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Posted Date: 30 April 2023

doi: 10.20944/preprints202304.1259.v1

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Article

Quantitative Loop-Mediated Isothermal Amplification Detection of *Ustilaginoidea virens* Causing Rice False Smut

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Abstract: Rice false smut caused by *Ustilaginoidea virens* is one of the most devastating diseases on rice worldwide, which results in serious reduction of rice quality and yield. As an airborne fungal disease, early diagnosis of rice false smut, monitoring the epidemics and distribution of its pathogens, is particularly important to management the infection. In this study, a quantitative loop-mediated isothermal amplification (q-LAMP) method for the *U. virens* detection and quantifying was developed. This method has higher sensitivity and efficiency compared to quantitative real-time PCR (q-PCR) method. The species-specific primer sets UV-2 used was design based on the unique sequence of *U. virens* ustiloxins biosynthetic gene (NCBI accession number: BR001221.1). The q-LAMP assay was able to detect a concentration of 6.4 spores/mL at an optimal reaction temperature of 63.4 °C within 60 min. Moreover, the q-LAMP assay can even achieve accurate quantitative detection when there were only 9 spores on the tape. A linearized equation for the standard curve, $y = -0.2866x + 13.829$ (x is the amplification time, the spore number = $10^{0.65y}$), was established for detection and quantifying of *U. virens*. In field detection applications, this q-LAMP method is more accurate and faster than traditional observation method. Collectively, this study has established a powerful and simple monitoring tool for *U. virens*, which provide valuable technical supports for forecast and management of rice false smut, and theoretical basis for precise fungicide application.

Keywords: rice false smut; quantitative loop-mediated isothermal amplification (q-LAMP); detection; ustiloxins biosynthetic gene

1. Introduction

Rice false smut is a disease on rice spikes that occurs from the flowering to the milking stage[1,2]. It's typical and visible symptom is the replacement of rice grains with false smut balls[3,4]. It occurs mainly in Asian countries such as China, Japan, Korea, the Philippines and India, and is one of the most devastating diseases in the world's major rice producing regions[5–8]. In recent years, due to the promotion of short-stalked compact and high-yielding rice varieties, indica-japonica interspecific hybrid rice combinations, changes in cultivation patterns, and the excessive use of nitrogen fertilizer during the tillering and gestation periods, the occurrence of rice false smut has become increasingly serious and has gradually risen from a previously minor or sporadic disease to become one of the three new major diseases of rice in China[9,10].

The damages caused not only result in decrease of rice quality and yields, but also the generation of mycotoxin ustiloxins on infected rice spikelets[11,12]. As antimitotic cyclopeptide mycotoxins, the ustiloxins produced within false smut ball can inhibit microtubule assembly and cell skeleton formation, which poses a serious threat to farmland preservation, ecosystems, as well as the safety of

humans and animals[13]. Strategies are therefore urgently needed to management of this devastating disease.

It is widely accepted that *Ustilaginoida virens* (telemorph *Villosiclava virens*) is the causing agent of rice false smut[14,15]. As a typical airborne disease, virulent pathogen spores land on the surface of a rice spikelet, germinate hyphae as well as false smut ball on the spikelet[12,16–19]. Thus, the epidemic of rice false smut is closely related to the amount of *U. virens* spores in the field, and rice false smut diagnosis combining of accurate detection and spore quantification is of significantly important for its prevention and management[5,20]. Traditionally, the microscopic counting of spores after capture is widely used in rice false smut diagnosis, however this method requires specialist taxonomic technicians[21]. Given that the complexity of environmental samples and human subjectivity, microscopic analysis is difficult to provide reliable data with high efficiency. Recently, quantitative real-time PCR (q-PCR) technique has been applied for the identification and quantification of pathogen in airborne disease early warning[22]. However, this technique is susceptible to interference from environmental dust and other pathogens, making it difficult to quantify the low concentrations of spores captured[21].

Loop-mediated isothermal amplification (LAMP) is a non-PCR-based nucleic acid amplification technique that can be used for the molecular detection of various bacteria, viruses, fungi in disease diagnosis[23–25]. The LAMP reaction is carried out at a constant temperature (between 60 and 65°C) in less than an hour by use of two pairs of primers, an inner primer (FIP/BIP) and an outer primer (F3/B3), to recognize specific nucleic acid sequences of monitored targets[26–28]. The quantitative-LAMP (q-LAMP) assay (DiaSorin S.p.A., Saluggia, Italy) is a technical improvement over the classical LAMP, which combine LAMP technology with the real-time fluorescence quantitative PCR technique[29]. It is based on the addition of nucleic acid fluorescent dyes (SYBR Green, etc.) or the synthesis of fluorescent probes (TaqMan probes, hybridization probes, etc.) resulting in a more sophisticated method suitable for the needs of field diagnosis[30]. In this study, we aimed to design and develop a specific and sensitive q-LAMP assay for detection and quantification *U. virens*, which can be applied in the early diagnosis of rice false smut for preventing the widespread spreading of this devastating airborne disease. And this study is the also first report describing a quantitative diagnostic test for detection of *U. virens* using q-LAMP.

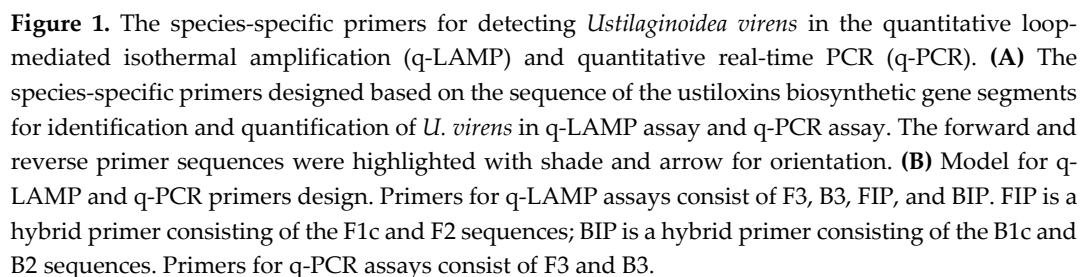
2. Results

2.1. Design of primers for *U. virens* detection

The best LAMP primers UV-2 were designed based on the ustiloxins biosynthetic gene sequence of *U. virens* (NCBI accession number: BR001221.1) that did not show any similarities to other sequences available in the National Center for Biotechnology Information (NCBI) GenBank database to allow specific amplification of *U. virens* (Figure 1, Table 1)[31–33]. Additionally, the primer sets UV-2 met the requirement that ΔG values of less than or equal to -4 Kcal/mol at the 3’end of F3/B3 and F2/B2 and 5’ends of F1c and B1c.

Table 1. The sequences of species-specific primers used in the quantitative loop-mediated isothermal amplification (q-LAMP) assay and quantitative real-time PCR (q-PCR) assay.

| Serial number | Sequence (5'-3') | |
|---------------|------------------|---------------------|
| UV-2 | F3 | GGCACAGCATGACAGGATG |
| | B3 | TGCTCCCACACTGGTAGT |



To optimize the q-LAMP assay system, the q-LAMP assay was carried out using the primer sets UV-2 at temperatures ranged from 61.8°C to 66°C. As shown in Figure 2, the fluorescence quantitative results found that the strongest fluorescence intensity and the shortest reaction time were obtained when the reaction temperature was 63.4°C (which reaching the amplification peak at 30 min). Thus, 63.4°C was chosen as the reaction temperature to carry out the optimal q-LAMP assays.

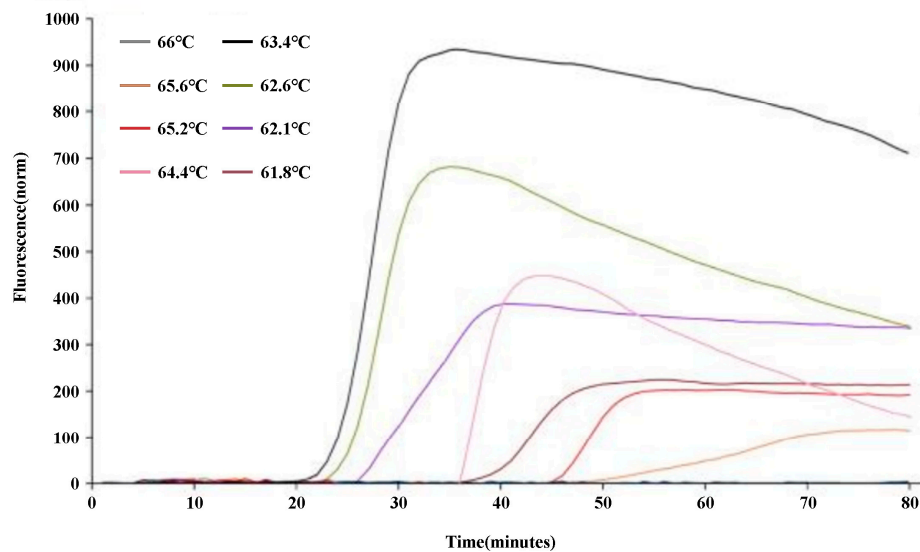


Figure 2. Optimization of the q-LAMP assay via reaction temperature screening. The influence of temperature ranged from 61.8°C to 66°C in the q-LAMP detection system showed that the strongest fluorescence intensity and the shortest reaction time were obtained at the 63.4°C (black line).

2.3. Specificity validation of the q-LAMP assay system

The specificity validation of design q-LAMP assay system (using primer sets UV-2 and 63.4°C as reaction temperature) was tested using *U. virens* stain and the other nine fungi. The results showed that fluorescence signals were detected in the samples with DNA template of *U. virens*, while the samples with DNA template of the other 9 fungi or ddH₂O (negative control) did not show any fluorescence signal (Figure 3), indicating that design q-LAMP assay system was highly specific for the detection of *U. virens*.

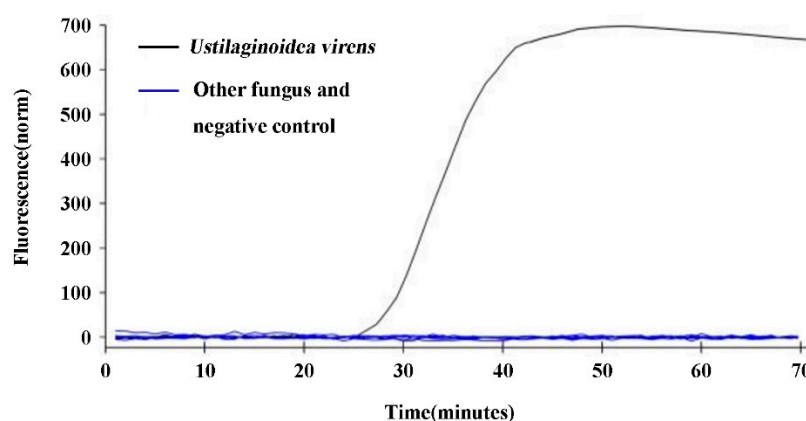


Figure 3. Specificity validation of the q-LAMP assay system. The q-LAMP assay system (using primer sets UV-2 and 63.4°C as reaction temperature) was highly specific for the detection of *U. virens*. The q-LAMP assay system showed that fluorescence signals were only detected in the samples with DNA template of *U. virens* (black line), while the samples with DNA template of the other 9 fungi (including *Fusarium fujikuroi*, *F. oxysporum*, *F. proliferatum*, *F. solani*, *F. graminearum*, *Penicillium* sp, *Pyricularia oryzae*, *Alternaria alternata* and *Rhizoctonia solani*) or negative control (nucleic acid-free water) did not show any fluorescence signal.

2.4. Sensitivity validation of the q-LAMP assay system

The sensitivity validation of the q-LAMP assay was determined using the genomic DNA of gradient dilution of *U. virens* spore as templates under optimal condition (using primer sets UV-2 and 63.4°C as reaction temperature). As shown in Table 2 and Figure 4, the fluorescence signals were detected in the samples with DNA template of 2×10^4 spores/mL, 4×10^3 spores/mL, 8×10^2 spores/mL, 1.6×10^2 spores/mL, 32 spores/mL and 6.4 spores/mL within 60 min, while no signals were detected in sample with DNA template of 1.28 spores/mL. Thus, theoretically q-LAMP assay was able to detect sample with concentration of 6.4 spores/mL. We also compared the sensitivity of q-LAMP assay system with quantitative real-time PCR (q-PCR) for *U. virens* detection. The q-PCR assay was carried out using the primer set F3/B3 and the effective amplification reactions were detected at samples with spore concentrations of 2×10^4 , 4×10^3 , 8×10^2 , 1.6×10^2 spores/mL, but not 32 spores/mL (Supplementary Figure S1), indicating the theoretically q-PCR assay was ineffective in detecting samples with spore concentration lower than 32 spores/mL. Thus, the q-LAMP assay system is more sensitive and efficient compared to q-PCR system.

Table 2. The time for reaching fluorescence signal peak and fluorescence signal detection in the q-LAMP assays for testing samples with known spore concentration.

| Spore concentration (spores/mL) | Time ^a (min) | Fluorescence signals ^b | The Log ₁₀ value of spore number |
|------------------------------------|-------------------------|-----------------------------------|--|
| 2×10^4 | 17.90 | + | 4.30 |
| 4×10^3 | 31.44 | + | 3.60 |
| 8×10^2 | 34.68 | + | 2.90 |
| 1.6×10^2 | 37.92 | + | 2.20 |
| 3.2×10^1 | 41.16 | + | 1.51 |
| 6.4 | 44.40 | + | 0.81 |
| 1.28 | / | - | 0.20 |
| CK ^c | / | - | / |

^a Time for reaching the amplification peak in the q-LAMP assays. ^b "+" indicates a successful fluorescence signal detection, "-" indicates no fluorescence signal detected in the q-LAMP assays. ^c The nucleic acid-free water was used negative control (CK) in the q-LAMP assay.

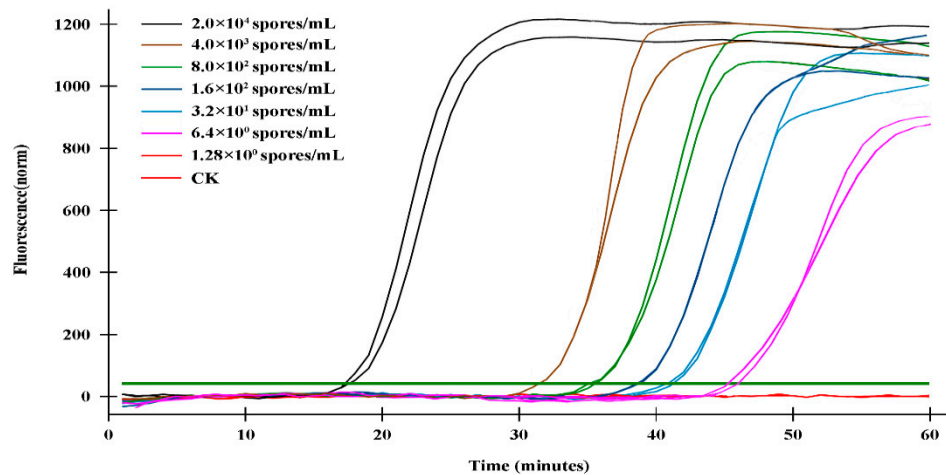


Figure 4. Sensitivity validation of q-LAMP system. The fluorescence signals in q-LAMP assays were detected in the samples with DNA template of 2×10^4 spores/mL, 4×10^3 spores/mL, 8×10^2 spores/mL, 1.6×10^2 spores/mL, 32 spores/mL and 6.4 spores/mL within 60 min, while no signals were detected in sample with DNA template of 1.28 spores/mL and CK. The bolded dark green line (horizontal) indicates fluorescence threshold. Fluorescence signals above this threshold marked as a successful detection for *U. virens* in q-LAMP assays.

2.5. Establishment of a DNA standard curve for q-LAMP detection of *U. virens*

A standard curve between the amplification time (x) and the \log_{10} value of spore number (y) was constructed based on the q-LAMP assay results listed in Table 2: $y = -0.2866x + 13.829$ (Figure 5), and the formula used for calculating spore number is: $10^{0.65y}$, and the correlation coefficient $R^2 = 0.9942$ showing a good linear relationship.

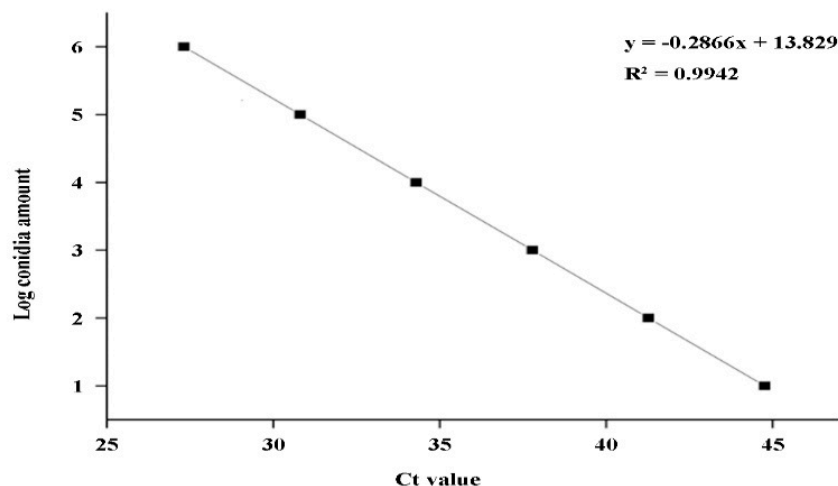


Figure 5. Standard curve of q-LAMP detection system. A standard curve between logarithmic values of the spore number (y) and the amplification time quantitated using the cycle threshold (Ct) values (x): $y = -0.2866x + 13.829$. The correlation coefficient (R^2) is 0.9942 showing a good linear relationship.

2.6. Application of q-LAMP assay for *U. virens* spore calculating

The standard curve of q-LAMP was applied to calculate *U. virens* spore number on tapes and each tape sample contained 450, 116, 29 and 9 manually added spores, respectively. As showed in Table 3 and Figure 6, the amplification times for the tested samples were 34.03, 37.12, 40.46 and 43.17

mins, corresponding to 446.07, 118.51, 28.29 and 8.85 predicted spores per tape, respectively, which is super close to the actual spore number on each Melinex tape. Thus, this q-LAMP system can efficiently quantitate *U. virens* spore number with high accuracy.

Table 3. Quantitative detection of *U. virens* spores using q-LAMP system.

| Manually added spores (spores/mL) | Time ^a (min) | Predictive spores (spores/mL) |
|-----------------------------------|-------------------------|-------------------------------|
| 450 | 34.03 | 446.07 |
| 116 | 37.12 | 118.51 |
| 29 | 40.46 | 28.29 |
| 9 | 43.17 | 8.85 |

^a Time for reaching the amplification peak in the q-LAMP assays.

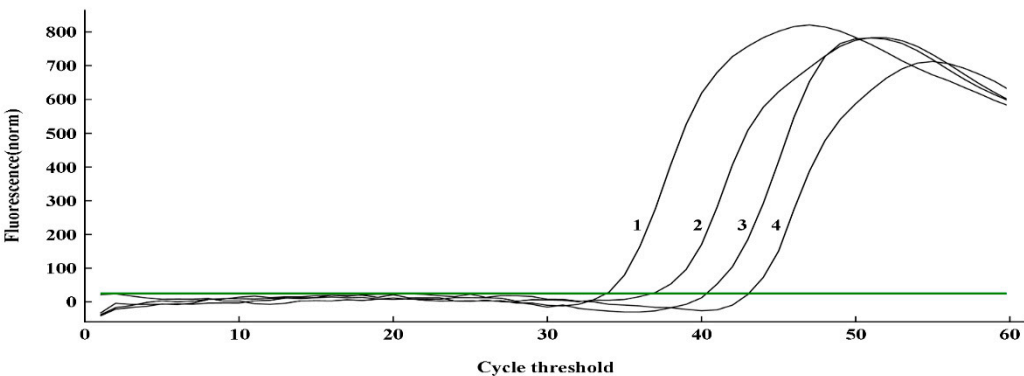


Figure 6. Quantitative detection of *U. virens* spores on Melinex tape using q-LAMP system. Serial numbers 1, 2, 3, and 4 represent 450, 116, 29, and 9 spores, respectively. The green line (horizontal) indicates fluorescence threshold. Fluorescence signals above this threshold marked as a successful detection for *U. virens* in q-LAMP assays.

2.7. Field application of q-LAMP assay system

The q-LAMP system results showed that spores of *U. virens* were first observed on August 27, 2018, while the results obtained by microscope showed that spores were observed for the first time on September 2. Then, the number of spores began to rise rapidly and reached its peak on September 20, and obvious symptoms of rice false trot were found in the field on September 25. In the next year (2019), the q-LAMP system and microscope manual observation were used to monitor the spores of *U. virens* in the field again. The results showed that the q-LAMP detection detected the spores for the first time on August 31, while the microscope observation found only a handful of spores on September 6, and then the concentration of spores reached its peak on September 30. The symptoms of rice false smut were found in the field on October 5. Through monitoring the dynamic change of spore number of *U. virens* in the field for two consecutive years(Figure 7), it was obviously found that the q-LAMP system was faster and more timely than the traditional microscope observation method.

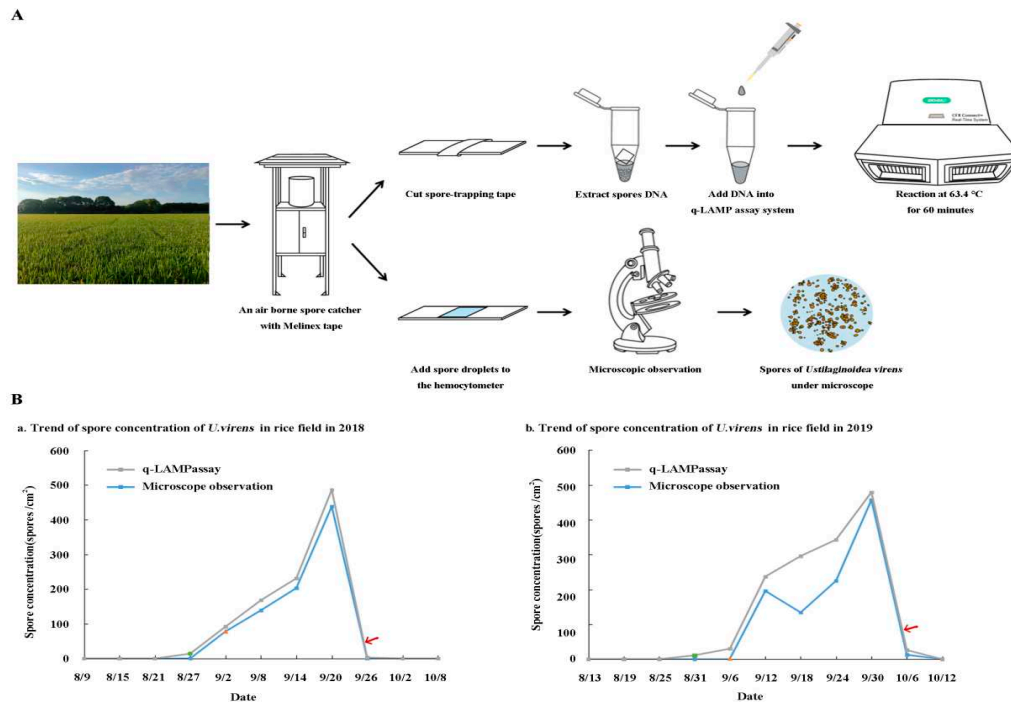


Figure 7. Field application of *U. virens* spores using q-LAMP system. **(A)** Flow chart of field *U. virens* spore sample detection, q-LAMP assay system and microscope observation were used for the collected samples respectively. **(B)** a and b are the results of *U. virens* spore concentration measured by different methods in rice fields in 2018 and 2019 respectively (q-LAMP assay system is the gray line, microscope observation method is the blue line), in which the green circle is the first detection of spores by q-LAMP assay system, the orange triangle is the first observation of spores by microscope observation, and the red arrow is the occurrence time of rice false smut in the field.

3. Discussion

Currently, rice false smut disease caused by *U. virens* is one of the most devastating rice diseases in China, as well as many other countries[34]. The occurrence of rice false smut disease not only results in decrease of rice quality and serious loss of rice yield, but also threatens food safety due to its production of toxic mycotoxins within false smut balls [10,35]. However, it has been found that the rice false smut disease is difficult to control. As a typical airborne disease, the epidemic of rice false smut is closely related to the amount of *U. virens* spores in the fields, thus early detection and warning are critical for preventing and mitigating rice false smut. In this study, a q-LAMP assay system was developed. The result showed that the species-specific primer sets UV-2 in q-LAMP assay system could correctly distinguish *U. virens* from the other 9 air-dispersal fungi including *Fusarium fujikuroi*, *F. oxysporum*, *F. proliferatum*, *F. solani*, *F. graminearum*, *Penicillium sp*, *Pyricularia oryzae*, *Alternaria alternata* and *Rhizoctonia solani* (Figure 3). Additionally, sensitivity validation found that the q-LAMP assay was able to detect a concentration of 6.4 *U. virens* spores/mL at an optimal reaction temperature of 63.4°C within 60 min (Figure 4), and the q-LAMP assay can even achieve accurate quantitative detection when there were only 9 *U. virens* spores on the Melinex tape (Figure 6). Moreover, there is a good linear relationship between the spore amount (y) and the amplification time (x) (Figure 5), which enables the accurate quantification of *U. virens* and provides early diagnosis of *U. virens* infection via q-LAMP assay.

For *U. virens* diagnosis, besides traditional disease diagnosis that includes identification of symptoms, isolation of pathogens and microscopic techniques, a conventional nested-PCR assay has been developed for detection *U. virens* in rice [36]. However, the nested-PCR has less sensitivity and cannot be used in accurate quantification of *U. virens*[37]. Recently, the q-PCR technique and q-LAMP assay have been applied for the identification and quantification of pathogen in disease diagnosis. In

this study, we have established these two systems for *U. virens* quantification. The q-PCR assay was carried out using the primer set F3/B3 and the effective amplification reactions were detected at samples with spore concentrations of 2×10^4 , 4×10^3 , 8×10^2 , 1.6×10^2 spores/mL, but not 32 spores/mL (Supplementary Figure S1), indicating a lower sensitivity of q-PCR for *U. virens* detection compared to q-LAMP assay system.

Rice false smut has no symptoms in the early stage and can only be identified in the late stage when the smut ball appears. Chemical control is the main means of rice false smut prevention and control[38]. The previous study showed that the first 4~15 d of ear bud breakage was the main period of control, and the first 4~7 d of control was the best[39]. If the key window stage for infection of *U. virens* on rice is not been grasped, the efficacy of management will be disappointment[16,39]. Therefore, for rice false smut that rely on airborne transmission, early detection and early warning can provide appropriate guidance for disease prevention and control. In this study, we collected spore samples of *U. virens* from the field for two consecutive years for q-LAMP assay system and microscope observation. Compared with manual observation, q-LAMP assay system could detect spores in the air more accurately and quickly, providing theoretical basis for precise fungicide application(Figure 7). Therefore q-LAMP assay with higher efficiency and sensitivity is a better choice for early diagnosis of rice false smut.

In conclusion, this is the first assay developed for the detection of *U. virens* using q-LAMP assays. Compared with other *U. virens* detection methods, the newly developed LAMP assay has stronger operability, specificity as well as sensitivity, and is more suitable for quantitative detection of *U. virens* in the early disease diagnosis.

4. Materials and Methods

4.1. Fungal isolates

Isolates of *Ustilaginoidea virens* and the 9 other fungal pathogens used in this study were isolated and identified in our lab, and the detail information of each fungus was listed in Table 4. Isolates were maintained on potato dextrose agar (PDA, prepared by 200 g potato, 20 g glucose and 20 g agar per 1 L pure water) slants at 4°C.

Table 4. The information of strains used in the specificity validation of the q-LAMP assay system.

| Species | Isolate NO. | Host | Origin |
|------------------------------|------------------------|-------|-----------------|
| <i>Fusarium fujikuroi</i> | / | Rice | Zhejiang, China |
| <i>F. oxysporum</i> | ACCC30927 ^a | Rice | Hainan, China |
| <i>F. proliferatum</i> | CICC2489 ^b | Rice | Anhui, China |
| <i>F. solani</i> | ACCC37119 | Rice | Hebei, China |
| <i>F. graminearum</i> | ACCC37680 | Wheat | Jiangxi, China |
| <i>Penicillium</i> sp. | ACCC31507 | Soil | Shandong, China |
| <i>Ustilaginoidea virens</i> | ACCC2711 | Rice | Hunan, China |
| <i>Pyricularia oryzae</i> | ACCC37631 | Rice | Fujian, China |
| <i>Alternaria alternata</i> | ACCC36843 | Rice | Hainan, China |
| <i>Rhizoctonia solani</i> | ACCC36246 | Rice | Beijing, China |

^a ACCC(Agricultural Culture Collection of China). ^b CICC (China Center of Industrial Culture Collection).

4.2. DNA templet preparation from mycelium and spores for q-PCR and q-LAMP analysis

For mycelial DNA templet preparation, after mycelia grew covering two-thirds of the PDA plate surfaces, the hyphae were then transferred to a mortar and ground with liquid nitrogen. The resultant powder was then placed into a 2-mL centrifuge tube and the mycelial DNA of each fungus was extracted using a Genomic DNA Kit (Sangon Biotech Co., Ltd, Shanghai) according to the manufacturer's instructions. The extracted DNA was used as DNA template in q-LAMP analyses and stored at -20°C. For spores DNA templet preparation, after growing on PDA medium at 25°C in darkness for 20 days, 5-mm diameter mycelial plugs taken from colony margin were placed into the potato sucrose (PS, prepared by 200 g potato and 20 g per 1 L pure water) medium at 25°C 150 rpm, in darkness for 7 days. Spores separated from medium with filtration through four layers of lens tissue and washed twice with distilled water. Then spores were diluted with 10% sodium dodecylsulfate (SDS) solution into a series of concentration gradients. 1-mL spore suspension sample with known concentration mixed with 200-μL 10% Chelex-100 solution[21], 50-μL 10% SDS solution and 0.4-g acid-washed glass beads was placed into a 2.0-mL centrifuge tube. The sample was lysed by Fast Prep Apparatus (JXFSTPRP-24L, Jingxin Technology, Shanghai, China) for 40s at speed of 6 m/s, placed in boiling water bath for 5 minutes. The grinding and heating steps were repeated for three times, after which the sample was placed on ice for 2 min. The cooled lysate was used directly as DNA template in q-PCR and q-LAMP analyses and stored at -20°C.

4.3. Design of q-LAMP primers for *U. virens* detection

Ustiloxin A and Ustiloxin B of *U. virens* are synthesized by ustiloxins biosynthetic gene that was found to be species-specific to *U. virens* [13,40]. Thus, the sequence of ustiloxins biosynthetic gene (NCBI accession number: BR001221.1) was chosen for q-LAMP primers design using Primer Explore V5 (online web service, <http://primerexplorer.jp/e/>) ensuring the specificity and accuracy of q-LAMP assay system for *U. virens* detection. The q-LAMP primers contain forward inner primer (FIP), backward inner primer (BIP), and two outer (F3 and B3) primers (Figure 1B). The primers were design according to the following rules: ΔG values of less than or equal to -4 Kcal/mol at the 3' end of F3/B3 and F2/B2 and 5' ends of F1c and B1c.

4.4. Determination of optimum condition of the q-LAMP assay

To better facilitate the efficiency of q-LAMP reaction, the LAMP reaction system was improved via screening for the optimal reaction temperature based on a reference from Notomi[41]. The LAMP reaction was carried out in the following reaction mixtures containing 0.25 μM·L⁻¹ of the primers FIP and BIP, 0.2 μM·L⁻¹ of the primers F3 and B3, 1.0 mM·L⁻¹ betaine, 2.0 mM·L⁻¹ dNTPs (Takara Bio Inc., 108 USA), 25 mM·L⁻¹ Tris-HCl (pH 8.8), 12.5 mM·L⁻¹ KCl, 12.5 mM·L⁻¹ (NH₄)₂SO₄, 10 mM·L⁻¹ MgCl₂, 0.125% (v/v) Triton X-100, 0.2 U·L⁻¹ of Bst DNA polymerase (New England Biolabs, 110 Beijing), 0.5 μL 1×SYBR Green I and 1 μL of DNA template extracted as described above, and the volume was adjusted to 25 μL with nucleic acid-free water. The screened reaction temperature gradients were 61.8°C, 62.1°C, 62.6°C, 63.4°C, 64.4°C, 65.2°C, 65.6°C and 66°C. LAMP reactions were performed using a Bio-Rad quantitative fluorescent PCR instrument (Bio-Rad CFX96, Hercules, California, USA) for 80 cycles each, each cycle for 60 s, and the reaction was terminated at 80°C for 10 min. Optimal reaction temperature screening experiments were repeated three times.

4.5. Validation of the specificity for q-LAMP assay systems

The specificity of the reaction system was tested by performing q-LAMP reactions at the optimal reaction temperature with UV-2 primers in above 25-μL reaction mixtures for 70 min. The assay results were compared with the DNA of *U. virens* and the 9 other fungi listed in Table 4. The nucleic acid-free water was set as negative control. The results were rigorously validated with the assessment that the detectable peak of fluorescence signals detected by Bio-Rad CFX96 as positive; no fluorescence signal as negative. The specificity testing experiment was repeated three times.

4.6. Sensitivity validation of q-LAMP and q-PCR assay systems

The sensitivity validation of q-LAMP reactions was performed at the optimal reaction temperature with UV-2 primers in above 25- μ L reaction mixtures for 60 min. 1 μ L of DNA lysate from *U. virens* spores with known concentration was used as a DNA template in the LAMP reaction system. The nucleic acid-free water was used as a DNA template in the negative control (CK). The detectable peak of fluorescence signals detected by Bio-Rad CFX96 was regarded as positive, while no fluorescence signal was regarded as negative. Sensitivity assay experiments were repeated three times. The sensitivity of the q-PCR reaction system was assayed by performing q-PCR amplification using primers UV-2 F3/B3. The q-PCR reaction system was 12.5 μ L SYBR® Premix Ex Taq II (Tli RNaseH Plus, 2 \times), 1.0 μ L of forward primer F3 (10 μ M), 1.0 μ L of reverse primer B3 (10.0 μ M), 1.0 μ L DNA template (in CK, nucleic acid-free water was used as DNA template), and the volume was adjusted to 25 μ L with nucleic acid-free water. The reaction conditions were: pre-denaturation at 95°C for 2 min, denaturation at 95°C for 5 s, annealing at 60°C for 30 s, extension at 72°C for 6 s. The fluorescence signal was collected during the extension for a total of 40 cycles, and finally the lysis curve was plotted. The detectable peak of fluorescence signals detected by Bio-Rad CFX96 was regarded as positive, while no fluorescence signal was regarded as negative. Sensitivity assay experiments were repeated three times.

4.7. Establishment of standard curves for q-LAMP assay systems

A standard curve was constructed using software SPSS 13.0 by analyzing the association of logarithmic values of the spore number (y) and the amplification time quantitated using the cycle threshold (Ct) values (x). The correlation coefficient R^2 was used for assessing the linear relationship between the spore number in sample (y) and amplification time (x).

4.8. Calculating of *U. virens* spore using q-LAMP system

Spores of *U. virens* were artificially added to each of the four Melinex tape (1 cm \times 2 cm) in the ultra-clean bench, with 450, 116, 29 and 9 spores in each tape. The collected spore-adsorbed Melinex tape was cut and placed into 2-mL centrifuge tubes, and the genomic DNA of the spores on the Melinex tape was then extracted according to the method mentioned above. 1 μ L of the cooled lysate was used directly as DNA template. q-LAMP was performed with the optimal reaction conditions in above 25- μ L reaction mixtures for 60 min, and the cycle threshold (Ct) values detected by Bio-Rad CFX96 was recorded as the amplification time (x). The linearized equation for the standard curve was used for converting the amplification time to the corresponding spore number. Then the calculated spore number was compared to the amount of actual added (listed above) to test the accuracy efficiency of this q-LAMP system.

4.9. Field application of q-LAMP assay by *U. virens*

An air borne spore catcher (DIANJIANG, DJ-0723) with Melinex tape was established in Yongyou 1540 cultivation area, Jiangtang Village, Jinhua City, Zhejiang Province for the collection of spores of *U. virens*. and samples were collected at six-day intervals for 11 consecutive times starting on August 9, 2018. Similarly, starting on August 13 of the following year (2019), 11 consecutive samples were collected every six days. The spores of *U. virens* were adsorbed to the Melinex tape and the tapes (1 cm \times 1 cm) with spores were cut and placed in a 2-mL centrifuge tube, and the conidial DNA was extracted according to the methods mentioned above. 1 μ L of the cooled lysate was used directly as DNA template. q-LAMP was performed with the optimal reaction conditions in above 25- μ L reaction mixtures for 60 min, and the amplification time was recorded. According to the established standard curve, the number of spores was calculated. The spore population of *U. virens* in the glass slide was recorded by q-LAMP assay at six-day intervals. Meanwhile, the spores of *U. virens* (1 cm \times 1 cm) adsorbed on the slide were suspended in 1-mL ddH₂O, and the spore suspension was counted using a hemocytometer under the microscope to determine the spore concentration.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1. Sensitivity validation of q-PCR detection system.

Author Contributions: Conceptualization, T.M. and C.Z.; methodology, C.Z., Y.Z., and X.L.; software, T.M. and Y.Z.; validation, T.M. and C.Z.; formal analysis, Y.Z., S.Z.; investigation, S.Z. and X.L.; writing—original draft preparation, Y.Z. and T.M.; writing—review and editing, T.M. and C.Z.; visualization, J.W. and X.L.; supervision, T.M. and C.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Key Research and Development Project of Zhejiang Province, China (2015C02019), Science & Technology Program of Agriculture and Country in Zhenhai District.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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