncontrolled expansion. To conduct monitoring or preventive control, it is vital to rapidly and accurately detect low-level pathogens that are adequate for the early diagnosis of leaf blight. In practice, the timely detection of leaf blight at the early stages

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of infection will be conducive to making decisions to control this plant blight. Therefore, there is a need to develop early detection and preventive technologies for leaf blight.

The traditional technology of *P. ananatis* detection includes pathogen isolation and species identification based on morphological characteristics, which is difficult, time-consuming, and requires professional knowledge, which greatly increases the difficulty of detection (Teresa et al. 2006; Kini et al. 2019; Mamede et al. 2020; Mamede et al. 2018). Currently, the polymerase chain reaction (PCR) (Asselin et al. 2016; Shin et al. 2022; Figueiredo et al. 2012), multiplex PCR (Kini et al. 2021; Bangratz et al. 2020), qReal-Time PCR (qRT-PCR) (Shabnam et al. 2019), loop-mediated isothermal amplification (LAMP) (Kossi et al. 2021), and other molecular detection techniques have already been applied to the detection of pathogenic bacteria. PCR and qRT-PCR greatly improve the specificity and sensitivity of the detection, but their detection time is long, and their operations are complicated with demands for expensive materials and devices; hence, they are difficult to apply directly to the field (Huang and Yan 2020). LAMP detection exhibits strong specificity and simple operation; however, its primer design is complex and prone to generating false positives.

Recombinase-aided amplification (RAA) is an innovative technique for the rapid amplification of isothermal nucleic acids. It mainly consists of three core enzymes (recombinase, single-stranded DNA-binding protein, and DNA polymerase) undergoing enzymatic reactions at low temperatures to achieve rapid amplification of DNA or RNA, as a molecular biology detection technology (Lu et al. 2010). RAA shares similar principles as recombinase polymerase amplification (RPA). The difference is that the recombinases in the two reactions come from different origins. RPA recombinase originates from bacteriophage T4, and RAA recombinase is from bacteria or fungus. In recent years, the RAA technique has been widely used for the rapid detection of viruses, bacteria, mycoplasmas, parasites, and other species (Li et al. 2019; Xue et al. 2020a, b; Sun et al. 2020; Hu et al. 2019). The lateral flow dipstick (LFD) is an endpoint detection technology for visualizing the observation of amplification products. It is the product of biotin and carboxyl fluorescein (FAM) labels amplified by RAA, which applies antigen antibody binding to form "fluorescein antibody-double labeled nucleic acid amplification product-colloidal gold composite" at the detection line. The results can be directly observed with the naked eye. The RAA-LFD method integrating RAA and LFD detection technology presented strong specificity, high sensitivity, fast response, visualized results, and low standards for devices and operators and is applicable for quick checks in non-laboratory environments.

Currently, RAA technology, as a powerful monitoring tool, has been broadly applied in many fields, such as early monitoring and screening of pathogenic microorganisms, detection and identification of crop diseases and pests, rapid entry-exit quarantine, and food safety (Zhu et al. 2018; Ma et al. 2020; Fu et al. 2019; Li et al. 2019; Lai et al. 2022; Ju et al. 2020; Ju et al. 2022; Wang et al. 2021; Gao et al. 2022); however, there are no reports on the detection and application of *P. ananatis* in China or abroad. By forming the RAA-LFD detection system for *P. ananatis*, this study provides technical support not only for the rapid detection and identification of plant pathogens but also for further application in quick checks in basic-level quarantine departments and resource-limited laboratories.

Materials and methods

Strains for testing

Various plant pathogens including *Pantoea ananatis*, *Xanthomonas oryzae* pv. *oryzae*, *Pantoea dispersa*, *Enterobacter cancerogenus*, *Xanthomonas oryzae* pv. *oryzicola*, and *Burkholderia glumae* selected in this study are important pathogens of rice, including nine strains of *P. ananatis* and one strain of other types of pathogens. The strains of *P. ananatis* were provided by the Laboratory of Disease-Resistance and Breeding, whereas the other strains were provided by the Research Laboratory of Intelligent Forecasting Technology.

Sample preparation

DNA extraction with the kit: Lines were drawn on the Na Plate and cultured overnight at 37°C, the ring scraper was inoculated to collect the bacterial solution, and the genomic DNA was extracted using a bacterial genomic DNA extraction kit following the manufacturer's instructions.

Diseased leaves: Approximately 1 cm of diseased leaves were cut into pieces and placed in a 1.5 ml centrifuge tube. Sterile water (1 ml) was added, and the mixture was soaked for 10 min. The crude extract was used as a template. Bacterial solution: The bacterial solution was used directly as a template.

Instruments, devices, and reagents

Thermostatic metal bath, Test dipstick method by RAA amplification kit (RAA-nfo) (T00001, Jiangsu Qitian Gene Company), and LFD test dipstick (MGHD 1, Milenia Biotec, Germany).

Design of primers and probes

The GyrB gene encodes the DNA gyrase B subunit, which is a conserved housekeeping gene with a single copy. According to the gene sequence of *P. ananatis* GyrB (GenBank No: MW981329.1) registered in the NCBI, DNAMAN software was used for sequence alignment, and Oligo software was applied to design primers and probes in the intraspecific conservative region. When integrated with the LFD, the 5' end of the downstream primer was labeled with biotin. The 5' end of the probe was labeled with the FAM fluorescent group, and the base 30 bp from the 5' end was replaced with Tetra-Hydro-Furan (THF). The detection primers and probes were synthesized by Shanghai Sangong Bioengineering Co. Ltd. (Shanghai, China).

Formation of RAA-LFD reaction system

The RAA reaction was performed using RAA-nfo kit following the manufacturer's instructions. The total volume of the reaction system was 50 μL including 15.2 μL of double distilled water (ddH_2O), 2.1 μL of each forward primer (10 $\mu\text{Mol/L}$) and reverse primer (10 $\mu\text{Mol/L}$), 0.6 μL of probe (10 $\mu\text{mol/L}$), 25.0 μL of basic buffer, 2.5 μL of DNA, all of which were added to the reaction tube after mixing to get dissolved fully, and finally, 2.5 μL of magnesium acetate was added to trigger the amplification reaction. The reaction conditions were maintained at constant temperature amplification of 39°C for 20 min. The amplification product was diluted with buffer, and a 10 μL drop was placed on the sample pad. The LFD sample pad end was inserted vertically into a 100 μL dipstick expansion buffer (Milenia GenLine Dipstick Assay Buffer), and the reaction was continued at room temperature for 2 min. A positive LFD test dipstick shows both the quality control line and the detection line, indicating that the sample contains the nucleic acid fragment to be detected. A negative result indicates that the sample lacks the target nucleic acid fragment or has a quantity lower than the minimum detection quantity indicated by the test dipstick if only the quality control line appears.

Specificity test

Using *P. ananatis* as the test target, we selected the common rice pathogenic bacteria described in "Materials and methods" as the control group, the bacterial solution of the pathogenic bacteria as the template, the DNA of *P. ananatis* as the positive control, and sterilized ultra-pure water as the negative control, to test the specificity of the RAA-LFD reaction system.

Sensitivity test

Genomic DNA of *P. ananatis* was diluted by the 10-fold gradient dilution method to 10 $\text{ng}\cdot\mu\text{L}^{-1}$, 1 $\text{ng}\cdot\mu\text{L}^{-1}$, 100 $\text{pg}\cdot\mu\text{L}^{-1}$, 10 $\text{pg}\cdot\mu\text{L}^{-1}$, 1 $\text{pg}\cdot\mu\text{L}^{-1}$, and 100 $\text{fg}\cdot\mu\text{L}^{-1}$, with sterilized ultrapure water as the negative control, and the sensitivity of the reaction system to the target gene was tested.

The pure culture of *P. ananatis* was diluted at 10^5 CFU/mL in a 10-fold gradient, 1 μL each was used as a template, and sterilized ultrapure water was used as a negative control to test the sensitivity of the formed reaction system to the pure culture of *P. ananatis*.

Usability test

To test the usability of the reaction system, diseased leaves from different regions of the Zhejiang Province were tested. A pure culture of *P. ananatis* was used as the positive control, and the sterilized ultrapure water was used as the negative control.

To test the effects of the reaction system on the detection of the diseased tissues, *P. ananatis* solution was prepared and inoculated on the rice leaves at the seedling stage. After activating the *Pantoea ananatis* strain, we cultured it overnight in PSA liquid medium with shaking. Then, we adjusted the inoculum concentration to an OD600 of 0.5 and inoculated rice leaves using the leaf-cutting method. Taking the tissues of the rice inoculation site, the template was prepared based on the method described in "Strains for testing", with a pure culture of *P. ananatis* as the positive control and sterilized ultrapure water as the negative control.

A bacterial suspension of 10^8 CFU/ml was inoculated to the rice using the leaf-cutting method, and water was used as the negative control. The samples were taken for examination 12h, 24h, 48h and 72h after the inoculation.

Analysis of applicable scope of detecting conditions

Amplification was performed at 33, 35, 37, 39, and 41 °C for 4, 8, 12, and 16 minutes, respectively. *P. ananatis* solution was used as the template, and sterilized water was used as the negative control. The developed RAA-LFD method was evaluated for its applicable temperature range and minimum amplification time, and the feasibility of temperature triggering amplification was also analyzed. we repeats three times for each RAA-LFD.

Table 1 List of primers and probes

Primer name	Primers and probe sequences (5'-3')	Amplicon length/bp
Leus-F1	GTCCAGATACCTTCTACGGCGCGACCTATG	98
Leus-R1	CATTCGGCAATAAAGTCAGCGAGGGCAGGA	
Leus-F2	TTCCTGTCTGGGTCGCGAACTTTGTGCTGA	102
Leus-R2	TCGGCAGATCGTATTTTCGTGGCAAACCTCCC	
GyrB-F3	TGGTATCCGCGCCTTTGTTGAGTACCTGAA	165
GyrB-R3-Bio	Biotin-TGTGGAATATTGTTTCGTGAAGCAGTAGATG	
GyrB-nfo-Probe	FAM-AAAACAAAACGCCTATTCACCCAACCGTAT-THF-CTATTTCTCAACCGA	
GyrB-F4	CATCTACTGCTTCACGAACAATATTCACCA	186
GyrB-R4	GACCGACACGACCGCAATCAGACCTTCACG	
GyrB-F5	CATCTACTGCTTCACGAACAATATTCACCA	263
GyrB-R5	TTTGCTGCTCAACGGCCGATTTCACCTCTG	

Results and analysis

Formation of RAA-LFD method for detecting *Pantoea ananatis*

Based on the principle of RAA primer design, four sets of primers (Table 1) were designed for the conserved region of the GyrB gene of *P. ananatis*, which ranged from B1 to B5. The primers were screened using the RAA Basic Kit. The results showed that the five groups of primers could amplify a single specific band, but the blank control heterobands of P1, P2, P4, and P5 were bright (Fig. 1a). Therefore, the P3 primer pair was selected for validation. The probe for the paper dipstick method was designed manually and the P3

downstream primer with a biotin marker was synthesized again. The designed primer and probe combination were verified according to the RAA amplification system and procedures with *P. ananatis* solution as a template. The results showed that in the reaction system containing *P. ananatis* solution, there were two bands on the test dipstick: one in the quality control area and the other in the detection area. In the negative control, there was only one band in the quality control area, and there was no dipstick in the detection area (Fig. 1b). Furthermore, the amplified products were detected by agarose gel electrophoresis. The results indicated that the bands amplified by the designed primer pairs were single and clear, and the size of the target fragment was 165 bp (Fig. 1b). The results revealed that the RAA-LFD detection

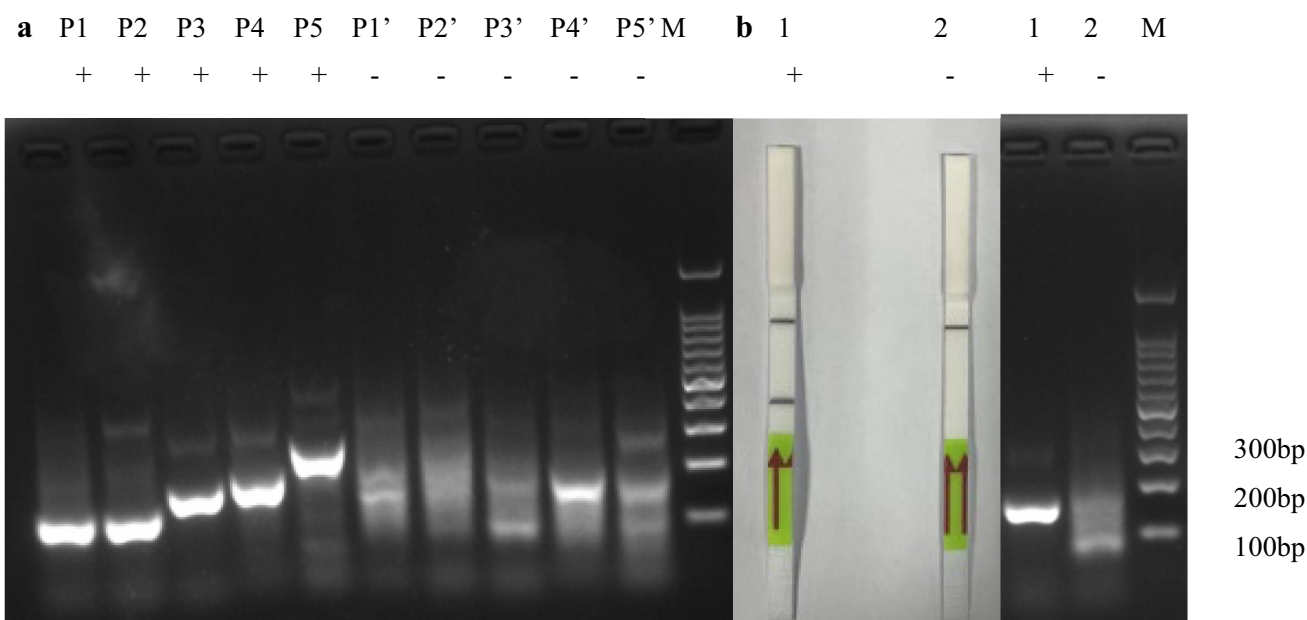


Fig. 1 Primers and probe selection for RAA-LFD method. **a** Primer selection for RAA-LFD. **b** Probe selection for RAA-LFD; +, *Pantoea ananatis* solution; -, ddH₂O

method for *P. ananatis* was preliminarily developed, and the combination of the designed primers and probes could be used for subsequent tests.

Evaluation of the specificity of RAA-LFD detection method for *Pantoea ananatis*

To verify the specificity of the combination of LFD-RPA primers and probes, RAA detection was performed on the tested *P. ananatis* strains and three common plant pathogens. After the reaction, the amplified product was detected using a flow cytometry dipstick. The results showed that the DNA and bacterial solution of *P. ananatis* pantomycetes were positive, with obvious detection lines in the detection area, whereas other pathogenic bacteria were negative, and no band was detected in the detection area (Fig. 2), indicating that the combination of RAA-LFD primers and probes designed in this study could specifically detect *P. ananatis*.

Sensitivity test of RAA-LFD method for detecting *Pantoea ananatis*

To validate the sensitivity of the RAA-LFD detection method, genomic DNA of *P. ananatis* diluted 10 times in the concentration gradient was used as the template for testing, and sterilized pure water was used as the negative control. Reference to the methods and standard curves of chen (2021). The results showed that all test dipsticks had visible control lines, and when the DNA concentration in the template was 10^7 – 10^2 copies/ μ L, the test dipstick presented a visible detection line (Fig. 3a), indicating that the minimum concentration of the RAA-LFD method for detecting genomic DNA of *P. ananatis* was 100 copies/ μ L.

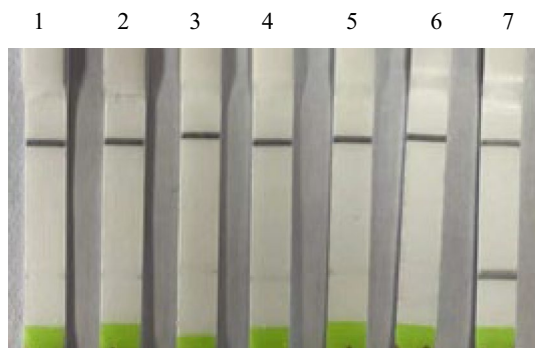


Fig. 2 Specificity analysis of the RAA-LFD method. **1** *Xanthomonas oryzae* pv. *Oryzae*. **2** *Pantoea dispersa*. **3** *M*Enterobacter cancerogenus. **4** *Xanthomonas oryzae* pv. *Oryzicola*. **5** *Burkholderia glumae*. **6** ddH₂O. **7** *Pantoea ananatis*

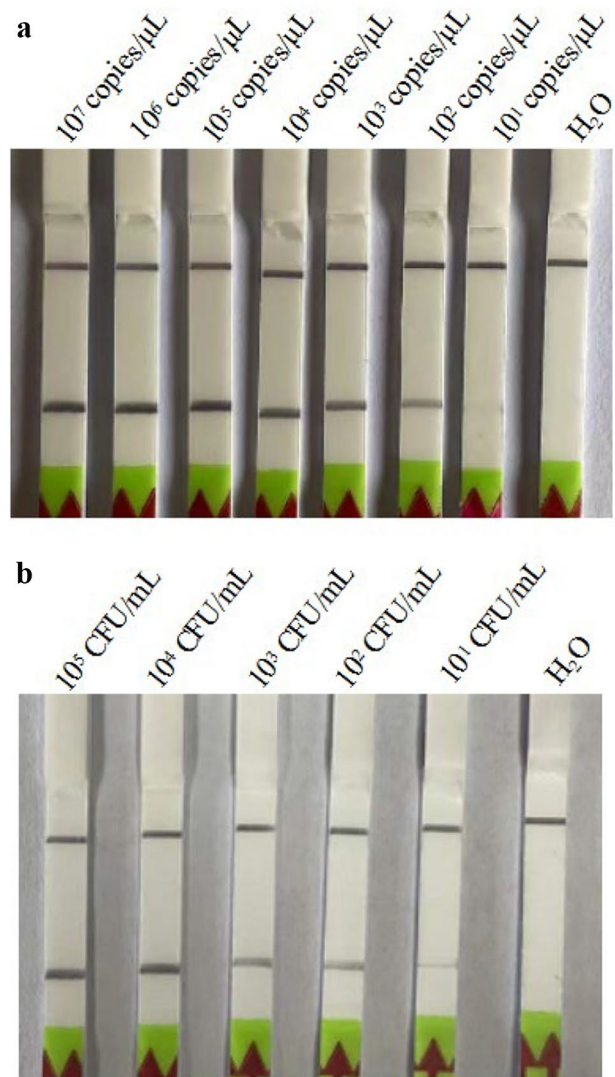


Fig. 3 Sensitivity analysis of the RAA-LFD method

The bacterial culture solution (10^5 CFU/mL) was diluted 10 times, and RAA-LFD detection was conducted on the diluted template. As shown in Fig. 3b, the band of the detection line tended to weaken with a decrease in the concentration of the bacterial solution. At a concentration of 10^2 CFU/mL, a visible detection line was still observed, indicating that the minimum concentration of the RAA-LFD method for detecting *P. ananatis* pure culture solution was 10^2 CFU/mL.

Usability test

The RAA-LFD primers designed in this study were used to amplify four diseased leaves from different regions of Zhejiang Jiangshu and Jiangxi provinces. The results showed

that the LFD of the four diseased samples had quality control and detection lines (Fig. 4a), indicating that the RAA-LFD method has good universality. The RAA-LFD primers were used to amplify nine strains of *P. ananatis*, and the LFD of the nine strain samples had quality control and detection lines (Fig. 4b).

According to the method described in "Strains for testing", the crude extract of rice tissue inoculated with *P.*

ananatis for different symptoms was used as a template for RAA amplification. LFD detection showed that the RAA-LFD method could accurately detect diseased tissues, indicating the incubation period of *P. ananatis* before symptoms appeared in the artificially inoculated rice tissues. These results were consistent with those of q-PCR (Fig. 4c).

According to the method in "Strains for testing", the rice tissues inoculated with *P. ananatis* at different times were cut into

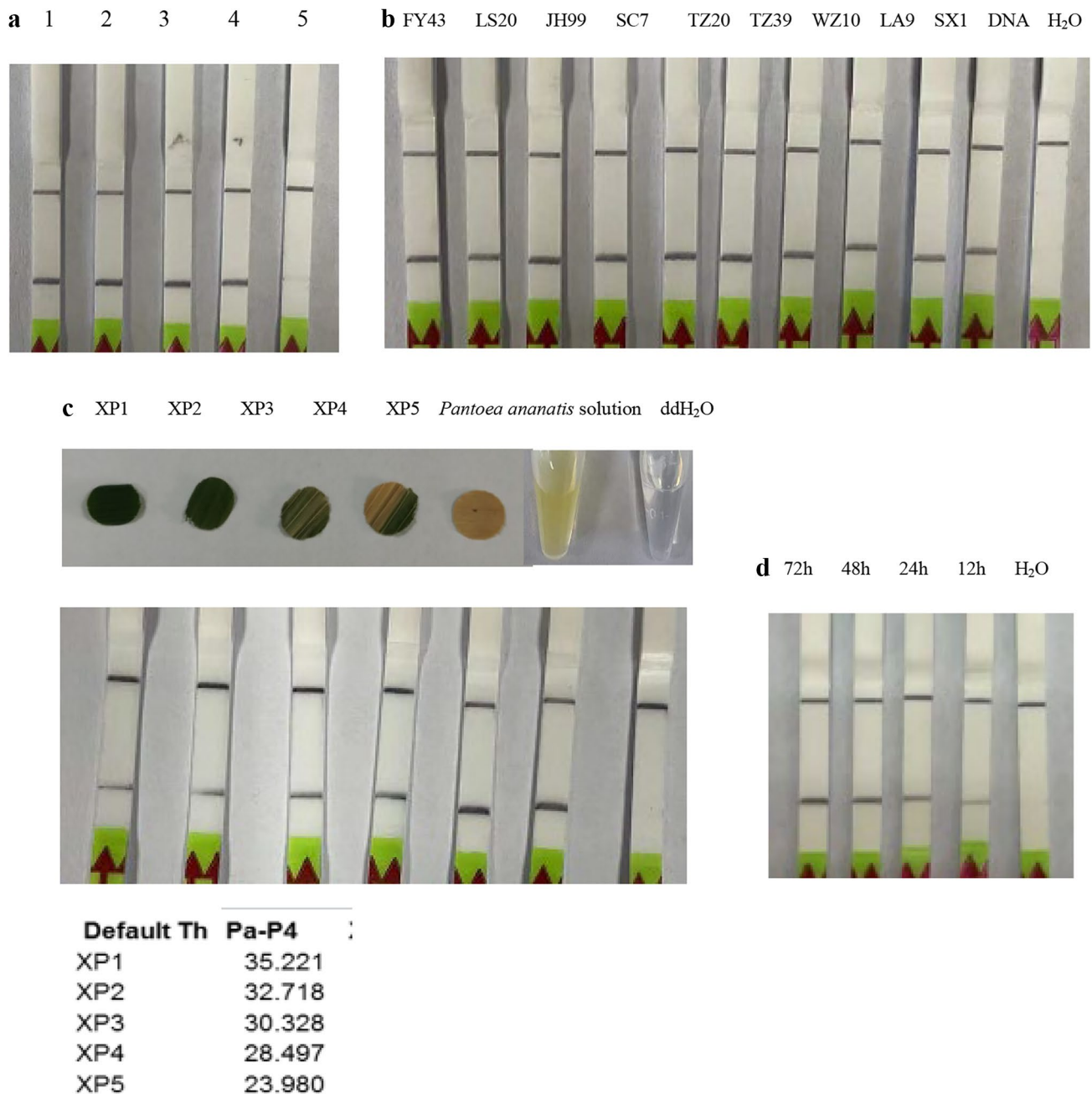


Fig. 4 Utility test of the RAA-LFD method. **a** 1, diseased leaves from FuYang; 2, diseased leaves from Hangzhou; 3, diseased leaves from Zhenjiang; 4, diseased leaves from Jiangxi; 5, H₂O. **b**, 9 strains of *P.*

ananatis; **c**, the crude extract of rice tissue inoculated with *P. ananatis* for different symptoms; **d**, diseased leaves from different inoculation time

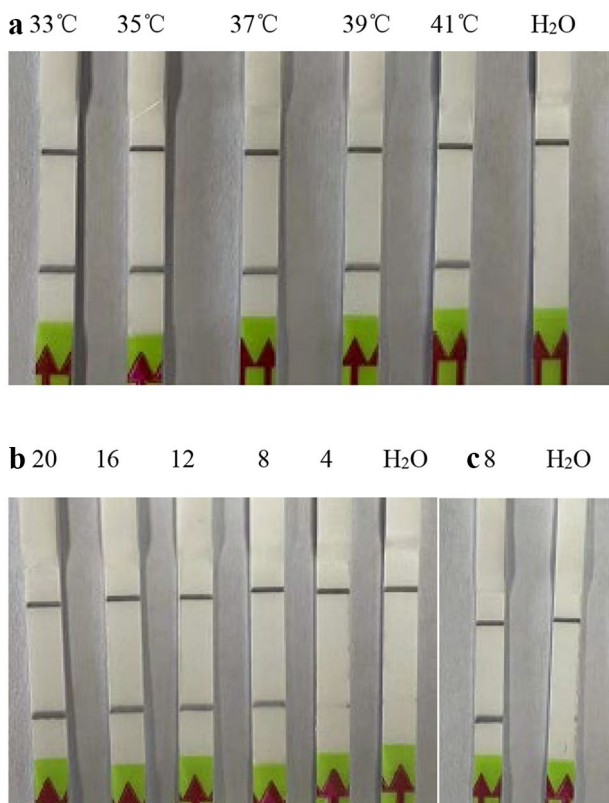


Fig. 5 Applicability analysis of the RAA-LFD method. **a** Amplification temperature analysis. **b** Amplification speed analysis. **c** Triggered by body temperature

pieces, and the tissue solution was extracted as the template for RAA. The LFD detection exhibited that when the tissue solution template was the rice tissues inoculated for 12h–72h, the LFDs displayed quality control strips and detection strips. The color of the detection lines deepened with time, indicating that the amount of bacteria accumulated (Fig. 4d).

Determination of detecting condition range of RAA-LFD method

In this study, we developed a simple and rapid qualitative identification technology that can be used outside the laboratory. Therefore, the scope of application of the developed RAA-LFD method was evaluated, including the amplification temperature and amplification duration. The detection ability of the RAA-LFD method at different temperatures of 33, 35, 37, 39, and 41 °C, was individually determined using *P. ananatis* solution as a template. The temperature range screening results revealed that the primer pair had a good detection effect at 33–41 °C (Fig. 5a). To improve detection efficiency, the detection effect of the RAA-LFD method after amplification for 4, 8, 12, 16, and 20 minutes at 39

°C was determined. The amplification duration experiment showed a fuzzy detection line when the RAA-LFD reagent was amplified for 4 minutes and the detection line was visible when it was amplified for 8 minutes (Fig. 5b). Without any heating module, the staff could efficiently complete amplification and accurate detection by holding the PCR tube in the palm of their hand for 8 minutes (Fig. 5c).

Conclusions

The RAA-LFD detection system developed in this study was not only simple and fast, but also had visualized results, good specificity, and high sensitivity. It is applicable for early detection and rapid field diagnosis, providing technical support for the early warning of *P. ananatis* in rice and on-site detection of field samples.

Discussion

Novel rice leaf blight is a new type of bacterial disease that has emerged in Zhejiang, Anhui, and other provinces in the middle and lower reaches of the Yangtze River in recent years. This has caused huge losses in grain production, and the degree of its damage is increasing every year (Yu et al. 2022a, b; Teresa et al. 2006), which has severely affected the safe production of rice and sustainable development of this industry. Rapid and accurate detection of leaf blight pathogens in plants is key means to effectively preventing and controlling leaf blight. However, the existing pathogen identification methods can only be performed in the laboratory, and there is a delay between sample collection and result detection. We developed a visual detection method based on RAA and LFD detection. The crude extract of diseased tissue can be directly used as a template, which significantly reduces the template preparation time and improves detection efficiency. In contrast to the visualized loop-mediated-isothermal amplification (LAMP) detection system, which requires 60 minutes amplification at 65 °C, this method can complete the detection of samples in 10 minutes at 37 °C with visual results (Shabnam et al. 2019). This RAA-LFD method can be initiated even at human body temperatures. The entire detection process does not require any complex devices and is easy to operate, making its application beyond the laboratory environment to achieve the rapid detection of new rice leaf blight on site.

Usually, early infected rice plants do not exhibit any symptoms. Therefore, the early diagnosis of blight is particularly important to avoid uncontrolled blight expansion. The RAA-LFD method can accurately detect *P. ananatis* in artificially inoculated rice tissues at an early stage of infection before symptoms. Its detection result

is consistent with that of fluorescent quantitative PCR; however, it does not require expensive devices or equipment and has a detection time of only 10 minutes. During detection using the RAA-LFD method, the DNA concentration of pathogenic bacteria is directly proportional to the color depth of the detection strip. The higher the DNA concentration, the deeper the color of the detection strips. Therefore, a colorimetric card displaying the color change of the detection strip can be prepared to establish the semi-quantitative rapid detection technology of RAA-LFD as a simple method for the real-time detection of pathogenic bacteria and rapid diagnosis of diseases.

Currently, the working principle of the LFD method for detecting RAA products is immuno-precipitation, and the primary cost involves the conjugated antibody on the dipstick. Reducing the quantity of combined monoclonal antibodies on the lateral flow dipstick could lower this cost. In addition, a microfluidic chip can be installed to replace the dipstick, which has already been successfully applied for rapid detection of SARS CoV-2 (Liu et al. 2021). At present, our team has been developing a filter paper dipstick method for extracting DNA and an isothermal amplification detection method based on a microfluidic paper-based chip with its supporting products and devices, which are expected to further reduce costs and improve efficiency.

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Author contributions Wang Aiyiing and Liu Shuhua conceived and designed the project. Wang Aiyiing conducted most of the experiments and wrote the manuscript. Luo Ju, Yang Baojun and, Tang Jian contributed part of the experiments and analyzed data. All the authors have read and approved the final manuscript.

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Data Availability All data included in this study are available upon request by contact with the corresponding author.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interests The authors declare that they have no competing interests.

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