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ORIGINAL ARTICLE

# Reverse transcription loop-mediated isothermal amplification to rapidly detect *Rice ragged stunt virus*

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Reverse transcription loop-mediated isothermal amplification (RT-LAMP);
RT-PCR

**Abstract** *Rice ragged stunt virus* (RRSV) is a very important virus that infects rice and causes serious yield losses in Asian countries and other major rice planting areas. Thus, it is urgent to establish an efficient and practical approach for identification and diagnosis in the field. Our results indicated that reverse transcription loop-mediated isothermal amplification (RT-LAMP) reactions are more efficient and sensitive than RT-PCR for RRSV detection. The optimal LAMP conditions were as follows: 0.4–1.2 μM internal primers, 0.2–0.25 μM external primers, 0.8 μM loop primers, and incubation at 62 °C or 63 °C for 30 min. Furthermore, the RT-LAMP primers specifically targeted RRSV virus and resulted in typical waterfall-like bands by gel electrophoresis and sigmoidal amplification curves. The primers could not be used to amplify other common plant viruses including *Papaya ringspot virus* (PRSV), *Rice yellow stunt virus* (RYSV), *Sorghum mosaic virus* (SrMV), *Cactus virus X* (CVX), *Melon yellow spot virus* (MYSV) and *Southern rice black-streaked dwarf virus* (SRBSDV). Ten-fold serial dilutions of RRSV cDNA indicated that RT-LAMP is much faster and at least ten times more sensitive than RT-PCR in detecting the virus. The waterfall-like product bands could be observed within one hour. In the field study, about 77% samples were identified as RRSV. RT-LAMP has many benefits over RT-PCR such as low cost and high accuracy, sensitivity,

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and specificity. This technology meets the requirements for rapid diagnosis of plant virus diseases in the field to best guide management practices for growers.



## 1. Introduction

*Rice ragged stunt virus* (RRSV), a member of Reoviridae, is mainly transmitted and spread by the brown plant hopper in a persistent manner (Cabauatan et al., 2009). It was first discovered in 1976 in Indonesia and the Philippines (Ling et al., 1978). In China, RRSV occurrence is very low in Hunan, Zhejiang, Fujian, and Guangdong provinces where it appears to spread by mechanical sap inoculation, grafting and vegetative propagation (Li et al., 2014; Wang et al., 2014). Symptoms of RRSV infection included curly and serrate rice leaves, swelled veins and abnormal protruding "tumors" or enations (Fig. 1).

Indicator plants, electron microscopy, enzyme-linked immunosorbent assays (ELISA), and RT-PCR are often used approaches to inspect viruses. However, these methods are slow, require high virus titer and/or sometimes yield false positives. RT-PCR is generally considered sensitive and reliable, but it necessitates a special thermocycler that cannot be practically used in the field. RT-LAMP is a novel approach based on autocycling strand displacement DNA synthesis. It was usually performed with a set of six specially designed primers that recognize the distinct sequences of the target under isothermal conditions. It has been used to detect a wide array of pathogens and transgenes in many different biological systems (Almasi et al., 2013; Aydin-Schmidt et al., 2014; Kil et al., 2015; Kimura et al., 2005; Notomi et al., 2000). It can detect target sequences at a constant temperature from 60 to 65 °C with high sensitivity and specificity. With the addition of a DNA binding dye such as SYBR Green I (Life Technologies, USA), the success of the reactions can be easily evaluated without downstream processing (Almasi et al., 2013; Peng et al., 2012; Shen et al., 2014a,b).

In this study, we aimed to establish an efficient RT-LAMP method to detect RRSV in infected rice. Our method may serve as a basis for seedling or propagule quarantine, and diagnosis of RRSV.

## 2. Materials and methods

### 2.1. Plant samples and reagents

Rice infected with RRSV was collected in the Hainan winter-breeding areas in Sanya, Lingshui and Qionghai in Hainan Island, China. Positive controls were provided by the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences. E.Z.N.A. RNA Isolation Kit was purchased from Omega Bio-Tek, Inc. (USA); *Bst* DNA polymerase, betaine solution, dNTPs, fluorescent dyes, and DL2000 DNA marker were purchased from the Guangzhou Gene Deaou Company (China); PrimeScript RT-PCR kit, RNase Inhibitor, and DEPC were purchased from Takara Bio (Japan).

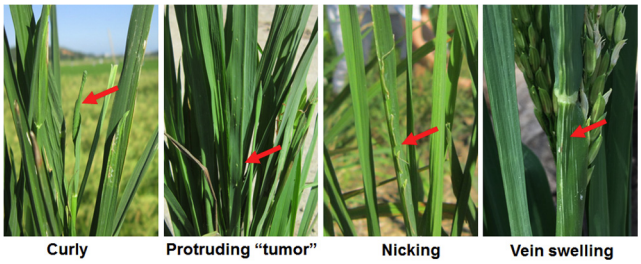


**Figure 1** Typical symptoms on rice infected by RRSV.

### 2.2. Primers

Using the RRSV genome sequence (NCBI accession No. GQ329711.1), we performed homology analysis with DNAMAN 7.0 (Lynnon Biosoft) to identify the unique sequences for primer design using Primer3 Input (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). Three pairs of specific primers for LAMP and one pair of primers for PCR were made (Table 1). All the primers were synthesized by the Shanghai Sangon Company (China).

### 2.3. Total RNA extraction, RT-PCR and RT-LAMP

Total RNA was extracted from ~200 mg of fresh rice leaf samples using the E.Z.N.A. RNA Isolation Kit according to the manufacturer's instructions and was stored at −80 °C for later use. RT-PCR was performed in a mixture containing 1.25 μM of random primers, 1.25 μM/L of oligo(dT)18, 0.25 mM of dNTPs, 1 μL of total RNA (50 ng), and added nuclease-free water to a final volume of 15 μL. First, the mixture was incubated at 65 °C for 5 min and chilled on ice for 2 min. Next, 0.5 μL of MLV reverse transcriptase (100 U), 0.5 μL of RNase Inhibitor (20 U) and 4 μL of 5X M-MLV buffer were added. Then, the mixture was incubated at 42 °C for 1 h followed by inactivation of the reverse transcriptase at 75 °C for 15 min and stored at −80 °C for later use. PCR was performed in a 20 μL reaction system including 1 μL cDNA template, 0.2 μL Pfu DNA polymerase (2.5 U/μL), 0.5 μL of 10 μM RRSV-P1/RRSV-P2 primers, 1 μL dNTPs, and added $ddH_2O$ to a final volume of 20 μL. The thermocycler program was performed as follows: pre-heated at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Eight microliters of PCR product was mixed with 1 μL GoldView dye for electrophoresis on a 1.5% agarose gel at 120 V for 15 min.

RT-LAMP was performed in a 25 μL system including 0.2 μM of external primers RRSV-F3 and RRSV-B3, 1.6 μM of internal primers RRSV-FIP and RRSV-BIP,

**Table 1** Primers used for RRSV virus detection by LAMP and RT-PCR.

| Primers | Sequences (5′-3′) | Genome position | Purpose |
|---|---|---|---|
| RRSV-F3 | GGAAGCACTTAAGAAGTTGTTG | 541–562 | RT-LAMP external primers |
| RRSV-B3 | CCACTTACACGTTCGCTT | 797–814 | |
| RRSV-FIP | GAGGGCCTCATCCTCGAGATATGCTGGATTGCAA GGTGATA | 647–667, 593–611 | RT-LAMP internal primers |
| RRSV-BIP | ACAACAAATGGCTTTTGCGGAGCTTAGATCGAAGG TTGATGGAA | 691–712, 746–766 | |
| RRSV-FLP | TCCTAGGCCAATGTTGTAAGTC | 622–643 | RT-LAMP loop primers |
| RRSV-BLP | GGAATTAGCTAGAGGAGCAGAG | 715–736 | |
| RRSV-P1 | TTCAACCAGCCGTCAGTAAAC | 197–217 | RT-PCR primers |
| RRSV-P2 | TACCTGTGTTCCAATTAGCCTCG | 883–905 | |

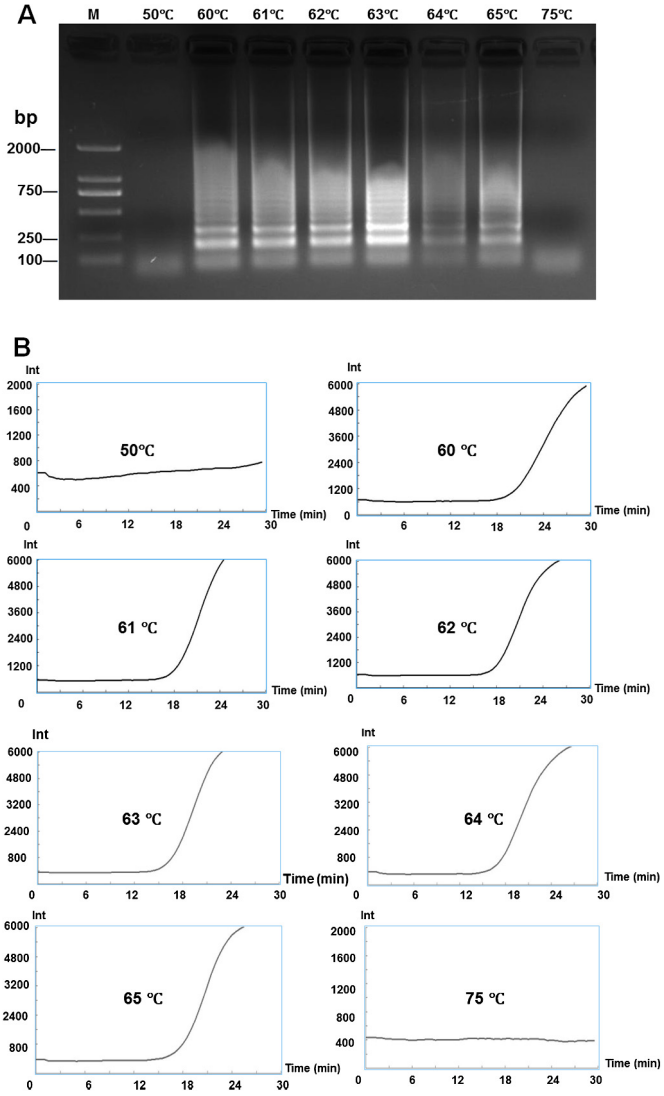


**Figure 2** RT-LAMP detection of RRSV under different reaction temperatures. RT-LAMP amplification products were subjected to 1.5% agarose gel electrophoresis (A) and their amplification curves were monitored under different temperatures (B). Lane M: DL2000 DNA Marker; other lanes were RT-LAMP products from different reaction temperatures: 50 °C, 60 °C, 61 °C, 62 °C, 63 °C, 64 °C, 65 °C, and 75 °C.

0.8 μM of loop primers RRSV-FLP and RRSV-BLP, 1.4 mM dNTPs, 0.8 M betaine, 6 mM $MgSO_4$, 2.5 μL 10X ThermoPol II (Mg-free) Reaction Buffer (20 mM Tris–HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100), 8 U *Bst* DNA polymerase (New England Biolabs, USA), 5 U AMV reverse transcriptase XL (Takara, Japan), 0.5 μL total RNA, and added nuclease-free water to a final volume of 25 μL. The mixture was covered with 20 μL mineral oil and was loaded on a 308 °C thermostat fluorescence detection system (Deaou Biological Technology, China). The reaction was performed at a certain temperature between 50 and 75 °C for at least 30 min, and then heated at 80 °C for 10 min to end the reaction. The amplification curve was checked to determine if there was RRSV virus in the plant sample. The products were inspected on a 1.5% agarose gel after electrophoresis at 120 V for 15 min.

### *2.4. RT-LAMP optimization*

To optimize the RT-LAMP reaction, we selected eight different reaction temperatures (50 °C, 60 °C, 61 °C, 62 °C, 63 °C, 64 °C, 65 °C, and 75 °C), four reaction times (30 min, 45 min, 60 min, and 75 min) and four different concentrations of primers (0.4 μM, 0.8 μM, 1.2 μM, and 1.6 μM for internal primers; 0.1 μM, 0.15 μM, 0.2 μM, and 0.25 μM for external primers; 0.1 μM, 0.2 μM, 0.4 μM, and 0.8 μM for loop primers) to compare the amplification effects of different reaction conditions on LAMP.

### *2.5. Comparison of the specificities and sensitivities of RT-LAMP and RT-PCR*

The cDNA from RRSV and six other plant viruses including *Rice yellow stunt virus* (RYSV), *Rice grassy stunt virus* (RGSV), *Sorghum mosaic virus* (SrMV), *Southern rice black-streaked dwarf virus* (SRBSDV), *Cactus virus X* (CVX), and *Melon yellow spot virus* (MYSV) were selected to determine the specificity of LAMP. The cDNA from rice leaves infected with RRSV was 10-fold serially diluted ($10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$, and $10^{-8}$).

### *2.6. Field diagnosis of rice virus using RT-PCR and RT-LAMP*

Twenty-six rice leaves from plants suspected to be infected with RRSV were collected in the field. RT-LAMP and RT-PCR were performed to determine RRSV infection.

## 3. Results

### *3.1. Optimization of the LAMP reaction to detect RRSV*

The reaction temperature, time, and suitable concentrations of three pairs of primers were optimized using total RNA extracted from plant leaves infected with RRSV. Successful RT-LAMP reactions can produce waterfall-like bands after gel electrophoresis. RT-LAMP was performed under different reaction temperatures from 50 °C to 75 °C. The results showed

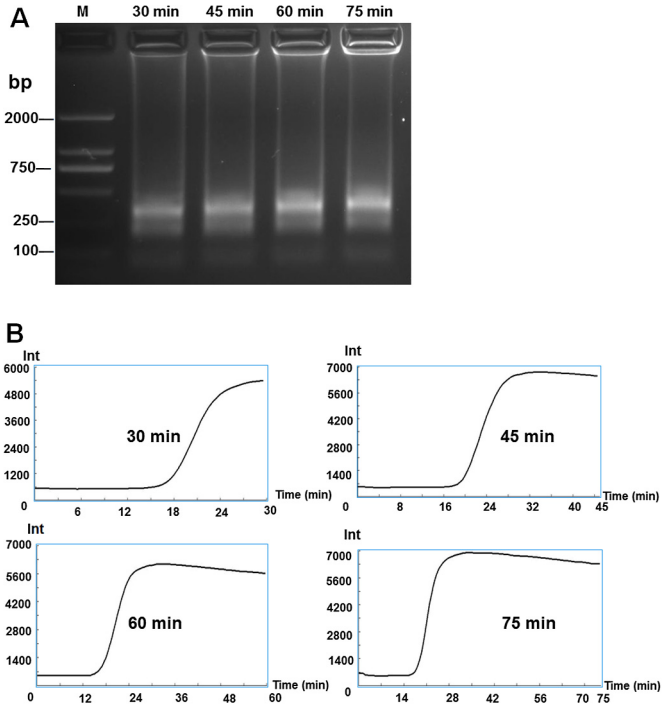


**Figure 3** RT-LAMP detection of RRSV under different reaction times. RT-LAMP amplification bands were subjected to 1.5% agarose gel electrophoresis (A) and the application curves under different reaction times (B). Lane M: DL2000 DNA Marker; other lanes were LAMP products from different reaction times: 30 min, 45 min, 60 min, and 75 min.

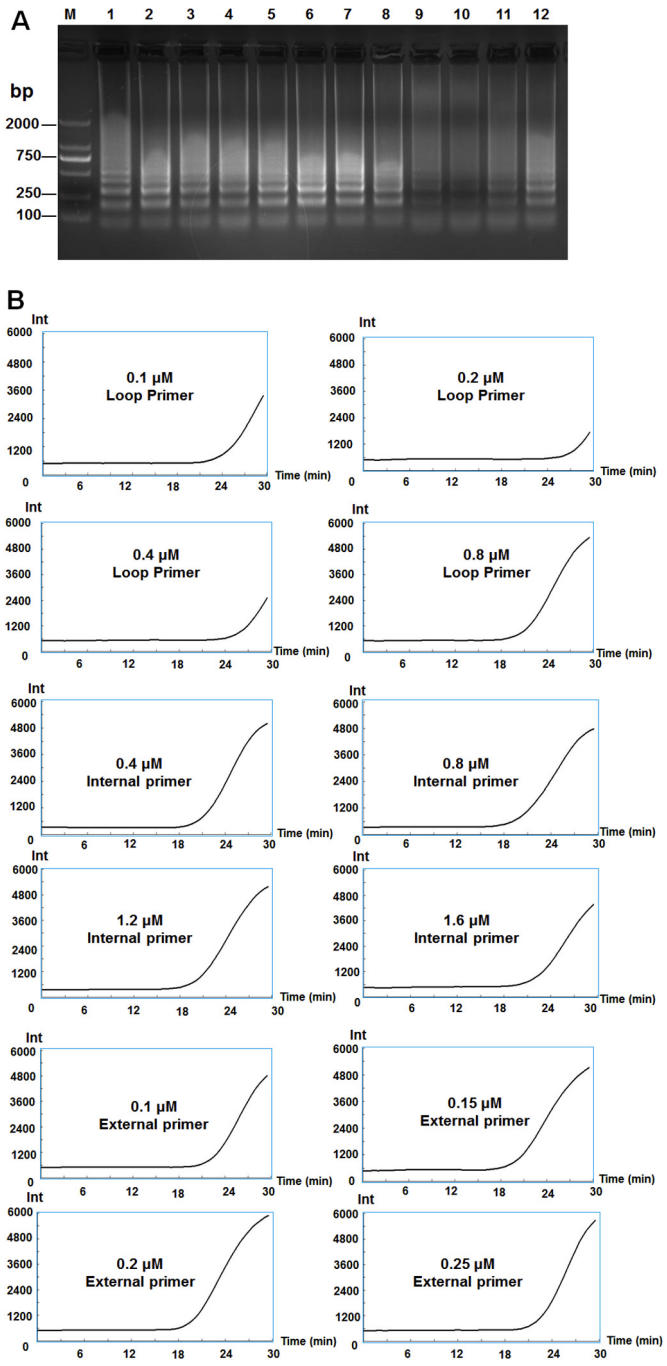


**Figure 4** RT-LAMP detection of RRSV by different concentrations of primer. RT-LAMP amplification products were subjected to 1.5% agarose gel electrophoresis (A) and the application curves with different concentrations of primers were monitored. (B) Lane M: DL2000 DNA Marker; other lanes were LAMP products from different concentrations of primers, lanes 1–4: 0.4 μM, 0.8 μM, 1.2 μM, and 1.6 μM of internal primers; lanes 5–8: 0.1 μM, 0.15 μM, 0.2 μM, and 0.25 μM external primers; lanes 9–12: 0.1 μM, 0.2 μM 0.4 μM, and 0.8 μM loop primers.

that no bands could be produced at 50 °C and 75 °C, and successful RT-LAMP reactions are from 60 °C to 65 °C. RT-LAMP at 63 °C produced very clear and bright stepwise bands, and a typical sigmoidal amplification curve (Fig. 2). Other optimization results indicated that 30 min was adequate for reaction completion and useful concentrations for the internal, external and loop primers were 0.4–1.2 μM, 0.2–0.25 μM, and 0.8 μM, respectively (Figs. 3 and 4). For field diagnosis, we chose 1.2 μM of internal primers, 0.25 μM of external primer, and 0.8 μM of loop primers to detect RRSV infection.

### *3.2. Specificity of RT-LAMP to detect RRSV*

Total RNA extracted from fresh tissues, each infected with RRSV or one of six other plant viruses (RYSV, RGSV, SrMV, SRBSDV, CVX, and MYSV), was used to evaluate the specificity of RT-LAMP with designed primers. Agarose gel

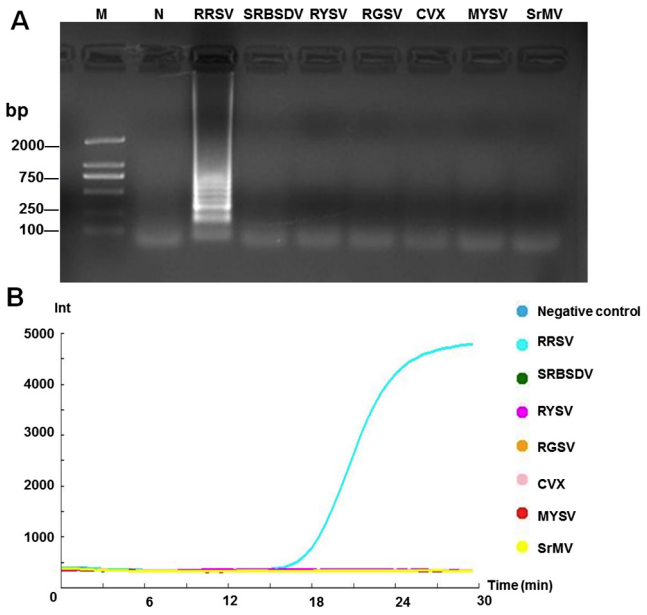


**Figure 5** Specificity of RT-LAMP to detect RRSV. RT-LAMP amplification products were subjected to 1.5% agarose gel electrophoresis (A) and the amplification curves were monitored. (B) Lane M: DL2000 DNA Marker; lane N: negative control; other lanes were RT-LAMP products with different plant viruses: *Cactus virus X* (CVX), *Melon yellow spot virus* (MYSV), *Rice ragged stunt virus* (RRSV), *Rice yellow spot virus* (RYSV), *Rice grassy stunt virus* (RGSV), *Sorghum mosaic virus* (SrMV), and *Southern rice black-streaked dwarf virus* (SRBSDV).

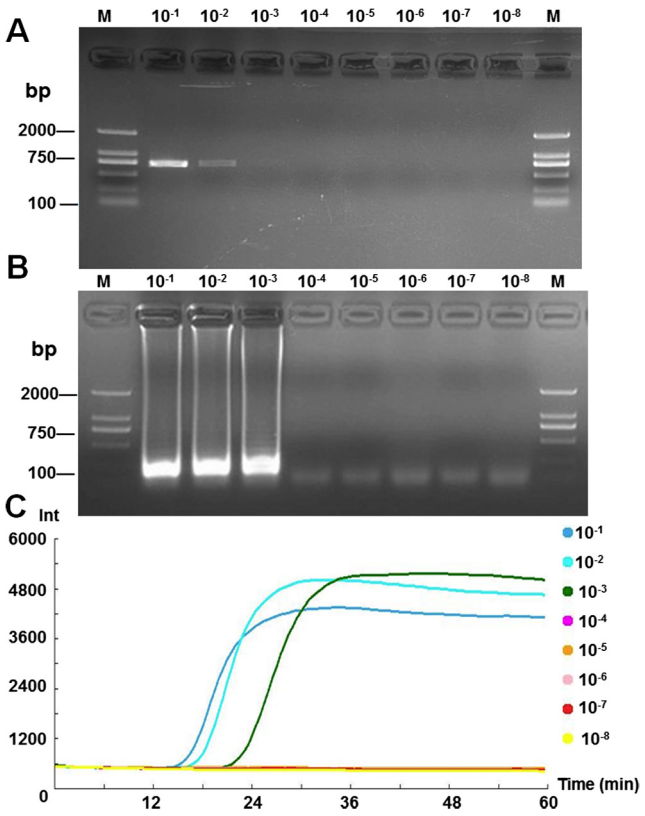


**Figure 6** Sensitivities of RT-PCR and RT-LAMP to detect RRSV. RT-PCR (A) and RT-LAMP (B) amplification bands were subjected to 1.5% agarose gel electrophoresis and RT-LAMP application curves in a 10-fold serial dilution. (C) Lane M: DL2000 DNA Marker; other lanes are serial dilution: $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$, and $10^{-8}$.

electrophoresis and RT-LAMP results indicated that only RRSV-infected tissues yielded the characteristic waterfall-like bands and a sigmoidal amplification curve while these were not detected within the other six plant viruses (Fig. 5).

### *3.3. Sensitivity comparison of RT-PCR and RT-LAMP for RRSV*

To compare the sensitivities of RT-PCR and RT-LAMP, the cDNA was serially 10-fold diluted (50–$5.0 \times 10^{-8}$ ng). RT-PCR could detect $10^{-1}$ and $10^{-2}$ diluted cDNA (Fig. 6A) while RT-LAMP could detect $10^{-1}$ to $10^{-3}$ diluted cDNA (Fig. 6B and C). Therefore, RT-LAMP was at least ten times more sensitive than RT-PCR.

### *3.4. Field diagnostic of RRSV by RT-PCR and RT-LAMP*

In order to test the possibility of RT-LAMP technology in practical applications, we collected and analyzed twenty-six samples suspected to be infected with RRSV to perform RT-LAMP. Altogether, 20 samples (77%) were diagnosed as being infected with RRSV (Fig. 7). These results were consistent with the RT-PCR results.

## 4. Discussion and conclusion

Assessment of rice virus disease incidence is done by visually inspecting the plants for symptoms, which happens when the disease is already prevalent. Consequently, this impacts the efficacy of subsequent control measures. Visual observation is not very reliable as similar symptoms can be due to very different reasons. Current detection methods are expensive and time-consuming, and sometimes unable to recognize low amounts of viruses. Using RT-LAMP to quickly detect RRSV would mean reduced misdiagnosis of rice diseases and timely delivery of pest management systems, translating into reduced costs from expenditure and misuse of pesticides.

In our study, the sensitivity of RT-LAMP technology was at least ten times higher than traditional RT-PCR methods. Moreover, RT-LAMP also indicated high specificity in detection of plant viruses. This is similar to what has been previously observed for other viruses (Chen et al., 2013; Fan et al., 2013; Liu et al., 2010; Wen et al., 2010). False positive rates can be further reduced by careful cleaning of workspaces and avoidance of sample aerosolization to prevent cross-contamination.

We established an efficient RT-LAMP protocol for RRSV detection that can be practically applied in field inspections. Due to its simplicity, rapidness and specificity over RT-PCR, RT-LAMP could be a preferred diagnostic tool for technical and non-technical personnel.

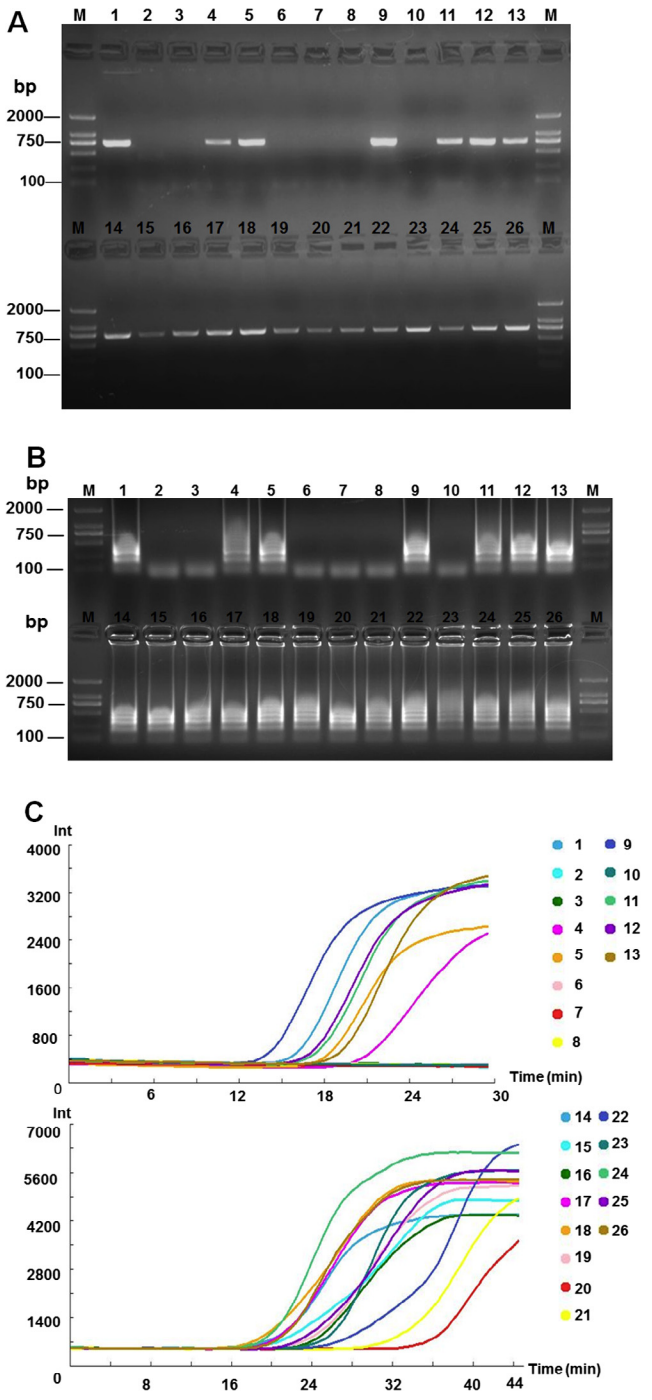


**Figure 7** Detection of suspected RRSV infection from twenty-six field samples. Products of field diagnosis of RRSV by RT-PCR and RT-LAMP were subjected to 1.5% agarose gel electrophoresis (A and B, respectively) and the LAMP application curves were monitored (C and D, respectively).

## Author contributions

GHY and APG conceived and designed the experiments. DWL and QXH performed the experiments. DWL, YLZ, and GHY analyzed the data. All the authors contributed to the writing of the paper. The authors have declared that no competing interests exist.

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