Development of Loop mediated isothermal amplification (LAMP) assay for the detection of Magnaporthe oryzae causing blast in rice

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Abstract

Rice is one of the most important nourishment crops providing a quarter of calories consumption. It alone contributes 23 per cent of calories consumed by people all over the world. Rice blast pathogen is an important ascomycetes fungus which causes severe yield losses up to 100 per cent under favorable climatic conditions. A field survey on rice blast disease revealed that the disease incidence was ranged from 50.1% - 72.46% with the highest disease incidence of 72.46% at Coimbatore district, Tamil Nadu, India. Totally seven isolates of *Magnaporthe oryzae* were collected and the identity was confirmed through morphological and molecular confirmation. A loop-mediated isothermal amplification assay was developed by targeting *Pita 2* gene sequence of *M. oryzae*. The assay developed was more sensitive as it detected the genomic DNA of *M. oryzae* up to 10 fg. The specificity of LAMP assay was proved by carrying out the assay with genomic DNA extracted from other fungal pathogens. Therefore, the LAMP assay developed will be helpful in rapid, specific and sensitive detection of rice blast pathogen at field level and will help in mitigating the disease incidence.

Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops next to wheat and also most important staple food, which contributes approximately 30 per cent of nutritional intake (Gnanamanickam, 2009). It is widely grown in India, China and the rest of Asia where the maximum of 92 per cent of world's rice is grown. India is the second largest country in rice production next to China. The rice crop suffers from number of diseases, among the different diseases infecting rice, blast is one of the most destructive disease around the world. This disease was incited by a fungal pathogen called *Magnaporthe oryzae* (Synonym: *Pyricularia oryzae* Cavara) which results in extensive yield losses. Every year the rice crop will face severe yield losses up to 100% under favourable conditions (Liu et al., 2013). The pathogen *M. oryzae* is an ascomycetes fungus encompasses hundreds of races (pathotypes) around the world (Jia et al., 2014). The pathogen is quite capable to infect the rice plants at any stage of its growth period from seedling to grain formation and causes multifarious infection like leaf blast, collar rot, nodal blast and neck blast or panicle blast (Gowda et al., 2015). Therefore, it is necessary to identify the pathogen at earlier stage of its infection which helps to take up the timely management practices.

The identification of pathogens through morphometric analysis and molecular confirmation through nucleic acid based detection like PCR were time consuming, laborious and require skilled labours (Notomi et al., 2000). The sensitivity of the techniques was adversely affected by PCR inhibitors, particularly when inoculum levels are low or near detection limits. Loop mediated isothermal amplification assay (LAMP) is an novel detection technique, which was applied for the successful detection of several plant pathogens (Notomi et al., 2000, Thiessen et al., 2016, Tomlinson et al., 2010, Villari et al., 2017). This method requires DNA polymerase, and a set of four specially designed primers that recognize six different regions on the target DNA template. This method has been widely applied under field condition for on-site detection, because of its low cost, high specificity, efficiency, simplicity of operation and rapidity (Niessen and Vogel, 2010). The entire reaction will be completed under isothermal condition in conventional water bath.

In order to mitigate the yield losses and to take up timely management practices, early detection and efficient diagnosis of plant diseases are needed. LAMP is a rapid technique emerging as a quick diagnostic tool for advanced detection and identification of plant diseases. While this helps to improve research on detection and reduces time for detecting plant pathogens during field analysis. In this study, we have developed a LAMP assay protocol for rapid, early, specific and sensitive detection of *M. oryzae* infecting rice for the first time in India.

Materials And Methods

Survey and pathogen isolation

A roving survey was conducted in Coimbatore and Erode districts of Tamil Nadu, India during *Kharif* season of 2018-19 in major rice growing areas to assess the incidence of rice blast disease. The rice blast disease incidence was assessed by utilizing the scale of IRRI, 1996 as follows. 0- No lesion observed (Highly Resistant), 1- Small brown specks of pin point size (or) larger brown specks without sporulating centre (Resistant), 2- Small roundish to slightly elongated, necrotic gray spots, about 1-2 mm in diameter, with a distinct brown margin (Moderately Resistant), 3- Lesion type is the same as in scale 2, but significant numbers of lesions are on the upper leaves (Moderately Resistant), 4- Typical susceptible blast lesions of 3 mm or longer, infecting less than 4% of leaf area (Moderately Susceptible), 5- Typical blast lesions infecting 4-10% of the leaf area (Moderately Susceptible), 6- Typical blast lesions infecting 11-25% of the leaf area (Susceptible), 7- Typical blast lesions infecting 26-50% of the leaf area (Susceptible), 8- Typical blast lesions infecting 51-75% of the leaf area many leaves are dead (Highly Susceptible), 9- Typical blast lesions infecting more than 75% leaf area affected (Highly Susceptible). Finally, by using McKinney (1923) formula, per cent disease index (PDI) of rice blast was calculated

$$PDI = \frac{\text{Sum of all numerical rating}}{\text{Total number of leaves observed}} \times \frac{100}{\text{Maximum grade in the score chart}}$$

A total of seven rice blast diseased leaf samples were collected from Coimbatore and Erode districts of Tamil Nadu, India. A fungus was constantly isolated from the blast infected leaves by tissue segment method on PDA medium. The cultures were maintained at 4°C for further identification and characterization.

Morphological and molecular characterization of *M. oryzae*

The actively growing mycelia were taken from the edge of 9 days old mother cultures of each isolate placed on PDA medium. The radial growth of different isolates was measured daily from the first day after inoculation until maximum growth on the Petri dishes. Radial growth of the isolates was compared on the 10th day after inoculation. The length and breadth of the conidia of seven *M. oryzae* isolates were measured using a light microscope of 400X magnification and photographed in Image Analyser. Molecular characterization of rice blast pathogen was done using fungal culture of all seven isolates. A fresh fungal culture from each isolates was inoculated into 100 ml of PDA broth. A 100 g of 14 day's old dried fungal mat was harvested from the broth and subjected to genomic DNA isolation by Cetyl Trimethyl Ammonium Bromide (CTAB) method. The genomic DNA was checked by gel electrophoresis and DNA concentrations of the samples were determined using a spectrophotometer (Nanodrop, ND-1000, Wilmington, DE) and stored at - 20°C for further use. The conventional PCR was performed at 20 µl mixture containing 2 µl of genomic DNA (~ 50 ng/ µl), 10 µl of TaKaRa master mix (2 X concentration) and 2 µl of each forward and reverse primers (20 pmol). The reaction was carried out in eppendorf thermocycler. The PCR amplification of ITS region consisted of an initial denaturation of 4 minutes at 94°C followed by 40 cycles of 2 minutes of denaturation at 94°C, 45 seconds of annealing at 53 °C, 2 minutes of extension at 72°C and a final extension for 10 minutes at 72°C. The PCR program for the amplification of *Pot 2 transposon* region consisted of an initial denaturation of 4 minutes at 94°C followed by 40 cycles of 45 seconds

of denaturation at 94°C, 45 seconds of annealing at 55°C, and 45 seconds of extension at 72°C. The final extension was done for 10 minutes at 72°C. The PCR amplified products were visualized under UV and the images were documented with an Alpha Imager EC (USA).

LAMP primers designing

The *Pita 2* gene of *M. oryzae* was selected as a target site for designing LAMP primers. The primer sequences were designed using Primer Explorer version 5.0 software on the Eikon Genome site. All parameters viz., GC content, melting temperature, distance between the primer ends were as per the default setting.

Optimization of LAMP reaction

LAMP assay was performed using *Magnaporthe oryzae* DNA as template to determine the optimum reaction temperature and time. To determine the optimum temperature, the assay was tested with a range of temperature (56 to 68 °C) using pure DNA of *M. oryzae* and to determine the optimum time, the assay was tested with a range of time (30 to 120 minutes). The reaction was terminated by heat inactivation at 80 °C for 2 minutes.

To optimize the concentration of MgSO₄ in the reaction mixture, a total of five different concentrations of Mg²⁺ (2.00, 4.00, 6.00, 8.00 and 10.00 mM) were tested along with nuclease free water without MgSO₄ (negative control). The concentration of other components such as LAMP primers, Thermophol reaction buffer, dNTPs, Bst DNA polymerase, betaine, hydroxynaphtholblue (HNB) indicator, DNA template and water in LAMP assay was kept constant. The tubes containing 25 µl reaction mixtures were incubated at 65°C for 60 minutes and reaction was terminated by heat inactivation at 80°C for 2 minutes. The results were reconfirmed by assessment using HNB, EtBr, as well as resolving in 1.0 per cent agarose gel electrophoresis.

LAMP specificity assay

The specificity of LAMP assay was determined using the total genomic DNA isolated from *M. oryzae* and other plant pathogens like *Helminthosporium oryzae* infecting rice (Brown spot), *Plasmopara viticola* infecting grapes (Downy Mildew), *Erysiphe necator* infecting grapes (Powdery mildew), *Fusarium oxysporum* f. sp. *cubense* infecting banana (Panama wilt), *Colletotrichum capsici* infecting chilli (Chilli anthracnose), *Pernoscleropsora sorghi* infecting sorghum (Sorghum downy mildew) and *Sclerospora graminicola* infecting bajra (Cumbu downy mildew) along with nuclease free water serves as negative control. The reaction mixtures (25 µl) with template DNA of different fungal pathogens in each tube were incubated at 65 °C for 60 minutes and reaction terminated by heat inactivation at 80 °C for 2 minutes. The specificity of the assay was assessed based on HNB-visualized color change, EtBr visualization and further confirmed with 1.0 per cent agarose gel electrophoresis.

LAMP sensitivity assay

The sensitivity of LAMP assay was evaluated by ten-fold serial dilution of purified genomic DNA of *M. oryzae* from 100 nano gram to 1 femto gram (100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg). The 25 µl of reaction mixtures with different concentration of serially diluted genomic DNA of *M. oryzae* in each tube

were incubated at 65°C for 60 minutes and reaction was terminated by heat inactivation at 80°C for 2 minutes. The sensitivity of the assay was assessed based on HNB-visualized color change, EtBr visualization and further confirmed with 1.0 per cent agarose gel electrophoresis.

Detection of *M. oryzae* by LAMP

The LAMP assay was performed in a 25 µl reaction mixture containing 1.4 µM each of the FIP and BIP primers, 0.2 µM each of F3 and B3 primers, 0.4 µM each of the LF and LB primers, 1.4 mM each of dNTPs, 2.5 µl of 10X Thermophol reaction buffer, 4 mM MgSO₄, 8U of Bst DNA polymerase (New England Biolabs, Ipswich, MA), 0.8 M betaine solution, 120 µM hydroxynaphtholblue (HNB) indicator and 2.5 µl of DNA (~100 ng) template. The reaction tubes were incubated at 65°C for 60 minutes and terminated by thermal inactivation at 80°C for 2 minutes in eppendorf thermal cycler. The LAMP amplicons were visualized by different strategies: (a) visualization under hydroxy naphthol blue (HNB), (b) visualization under ethidium bromide (EtBr) and (c) 1.0 per cent agarose gel electrophoresis.

Results

Survey and pathogen isolation

Field survey results indicated that the highest incidence of rice blast infection was observed in Poosaripalayam village of Coimbatore district with PDI of 72.46%, followed by 67.75%, 60.63% in Booluvampatti and Bhavanisagar, respectively. The Gobichettipalayam village of Erode district showed the lowest incidence of 50.10% (Table 1). A fungus was constantly isolated from the rice leaf samples showing spindle shaped lesions on PDA medium. A total of seven isolates were isolated from infected leaf samples and stored at 4°C for characterization (Figure 1).

Morphological and molecular characterization of *M. oryzae*

The fungus mycelium was hyaline, septate and branched. The conidiophores were thin walled, slender and unbranched. The fungus produced pyriform conidia with 2 septation and hilum at base (Figure 2). The genomic DNA samples of *M. oryzae* obtained from mycelium of all the seven isolates were subjected to PCR amplification using universal primers ITS 1 and ITS 4. PCR amplification yielded a fragment of expected amplicon size of 550 bp (Figure 3). The amplified products of all the isolates was sequenced at Eurofins genomics India Pvt. Ltd. and confirmed as *M. oryzae* by comparing with the sequences already deposited in NCBI database. The comparison of nucleotide sequences showed an identity of 97-100% with the *M. oryzae* isolates available in GenBank (CP034204, MH859782, MF583153, LK932250, MF583148, KJ522980 and KM816801). The sequences of all seven isolates were deposited in GenBank and accession numbers were obtained (Table 2). The genomic DNA samples were further amplified using species specific primer set targeting *Pot 2 transposon* gene. The PCR amplification generated an amplicon size of approximately 680 bp for all the seven isolates authenticating the fungus as *M. oryzae* (Figure 4).

LAMP primers designing

The *Pita 2* gene sequence (GenBank accession no: AB607344) of *M. oryzae* was selected as the target region for designing of LAMP primers. We hypothesized that targeting the specific gene sequence (*Pita 2*) through LAMP assay may be more appropriate for accurate detection with 100 per cent efficiency. There are totally six primers (inner primers (FIP and BIP), outer primers (F3 and B3) and Loop primers (F loop and B loop)) designed using Primer Explorer v.5 software. The designed primers were projected in Table 3.

LAMP optimization

The optimum LAMP reaction conditions such as temperature and time for the detection of *Magnaporthe oryzae* were determined. Among the five different temperatures tested, positive reaction of sky blue color change was observed at almost all the temperature except at 56°C. However, the amplification efficiency exhibited a strong increase at 65°C, where the discrete ladder like pattern was more consistent and emitted a strong fluorescence under EtBr visualization (Table 4 and Figure 5). When the LAMP was performed at 65°C with range of test time, colour change was observed at two different temperatures. However, the amplification efficiency exhibited a strong increase at 60 minutes of incubation time under HNB visualization, EtBr fluorescence and the discrete ladder like pattern was more consistent at the same test time (Table 5 and Figure 6).

Among five different concentrations of Mg^{2+} (2.00, 4.00, 6.00, 8.00 and 10.00 mM) tested, the colour change was observed only at 4.00, 6.00 and 8.00 mM of Mg^{2+} . However, the strong sky blue colour development was observed at 4.00 mM of Mg^{2+} with bright fluorescence under EtBr visualization. The discrete ladder like pattern was more intense at the same test concentration indicating that the optimum concentration of $MgSO_4$ for the effective detection of *Magnaporthe oryzae* was 4.00 mM (Table 6 and Figure 7).

LAMP specificity assay

The specificity of LAMP assay was tested with 8 fungal pathogens with two indicator dyes like HNB and EtBr. The genomic DNA of fungal pathogens like *Magnaporthe oryzae*, *Helminthosporium oryzae*, *Plasmopara viticola*, *Fusarium oxysporum* f.sp. *cubense*, *Colletotrichum capsici*, *Erysiphe necator*, *Pernoscleropsora sorghi* and *Sclerospora graminicola* were subjected to LAMP assay. After incubation of LAMP reaction mixture at 65 °C for 60 minutes, a strong sky blue color development was observed only with *M. oryzae* DNA. However, LAMP reactions with the templates of other fungal isolates remained violet, indicating the absence of target DNA. A similar kind of results was obtained with the LAMP assay performed using EtBr indicator dye. The LAMP reaction with *M. oryzae* DNA developed a strong fluorescence, whereas the LAMP reaction with other non target pathogens did not produce fluorescence. The above results were confirmed using agarose gel electrophoresis. A discrete intense ladder like banding pattern was observed only in LAMP products with *M. oryzae* DNA. Ladder like banding pattern was absent in LAMP products with other fungal isolates and nuclease free water (negative control). This indicated that the developed LAMP assay was highly specific for the detection of *M. oryzae* (Table 7 and Figure 8).

LAMP sensitivity assay

To determine the detection limit of LAMP assay, a sensitivity test was performed with different DNA concentration of *M. oryzae* with two indicator dyes namely HNB and EtBr. The results showed that the sky blue color development was visible in almost all the concentration of genomic DNA tested, except at 1 fg. A similar kind of results was obtained while performing the test with EtBr dye. LAMP reaction with EtBr produced fluorescence from 100 ng to 10 fg except 1 fg of genomic DNA. The above results were confirmed using agarose gel electrophoresis assay where discrete ladder-like banding pattern was visible upto 10 fg DNA concentration. These results indicated that the developed LAMP assay could detect up to 10 fg of DNA of *M. oryzae* (Figure 9).

Detection of *M. oryzae* by LAMP

The LAMP assay specific to *M. oryzae* was developed and tested against genomic DNA of seven isolates of *M. oryzae* study isolates with two indicator dyes such as HNB and EtBr. The strong sky blue color development was observed in all the tubes containing DNA of *M. oryzae* isolates. However, the tube with nuclease free water as a negative control did not showed any sky blue color development, indicating the absence of *M. oryzae* DNA.

A similar kind of results was obtained in LAMP reaction with EtBr indicator dye, where a strong fluorescence was observed in all the tubes containing genomic DNA of *M. oryzae*. The absence of fluorescence in the tube with nuclease free water indicated the absence of target DNA. The above LAMP products were confirmed with 1 per cent agarose gel electrophoresis assay. Agarose gel electrophoresis assay yielded a discrete ladder-like banding pattern for genomic DNA from *M. oryzae* isolates. Whereas no such ladder-like banding pattern was observed with nuclease free water (Figure 10).

Discussion

M. oryzae causing rice blast disease is a major threat to rice cultivation at global level. A quick and on-site detection of pathogens will bring down the chances for the failure of rice crop thereby improves the yield. The conventional detection of rice blast pathogen based on symptoms and PCR which are time consuming and require skilled labor. LAMP assay is a rapid and more sensitive detection technique and it can be detected more number of samples at a time. Recently, many plant pathogens viz., *Magnaporthe grisea*, *Alternaria solani*, *Plasmopara viticola*, *Erysiphe necator*, *Puccinia graminis* f.sp. *tritici* has been successfully detected using LAMP assay. It has several advantages over conventional PCR assay such as amplification of DNA at very low concentration, specific detection within 20 to 60 minutes, can be performed using water bath and visual interpretation of results with the help of indicator dyes. In present study, we strongly recommend LAMP assay for the on-site detection of rice blast pathogen, since it requires minimum quantity of DNA (10 fg) which is much lesser than that of the quantity required for conventional PCR. To the best of our knowledge, this is the first report of application of LAMP assay in India for the detection of *M. oryzae* infecting rice.

LAMP assay can detect the presence of pathogen within in 60 minutes by specific amplification of DNA which is more reliable and less time bound. Therefore, it can be used as a rapid diagnostic kit. It amplifies DNA under isothermal conditions and therefore, visual observation in a single reaction is possible. The time consumed in reconfirmation of amplified products (positive reaction) under gel electrophoresis can be eliminated due to change in turbidity of the reaction mixture because of the deposition of magnesium pyrophosphate which serves as a simple identification technique under naked eye. LAMP primers are designed in such a way that it can recognize six distinct regions of the target site leaving no space for false positive or false negative results. In PCR, only two

regions can be recognized which results in more chances of amplifying false positives and false negatives and leaving the accuracy of detection under question. In present study, a set of six primers were designed using Primer Explorer v.5 software to target the *Pita 2* gene specific to *M. oryzae* and there is no cross reaction observed. The designed LAMP primers were highly sensitive and more specific in detecting *M. oryzae* thereby differentiating them from other tested pathogens. Ortega et al., (2018) designed LAMP primers for the detection of *M. oryzae* in rice seeds by targeting *calmodulin* gene. Likewise, Villari et al., (2017) designed LAMP primers targeting the gene sequence *LPKY 97- 1* of *M. oryzae* isolates from rye grass using primer explorer v.4 software tool which is in accordance with our present study.

The LAMP reaction conditions like incubation temperature, time and MgSO_4 concentration were optimized for the effective detection of *Pita 2* gene of *M. oryzae*. The temperature is highly essential in strand displacement activity. Both a high or low temperature conditions may results in development of false positives and false negatives in LAMP assay. Therefore, an optimum temperature condition is highly essential for reliable detection of target pathogen. In this present study, the optimum temperature was found to be 65°C which is in accordance the results of Patel et al., (2015) who identified the optimum temperature for the detection of *Rhizoctonia solani* as 65°C . Similarly, Dong et al., (2015) found the optimum temperature condition as 64.5°C for the detection of *Phytophthora capsici* using range of temperature from 62°C - 65°C . Lan et al., (2018) developed LAMP assay for sensitive and specific detection of *Fusarium oxysporum* f.sp. *cucumerinum* Owen causing *Fusarium* wilt in cucumber within 60 minutes of incubation. The LAMP assay developed by Ghosh et al., (2016) to detect *Fusarium oxysporum* f.sp. *ciceris* causing *Fusarium* wilt in chickpea requires 60 minutes of incubation which is in concurrence with our present study.

The concentration of MgSO_4 is the most crucial component in LAMP mixture, since it highly influences the assay results when added in little quantities and also gives irrelevant results like positive amplification of negative samples when added in more amounts. This is because of binding of primers to an incorrect template. Hence, an optimum amount is highly essential for the best outcome of LAMP assay (Cao et al., 2016). In this present study, the optimum concentration of MgSO_4 for the effective detection of *M. oryzae* was standardized as 4.00 mM. The results of our study was on par with the results of Duan et al., (2014) who has evaluated different concentration of MgSO_4 from 2.0-4.50 mM and found that optimum concentration of MgSO_4 for detection of *Colletotrichum falcatum* (red rot of sugarcane) was 4.0 mM. Similarly, Cao et al., (2016) evaluated the MgSO_4 concentration from 5.0 - 8.0 mM for LAMP based diagnosis of *Ustilago maydis* (common smut of corn). They optimized that 6.0 mM to 8.0 mM were effective for the detection of *Ustilago maydis*. The LAMP assay is highly sensitive in nature and it can detect the target pathogen even in samples with low concentration of DNA. In our present study, the LAMP assay developed for the detection of *M.oryzae* has an ability to detect upto 10 fg of DNA concentration. Our results are in concurrence with the LAMP assay developed by Zhang et al., (2019) for the detection of *Fusarium fujikuroi* infecting rice crop, wherein the assay could detect the presence of pathogen at 10 fg level of DNA concentration.

LAMP assay serves as a promising diagnostic kit, by forecasting the pathogen in advance. This will aid in designing of effective prediction models for the outbreak of diseases. Since, the early detection of the pathogen is achieved the protective measures can be taken much earlier by application of prophylactic fungicides.

Declarations

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Conflicts of interest/Competing interests:

The authors declare that they no conflicts of interest.

Ethics statement:

There was no involvement of Human Participants and/or Animals in this research and hence ethical approval is not required

Consent to Participate (Ethics)

- All participants were informed and agreed.

Consent to Publish (Ethics) -

All the authors agreed to publish

Authors' contributions:

NG-survey, experimentation, analysis, validation, writing, AK-conceptualization, survey, methodology, writing, original draft preparation, VGM- conceptualization, methodology, writing, SR-methodology, writing, review and editing, MK-validation, analysis and LR- methodology, writing and editing.

Availability of data and material:

All the data generated or analyzed during this study are included in this manuscript.

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Tables

Table 1

Collection of blast infected rice leaves from different places of Coimbatore and Erode district

S.no	District	Isolation code	Location	Variety	Latitude(°N)	Longitude(°E)	% Disease index
1.	Coimbatore	CBE 1	Veerakeralam	CO 39	10.5943 ° N	76.5459 ° E	58.23 ^d
2.	Coimbatore	CBE 2	Poosaripalayam	CO 39	11.0073 ° N	76.9110 ° E	72.46 ^a
3.	Coimbatore	CBE 3	Anaimalai	CO 43	10.5826 ° N	76.9528 ° E	51.80 ^f
4.	Coimbatore	CBE 4	Booluvampatti	CO 46	10.9921 ° N	76.7221 ° E	67.75 ^b
5.	Erode	BSR	Bhavanisagar	Bhavani	11.288 ° N	77.722 ° E	60.63 ^c
6.	Erode	SAT	Sathyamangalam	CO 46	11.5048 ° N	77.2384 ° E	54.29 ^e
7.	Erode	GB	Gobichettipalayam	CO 46	11.4504 ° N	77.4300 ° E	50.10 ^g
							CD (0.05) = 1.74

Note: * Mean of three replications.

Means in a column followed by same superscript letters are not significantly different according to DMRT at $P \leq 0.05$.

t according to DMRT at $P \leq 0.05$.

Table 2

Accession number of ITS region of study isolates of *Magnaporthe oryzae*

S. No.	Place	Study isolate name	Accession number
1.	Veerakeralam	CBE 1	MK880270
2.	Poosaripalayam	CBE 2	MK880271
3.	Anaimalai	CBE 3	MK937810
4.	Booluvampatti	CBE 4	MN067714
5.	Bhavanisagar	BSR	MK880269
6.	Sathyamangalam	SAT	MK937811
7.	Gobichettipalayam	GB	MN067715

Table 3

Sequences of LAMP primers designed

LAMP assay (gene target)	Primer	Primer sequence (5'-3')	Length
AVR Pita 2	FIP	GCGACGTCGATAAAGGTATCACCCCATGAGATCACCAGACC	41 nt
	BIP	AGCTCAGAACTTTGTTTGTTTCCTTCATAGCACGGAAGCTAGT	43 nt
	F3	CCTAATTACTGTGACATTTGGA	22 nt
	B3	GTAATTGTCACGGCCAGG	18 nt
	F Loop	GGTCAGGGGTCAGGGTTAG	19 nt
	B Loop	GAGTAGAATCTTCGTCGATAGATGC	25 nt

Table 4

Optimization of incubation temperature in LAMP assay for the detection of *M. oryzae*

S. No.	Temperature (°C)	Colour change with HNB	Fluorescence with EtBr	DNA amplification*
1.	56	Violet	No fluorescence	–
2.	59	Weak Sky blue	Very dull fluorescence	+
3.	62	Mild Skyblue	Dull fluorescence	++
4.	65	Strong Sky blue	Bright fluorescence	+++
5.	68	Weak Sky blue	Very dull fluorescence	+

*Where,

+++ : Strong DNA amplification

++ : Mild DNA amplification

+ : Weak DNA amplification

- : No amplification

Table 5

Optimization of incubation time in LAMP assay for the detection of *M. oryzae*

S. No.	Incubation Time (min.)	DNA amplification*
1.	30	–
2.	60	++
3.	90	+
4.	120	–
*Where, ++ : Strong DNA amplification + : Mild DNA amplification - : No amplification		

Table 6

Optimization of MgSO₄ concentration in LAMP assay for the detection of *M. oryzae*

S. No.	MgSO ₄ concentration (mM)	Colour change with HNB	Fluorescence with EtBr	DNA amplification*
1.	2	Violet	No fluorescence	–
2.	4	Strong Sky blue	Bright fluorescence	+++
3.	6	Mild Sky blue	Dull fluorescence	++
4.	8	Weak Sky blue	Very dull fluorescence	+
5.	10	Violet	No fluorescence	–
6.	0	violet	No fluorescence	–

*Where,

+++ : Strong DNA amplification

++ : Mild DNA amplification

+ : Weak DNA amplification

- : No amplification

Table 7

Specificity of LAMP assay for the detection of *M. oryzae*

S. No.	Cultures	Host	Accession no.	DNA amplification*
1.	<i>Magnaporthe oryzae</i>	Rice	MK880271	+
2.	<i>Helminthosporium oryzae</i>	Rice	MN901494	–
3.	<i>Plasmopara viticola</i>	Grapes	GQ258976	–
4.	<i>Erysiphe necator</i>	Grapes	MK637521	–
5.	<i>Fusarium oxysporum f.sp. cubense</i>	Banana	MF576349	–
6.	<i>Colletotrichum capsici</i>	Chilli	MF280216	–
7.	<i>Pernoscleropsora sorghi</i>	Sorghum	MK453403	–
8.	<i>Sclerospora graminicola</i>	Bajra	MK453404	–
9.	Negative control (Nuclease free water)	-		–
*Where, + : Positive amplification - : No amplification				

Figures

Figure 1

Collection of blast infected rice leaves from farmer's field.

Legend - (a) Development of individual spots at early stage of infection (b) Spindle shaped lesions on rice leaves (c) Drying of leaves

Figure 2

Morphological characterization of *M. oryzae* conidiophore at 400X magnification.

Legend- (a) Hyaline, septate conidiophore with conidia. (b) Three celled pyriform conidia with hilum at base

Figure 3

PCR amplification of ITS region of *Magnaporthe oryzae*.

Legend - Agarose gel electrophoresis of PCR amplicons of ITS region of *Magnaporthe oryzae* isolates infecting rice. Total DNA isolated from fungal mycelium on PDA medium used as template. The DNA samples analysed are from mycelium of fungal isolates on PDA medium from Lane 1- CBE 1, Lane 2- CBE 2, Lane 3- CBE 3, Lane 4- CBE 4, Lane 5- BSR, Lane 6- SAT, Lane 7- GB, Lane 8- positive control (*Magnaporthe oryzae*), Lane 9- nuclease free water (negative control)

Figure 4

PCR amplification of *Pot 2 transposon* region of *Magnaporthe oryzae*.

Legend - Agarose gel electrophoresis of PCR amplicons of *Pot 2 transposon* region of *Magnaporthe oryzae* isolates infecting rice. Total DNA isolated from fungal mycelium on PDA medium used as template. The DNA samples analysed are from mycelium of fungal isolates on PDA medium from Lane 1- CBE 1, Lane 2- CBE 2, Lane

3- CBE 3, Lane 4- CBE 4, Lane 5- BSR, Lane 6- SAT, Lane 7- GB, Lane 8- positive control (*Magnaporthe oryzae*), Lane 9- nuclease free water (negative control)

Figure 5

Optimization of incubation temperature in LAMP assay for the detection of *Magnaporthe oryzae*.

Legend - Optimization of incubation temperature for the detection of *Pita 2* gene of *Magnaporthe oryzae*. Assessment was based on HNB visualization, EtBr fluorescence and agarose gel electrophoresis. Total DNA

isolated from fungal conidia on PDA medium used as template. The DNA samples analysed are from Lane details: Lane 1- 56 °C, Lane 2 – 59 °C, Lane 3- 62 °C, Lane 4- 65 °C, Lane 5- 68 °C.

Figure 6

Optimization of incubation time in LAMP assay for the detection of *Magnaporthe oryzae*.

Legend -Optimization of incubation time for the detection of *Pita 2* gene of *Magnaporthe oryzae*. Assessment was based on HNB visualization, EtBr fluorescence and agarose gel electrophoresis. Total DNA isolated from

fungal conidia on PDA medium used as template. The DNA samples analysed are from Lane details: Lane 1- 30 minutes, Lane 2 – 90 minutes, Lane 3- 60 minutes, Lane 4- 120 minutes.

Figure 7

Optimization of MgSO_4 concentration in LAMP assay for the detection of *Magnaporthe oryzae*.

Legend - Optimization of MgSO_4 concentration for the detection of *Pita 2* gene of *Magnaporthe oryzae*. Assessment was based on HNB visualization, EtBr fluorescence and agarose gel electrophoresis. Total DNA

isolated from fungal conidia on PDA medium used as template. The DNA samples analysed are from Lane details: Lane 1- 2 mM MgSO₄, Lane 2 – 4 mM MgSO₄, Lane 3- 6 mM MgSO₄, Lane 4- 8 mM MgSO₄, Lane 5- 10 mM MgSO₄, Lane 6- nuclease free water (negative control)

Figure 8
Specificity of the LAMP assay for the detection of *Magnaporthe oryzae*.

Legend -Specificity of the LAMP assay targeting *Pita 2* gene, colour visualization, EtBr visualization and agarose gel electrophoresis. Total DNA isolated from fungal conidia on PDA medium used as template. The DNA samples analysed are from Lane details: Lane 1- *Magnaporthe oryzae*, Lane 2- *Helminthosporium oryzae*, Lane 3- *Plasmopara viticola*, Lane 4- *Erysiphe necator*, Lane 5- *Fusarium oxysporum f.sp. cubense*, Lane 6- *Colletotrichum capsici*, Lane 7- *Pernoscleropsora sorghi*, Lane 8- *Sclerospora graminicola*, Lane 9- nuclease free water (negative control)

Figure 9

Sensitivity of the LAMP assay for the detection of *Magnaporthe oryzae*.

Legend - Sensitivity of the LAMP assay targeting *Pita 2* gene in serially diluted genomic DNA of *Magnaporthe oryzae*, colour visualization, EtBr visualization and agarose gel electrophoresis. Total DNA isolated from fungal conidia on PDA medium used as template. The DNA samples analysed are from Lane 1- 100 ng, Lane 2- 10 ng, Lane 3- 1 ng, Lane 4- 100 pg, Lane 5- 10 pg, Lane 6- 1 pg, Lane 7- 100 fg, Lane 8- 10 fg, Lane 9- 1 fg, Lane 10- nuclease free water (negative control)

Figure 10

LAMP assay for the detection of *Magnaporthe oryzae*.

Legend - *Magnaporthe oryzae* detection in LAMP, colour visualization, EtBr visualization and agarose gel electrophoresis. Total DNA isolated from fungal conidia on PDA medium used as template. The DNA samples analysed are from Lane 1- CBE 1, Lane 2- CBE 2, Lane 3- CBE 3, Lane 4- CBE 4, Lane 5- BSR, Lane 6- SAT, Lane 7- GB, Lane 8- nuclease free water (negative control)