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Development of a Loop-mediated Isothermal Amplification for the Detection of *Burkholderia glumae*

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DEVELOPMENT OF A LOOP-MEDIATED ISOTHERMAL AMPLIFICATION
FOR THE DETECTION OF *BURKHOLDERIA GLUMAE*

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Plant Pathology and Crop Physiology

by

Maria A. Caldera Dominguez
B.S., Escuela Agrícola Panamericana, El Zamorano, 2011
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I dedicate this thesis to my parents Marlene Dominguez and Roberto Caldera and my grandmother Lucrecia Del Socorro and my aunt Maria Dominguez, who always supported me during this journey and who taught me that one of the biggest treasures is knowledge. I will always be grateful to them.

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ABSTRACT

Bacterial panicle blight is among the three most limiting diseases of rice production in the United States. Yield loss up to 40% has been reported from rice fields severely infested with bacterial panicle blight. The disease is caused by *Burkholderia glumae* and *B. gladioli*. Although, both species produce similar symptoms and are closely related, but *B. glumae* strains are more aggressive and cause more severe symptoms on rice. Bacterial panicle blight is difficult to manage in the absence of effective chemical control measures. Rapid and early disease detection is needed to avoid severe yield losses. Several techniques have been developed for bacterial identification, but these methods are time consuming and some require high-precision instruments for amplification and analysis of target DNA. We developed a Loop-Mediated Isothermal Amplification (LAMP) protocol for rapid detection of *B. glumae* using a set of six primers from the *gyrB* housekeeping gene. Several commercially available dyes including, PicoGreen, Hydroxynaphthol Blue (HNB) and Calcein were compared to analyze the LAMP product. The LAMP detection method resulted in rapid and accurate detection of *B. glumae*. Among the different detection dyes, PicoGreen and HNB produced reliable results in the detection of *B. glumae*. Although, both produced accurate results; however, HNB is more cost-effective.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Economic Impact and Disease Distribution

Rice is one of the most important crops grown worldwide and it is the primary food source for more than half of the world population (Redoña, 2004). China is the major rice producing country, followed by India, Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Philippines, Brazil and Pakistan (FAOSTAT, 2012). The United States rank 13th in rice production in the world. Its rice production and consumption have heavily increased between 1960 and 2012 (Ray and Schaffer, 2013). The United States produced 12.35 million tons of rice in 2011 (Childs, 2012). Rice production is mainly concentrated in the southern United States (Arkansas, Mississippi, Missouri, Louisiana and Texas) and the Sacramento Valley of California (USDA, 2013). In 2000, rice in Arkansas, Louisiana and Mississippi ranked among the top five agricultural commodities (Livezey and Foreman, 2004). Over the years, several new diseases have adversely affected the rice production in rice growing areas of the world, but more recently, bacterial panicle blight (BPB) has become an economically important disease responsible for causing severe yield losses in rice. Yield loss up to 40% has been reported from rice fields severely infested with BPB in the United States and Asia (Chien *et al.*, 1987; Tsushima, 1996 and Ham and Groth, 2011), and 40-75% in Panama, Nicaragua, Colombia, Venezuela, Costa Rica and Dominican Republic (Salamanca, 2011).

Bacterial panicle blight of rice is caused by *Burkholderia glumae* and *B. gladioli*. *Burkholderia glumae* was first described as the cause of grain rotting and seedling blight of rice in Japan (Goto and Ohata, 1956). Since then, *B. glumae* has spread to other rice growing regions of the world including the United States, east and southeast Asia and South America (Chien and Chang, 1987; Cottyn *et al.*, 1996; Shahjahan *et al.*, 1998 and Ziegler, 1990).

For several years, rice panicle blight has been observed in the southern United States, but it was considered an unknown disorder (Groth *et al.*, 1991).

Finally, during 1996-1997, *B. glumae* was confirmed the causal organism of BPB of rice (Rush *et al.*, 1998 and Shahjahan *et al.*, 1998). Nandakumar *et al.* (2009) demonstrated that BPB is caused by *B. glumae* and *B. gladioli* in the southern United States. The symptoms of BPB include seedling blight, sheath rot and panicle blight (Chien *et al.*, 1987 and Tsushima, 1996). Symptoms found on the leaf sheath include long, vertical, grayish lesions surrounded by dark reddish brown margin. Shahjahan *et al.* (2000) observed that the stems below the infected grains remained green. They also reported that grain discoloration and green stems are the most important diagnostics characteristics of BPB. *Burkholderia glumae* has been reported as a seed-borne and soil-borne bacterium causing seedling blight, sheath rot and panicle blight in other parts of the world, but in the United States it has been reported to be associated with symptoms such as floret blight, unfilled grains and abortion of kernels (Trung *et al.*, 1993 and Groth *et al.*, 2009).

Management of BPB with oxolinic acid has been reported, however, oxolinic acid-resistant *B. glumae* strains have been isolated from rice in Japan (Hikichi *et al.*, 1998). Furthermore, oxolinic acid is not labeled for rice in the United States. Most commercially available rice varieties are susceptible to BPB, with the exception of Jupiter, which is partially resistance and two resistant lines designated as LM1 and LMT (Groth *et al.*, 2007).

1.2 Disease Epidemiology

The bacterium multiplies on the surface of panicles at initiating stage and infects the florets after emergence (Goto and Ohata, 1956).

High night time temperature and humidity favor disease development (Tsushima *et al.*, 1985; Zeigler and Alvarez, 1990 and Mew, 1992). Tsushima *et al.* (1995) established that high humidity at the flowering stage played an important role in spikelet infection. Presence of bacterial cells on the leaf sheath also played an important role in primary infection of the emerging panicles (Tsushima *et al.*, 1991 and Tsushima *et al.*, 1996).

Tsushima *et al.* (1985) demonstrated that the rate of infection is directly proportional to the inoculum density. They also reported that the disease progressed in a circular pattern with fewer affected plants on the edges in the field.

1.3 *Burkholderia glumae*

Burkholderia glumae was initially classified as *Pseudomonas glumae* (Kurita and Tabei, 1967). The bacterium is rod-shaped, gram negative, non-endospore producer and possesses two to four polar flagella. It produces slightly yellow or milky white colonies on potato dextrose agar at 30-35°C. The growth of bacterium ceases below 11°C and above 40°C (Asuyama, 1939 and Hashioka, 1969).

Fory *et al.* (2013) observed that after 48 hours of incubation on Kings B agar medium, *B. glumae* strains appeared as convex colonies with pronounced halo zone, usually with irregular margins and an average diameter of 0.6 mm. They also reported that *B. gladioli* colonies were slightly domed, smooth and shiny, with regular, circular margins and a larger average diameter of 1.6 mm. Also, Yuan *et al.* (2004) observed that convex colony was a common morphological characteristic among *Burkholderia* species.

1.4 *Burkholderia glumae* Detection Methods

Several selective and semi-selective media have been developed for the detection of *B. glumae* including: semi selective (SP-G) (Tsushima *et al.*, 1986), selective media (SMART)

(Kawanishi *et al.*, 2011) and CCNT media (Kawaradani *et al.*, 2000). However, conventional methods including colony morphology or disease symptoms are less reliable and time consuming and serological or molecular techniques may be required to detect and confirm the identity of the pathogen in question. Biochemical methods such as BIOLOG can be used to identify bacterial pathogens, but the assay requires pure culture of the bacterium and is time consuming (Cottyn *et al.*, 1996).

Enzyme-Linked Immunosorbent Assay (ELISA) is a serological test based on the use of antibodies specific for detection of pathogens. However, sensitivity is lower than 10^5 cells per ml (Agdia, Inc., 2008).

Fatty Acid Methyl Esters (FAME) has been used for bacterium identification, but it requires the use of an automated gas chromatograph and the process involves several steps including harvesting, saponification, methylation, extraction and gas chromatography. After obtaining the fatty acids profile, it is compared to profiles present in the FAME database (Sasser, 1990 and Zhu *et al.*, 2008).

Polymerase chain reaction (PCR), real-time PCR and multiplex PCR are molecular methods that can identify pathogens at very low concentration (Takeuchi *et al.*, 1997 and Maeda *et al.*, 2006). However, PCR based methods require high precision instruments for amplifying the target DNA and post-PCR analysis of the amplified DNA in case of conventional PCR.

Real-time PCR has the ability to amplify the target DNA in short period of time and does not require gel electrophoresis to visualize amplified DNA (Walker, 2002). Real-time PCR has many advantages including sensitivity, quantitative aspects, specificity and lower contamination; however, real-time PCR requires expensive equipment and specific probes (Parida *et al.*, 2008).

1.5 Loop-Mediated Isothermal Amplification

Loop-mediated isothermal amplification also known as LAMP rapidly amplifies target DNA with high specificity and efficiency under isothermal conditions over a range of 60-65°C, using a strand displacement reaction (Notomi *et al.*, 2000). The strand displacement reaction has the ability to displace downstream DNA produced during DNA synthesis (New England Biolabs, 2014).

The method employs a DNA polymerase and a set of four to six specially designed primers that recognize a total of six distinct sites on the target DNA (Notomi *et al.*, 2000 and Nagamine *et al.*, 2002).

LAMP has shown accurate results in medical sciences for the detection of bacterial pathogens in specimens from humans and animals (Iwamoto *et al.*, 2003; Savan *et al.*, 2004; Song *et al.*, 2005; Dukes *et al.*, 2006; En *et al.*, 2008 and Wastling *et al.*, 2010). This technique also has been used for the detection of plant pathogenic bacteria including *Ralstonia solanacearum* (Kubota *et al.*, 2008), *Xylella fastidiosa* (Harper *et al.*, 2010), *Pectobacterium atrosepticum* (Li *et al.*, 2011), *Erwinia amylovora* (Temple and Johnson, 2011) and *Candidatus Liberibacter solanacearum* (Ravindran *et al.*, 2012).

LAMP can amplify and detect a gene by incubating the reaction at a single temperature (Notomi *et al.*, 2000).

Unlike other nucleic acid amplification techniques, LAMP has the advantage of greater simplicity with good amplification efficiency (Parida *et al.*, 2008). LAMP does not require a thermo cycler and amplification can be readily achieved in a standard heat-block or a water-bath (Notomi *et al.*, 2000 and Tomlinson and Boonham, 2008). In addition, gel electrophoresis to visualize the amplified DNA is not required (Tomlinson and Boonham, 2008). LAMP amplified

product can be visualized using intercalator dyes including PicoGreen, SYBR Green I and ethidium bromide and metal (e.g. magnesium) indicator dyes such as Calcein and HNB (Parida *et al.*, 2008).

1.6 LAMP Principle

The principle of LAMP is based on auto-cycling strand displacement DNA synthesis facilitated by *Bst* (*Bacillus stearothermophilus*) DNA polymerase by using a set of four to six primers, including forward inner primer (FIP) and backward inner primer (BIP), outer primers (F3 and B3) and loop primers (LF and LB) (Notomi *et al.*, 2000). The loop primers help accelerate the amplification reaction by binding to additional sites that are not accessed by both inner primers (Nagamine and Notomi, 2002).

The forward inner primer anneals to initiate the first strand synthesis of the target sequence by the *Bst* DNA polymerase. The forward outer primer anneals to the complementary region F3c, outside of FIP, on the target sequence and displaces the synthesized first strand. The released strand forms a stem-loop structure at the 5' end because of the complementary sequence to the F1 region contained in the FIP primer. This released single strand serves as a template for BIP, similarly to FIP process the B3 primer anneals to its complementary region B3c.

Starting from the 3' end of the BIP, synthesis of the complementary DNA starts and reverts the loop structure formed by F1 and F1c regions into a linear structure.

A double stranded DNA is produced from the process previously described. The BIP-linked complementary strand previously displaced forms a structure with stem-loops at each end, resulting in a dumbbell structure. The formation of the dumbbell-like product is essential for LAMP to establish isothermal amplification because the loop structures are always single stranded and can be annealed by FIP or BIP. The formation of the loop structure can lead to the

elimination of the denaturing step, which is otherwise indispensable in PCR for obtaining single-stranded DNA (Notomi *et al.*, 2000).

After dumbbell-like structure formation, a cyclic reaction is established between the dumbbell-like structure and its complementary product, employing inner primers. The product is made of different size structures consisting of alternately inverted repeats of the target sequence of the same strand (Figure 1), producing a cauliflower-like structure (Notomi *et al.*, 2000).

When amplification is achieved, LAMP generates large amounts of DNA strands that contain multiple copies of the target DNA. During this reaction magnesium pyrophosphate is produced as a by-product in the form of an insoluble white precipitate which becomes visible when the reaction is terminated (Mori *et al.*, 2001).

1.7 Detection of LAMP Product

Several different methods have been developed to detect LAMP products.

1.7.1 Turbidity

Initially, turbidity of the final product was used to detect positive amplification of DNA (Notomi *et al.*, 2000; Yoshida *et al.*, 2005 and Parida *et al.*, 2008). Parida *et al.* (2008) reported that to observe the turbidity in the form of a white precipitate, DNA yield in excess of 4 μ g is required.

They also found that the LAMP reaction can produce DNA yields of $\geq 10 \mu\text{g}$ compared to 0.2 μg produced by conventional PCR in 25 μL reaction. On the basis of this reaction, Tomita *et al.* (2008) developed a colorimetric method for detection of LAMP reaction by adding Calcein (fluorescence metal indicator) to the pre-reaction solution in conjunction with manganese ions.

LAMP Principle

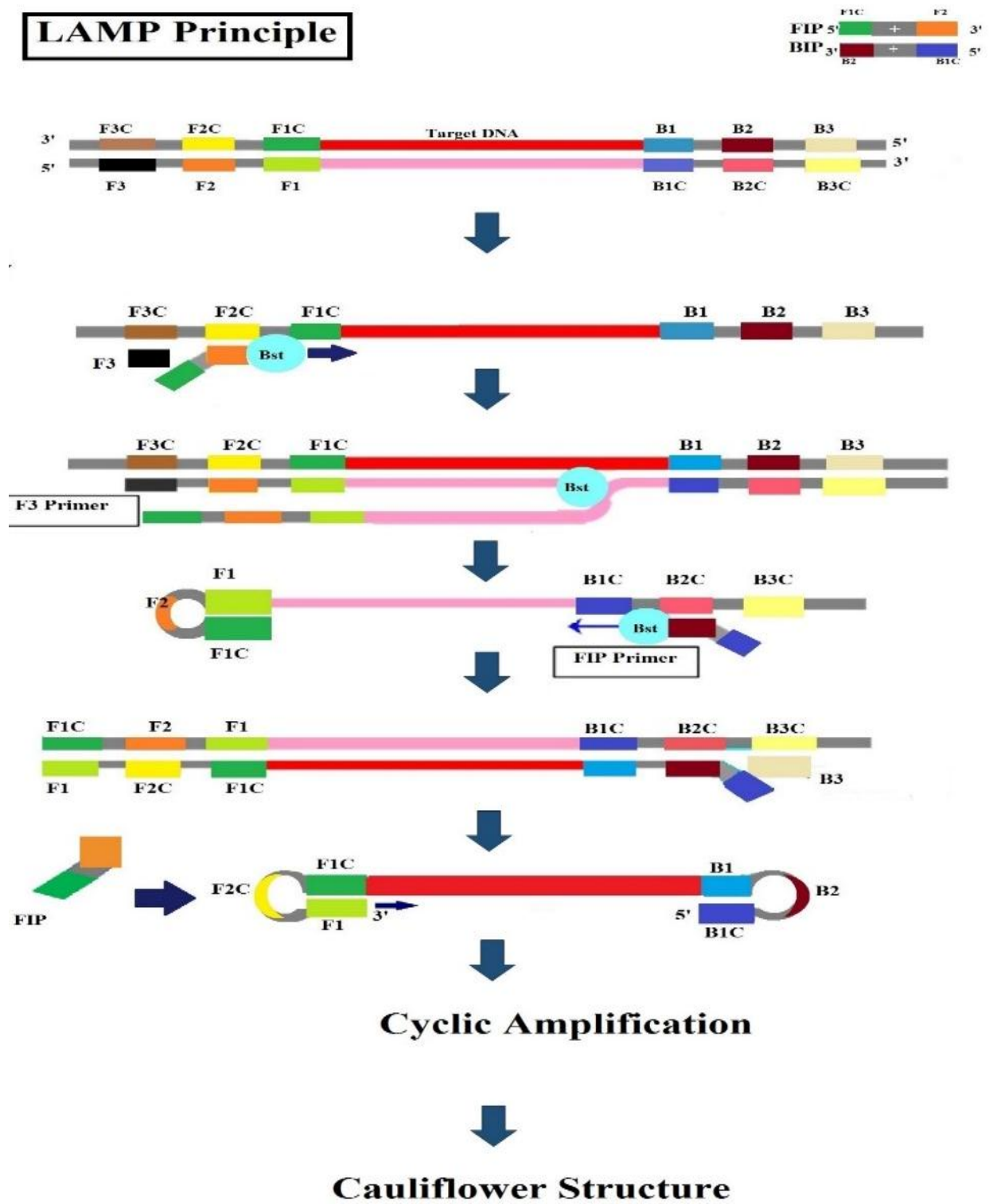


Figure 1. Principle of LAMP method (adopted from Notomi *et al.*, 2008).

Amplified DNA also can be detected by intercalating dyes such as, ethidium bromide (Pham *et al.*, 2005), SYBR green I (Iwamoto *et al.*, 2003), Hydroxynaphthol Blue (HNB) (Goto *et al.*, 2009) and PicoGreen (Dukes *et al.*, 2006 and Tsai *et al.*, 2009). In addition, to confirm that the DNA amplified in the reaction is the target DNA and not the non-specific amplification or primer dimers, the final product can be visualized on the gel to obtain the characteristic banding pattern defined by the number of target sequence copies (Tomlinson and Boonham, 2008).

1.7.2 Calcein and MnCl₂

Calcein is a fluorescent dye also known as fluorexon. It has an excitation and emission wavelengths of 495 and 515 nm, respectively. Calcein can be used as a Ca²⁺ or Mg²⁺ indicator in alkaline pH and can be quenched strongly by Co²⁺, Ni²⁺ and Cu²⁺ and significantly by Fe³⁺ and Mn²⁺ at neutral pH (Zhang *et al.*, 2012).

Calcein is soluble in Dimethyl sulfoxide (DMSO) and Dimethyl formamide (DMF) and it is slightly water soluble (pH > 6). Calcein is light sensitive and must be stored at 4°C (Green, 1990). Previous report has shown that Calcein only produces a strong fluorescence signal in neutral buffer (Yu *et al.*, 2008). Therefore, it is a good indicator for techniques that involve amplification of DNA at neutral pH.

Calcein is quenched in the pre-reaction by manganese. As the LAMP reaction proceeds, pyrophosphate ions (PPi) are produced as a by-product of the *Bst* DNA polymerase reaction; PPi subsequently form a manganese pyrophosphate complex, causing the removal of the manganese ion from Calcein, because PPi are stronger base than Calcein. Consequently, free Calcein combines with magnesium ions and produce bright fluorescence under UV light. As a result, the presence of fluorescence indicates the presence of the target DNA (Tomita *et al.*, 2008).

1.7.3 Hydroxynaphthol Blue

HNB has been reported to be useful as a colorimetric indicator for the titration of Ca^{2+} ions at pH 13.0 and Mg^{2+} ions at pH 10 (Ito and Ueno, 1970). Goto *et al.* (2009) hypothesized that HNB could be a novel indicator for LAMP reaction by monitoring the change in the Mg^{2+} ion concentration since *Bst* DNA polymerase synthesizes DNA under alkaline conditions (pH 8.8 at 25°C). This indicator changes color depending on the pH of the solution; it was reported that when the solution contained 8 mM Mg^{2+} and no dNTPs, its color was magenta at pH 8.6 to 9.0 and violet at pH 8.4. After the addition of 1.4 mM dNTPs to this solution, the color of HNB changed from magenta to violet regardless of the pH (Goto *et al.*, 2009). This change in color is induced by chelation of Mg^{2+} ions by dNTPs.

Also, it has been reported that HNB is purple at Mg^{2+} concentrations of 6 mM or higher, but as DNA synthesis progresses, the concentration of Mg^{2+} decreases, and when it reaches below 6 mM, the color of HNB changes to sky blue, indicating a positive reaction.

1.7.4 PicoGreen

PicoGreen is a fluorescent probe that binds to double stranded DNA and forms a highly luminescent complex. It has characteristics similar to SYBR Green I.

It has a maximum excitation at 480 nm and an emission peak at 520 nm. Dragan *et al.* (2010) reported that when PicoGreen binds to double stranded DNA, fluorescence is extremely high. They also observed that PicoGreen shows similar fluorescence enhancement when binds to short 16 bp DNA and with highly polymeric DNA. Based on their DNA assays, sensitivity to DNA was demonstrated at a concentration of ≈ 1 pg/ml.

1.8 LAMP Reagents

LAMP reagents vary in some major aspects to one of the techniques more widely used, conventional PCR, for the detection of *B. glumae*. The use of six primers provide a greater level of target specificity than the one achieved with two primers normally used in PCR (Notomi *et al.*, 2000) as well as the use of a DNA polymerase with strand displacement activity (*Bst* DNA polymerase) under isothermal conditions and lower temperature (e.g. 60-65°C). *Bst* DNA polymerase contains the 5'→3' polymerase activity, but lacks 5'→3' exonuclease activity (New England Biolabs, Beverly, MA). The enzyme has a heat-resistant property and a strand-displacement type DNA polymerase activity, which synthesizes a new DNA strand while separates the hydrogen bond of the double stranded template DNA. Since the strand-displacement DNA polymerase does not require dissociation of double-stranded DNA, the DNA can be synthesized at a constant temperature.

Optimum temperature for *Bst* DNA polymerase ranges from 50-65°C, which facilitates primer annealing and enhance tolerance to inhibitors typically found in diagnostic samples; an advantage compared to other polymerases. However, inhibition above 65°C and inefficient incorporation of dNTPs has been reported (Tanner, New England Biolabs, Inc.). The DNA polymerase is supplied with Thermopol buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton®-X-100, pH 8.8 @ 25°C) that ensures polymerase activity.

The concentration of dNTPs in the PCR reaction mixture also plays an important role in achieving the desired results. Slight deviation from the required concentration has led to dramatic increase of misincorporation levels of dNTPs (McPherson and Moller, 2000). In most of the publications about LAMP, dNTPs concentration is very high (1.4 mM) compared to conventional PCR (Li *et al.*, 2011).

The presence of magnesium source (MgSO_4) in conventional PCR is required for polymerase activity, primer annealing, strand dissociation and product specificity. Previous reports on conventional PCR have demonstrated that optimum concentrations of MgSO_4 should contain 0.5 to 2.5 mM over the total dNTPs concentration (Innis and Gelfand, 1990). Previous LAMP procedures concentrations up to 2.5 mM have been reported (Ravindran *et al.*, 2012). In LAMP assays, magnesium concentration can also interfere with dye reactions (Goto *et al.*, 2009 and Dasa *et al.*, 2012).

Previous studies have reported that the addition of enhancers such as Betaine improves yield and specificity in GC rich regions and decreases the production of undesired products (Reese *et al.*, 1993; Henke *et al.*, 1997 and Jensen *et al.*, 2010).

DNA regions rich in GC concentration can sometimes be problematic due to inefficient separation of DNA strands or the formation of secondary structures. Betaine decreases the amount of energy necessary to separate DNA strands (Rees *et al.*, 1993).

CHAPTER 2 MATERIAL AND METHODS

2.1 LAMP Primer Design

There are 4,964 nucleotide sequences, for *B. glumae*, of variable lengths available in the National Center for Biotechnology Information (NCBI) GenBank database. Taking into consideration the large amount of sequence data, regions used for previous PCR assays and housekeeping genes (Takeuchi *et al.*, 1997; Maeda *et al.*, 2006 and Sayler *et al.*, 2006) that have resulted in accurate detection of *B. glumae* were used in this study for the development of LAMP primers.

Several genes were selected as promising sequences for LAMP primer design including, 16-23S rDNA Intergenic Spacer Region, *rpoD*, and *gyrB* housekeeping genes. The 16-23S rDNA Intergenic Spacer Region of *B. glumae* used in this study was previously used to design primers for conventional PCR (Takeuchi *et al.*, 1997).

16-23S rDNA was suitable for LAMP primer design because it contained 51.7% GC content. Housekeeping genes evaluated (*rpoD* and *gyrB*) also contained high GC content (64-65%). GC content is one of the most important criteria in order to design an ideal LAMP primer set; it must be around 50-60%. Primers with a higher number of Gs and Cs are more stably paired with the template at a given temperature (e.g. 60°C-65°C) than primers of the same length that have higher number of As and Ts.

From *gyrB* and 16-23S rDNA Intergenic Spacer Region of *B. glumae*, only 294 bp and 306 bp were used respectively, due to the required distance between LAMP primers, GC content and alignment with closely related species.

The first set of LAMP primers for *B. glumae* were designed based on Takeuchi *et al.* (1997) sequence of the 16-23S rDNA Spacer Region for the detection of *B. glumae*, which amplifies an approximate 400 bp fragment (Figure 2). Based on these specifications, part of the amplicon region from 280-589 bp was used to generate oligonucleotide primers using PREMIER Biosoft LAMP designer software (Figure 3).

```

GCCGATCGGAAGGTGCGGCTGGATCACCTCCTTTCTCGAGCTTATTCC
GCATACATTGAGCGTTCACGCTTATCGGCTGTTGATCAAGACAGACTCA
GGGGTCTGTAGCTCAGTCGGTTAGAGCACCGTCTTGATAAGGCGGGGG
TCGTTGGTTCGAATCCAACCAGACCCACCAATTGTCTGACACGGAACAC
CTGGGTAAGTCTCTGTAGGGAAGGGGGGCATAGCTCAGCTGGGAGAGCAC
CTGCTTTGCAAGCAGGGGGTCGTCGGTTCGATCCCGTCTGCCTCCACCA
ATCTTCAATGACAAACGTTTCAGGGATGCTGAGCAGTTGTCATTGGCGAT
TGAGCCAGTCAGAGGATGAGCGAGAAGTAGCGCTTATCGGCTGTCGTT
CTTTAACAATCTGGAAGAAGTAGTAAAGTGGATAGCGGAAGCGCTTTT
GAGATGGGCGTGGAAGTTATCCGGGTTGTGATTGTATCGATGTATCTCA
AGATGATTTCGAACGCAAGTTCGGCTCAATTGGAATACGGCACAAATGC
GAGAACTCAACCTGTAGCGGCTGTCATCTCTTCGGGAGAGCGAGACAG
ACTCGTTATAGGGTCAAGCGAACAAGTGCATGT
  
```

Figure 2. Nucleotide sequence of 16-23S rDNA Intergenic Spacer Region (GenBank accession no. D87080.1) and location of forward and reverse LAMP primers (adapted from Takeuchi *et al.*, 1997). Forward and reverse primers adapted from Takeuchi *et al.* (1997) are indicated in yellow and LAMP primers (Forward and backward) designed in this study are indicated in green color.

```

                F3
280 TCT GCCTCCACCAATCTTCAAT GACAAACGTTTCAGGGATGCTGAGCAGTTGTCATT
                F2                               Loop F
GGCGATTGAGCCAGTCAGAGGATGAGCGAGAAGTAGCGCTTATCGGCTGTCGTTCTT
                F1
TAACAATCTGGAAGAAGTAGTAAGTGGATAGCGGAAGCCCTTATTTGAGATGGG
                B1
CGTGGAAGTTATCCGGGTTGTGATTGTATCGATGTATCTCAAGATGATTTCGAACGCA
                Loop B                               B2
AGTTCGGCTCAATTGGAATACGGCACAAATGCGAGAACTCAACCTGTAGCGGCTGT
                B3
CATCTCTTCGGAGAGCGGAGACAGACTC 589
  
```

Figure 3. Nucleotide sequence of 16-23S rDNA Intergenic Spacer Region (GenBank accession no. D87080.1) and location of the LAMP primers designed with PREMIER Biosoft LAMP designer.

A second set of LAMP primers for *B. glumae* were designed by aligning 16-23S rDNA regions of three closely related *Burkholderia* species including *B. glumae* (GenBank accession no. D87080), *B. gladioli* (GenBank accession no. D87081) and *B. plantarii* (GenBank accession no. D87079) using the NCBI software. A third set of LAMP primers for *B. glumae* were designed from the *gyrB* region of *B. glumae* sequence used by Maeda *et al.* (2006) to design primers for the phylogenetic study and multiplex PCR-based detection among *B. plantarii*, *B. glumae* and *B. gladioli* using *gyrB* sequence (Figure 4 and Figure 5). Different parameters used for designing LAMP primers are shown in Table 1 and PCR and LAMP primers used in this study are shown in Table 2.

The image shows a nucleotide sequence of the *gyrB* gene for DNA gyrase subunit B. The sequence is presented in a single line with line breaks. Several regions are highlighted with colored boxes to indicate primer locations:

- A yellow box highlights the sequence: GAAGTGTCGCCGATGGAG
- A green box highlights the sequence: CATCATCGGCGAGAA
- A green box highlights the sequence: GGAC
- A yellow box highlights the sequence: ATGCGTGCTGTCGGTGAAGG

The full sequence shown is: GAAGTGTCGCCGATGGAGGTCGTCGGCGAGACCGAGAACCGCGGCACC GAAGTGCATTTTCATGGCCGATCCGACCATCTTCGGCACCGTTCGAATACC ACTACGACATCCTCGCCAAGCGGATTCGCGAGCTGTCGTTCTCAACAA CGGCGTGCGCATCCGCCTGACCGACCAGCGCAGCGGCAAGGAAGACGA TTTCGCGTTTCGTGGGCGGCGTGAAGGGCTTCGTCGAGTACATCAACAA GACCAAGAGCGTGCTGCACCCGACCATCTTCCA CATCATCGGCGAGAA GGACGGCGTGGGGGTGGAAGTGGCGATGCAGTGGAACGACAGCTACA ACGAGAACGTGCTGTGTTTCACCAACAACATCCCGCAGCGCGACGGCG GCACCCACCTGACCGGCCTGCGCGCGGCGATGACGCGCGTGATCAACA AGTACATCGTCGACAACGAGATCGCCAAGAAGGCCAAGGTGGAGACGT CGGGCGACGACATGCGCGAAGGGCTCTCATGCGTGCTGTCGGTGAAGG

Figure 4. Nucleotide sequence of the *gyrB* gene for DNA gyrase subunit B and location of the forward and reverse LAMP primers and Maeda *et al.*, 2006 primers. Forward and reverse primers are indicated in yellow (Maeda *et al.*, 2006) and forward and reverse LAMP primers are indicated in green color.

```

GAAGTGTCGC CGATGGAGGT CGTCGGCGAG ACCGAGAACC GCGGCACCGA AGTGCATTTC ATGGCCGATC
CGACCATCTT CGGCACCGTC GAATACCACT ACGACATCCT CGCCAAGCGG ATTCCGCGAGC TGTCGATCCT
CAACAACGGC GTGCGCATCC GCCTGACCGA CCAGCGCAGC GGCAAGGAAG ACGATTTCGC GTTCGTGGGC
GGCGTGAAGG GCTTCGTCGA GTACATCAAC AAGACCAAGA GCGTGTGCA CCCGACCATC TTCCACATCA
TCGGCGAGAA GGACGGCGTG GGGGTGGAAG TGGCGATGCA GTGGAACGAC AGCTACAACG AGAACGTGCT

```

Figure 5. Nucleotide sequence of the *gyrB* gene for DNA gyrase subunit B and location of the LAMP primers along the sequence using PREMIER Biosoft software.

Table 1 - Different parameters used for LAMP *gyrB* primer design using PREMIER Biosoft LAMP designer.

Parameter	Value
Nucleic acid concentration (nM)	100
Monovalent ion concentration (mM)	30
Free Mg ²⁺ ion concentration (mM)	3
dNTPs concentration (mM)	0.4
Total Na ⁺ equivalent (mM)	223.76
Temperature for free energy calculation	60°C

Table 2 - PCR and LAMP primers used in this study.

Primer	Target Region	Sequence (5'-3')	Length (nt)	Reference
BGFW	16-23S rDNA	ACA CGG AAC ACC TGG GTA	18	Takeuchi <i>et al.</i> ,1997
BGRV	16-23S rDNA	TCG CTC TCC CGA AGA GAT	18	Takeuchi <i>et al.</i> ,1997
F3BGLU16	16-23S rDNA	GCC TCC ACC AAT CTT CAA T	18	This study
B3BGLU16	16-23S rDNA	GAG TCT GTC TCG CTC TCC	18	This study
FIPBGLU16	16-23S rDNA	CGC TTC CGC TAT CCA CTT AGC CAG TCA GAG GAT GAG	36	This study
BIPBGLU16	16-23S rDNA	TCA AGA TGA TTC GAA CGC AAG TCT ACA GGT TGA GTT CTC GC	41	This study
LOOPFBGLU16	16-23S rDNA	AGA ACG ACA GCC GAT AAG C	19	This study
LOOPBBGLU16	16-23S rDNA	GCT CAA TTG GAA TAC GGC AC	20	This study
F3	<i>gyrB</i> of <i>B. glumae</i>	GAA GTG TCG CCG ATG GAG	18	Maeda <i>et al.</i> , 2006
B3	<i>gyrB</i> of <i>B. glumae</i>	CTC GCC GAT GAT GTG GAA G	19	This study
FIP	<i>gyrB</i> of <i>B. glumae</i>	CAC GCC GTT GTT GAG GAA CTT CGG CAC CGT CGA ATA C	37	This study
BIP	<i>gyrB</i> of <i>B. glumae</i>	GCG GCA AGG AAG ACG ATT CAC GCT CTT GGT CTT GTT GA	38	This study
Loop-F	<i>gyrB</i> of <i>B. glumae</i>	TTG GC GAG GAT GTC GTA GT	19	This study
Loop-B	<i>gyrB</i> of <i>B. glumae</i>	AAG GGC TTC GTC GAG TAC A	19	This study
glu-F	<i>gyrB</i> of <i>B. glumae</i>	GAA GTG TCG CCG ATG GAG	18	Maeda <i>et al.</i> , 2006
glu-B	<i>gyrB</i> of <i>B. glumae</i>	CCT TCA CCG ACA GCA CGC AT	20	Maeda <i>et al.</i> , 2006

All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

2.2 DNA Extraction

Burkholderia glumae, *B. gladioli*, *B. plantarii*, *Pseudomonas syringae*, *Xanthomonas campestris* and *Xanthomonas campestris* pv. *vignicola* were provided by Jong Hyun Ham and Hari Karki (Phytobacteriology Laboratory, LSU AgCenter). Bacterial strains were maintained on LB agar at 30°C.

DNA from pure cultures of bacterial isolates and asymptomatic and symptomatic Trenasse and Lemont rice seeds (provided by Phytobacteriology Laboratory, LSU AgCenter) were extracted with DNeasy Plant Mini Kit according to manufacturer's guidelines (QIAGEN Inc, Valencia, CA) as well as symptomatic rice seeds provided by Donald Groth, LSU AgCenter. DNA was quantified with NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, NC) by absorbance at 260 nm and further diluted to 50 ng/μL for LAMP amplification assays. Bacterial strains used in this study are listed in Table 3.

2.3 Bacterial Strains Identity Confirmation

Burkholderia glumae and *B. gladioli* strains were confirmed with conventional PCR using species specific primers, GL-13f (5'-ACACGGAACACCTGGGTA-3') and GL-14r (5'-TCGCTCTCCCGAAGAGAT-3') for *B. glumae* and GLA-f (5'-CGAGCTAATACCGCGAAA-3') and GLA-r (5'-AGACTCGAGTCAACTGA-3') for *B. gladioli* (Takeuchi *et al.*, 1997 and Furuya *et al.*, 2002). Reaction mixture (25 μL) consisted of 12.5 μL GoTaq® Green Master Mix (Promega Co.), 0.4 μM of each forward and reverse primer, 1 μL of DNA template and 9.5 μL of nuclease free water. The PCR conditions consisted of initial denaturation at 95°C for 2 min and 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 40 s and a final elongation at 72°C for 7 min. The PCR products were visualized using 1% agarose gel.

Table 3 - Bacterial isolates used in this study.

Name	Strain	Description and Origin	Reference
<i>B. glumae</i>	106sh-5	Avirulent, Louisiana	Nandakumar <i>et al.</i> , 2009
	237gr-5	Avirulent, Louisiana	Nandakumar <i>et al.</i> , 2009
	366gr-2	Avirulent, Arkansas	Nandakumar <i>et al.</i> , 2009
	257sh-1	Avirulent, Louisiana	Nandakumar <i>et al.</i> , 2009
	396gr-2	Avirulent, Arkansas	Nandakumar <i>et al.</i> , 2009
	961149-4-4	Avirulent, Louisiana	Nandakumar <i>et al.</i> , 2009
	99sh-7	Avirulent, Louisiana	Nandakumar <i>et al.</i> , 2009
	201sh-1	Virulent, Louisiana	Nandakumar <i>et al.</i> , 2009
	336gr-1	Virulent, Louisiana	Nandakumar <i>et al.</i> , 2009
	117g1-7a	Virulent, Louisiana	Nandakumar <i>et al.</i> , 2009
	189 gr-8	Virulent, Texas	Nandakumar <i>et al.</i> , 2009
<i>B. gladioli</i>	372 gr-1	Arkansas	Yuan, 2004
	170sh-1	Louisiana	Yuan, 2004
	223gr-1	Louisiana	Yuan, 2004
<i>B. plantarii</i>	254-gr5	Louisiana	Yuan, 2004
<i>X. campestris</i>		Louisiana	Isolated by Clark, 1986
<i>X. campestris</i> pv. <i>vignicola</i>		Louisiana	Isolated by Clark, 1995
<i>P. syringae</i>		Louisiana	Isolated by Clark, 1986

2.4 LAMP Primer Specificity

The six primers were tested in sets of two (forward and reverse), using conventional PCR, and then the PCR product was separated by agarose (1%) gel electrophoresis.

Amplification for the Loop primers (LF and LB) was not expected. This experiment was performed using the *gyrB* and 16-23S rDNA LAMP primers.

2.5 LAMP Reaction

LAMP reactions (25 μ L) were performed in PCR tubes. Master mix containing Thermopol buffer, *Bst* DNA polymerase (M0275S, New England Biolabs), Betaine solution (B0300-5VL, Sigma Aldrich), MgSO₄, dNTPs (DNTP10-1KT, Sigma Aldrich) and six primers were prepared (Table 4). Two assays were performed according to Ravindran *et al.* (2012) using the 16-23S rDNA LAMP primers. The first mixture following the same concentration as Ravindran *et al.* (2012) (Table 4) and the second mixture using lower concentration of reagents (Table 5) were prepared. For the LAMP product visualization, Calcein and MnCl₂ were added to the master mix according to Tables 4 and 5.

Table 4 - Composition of LAMP reaction mixture adopted from Ravindran *et al.*, 2012.

Components	Working conc.	Required conc.	Required vol/reaction (μ L)
Nuclease free water			6.20
Thermopol buffer ^a	10X	2X	5.00
Betaine	5 M	1.6 M	4.00
MgSO ₄	100 mM	12 mM	1.50
dNTPs	25 mM	2.8 mM	1.40
F3 ^c	100 pM	10 pM	0.10
B3 ^c	100 pM	10 pM	0.10
FIP ^c	100 pM	40 pM	0.20
BIP ^c	100 pM	40 pM	0.20
LF ^c	100 pM	20 pM	0.40
LB ^c	100 pM	20 pM	0.40
MnCl ₂	20 mM	1 mM	1.25
Calcein	1 mM	50 μ M	1.25
8 units of <i>Bst</i> DNA polymerase ^{a,b}			1.00
Template DNA (~50 ng/ μ l)			2.00
Total reaction volume			25.00

^aPurchased from New England BioLabs

^b*Bacillus stearothermophilus* DNA polymerase

^cPrimers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA)

Table 5 - Modified composition of LAMP reaction mixture.

Components	Working conc.	Required conc.	Required vol/reaction(μ L)
Nuclease free water			6.2
Thermopol buffer ^a	2 X	0.4X	5
Betaine	1.6 M	0.25 M	4
MgSO ₄	12 mM	0.7 mM	1.5
dNTPs	10 mM	0.5 mM	1.4
F3 ^c	100 μ M	0.4 μ M	0.1
B3 ^c	100 μ M	0.4 μ M	0.1
FIP ^c	100 μ M	1.6 μ M	0.4
BIP ^c	100 μ M	1.6 μ M	0.4
LF ^c	100 μ M	0.8 μ M	0.2
LB ^c	100 μ M	0.8 μ M	0.2
MnCl ₂	20 mM	1 mM	1.25
Calcein	1 mM	0.05 mM	1.25
8 units of Bst DNA polymerase ^{a,b}			1
Template DNA (~50 ng/ μ l)			2
Total reaction volume			25

^aPurchased from New England BioLabs

^b*Bacillus stearothermophilus* DNA polymerase

^cPrimers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA)

Accordingly, the F3 and B3 primers, required only for the initial displacement reaction, were added at a lower (4-fold) concentration than the FIP and BIP primers. The LF and LB primers are not required for DNA synthesis and can be omitted from the reaction, but they help reduce the amplification time (Nagamine *et al.*, 2002).

Bst DNA polymerase was added to the master mix and then 23 μ L aliquot of the mix was dispensed to each sample tube, and 2 μ L of bacterial DNA (50 ng/ μ L) was added. Each reaction was carefully mixed after the addition of the DNA into the mix. A third mixture was performed using 8 mM of MgSO₄ and 1.4 mM dNTPs using the *gyrB* LAMP primers (Table 6). For LAMP product visualization, 2 μ L of Quant-iT™ PicoGreen® dsDNA was added 60 minutes post-reaction. Another assay was performed using the same components as before except that 3 mM of Hydroxynaphthol Blue was added pre-reaction to the master mix.

Optimal reaction conditions are listed in Table 6. The optimization of the LAMP protocol was conducted using DNA extracted from *B. glumae* culture grown on LB agar at 30°C and from *B. glumae* infected seeds and DNA extracted from other bacterial cultures (Table 3) as the negative control. LAMP *gyrB* primers were added in concentrations as recommended previously (Table 6).

Table 6 - Composition of LAMP reaction mixture used for *gyrB* LAMP Primers.

Components	Working Conc.	Required conc.	Required vol/react.(μ L)
Nuclease free water			7.9
Thermopol buffer ^a	10x	1x	2.5
Betaine	5 M	0.8 M	4
MgSO ₄	100 mM	6 mM	1.5
dNTPs	10 mM	1.4 mM	3.5
F3 ^c	50 μ M	10	0.1
B3 ^c	50 μ M	10	0.1
FIP ^c	50 μ M	40	0.8
BIP ^c	50 μ M	40	0.8
LF ^c	50 μ M	20	0.4
LB ^c	50 μ M	20	0.4
8 units of <i>Bst</i> DNA polymerase ^{a,b}			1
Template DNA (50 ng/ μ l)			2
Total reaction volume			25

^a Purchased from New England Bio Labs

^b *Bacillus stearothermophilus* DNA polymerase

^c Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

2.6 Direct Analysis of LAMP Products

LAMP amplicons were directly detected by checking the formation of magnesium pyrophosphate in the reaction tubes; turbidity indicated a positive reaction, whereas a negative result remained clear. To improve the visualization of magnesium pyrophosphate, samples were centrifuged for 15 seconds using an Eppendorf 5424 centrifuge to look for pellet formation.

Detection was also enhanced by the addition of 1 mM of MnCl₂ and 50 µM of Calcein to the master mix, which results in the formation of a fluorescent green metal indicator. A positive reaction produced green fluorescence and a negative reaction did not produce fluorescence. Amplified products were also detected using 2 µL PicoGreen post-reaction and 3 mM of HNB pre-reaction.

In daylight, positive results with PicoGreen were detected by an orange to green color change and fluorescence for positive results and no fluorescence for negative results under UV light, and by violet to blue color change using HNB. LAMP product was confirmed by 2% agarose gel electrophoresis at 85 V for 1 hour, revealing a characteristic ladder pattern for positive results.

2.7 Optimum Temperature and Time

LAMP products were detected using 2% agarose gel after 60 min amplification and 5 minutes of heat-inactivation at 80°C. The assay was performed at 50°C, 60°C and 70°C using 16-23S rDNA and at 60°C and 65°C using *gyrB* LAMP primers. *Burkholderia gladioli* and water were used as negative controls.

LAMP primers from both regions were tested at three different times (30, 45 and 60 min) at 60°C. After amplification at 60°C for 1 h, *Bst* DNA polymerase heat inactivation was tested at 80°C at 2, 5 and 10 min.

2.8 LAMP Specificity and Sensitivity

For the sensitivity assay using *gyrB* LAMP primer set, a LAMP reaction was performed using a serial dilution of DNA extracted from *B. glumae* (336 gr-1). The DNA sample was diluted 10-fold from 10² (100 ng) to 10⁻⁶ (0.000001 ng). The presence of LAMP product was

determined by naked eye using PicoGreen post-reaction, and by running the products on a 2% agarose gel to determine characteristic ladder patterns.

For the specificity assay, LAMP *gyrB* primers were tested using DNA samples from closely related bacterial species including: *B. gladioli* strains and *B. plantarii* (Table 3). Other bacteria species including *X. campestris*, *X. campestris* pv. *vignicola*, *P. syringae* and seven avirulent and four virulent strains of *B. glumae* were also included in the assay.

CHAPTER 3 RESULTS AND DISCUSSION

3.1 LAMP 16-23S rDNA Primer Design

Outer and loop primers from the 16-23S rDNA Intergenic Spacer Region produced promising results by amplifying DNA from *B. glumae* strains (Figures 6, 7 and 8). As expected, loop primers did not amplify any product (Data not shown).

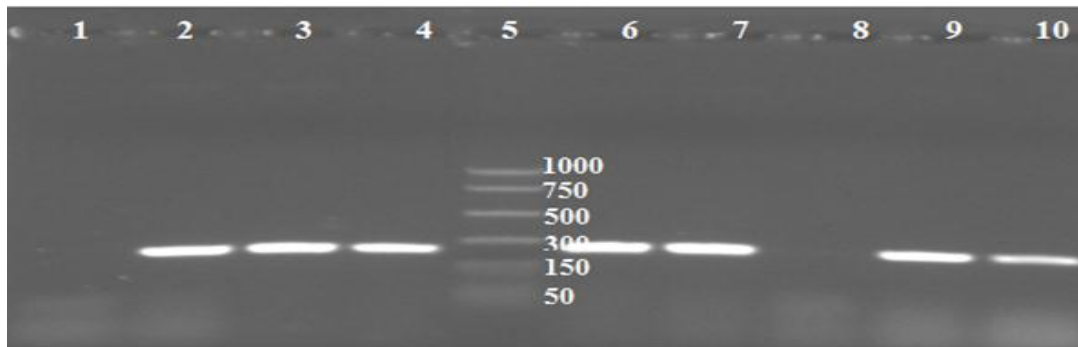


Figure 6. Agarose gel (1%) showing PCR products, using LAMP 16-23S rDNA outer primers, from *B. glumae* strains. Lane1, water; lanes 2-4, 336gr-2; 366gr-2; 106sh-5; lanes 6-7, 117g17-a; 189gr-8; lane 8, *B. gladioli* and lanes 9 and10, 201sh-1 and 396gr-2.

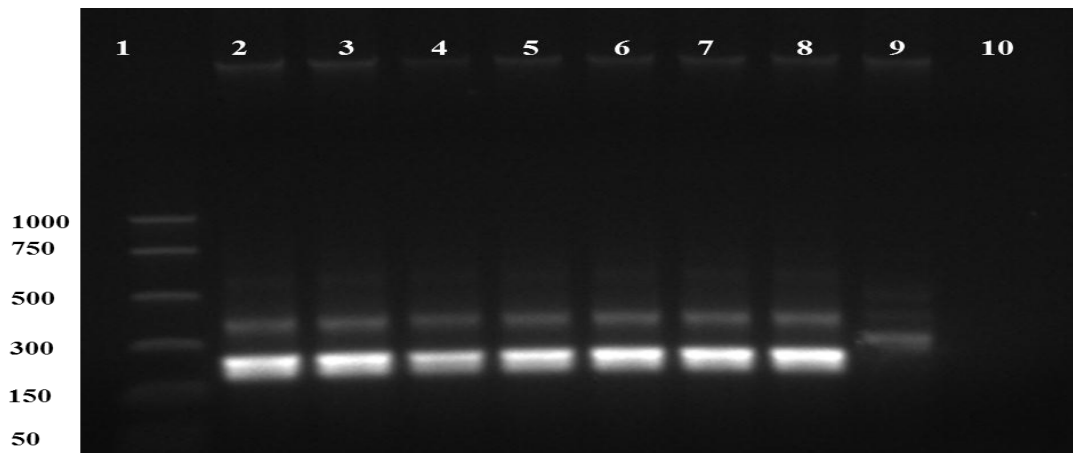


Figure 7. Agarose gel (1%) showing PCR products, using LAMP 16-23S rDNA inner primers , from *B. glumae* strains. Lane 1, Bench Top DNA marker; Lanes 2-8, *B. glumae* strains, 336gr-2; 366gr-2; 106sh-5; 117g17-a; 189gr-8; 201sh-1 and 396gr-2, respectively. Lanes 9 and 10, *B. gladioli* and water.

3.1.1 LAMP 16-23S rDNA Optimum Temperature

LAMP 16-23S rDNA amplified products were visualized using 2 % agarose gel and, amplification for *B. glumae* was observed at 60°C (Figure 8).

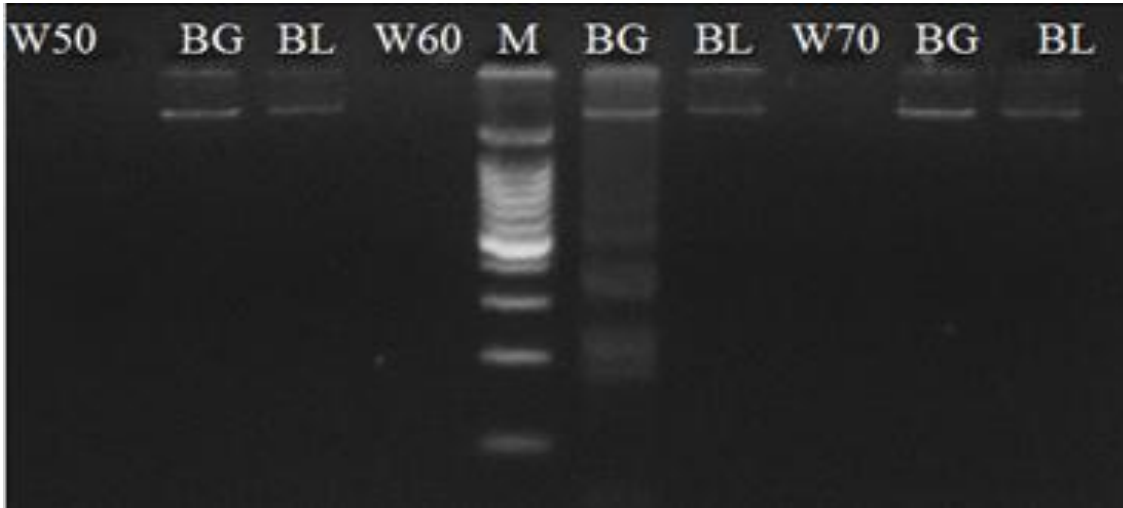


Figure 8. Agarose gel (2%) showing optimum reaction temperature for LAMP assays using primers from 16-23S rDNA from *B. glumae*. LAMP products were detected after 60 minutes amplification with 100 ng of DNA template at 50°C, 60°C, and 70°C. BG, *B. glumae*; BL, *B. gladioli*; W, water and M, 1.5 kb DNA marker.

LAMP 16-23S rDNA reaction with concentrations shown in Table 5 amplified the target DNA and the product was checked under UV light. After 2 h of amplification followed by heat-inactivation, bright fluorescence was emitted by the sample containing only *B. glumae* DNA and the characteristic multiple band pattern was visualized using 2% agarose gel (Data not shown). This result was not reproducible.

When the same reaction was performed for 1 h at 60°C and *Bst* DNA polymerase was heat inactivated at 80°C for 10 min, all the samples emitted fluorescence under UV light (Data not shown) but multiple bands pattern just appeared in *B. glumae* sample.

3.1.2 LAMP 16-23S rDNA Primers and HNB

Low concentrations of magnesium did not change color using HNB, because the master mix was already blue, pre-reaction. However, a weak difference was found between *B. glumae* samples and other bacterial strains (Figure 9). Reactions were confirmed using 2 % agarose gel revealing multiple bands for *B. glumae* DNA (Figure 10).



Figure 9. LAMP 16-23S rDNA products after 1 h amplification at 60°C using 120 μ M of HNB. Tube 1, Water; tubes 2 and 3, *B. glumae*; tube 4, *B. gladioli* and tube 5, *B. plantarii*.

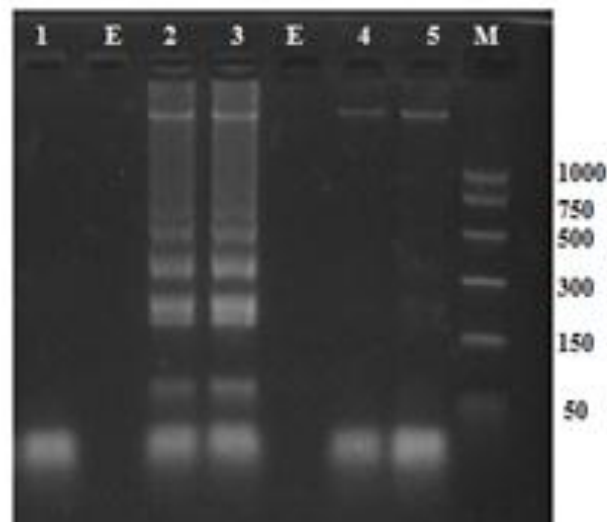


Figure 10. Agarose gel (2%) showing LAMP 16-23S rDNA products from HNB assay. Lane 1, water; lanes 2 and 3, *B. glumae*; lane 4, *B. gladioli* and lane 5, *B. plantarii*. E, empty and M, Bench Top DNA marker.

The same reaction using lower concentrations of magnesium was performed using avirulent and virulent strains of *B. glumae* and no difference was found (Figure 11).

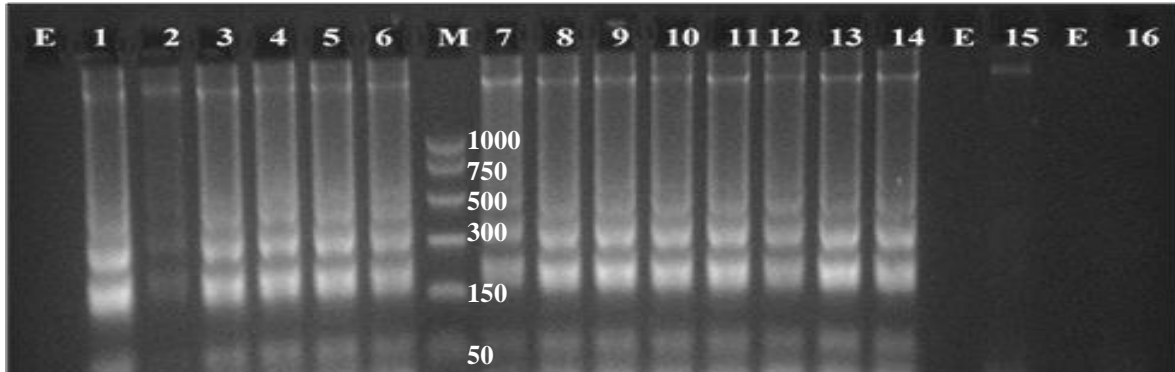


Figure 11. Agarose gel (2%) showing LAMP 16-23S rDNA products of avirulent and virulent *B. glumae* strains. Lanes 1-6, virulent strains of *B. glumae*, 336gr1; 201sh-1; 117g1-7-a; 191sh-6; 189gr-8, and 957856-41-c, respectively. Lanes 7-14, avirulent strains of *B. glumae*, 366gr-2; 106sh-5; 237gr-5; 396gr-2; 257sh-1; 99sh-7; 395gr-2 and 261-gr-9, respectively; lane 15, *B. gladioli* and lane 16, water. E, empty and M, Bench Top DNA marker.

3.2 LAMP *gyrB* Primer Design

LAMP *gyrB* preliminary primer testing was done using conventional PCR (Figure 12) and expected results were achieved, getting amplified products in outer and inner primers for *B. glumae* DNA and no amplification with loop primers.



Figure 12. Agarose gel (1%) showing PCR product using LAMP *gyrB* outer inner and loop primers). W, water; GLA, *B. gladioli*; GLU, *B. glumae* and M, 1.5 kb DNA marker.

3.2.1 LAMP *gyrB* Optimum Temperature

Optimum temperature was tested using two different temperatures (60 and 65°C), but no difference was observed between the two temperatures (Figure 13).

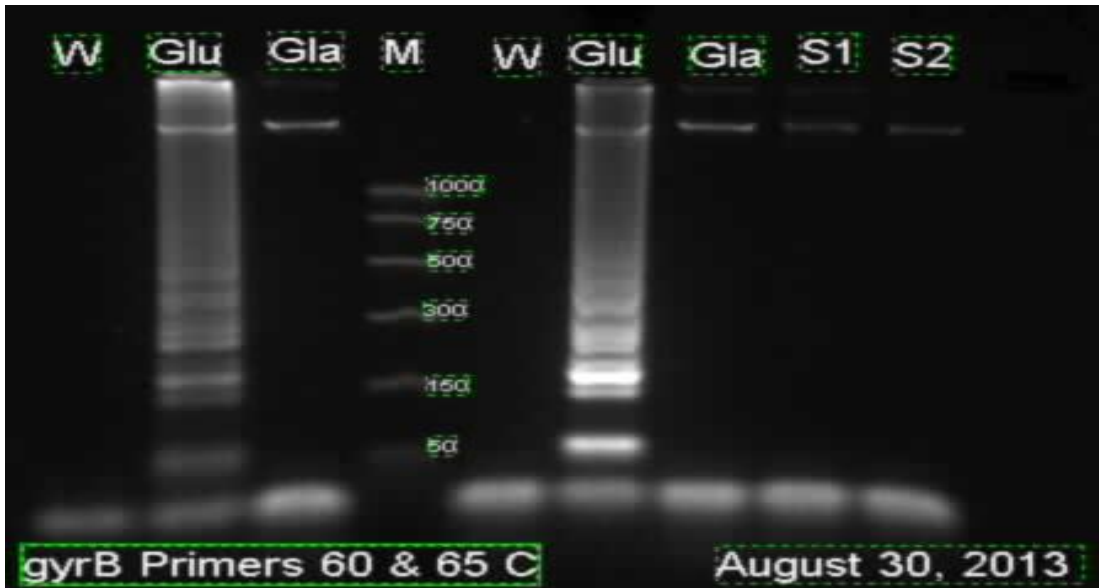


Figure 13. Agarose gel (2%) showing optimum reaction temperature for LAMP assays using *B. glumae gyrB* primers. W, water; GLU, *B. glumae*; GLA, *B. gladioli*; S1, *P. syringae* at 60°C and S2, *P. syringae* at 65°C. The first three lanes were amplified at 60°C and the following lanes at 65°C.

3.2.2 LAMP *gyrB* Primers and PicoGreen

The same reaction was performed using concentrations shown in Table 6 at 60°C for 1 h and inactivated for 5 min at 80°C. Two microliters of PicoGreen was added to each sample post-reaction. In daylight, positive results were detected by an orange to green color change (Figure 14A) and strong fluorescence under UV light for positive results (Figure 14B). Confirmation was done using 2% agarose gel, revealing multiple bands for *B. glumae* DNA (Figure 14C).

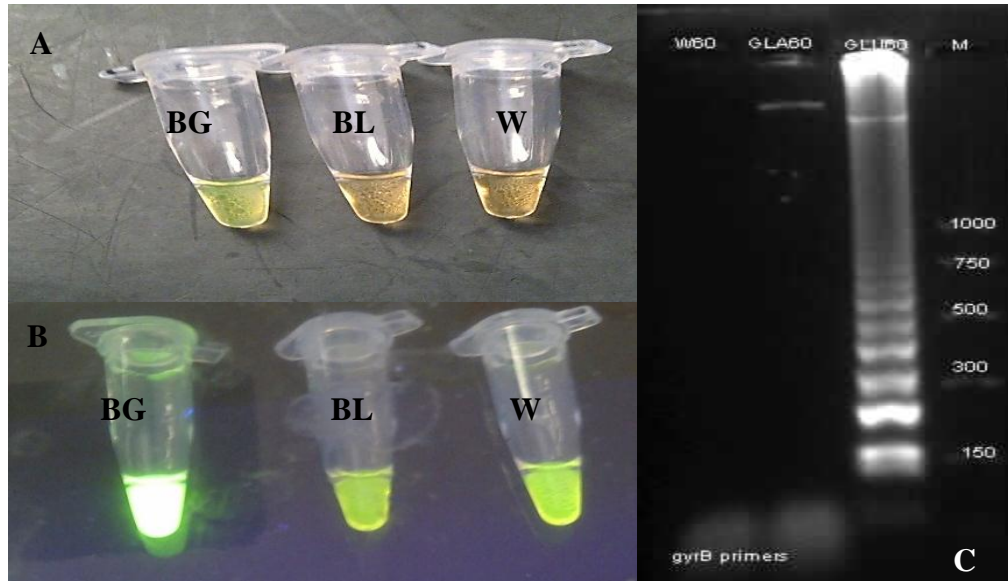


Figure 14. An example of positive and negative LAMP *gyrB* results using PicoGreen as an indicator in daylight (A), under UV light (B) and confirmation by 2 % agarose gel (C). BG, *B. glumae*; BL, *B. gladioli* and W, water.

Similar results were observed when PicoGreen was used with different strains of *B. glumae* and orange to green color changed in positively amplified tubes (Figure 15) and the multiple banding pattern was observed for strains of *B. glumae* (Figure 16).

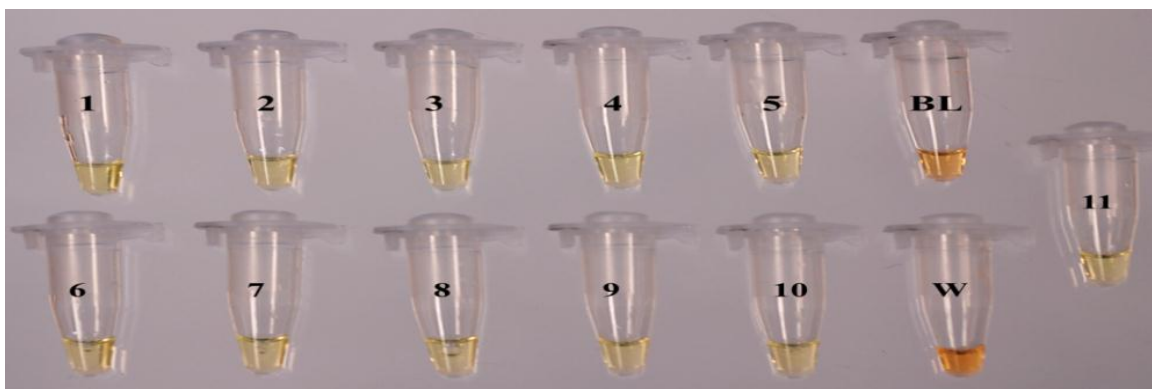


Figure 15. Avirulent and virulent strains of *B. glumae* showing positive results with *gyrB* LAMP primers using PicoGreen as an indicator in daylight. Lanes 1-7 avirulent strains, 106sh-5; 237gr - 5, 366gr-2; 396 gr-2; 961149-4 and 99sh-7. Lanes 8-11 virulent strains of *B. glumae*, 201sh-1; 336gr-1; 117g1-7a and 189gr-8. W, water and BL, *B. gladioli*.

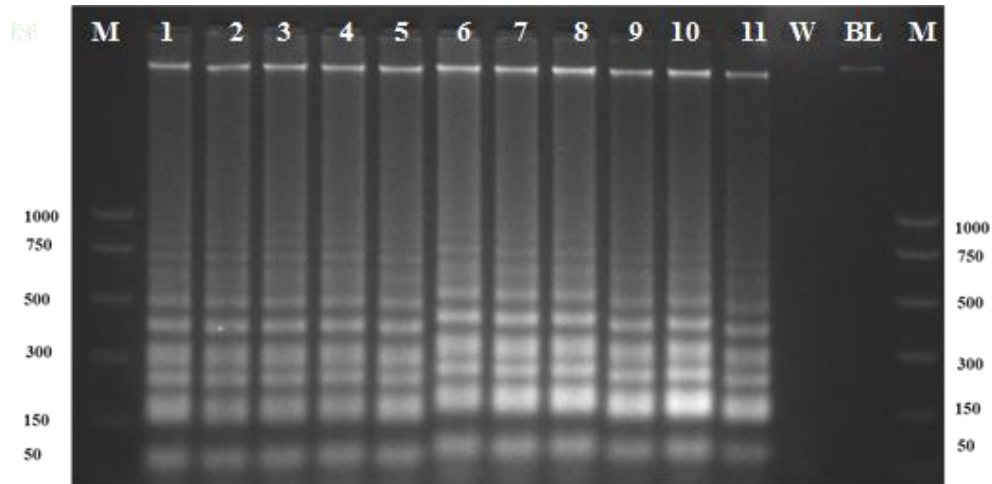


Figure 16. Confirmation of LAMP *gyrB* product using PicoGreen as an indicator. Lanes 1-7 *B. glumae* avirulent strains, 106sh-5; 237gr -5; 366gr-2; 396 gr-2; 961149-4 and 99sh-7. Lanes 8-11, virulent strains, 201sh-1; 336gr-1; 117g1-7a and 189gr-8. W, water; BL, *B. gladioli*; M, Bench Top DNA marker.

Amplified product was detected with 10^{-3} dilution (0.01 ng) using 2% agarose gel (Figure 17). The multiple banding pattern was not clear at 10^{-4} dilution (0.001 ng) of *B. glumae* DNA, however PicoGreen still detected the amplified product (Figure 18).

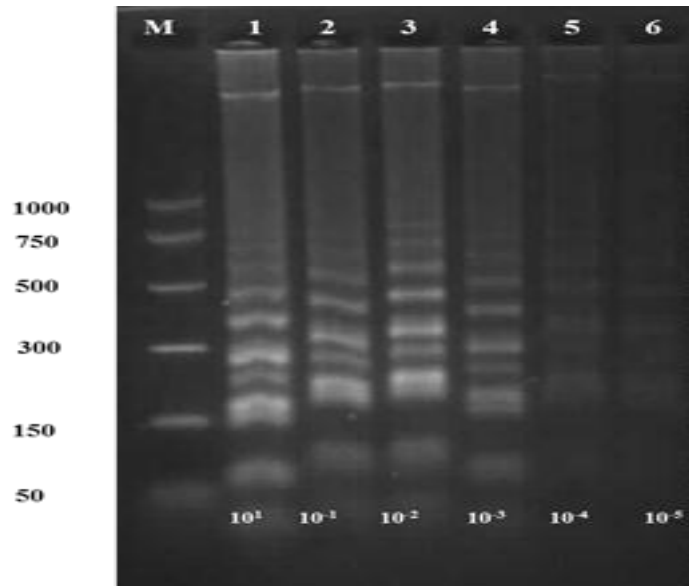


Figure 17. Agarose gel (2%) showing serial dilutions of *B. glumae* DNA amplified by LAMP *gyrB* primers. M, Bench Top DNA marker.

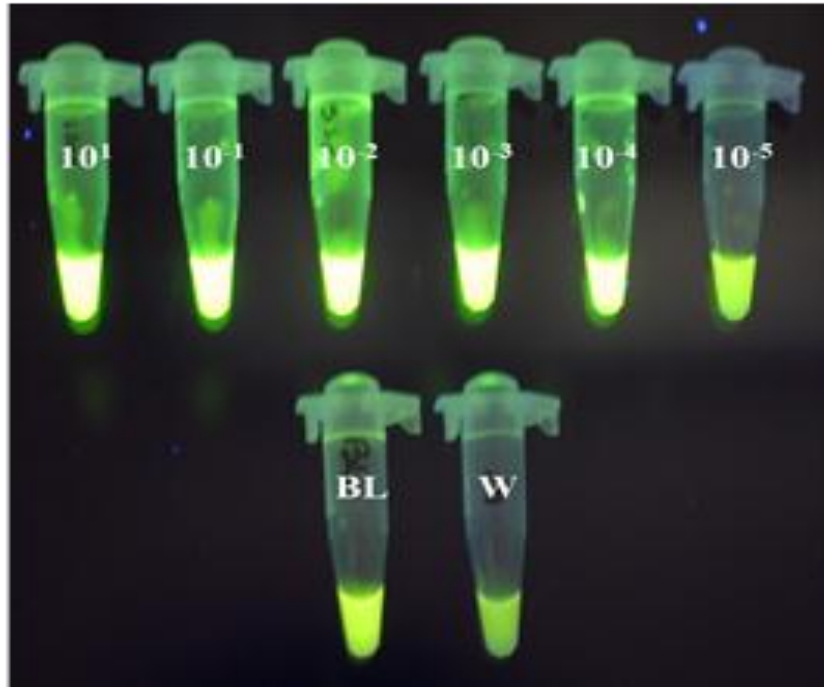


Figure 18. Serial dilutions of LAMP *gyrB* product using, PicoGreen post-reaction as an indicator, under UV light. BL, *B. gladioli* and W, water.

PicoGreen assay using DNA from rice seeds revealed that three samples were positive for *B. glumae* (Figures 19, 20 and 21).



Figure 19. LAMP *gyrB* product from asymptomatic and symptomatic rice seeds using PicoGreen as an indicator in daylight . Tubes 1-8 symptomatic and tubes 9-16 asymptomatic Trenasse rice seeds. Tube 17 symptomatic and Tube 18 asymptomatic Lemont rice seeds. W, water and BL, *B. gladioli*.

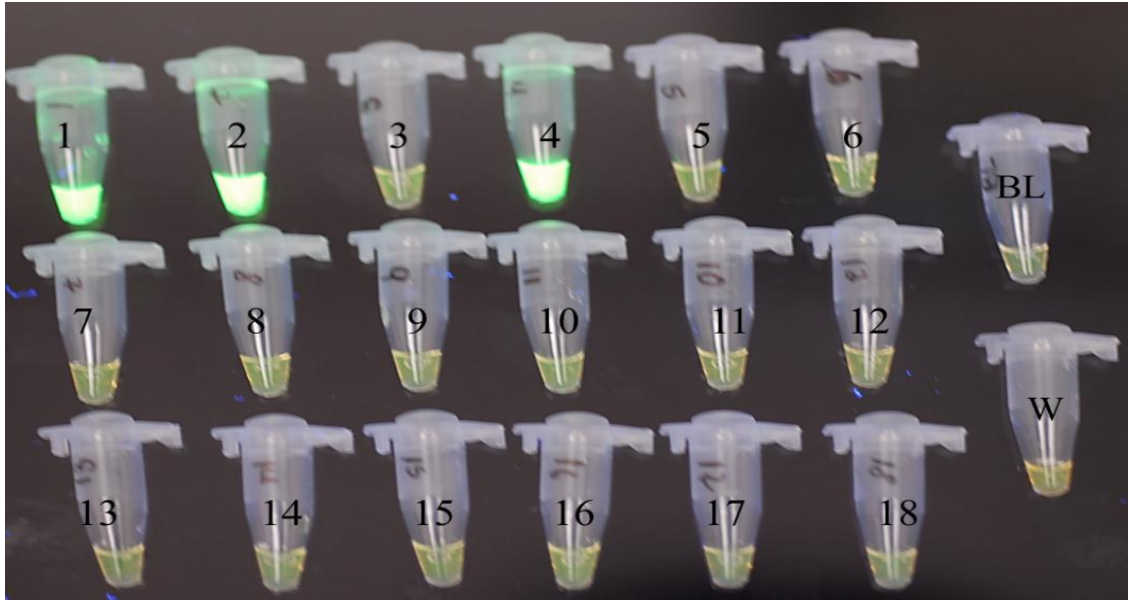


Figure 20. LAMP *gyrB* product from asymptomatic and symptomatic rice seeds using PicoGreen as an indicator under UV light. Tubes 1-8 symptomatic and tubes 9-16 asymptomatic Trenasse rice seeds. Tube 17 symptomatic and Tube 18 asymptomatic Lemont rice seeds. W, water and BL, *B. gladioli*.

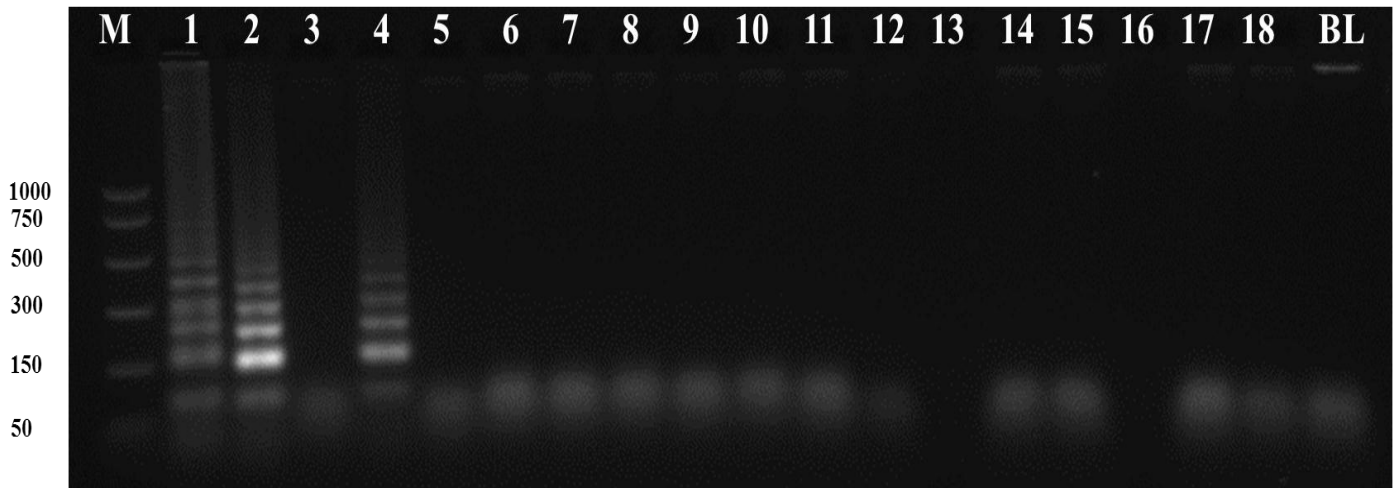


Figure 21. Confirmation of LAMP *gyrB* product from asymptomatic and symptomatic rice seeds. Tubes 1-8 symptomatic and tubes 9-16 asymptomatic Trenasse rice seeds. Tube 17 symptomatic and Tube 18 asymptomatic Lemont rice seeds. BL, *B. gladioli* and M, Bench Top DNA marker.

Post-reaction assays sometimes lead to contamination; However, PicoGreen was found to be reproducible and false positives were not found (Figures 22 and 23).



Figure 22. LAMP *gyrB* product using PicoGreen post-reaction in daylight. BG, *B. glumae*; BL, *B. gladioli*; BP, *B. plantarii*; PSE: *P. syringae*; XC, *X. campestris*; W, water.

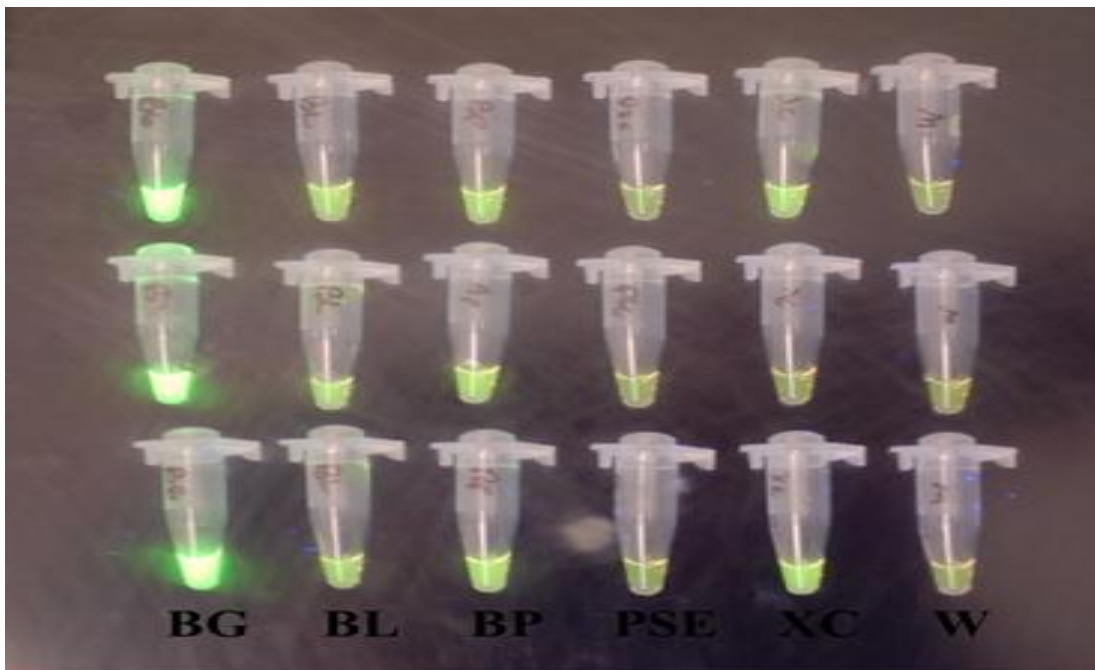


Figure 23. LAMP *gyrB* product using PicoGreen post-reaction under UV light. BG, *B. glumae*; BL, *B. gladioli*; BP, *B. plantarii*; PSE, *P. syringae*; XC, *X. campestris*; W, water.

3.2.3 LAMP *gyrB* Primers and HNB

The same assay was performed using HNB pre-reaction and positive amplification was observed by change in color from violet to sky blue. Tubes containing water and *B. gladioli* remained violet (Figure 24).

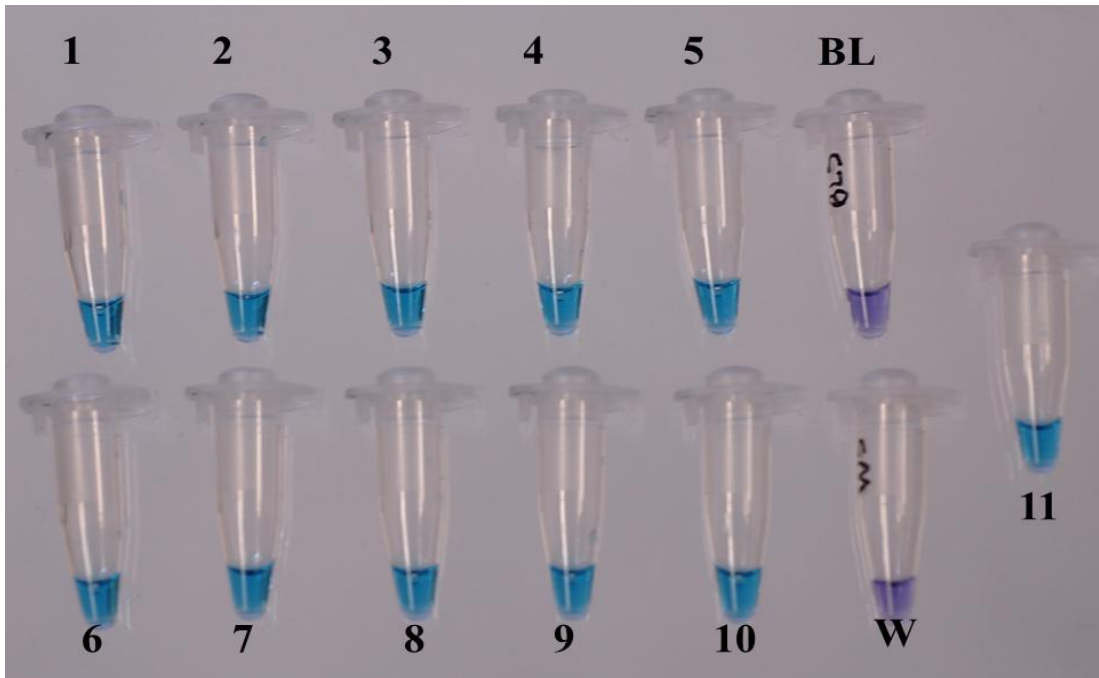


Figure 24. Avirulent and virulent strains of *B. glumae* showing positive results with *gyrB* LAMP primers using HNB as an indicator. Tubes 1-7 *B. glumae* avirulent strains, 106sh-5; 237gr -5; 366gr-2; 396 gr-2; 961149-4 and 99sh-7. Tubes 8-11, virulent strains, 201sh-1; 336gr-1; 117g1-7a and 189gr-8. W, water and BL, *B. gladioli*

LAMP *gyrB* amplification using HNB was confirmed using 2% agarose gel and multiple banding pattern was observed in all the positive samples (Figure 25). HNB assay was replicated several times and was found to be reproducible (Figures 26 and 27).

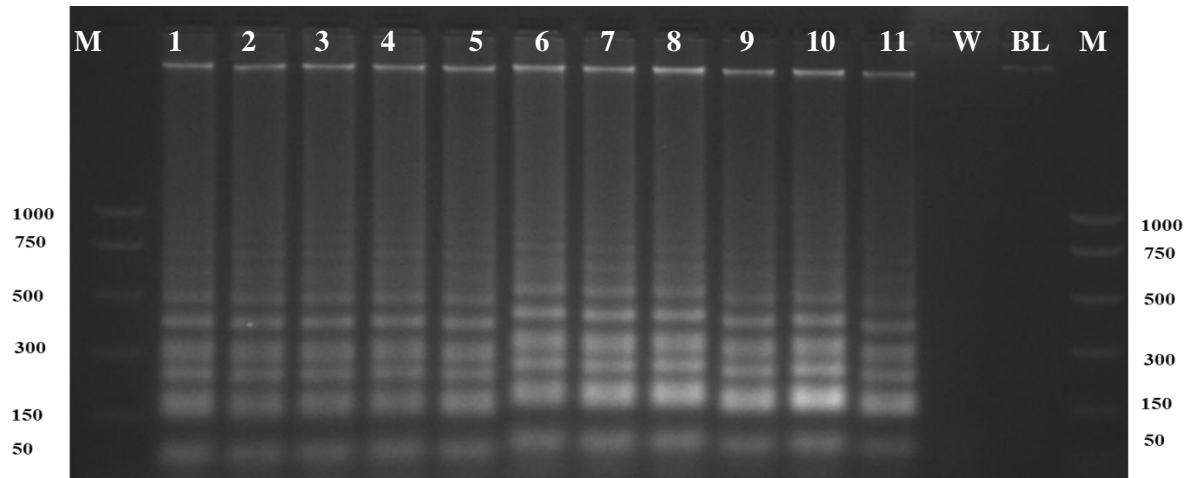


Figure 25. Confirmation of LAMP *gyrB* product using HNB as an indicator. Lanes 1-7 *B. glumae* avirulent strains, 106sh-5; 237gr-5; 366gr-2; 396 gr-2; 961149-4 and 99sh-7. lanes 8-11, virulent strains, 201sh-1; 336gr-1; 117g1-7a and 189gr-8. W, water; BL, *B. gladioli*; M, Bench Top DNA marker.

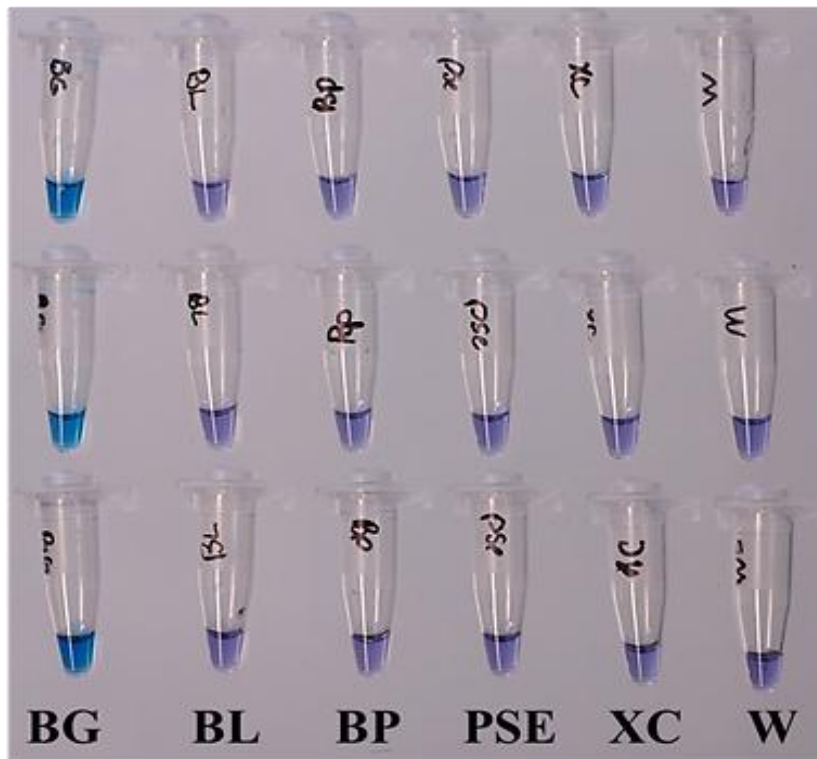


Figure 26. LAMP *gyrB* product using HNB. BG, *B. glumae*; BL, *B. gladioli*; BP, *B. plantarii*; PSE, *P. syringae*; XC, *X. campestris*; W, water.

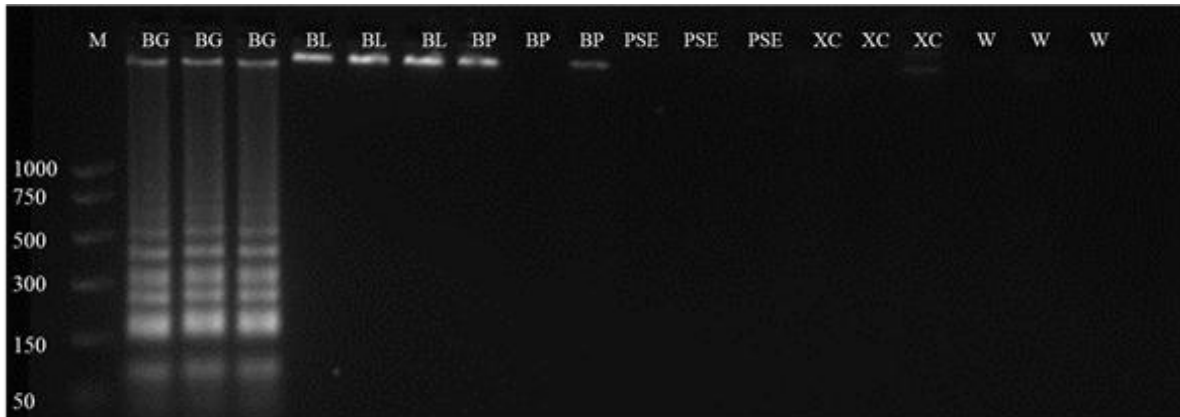


Figure 27. Confirmation of LAMP *gyrB* product by agarose (2%) gel. BG, *B. glumae*; BL, *B. gladioli*; BP, *B. plantarii*; PSE, *P. syringae*; XC, *X. campestris*; W, water and M, Bench Top DNA marker.

3.2.4 LAMP *gyrB* Primers Optimum Time

LAMP *gyrB* primers were tested at 30, 45 and 60 min, amplifying *B. glumae* DNA at 45 and 60 min. (Figure 28).

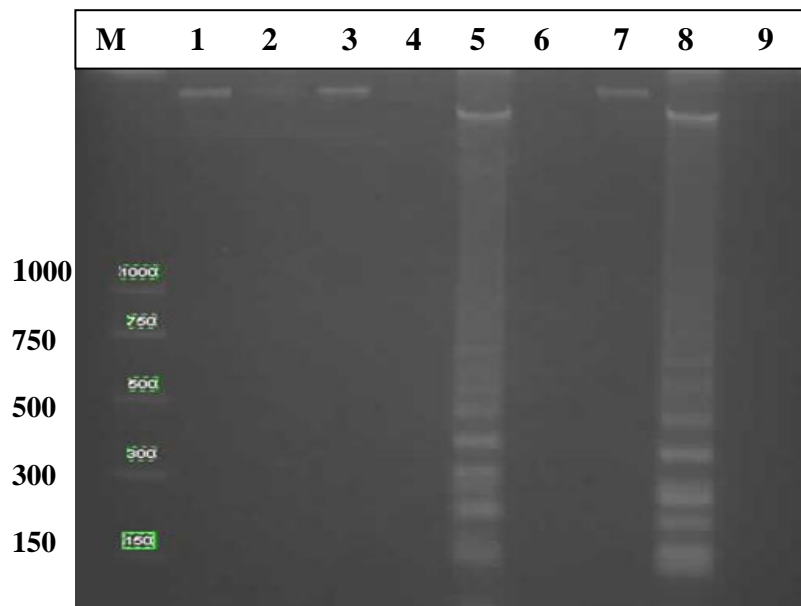


Figure 28. Agarose gel (2%) showing LAMP *gyrB* amplified product at 30, 45 and 60 minutes. Lanes 1-3, *B. gladioli*; water, and *B. glumae* incubated at 60°C for 30 min, lanes 4-6, *B. gladioli*; *B. glumae* and water incubated for 45 min and lanes 7-9, *B. gladioli*; *B. glumae* and water incubated for 60 min. M, Bench Top DNA marker.

3.3 Optimal Conditions for Detection of *B. glumae* using LAMP

Optimum concentration of *Bst* DNA polymerase, MgSO₄, and Betaine were found to be 8 Units per reaction, 8 mM, and 0.8 M, respectively. LAMP amplification at 50 and 70°C was not observed (Figure 8). Positive amplification was observed at both 60 and 65°C using LAMP *gyrB* primers (Figure 13).

LAMP reactions with *gyrB* primers required a minimum of 45 min incubation to achieve positive amplification. No amplification was observed when reactions were incubated for 30 min (Figure 28).

Bst DNA polymerase inactivation at 80°C was evaluated at 10 and 5 min. No difference was observed between the two inactivation times, and five min inactivation time was chosen to decrease the reaction time.

Primers from the 16-23S rDNA Intergenic Spacer Region of *B. glumae* produced successful amplification and were specific for *B. glumae* using the concentrations shown in Table 5, but positive reactions could not be detected using HNB (Figure 9) and Calcein (Data not shown).

In addition, the master mix pre-reaction was dark blue due to the low levels of magnesium sulfate and dNTPs in the reaction. Therefore, the 16-23S rDNA primers were not used for further studies. Concentrations from Table 4 were evaluated as well, but it amplified DNA from *B. gladioli* as well, due the high concentration of magnesium and the lack of specificity of the 16-23S rDNA LAMP primers for *B. glumae*.

3.4 Visual Detection of LAMP Products

Different methods for the amplified product were evaluated. Turbidity was not observed with any set of primers.

Detection of LAMP products with Calcein produced variable results. Fluorescence was observed in negative controls as well.

Successful amplification using HNB was detected by a color change from purple to blue in tubes containing *B. glumae* DNA (Figures 9, 24 and 26). No color change was observed in tubes containing water or *B. gladioli* DNA. HNB and PicoGreen assays were reproducible (Figures 22, 23, 26 and 27) and were able to detect the amplified product from symptomatic rice seeds (Figures 22 and 23).

3.5 Specificity of LAMP

The specificity of LAMP using the *gyrB* LAMP primer set was confirmed using DNA samples from closely related bacterial species such as *B. gladioli* and *B. plantarii*. Multiple alignments of the amplicon and primer sequences corresponding to the region used for selection of the *gyrB* LAMP primers indicated that there was ~93 % similar to *B. gladioli*. The primer set did not amplify products from other *Burkholderia* species (e.g. *B. gladioli* and *B. plantarii*) as well as other bacterial isolates shown in Table 3.

Using lower concentrations of MgSO₄, LAMP 16-23SrDNA primers could amplify just *B. glumae* making the assay specific for amplification of this bacterium. When the magnesium concentration was raised these primers amplified DNA from *B. gladioli* as well, but no amplification was observed using other bacterial isolates shown in Table 3.

Conventional PCR using 16-23S rDNA primers (Takeuchi *et al.*, 1997) amplified DNA from eleven *B. glumae* strains.

As well conventional PCR primers from *B. gladioli* 16-23S rDNA region (Furuya *et al.*, 2002) were used to confirm *B. gladioli* strains (170sh-1, 223gr-1 and 372gr-1). These experiments confirmed that the *gyrB* LAMP primers are specific for *B. glumae* strains.

3.6 Sensitivity of LAMP

The sensitivity of LAMP to detect *B. glumae* DNA was compared using a 10-fold dilution series from 10^2 (100 ng) to 10^{-6} DNA (0.000001 ng). Amplified product was detected with 10^{-3} dilution (0.01 ng) using 2% agarose gel and 2 μ L of PicoGreen post-reaction. The multiple banding pattern was not clear at 10^{-4} dilution (0.001 ng) of *B. glumae* DNA, however PicoGreen still detected the amplified product (Figure 18). DNA product less than 100 ng was not detected using HNB.

3.7 Discussion

This study provides the nucleotide sequences for the LAMP primer set, *gyrB* LAMP, and describes a complete protocol for LAMP detection of *B. glumae*. Furthermore, this study discusses primer design, reaction optimization, as well as reduction in time required for successful *B. glumae* DNA amplification and the visualization of the amplified LAMP product.

In spite of the high degree of similarity among *Burkholderia* species (60-90%) and *B. gladioli*, *B. glumae* and *B. plantarii* content of 81-90% degree similarity, specific primers were designed from the *gyrB* region of *B. glumae*. This gene encodes the β -subunit polypeptide of DNA gyrase and it is estimated to evolve much faster than the 16S rDNA gene (Yamamoto and Harayama, 1998). It was reported from previous studies that this housekeeping gene showed no diversity among 41 strains of *B. glumae* (Maeda *et al.*, 2006). This study confirmed previous results using eleven different strains of *B. glumae* and showed no difference among strains in DNA synthesis and visualization of LAMP products.

The results using the *gyrB* LAMP primer set and the optimized LAMP protocol validated that the method effectively detected *B. glumae*. Our LAMP protocol can be accomplished in a water bath at 60°C within 45-60 min and post-reaction processes are not required. LAMP amplified product can be visualized by the naked eye or under UV light using HNB or PicoGreen, respectively. These results emphasize the significant advantages of the LAMP technique i.e. accuracy and speed.

LAMP *gyrB* primers amplified *B. glumae* DNA at 60 and 65°C and amplification was not observed at 50 and 70°C. Optimal annealing temperature is determined by the melting temperature of the primer. If the temperature is too high the primers will melt, and if the annealing temperature is too low the primers may anneal nonspecifically. Also *Bst* DNA polymerase efficiency is around 20% at 70°C and 30-45% at 50°C and 100% at 60-65°C.

Several studies have been reported using expensive real-time turbidimeter for the detection of the LAMP product (Parida *et al.*, 2004). In fact the use of expensive equipment takes away one of the main focus of LAMP technique and decreases the limit of use in developing countries, where this technique can be of high importance. Turbidity in LAMP reaction mixtures is caused by production of insoluble magnesium pyrophosphate, a by-product of DNA synthesis (Notomi *et al.*, 2002 and 2008). Since the production of magnesium pyrophosphate is directly associated with the amount of DNA synthesized (Mori *et al.*, 2001), the absence of turbidity in the *B. glumae* LAMP reaction mixture could be related to insufficient amplification of *B. glumae* DNA.

Due the absence of turbidity in preliminary assays, it was intended to develop an easy and fast protocol for the detection of *B. glumae*, using metal indicator and intercalator dyes such as HNB, Calcein and PicoGreen, respectively.

The performances of LAMP assays using HNB, PicoGreen, and Calcein were compared by reliability and sensitive. Several previous reports have proposed Calcein as a good indicator dye (Ravindran *et al.*, 2012), but a lot of false positives and variable results were obtained in this study.

It was believed that color change in positive amplification occurred due to the detection of magnesium pyrophosphate by removing a manganese quencher from the Calcein-manganese complex (Tomita *et al.*, 2008). However, the interaction between DNA and Calcein had not been reported until Zhang *et al.* (2012) reported that there exists an obvious interaction between Calcein and dsDNA. LAMP inner primers are susceptible to form primer dimers due to their structures and lengths, suggesting that Calcein emitted fluorescence because it was able to detect primer dimers produced by the inner primers during the LAMP reaction.

LAMP is a method that permits a high risk of cross contamination of samples by aerosolized product (Zhang *et al.*, 2012). In order to avoid cross contamination, methods that allow the incorporation of the dye pre-reaction were developed such as HNB. This study showed that LAMP protocol using HNB was accurate and reliable as compared to other methods in which tubes are opened post reaction to add PicoGreen. HNB produced consistent and visual color change in positively amplified reactions and did not require UV to analyze the results. In addition, HNB is more cost effective than PicoGreen. The only disadvantage using HNB as an indicator is that it was unable to detect amplified product less than 100 ng.

PicoGreen showed accurate results with high sensitivity (until 0.001 ng) and results can be seen in daylight and under UV light. Both techniques were able to detect *B. glumae* in symptomatic rice seeds and HNB was able to detect the amplified product by adding more DNA to the reaction.

Our LAMP *gyrB* protocol offers two reliable and simple methods for the detection of *B. glumae*. PicoGreen is accurate and highly sensitive, but poses a high risk of post reaction contamination, whereas HNB is accurate, cheaper and omits the risk of post reaction cross contamination, but has low sensitivity.

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