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Validation and optimization of the loop-mediated isothermal amplification (LAMP) technique for rapid detection of wheat stripe mosaic virus, a wheat-infecting pathogen

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ABSTRACT

Background: Wheat stripe mosaic virus (WhSMV) is a significant wheat pathogen that causes substantial yield losses in Brazil and other countries. Although several detection methods are available, reliable and efficient tools for on-site WhSMV detection are currently lacking. In this study, a Loop-Mediated Isothermal Amplification (LAMP) method was developed for rapid and reliable field detection of WhSMV. We designed WhSMV-specific primers for the LAMP assay and optimized reaction conditions for increased sensitivity and specificity using infected plant samples.

Results: We have developed a diagnostic method utilizing the Loop-Mediated Isothermal Amplification (LAMP) technique capable of rapidly and reliably detecting WhSMV. The LAMP assay has been optimized to enhance sensitivity, specificity, and cost-effectiveness.

Conclusion: The LAMP assay described here represents a valuable tool for early WhSMV detection, serving to mitigate the adverse economic and social impacts of this viral pathogen. By enabling swift and accurate identification, this assay can significantly improve the sustainability of cereal production systems, safeguarding crop yields against the detrimental effects of WhSMV.

1. Introduction

Wheat (*Triticum aestivum L.*) is one of the most cultivated cereals globally. In Brazil, wheat is grown during the winter and spring, primarily in the three Southern states: Paraná (PR), Santa Catarina (SC) and Rio Grande do Sul (RS). Viral diseases are important factors affecting wheat in Brazil.

Wheat stripe mosaic virus (WhSMV) is a plant-pathogenic virus consisting of two segments of polyadenylated single-strand ssRNA (RNA1 and RNA2), found in Southern Brazil,[1] Paraguay,[2] and South Africa,[3] and causes considerable yield loss in infected wheat and other cereal crops. The primary mode of transmission is through the plasmodiophorid *Polymyxa graminis*, an obligate plant parasitic soil inhabitant that infects the roots of wheat and other cereals.[1] The development of a point-of-care diagnostic tool for WhSMV is crucial to enable early detection and the implementation of effective control measures. Such a tool would help mitigate the virus's economic and social impacts and enhance the sustainability of cereal production systems.[4] In addition, this diagnostic tool will be fundamental for characterizing wheat genotypes with respect to WhSMV resistance, as current characterization relies solely on plant symptoms.[5]

Other methodologies are available for WhSMV detection, include serological, molecular, and imaging techniques.[1,6] Serological approaches involve the utilization of virus-specific antibodies to detect WhSMV in plant tissues or extracts, with the enzyme-linked immunosorbent assay (ELISA) often being employed. Nevertheless, the efficacy of ELISA in terms of sensitivity and specificity might be influenced by potential cross-reactivity with other viruses and variations in viral concentration.[6] ELISA is not considered a point-of-care assay and relies on using laboratory infrastructure for the assay.[7]

Abbreviations: BYDV, barley yellow dwarf virus; ELISA, Enzyme-Linked Immunosorbent Assay; LAMP, Loop-Mediated Isothermal Amplification; M – MLV RT, moloney murine leukemia virus reverse transcriptase; OYDV, onion yellow dwarf virus; PCR, Polymerase Chain Reaction; RSNV, rice stripe necrosis virus; RT-PCR, Reverse Transcription-PCR; VR, Viral replication; WhSMV, wheat stripe mosaic virus.

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Molecular methods, such as polymerase chain reaction (PCR) or reverse transcription PCR (RT-PCR), enable the detection of viral nucleic acids (RNA or DNA), with high sensitivity and specificity.[8] However, applying such molecular methods can be time-consuming, necessitate specialized equipment and expertise, and are not practical for on-site field detection of WhSMV in wheat crops.[9]

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification technique that has emerged as a promising alternative to PCR-based methods for the detection of viral nucleic acids, including RNA or DNA.[10] LAMP is a single-tube reaction that amplifies DNA sequences under isothermal conditions. This technology offers several advantages over PCR-based methods, including high sensitivity and specificity and simple instrumentation requirements.[11] These features make LAMP a useful tool for rapid and reliable field detection of viral pathogens, with potential applications in crop protection, disease diagnosis, and public health surveillance.[12–14]

This study focused on developing a LAMP protocol for the point-of-care detection of WhSMV. By designing WhSMV-specific primers and fine-tuning the reaction conditions, we successfully optimized the LAMP assay for enhanced sensitivity and specificity using infected wheat plant samples. The significance of this research lies in utilizing LAMP as an alternative to traditional PCR-based methods, offering numerous advantages such as heightened sensitivity, specificity, rapid detection, and simplified instrumentation requirements.

The application of LAMP for in-field detection of viral pathogens holds immense potential not only in crop protection but also in disease diagnosis and public health surveillance. This study paves the way for practical and efficient WhSMV detection strategies, ultimately contributing to the mitigation of economic losses, the preservation of cereal production systems, and the overall improvement of agricultural sustainability.

2. Materials and methods

2.1. Sample collection

Wheat samples, provided by the Plant Virology Laboratory of Centro de Ciências Agroveterinárias - Universidade do Estado de Santa Catarina (CAV-UDESC) were collected from the symptomatic areas of Embrapa Trigo in Passo Fundo, RS, located at coordinates 28° 13′S; 52°24′W and an altitude of 648 m. The selection of this location was based on a documented history of the disease and viral vector, with the cultivars exhibiting characteristic mosaic symptoms (Fig. S1). Wheat leaves were tested using RT-PCR to confirm their respective virus infection status. Leaf and stem parts of the plant were collected and stored at −80 °C for subsequent RNA extraction.

The control wheat samples used in this study comprised the same cultivar, age, and growth conditions, except that they remained non-infected.

For specificity studies, rice stripe necrosis virus (RSNV), isolated from rice (Oryza sativa), served as the control in LAMP specificity assays. In RT-LAMP assays, wheat samples infected with barley yellow dwarf virus (BYDV) were employed as field controls for experimental validation.

2.2. Primer design

In this study, conserved regions of the RNA sequence coding the viral replication (VR) polyprotein and coat protein (CP) genes (Figs. 1 and 2) were utilized as the foundation for designing RT-LAMP primers for WhSMV detection (Table 1). The designed primers hybridized to VR or CP target genes, (GenBank -accession numbers MH151795 and MH151801).[1] The primer design was executed using the Primer Explorer V5 program (Fujitsu Ltd. Tokyo, Japan, https://primerexplorer.jp/lampv5e/inde.html) as recommended by the Eiken GENOME SITE. Modifications were made, specifically adjusting the amount of G and C (40–60 %) to ensure a primer Tm between 60 and 65 °C and a ΔG value greater than − 4 to improve stability at the 5′ end.

The primer set “A” included primers F3, B3, FIP (F1c + F2), BIP (B1c and B2), LF, and LB, recognizing a total of eight sequences of the VR gene (Fig. 1) (Table S1). Set B detected the CP gene (ORF 2 of RNA 2) (Table S2), resulting in the use of seven primers (F3, B3, FIP (F1c + F2), BIP (B1c and B2) and LF) (Fig. 2). Due to restrictions imposed by the software, it was not possible to design the Loop Backward (LB) primer for ORF 2.

2.3. Total nucleic acid extraction

Total nucleic acid extraction was performed from wheat leaves and stems (100 mg total weight) using the TRIZOL reagent, following the protocol suggested by the manufacturer (Thermo Fisher). Subsequently, the pellet was resuspended in 30 μL of RNase-free H_2O. The sample concentration was determined using the NanoDrop equipment (NanoDrop 2000, Thermo Scientific), and the integrity of the molecules was evaluated by electrophoresis in a 1 % agarose gel. Finally, the samples were stored at −80 °C.

2.4. Reverse transcription reaction

The synthesis of complementary deoxyribonucleic acid (cDNA) was performed using M−MLV Reverse Transcriptase, 2 μg of RNA, and 0.5 μg of oligo dT primer, following the manufacturer's instructions (M−MLV Reverse Transcriptase -Promega Corporation, USA). The cDNA was treated with the ribonuclease inhibitor enzyme RNaseOUT™ (Invitrogen, Life Technologies, Carlsbad, USA) and stored at −80 °C.

2.5. Cloning, sequencing, and sequence analysis

For the synthesis of single-stranded viral cDNA, M−MLV Reverse Transcriptase, RNase H Minus (Promega Biotecnologia do Brasil, Ltd., São Paulo, Brazil) was employed, using random primers (Promega). The external primers (F3 and B3) of the LAMP assay, flanking the target gene, were utilized to verify the presence of the viral gene.[15] Following reverse transcription, 2 μL of cDNA was used for PCR amplification, in a total volume of 25 μL. The reaction mixture consisted of 1 μL of each external primer (10 μM), 10 μL of 5X PCR buffer, 4 μL of $MgCl_2$ (25 mM), 1 μL of dNTP (10 mM), 0.25 μL of GoTaq DNA Polymerase (5 U/μL) (Promega), and Milli-Q ultrapure water to reach the total volume. Thermocycler parameters were set as follows: an initial denaturation cycle at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, primer annealing at 62 °C for 30 s, extension at 72 °C for 20 s, and a final extension cycle at 72 °C for 5 min.

The PCR product was purified using the QIAquick PCR Purification Kit (QIAGEN). Subsequently, the purified product was cloned into the pGEM-T Easy plasmid (Promega) following the manufacturer's protocol and incubated overnight at 4 °C. Once the vector with the insert was obtained, it was transformed into electrocompetent *Escherichia coli* Top10F® cells (Invitrogen).

The bacteria were inoculated in LB-agar culture medium containing ampicillin (100 μg/mL), 100 μL of IPTG (100 mg/mL), and 20 μL of X-Gal (50 mg/mL) (Promega, Fischer-Biotech) and incubated at 37 °C overnight. Selected colonies were added to 1 ml of LB medium containing ampicillin (50 μg/ml) and incubated at 37 °C with shaking at 200 rpm overnight. After bacterial colony growth, DNA was obtained using a DNA extraction kit (QIAprep® Spin MIniprep Kit 250; Promega, GER).

The extracted DNA was subjected to PCR using primers (F3 and B3, Fig. 1), which anneal to flanking sequences in the inserted DNA fragment, followed by sequencing.

The sequencing was carried out at ACTGene Analytical Moleculars Ltda. (Rio Grande do Sul, Brasil). Sequences were analyzed using Basic Local Alignment Search Tool (BLAST) and MEGA 11 software for identification of viral species.

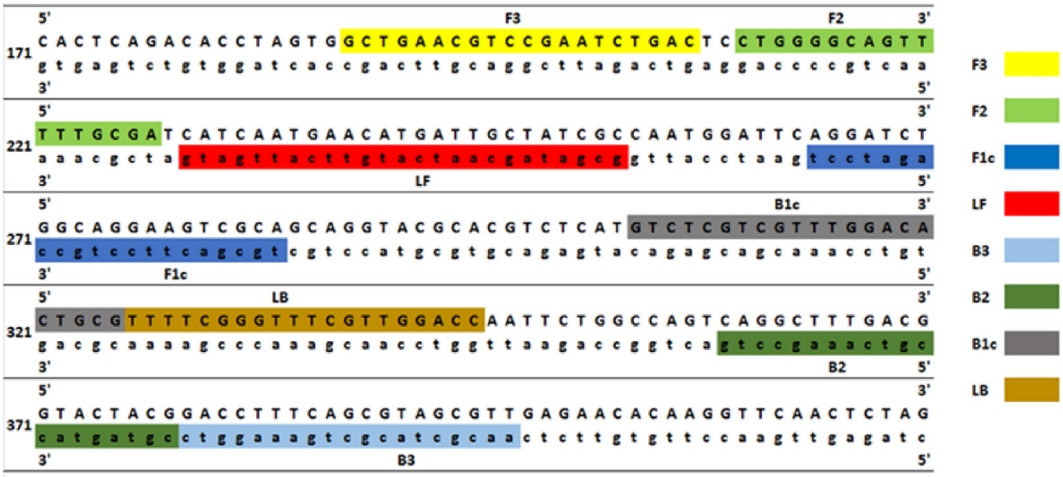

Fig. 1. The RNA1 sequence (GenBank accession number MH151795) is depicted as a double-stranded DNA segment in the figure. The sense strand (5′ to 3′, uppercase) and the antisense strand (3′ to 5´, lowercase) are highlighted to illustrate the specific DNA fragments identified by the software. These fragments were used to design set A of LAMP primers.

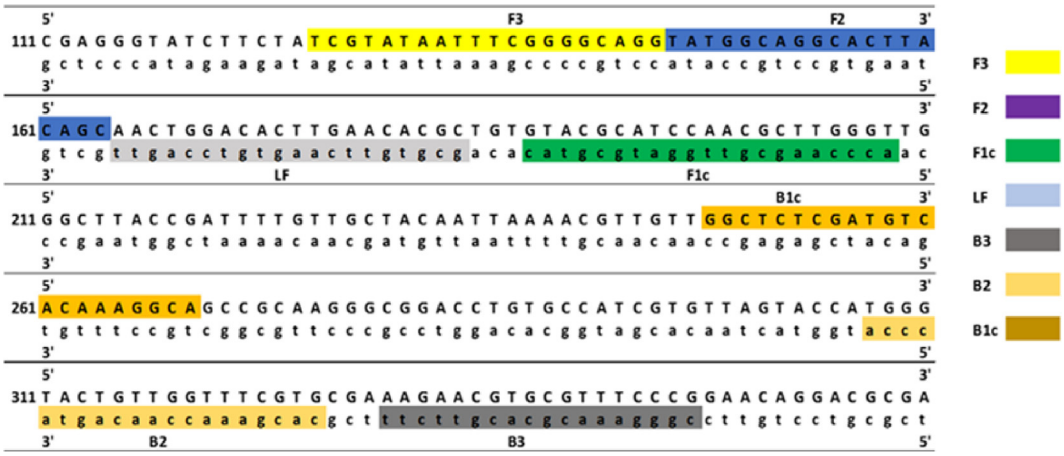

Fig. 2. The RNA2 sequence (GenBank accession number MH151801) is depicted as a double-stranded DNA segment in the figure. The sense strand (5′ to 3′) and the antisense strand (3′ to 5´) are highlighted to illustrate the specific DNA fragments identified by the software. These fragments were used to design the set B of LAMP primers.

Table 1
Primer sequences (set A) designed to target the gene associated with WhSMV viral replication (RNA 1 Genebank accession number MH151795).

Identification: A Dimer (Minimum) ΔG = −2.03					
Primer	**Melting Temperature (Tm) ˚C**	**Sequence (5′–3′)**	**Genome Position (5′–3′)**	**5′ΔG**	**3′ΔG**
F3	60.32	GCTGAACGTCCGAATCTGAC	188–207	−5.26	−4.76
B3	59.59	AACGCTACGCTGAAAGGTC	379–397	−6.07	−4.85
FIP		TGCGACTTCCTGCCAGATCCTCTGGGGCAGTTTTTGCGA			
BIP		GTCTCGTCGTTTGGACACTGCGCGTAGTACCGTCAAAGCCTG			
F2	60.15	CTGGGGCAGTTTTTGCGA	210–227	−6.29	−6.10
F1c	65.54	TGCGACTTCCTGCCAGATCCT	264–284	−6.59	−4.59
B2	59.74	CGTAGTACCGTCAAAGCCTG	359–378	−4.90	−6.08
B1c	65.45	GTCTCGTCGTTTGGACACTGCG	304–325	−5.53	−6.57
LF	60.87	GCGATAGCAATCATGTTCATTGATG	229–253	−5.16	−4.07
LB	60.56	TTTTCGGGTTTCGTTGGACC	326–345	−4.47	−5.86

2.6. LAMP reaction

LAMP reactions were conducted with cDNA produced from RT reactions detailed above. To select the best primer set among groups “A” and “B”, LAMP reactions were performed as previously described by Notomi et al.,.[10] The “A” reaction contained 8 primers (Table S1) responsible for recognizing the viral replication protein gene (RV), while the “B” reaction consisted of a set of 7 primers (Table S2) responsible for the synthesis of the envelope protein (CP). Each LAMP reaction was conducted in a total volume of 25 μL, comprising 2.5 μL of 10x Bst reaction buffer, 0.32 mM dNTPs, 0.64 M betaine, 0.2 μM F3 and 0.2 μM B3 (A and B), 0.64 μM FIB and 0.64 μM BIP (A and B),

0.32 μM LF (A and B) and 0.32 μM LB (A), 8 mM $MgSO_4$, 0.75 μL cDNA (1/10 dilution), 2 U/μL *Bacillus stearothermophilus* (Bst) 2.0 turbo DNA polymerase (Cellco, São Paulo, Brazil), and ultrapure Milli-Q water to complete the total volume for each reaction.

Betaine is a destabilizing agent of the DNA double helix structure and facilitates the denaturation of double-stranded DNA, being beneficial for the LAMP technique that operates at constant and relatively low temperatures. The addition of betaine to the LAMP reaction helps to improve amplification efficiency and overcome possible inhibitions that may affect the reaction.[16–17] After determining the optimal incubation temperature, $MgSO_4$ concentration, and betaine concentration, the shortest reaction duration was investigated to define the best set of primers that recognize the WhSMV virus.

LAMP amplification products (25 μL) were examined by direct visual inspection of the reaction tube before and after the introduction of SYBR Green I (Bio-Rad Laboratórios Brasil Ltda, Santo Amaro, SP, Brazil) in a dilution of 1:1,000 TE (v/v). In addition, LAMP amplification products (25 μL) were detected regularly using agarose gel electrophoresis (1.5 % agarose; TAE).

2.7. Real-time quantitative PCR (qPCR)

The qPCR (Quantitative Polymerase Chain Reaction - qPCR) reaction[18] was performed using cDNA samples and the external primer pair (F3/B3) from set "A" (Table 1), which were used to amplify the fragment corresponding to RP (RNA 1 - ORF 1) of WhSMV virus (Genbank accession number MH151795) and quantify the viral load of the samples to evaluate the sensitivity of the LAMP technique. The qPCR reactions were carried out using the SYBR Green PCR Master Mix Kit (Table S3) (Applied Biosystems, Carlsbad, USA) according to the manufacturer's instructions.

The reaction involved an initial denaturation step at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing and extension at 62 °C for 1 min, and a final step at 95 °C for 15 s. The analysis of the melting curve was performed with a slow increase from 62 °C to 95 °C at a rate of 1 °C per 5 s. To quantify the viral load, the Cq values (quantification cycle) obtained from each sample were compared with the corresponding standard curve.[19] For result analysis, StepOne™ Software V2.1 was used.

2.8. RT LAMP sensitivity and specificity

WhSMV is an RNA virus and therefore, we tested the RT-LAMP reaction for the development of a single tube point-of-care test. In these cases, RNA samples (1 μg / μL) were subjected to an RT-LAMP reaction as follows. The RT-LAMP assays were carried out in a final volume of 25 μL, containing 2.5 μL of 10X Bst 2.0 turbo buffer (Cellco), 8 mM $MgSO_4$, 0.5 mM dNTP, 0.64 M Betaine, 0.64 μM FIP and 0.64 μM BIP, 0.2 μM F3 and 0.2 μM B3, 0.32 μM LF and 0.32 μM LB, 0.45 μM $MgCl_2$, 1.8 μM DTT (Ludwig), 200 U of moloney murine leukemia virus reverse transcriptase (M−MLV RT), 8 U of *Bacillus stearothermophilus* (Bst) 2.0 turbo DNA polymerase (Cellco), 1.0 μL of RNA extract, and 4.0 μL of nucleases-free water. RNA extracts from non-infected wheat leaves were used as a control.

The RT-LAMP reactions were incubated at an optimal temperature for amplification, and following electrophoresis, the resulting bands were observed as an indicator of amplification.

To test LAMP reaction specificity, RNA samples from the rice stripe necrosis virus (RSNV virus from the Benyviridae family) were used as control.[1,20] RNA extracted from WhSMV-infected wheat leaves and non-infected wheat leaves were used as control. Three unidentified samples, two of which were infected with RSNV virus RNA (from the Benyviridae family) extracted from wheat roots and stems, and one sample infected with WhSMV RNA extracted from wheat leaves, were tested as a double-blind study.

2.9. Detection of WhSMV in the field with the RT-LAMP technique

In this study, the effectiveness of the RT-LAMP technique was evaluated, simulating field collection conditions using the pipette tip method, a variation of the toothpick method [21]. Due to the absorbent nature of toothpicks, which could affect the RT-LAMP reaction mixture, we replaced them with a 10 μL pipette tip. This pipette tip was employed to puncture the tissue of the sample (leaf) with 10–15 perforations. Method is referred here as the "pipette tip method". The reaction conditions are shown in Table S4. The sample was then immersed in a microtube containing the RT-LAMP reagents. The study focused on three different plants: a barley yellow dwarf virus (BYDV)-infected wheat plant, a non-infected wheat plant (*Triticum aestivum L.*), and a WhSMV-infected triticale (a hybrid of wheat and rye).

3. Results

3.1. Confirmation of viral identities

Sequences from cloned samples of the WhSMV virus were submitted to the National Center for Biotechnology Information (NCBI) GenBank and analyzed using the nucleotide-nucleotide BLAST (Blastn) tool. The analysis revealed that the fragments shared 98.18 % identity, compared to WhSMV isolate (GenBank MH151795.1 and MH151801).

3.2. LAMP reaction

Initially, experiments were conducted to determine the optimal reaction conditions. Thus, the reactions were tested for both primer sets (Tables 1 and 2), at constant temperature of 60 °C.

Previous studies demonstrated that increasing the reaction time beyond 2 h increases the occurrence of non-specific amplification, therefore setting reaction times to less than 120 min reduces the possibility of false positives [22] . To determine the minimum necessary time for accurate detection, tests were performed for 30, 60, or 90 min (Fig. 3A). However, the negative control reaction was maintained for 90 min to guarantee the absence of amplification.

Most previous studies conducted with the LAMP technique employed a concentration of 8 U of Bst DNA polymerase per reaction.[10,23–25] To reduce costs, the reduction of Bst DNA polymerase concentration in the LAMP reaction was investigated. In Fig. S2, the absence of amplification can be observed in the negative controls using 8 U of the Bst enzyme (reaction with cDNA from uninfected wheat, cultivated in the virology laboratory (UDESC)). Infected samples were tested using different enzyme concentrations specifically 8 U, 6 U, 4 U, and 2 U per reaction, with 2 U demonstrating efficiency in amplifying the target gene.

To assess the specificity of the primers designed for WhSMV detection in our LAMP reactions, we employed rice stripe necrosis virus (RSNV) cDNA as a negative control. RSNV was chosen because it shares a 51 % genetic similarity with WhSMV and is transmitted by the same vector.[1] This control helps us verify that our primers accurately distinguish WhSMV from closely related viruses like RSNV.

In Fig. 3A, agarose electrophoresis highlights DNA amplification (left lanes) using replication (RV) primers, starting from 30 min. Amplification of the coat protein (CP) fragment (right lanes) required a minimum of 60 min. Primer set A, with an additional Loop primer, demonstrated faster amplification, therefore, we decided to proceed with Primer set A for additional testing. Furthermore, Fig. 3B demonstrates the effective amplification of the WhSMV RNA1 gene using as little as 0.5 U/reaction of Bst DNA polymerase and primer set A. In this experiment, the reaction was optimized to enhance cost-effectiveness and to test sensitivity with reduced quantities of the Bst DNA polymerase enzyme, which is a cost-sensitive component for large-scale

Table 2
Primer sequences (set B) designed to target the gene associated with the WhSMV capsid protein (RNA 1, Genebank accession number MH151801).

Identification: B Dimer (Minimum) ΔG = -1,66					
Primer	**Melting Temperature (Tm) °C**	**Sequence (5′–3′)**	**Genome Position (5′–3′)**	**5′ΔG**	**3′ΔG**
F3	59.1	TCGTATAATTTCGGGGCAGG	126–145	−4.31	−6.69
B3	60.01	CGGGAAACGCACGTTCTT	330–347	−6.14	−4.01
FIP		ACCCAAGCGTTGGATGCGTACTATGGCAGGCACTTACAGC			
BIP		GGCTCTCGATGTCACAAAGGCACACGAAACCAACAGTACCCA			
F2	59.18	TATGGCAGGCACTTACAGC	146–164	−4.98	−4.98
F1c	65.17	ACCCAAGCGTTGGATGCGTAC	188–208	−5.51	−5.91
B2	59.66	CACGAAACCAACAGTACCCA	307–326	−5.35	−5.09
B1c	64.81	GGCTCTCGATGTCACAAAGGCA	248–269	−5.93	−5.75
LF	60.49	GCGATAGCAATCATGTTCATTGATG	165–184	−6.73	−5.00

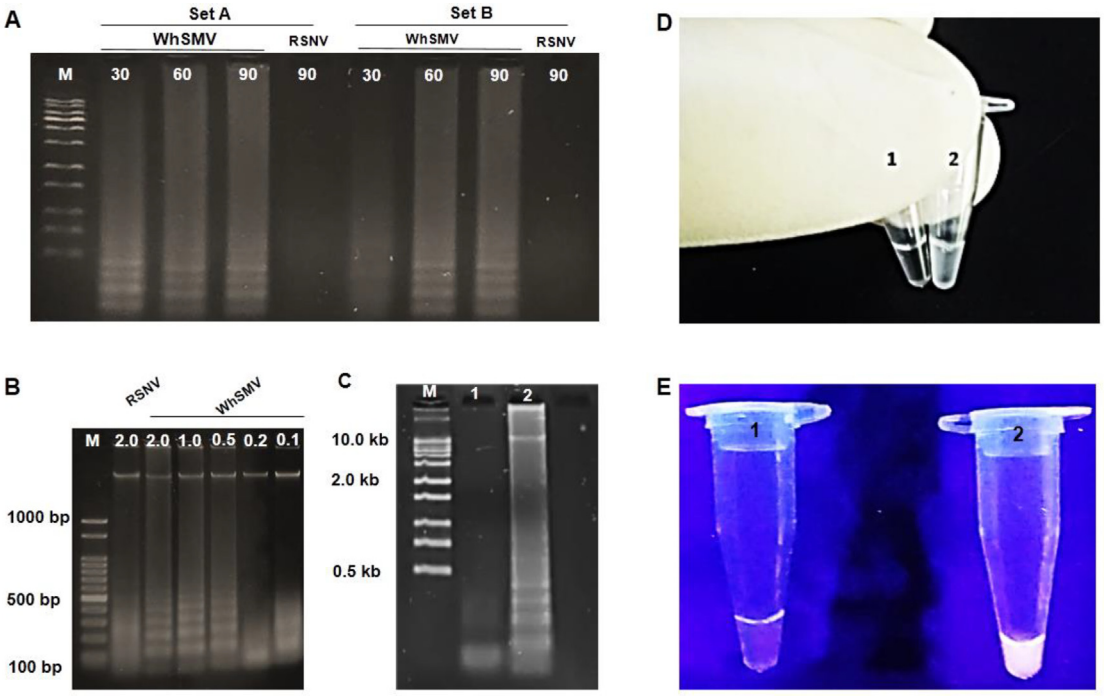

Fig. 3. LAMP Amplification and Optimization. In panel A, agarose gel electrophoresis displays LAMP products for WhSMV using primer sets A and B, showing amplification from 30 to 90 min for WhSMV cDNA and 90 min for the RSNV control virus cDNA. In panel B, agarose gel electrophoresis displays the optimization of Bst DNA polymerase enzyme concentrations ranging from 2.0 to 0.125 units, respectively to WhSMV, and 2.0 units to RSNV. In panel C, the LAMP optimization test distinguishes RSNV negative samples (Lane 1) from WhSMV positive samples (Lane 2), on agarose gel electrophoresis. Panel D shows visual inspection, indicating turbidity andnucleic acid presence in a collection tube, visible to the naked eye. In panel E, LAMP detection using SYBR Green was excited by UV light at 302 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

testing. As depicted in Fig. 3B, the utilization of 0.5 U of this enzyme per reaction successfully amplified samples infected with the WhSMV virus. However, for added safety margin, the utilization of 0.75 U per reaction was established.

Fig. 3C shows the reaction specificity, where amplification can only be observed in lane 2, which contains WhSMV cDNA from infected samples as compared to lane 1 which contains cDNA from RSNV infected samples.

The LAMP technique enables visual interpretation of molecular results, expediting diagnoses in labs or point-of-care setups. In Fig. 3D, microtube 1 (RSNV virus cDNA) appears clear, while microtube 2 (WhSMV) displays heightened turbidity due to magnesium pyrophosphate release post-reaction.

However, relying solely on turbidity for amplification assessment is error-prone and unsuitable as a primary LAMP detection method. A superior option involves fluorescent agents like the double-stranded DNA intercalator SYBR Green. It necessitates a UV light source at 302 nm for detection.[26]

In Fig. 3E, samples were tested using SYBR Green. Notably, microtube 1 (RSNV) exhibited no luminescence, unlike microtube 2 (WhSMV). Positive samples exhibit fluorescence under UV light excitation (3E). To ensure authenticity, an additional 3 μL of SYBR Green was added to microtube 1 (RSNV) compared to tube 2 (WhSMV), without altering the outcome.

3.3. Real-time amplification efficiency curve – qPCR and sensitivity of LAMP assay compared to previous methods

The analysis of the standard curve by qPCR technique allows for the quantification of viral load in samples infected with WhSMV. To quantify the viral load of WhSMV in each sample with the target gene, the linear equation for quantitative qPCR y = -3.4831x + 40.157 was used.[27] Results are shown in Graphic S1, where Cq values are correlated with the number of viral copies per sample. For the target gene RNA 1, the external primers “A” demonstrated amplification sensitiv-

ity in samples with a concentration of 20 pg, which contained an approximate value of 200 viral copies.

The sensitivity analysis of the LAMP technique was performed by comparing the number of viral copies quantified per sample using the qPCR technique. Six different viral load points were selected, including 10^0, 10^1, 10^2, 10^3, 10^4, and 10^5 copies, to assess the sensitivity of the LAMP assay. These samples were subjected to the LAMP technique following the standardized protocol established in this study. The reactions were incubated at 60 °C for 40 min using the external primers "A."

In Fig. 4A, the results of LAMP amplification are present, showing the detection of ~ 200 copies of the target gene sequence. This observation demonstrates the high sensitivity of the LAMP technique in detecting and amplifying viral genetic material. The results were visualized using 1.5 % agarose gel electrophoresis, confirming the successful amplification of the target gene fragment.

3.4. RT-LAMP assays for WhSMV detection

Several RT-LAMP parameters were tested to determine the optimal reaction conditions for the identification of WhSMV. Positive controls containing WhSMV RNA and negative controls with genetic material from non-infected wheat plants were utilized. The set of primers "A," targeting viral replication (RV) and providing faster amplification results, was tested at an isothermal temperature of 60 °C with varied reaction times of 40 and 80 min.

As shown in Fig. S3, amplification was achieved from the positive control (+) using either a 40-min (left lane) or 80-min (right lane).

In the RT-LAMP technique, the Bst DNA polymerase enzyme was used at a concentration of 8 U per reaction[28] as recommended by the manufacturer (Cellco; São Paulo; Brazil). After standardization and verification of the technique's specificity, RT-LAMP reactions were performed with different enzyme concentrations to detect WhSMV, aiming to reduce the cost of this reagent per reaction.

To detect the WhSMV virus, the RT-LAMP technique requires a reverse transcription step, where cDNA is synthesized from RNA.[10] Therefore, two enzymes, a strand displacement polymerase, and a reverse transcriptase (RT) enzyme, are crucial components for performing this technique.

The moloney murine leukemia virus reverse transcriptase (M−MLV RT) is often used in many studies, at a concentration of 200 U (Promega; São Paulo; Brazil) per reaction.[2] In the present study, the M−MLV (RT) enzyme was chosen due to its cost-effectiveness. However, the reactions were performed with reduced concentrations of 100 U and 50 U. In Fig. 4B, the use of 50 U was observed to be sufficient to obtain a positive result.

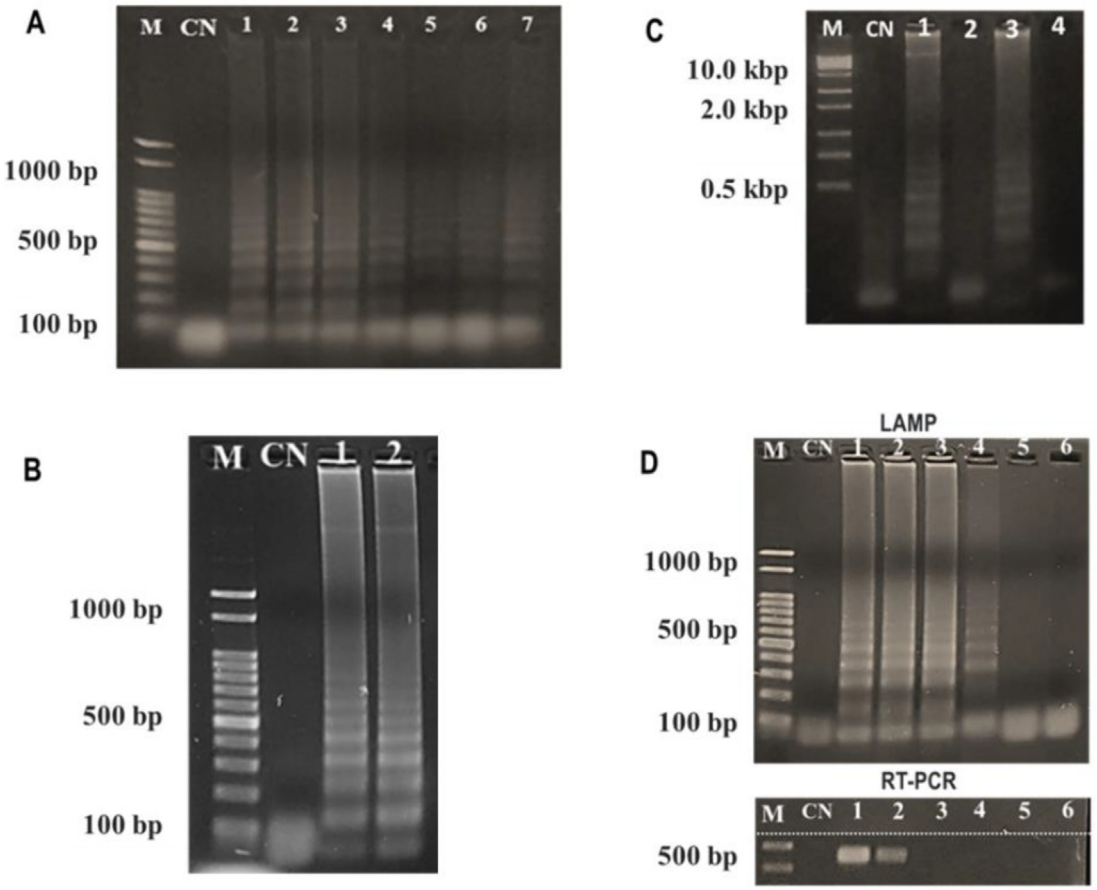

Fig. 4. In A, the sensitivity test of the LAMP reaction for viral load amplification of the WhSMV virus. Lane M: marker; CN is negative control with the uninfected wheat sample; lanes 1–7: samples with different copy amounts of WhSMV virus RNA (1) 585,475; (2) 28,923; (3) 14,075; (4) 2,498; (5) 1,206; (6) 782 and (7) 202. In B, the optimization of the M−MLV enzyme for the amplification of target RNA 1 gene with different concentrations of 100 and 50 U, respectively at lanes 1 and 2. Gel C shows RT-LAMP assays for WhSMV detection in wheat root contaminated with WhSMV virus at 80 ng/uL-lane 1 and 10 ng/μL-lane 3. Lanes 2 and 4 are RNA extraction samples from RSNV- spiked wheat root. In D, the WhSMV detection sensitivity comparison among RT-LAMP and RT-PCR.: Agarose gel electrophoresis illustrating the sensitivity of the LAMP reaction over target dilution, ranging from 1 Ăμg (μg) to 1 zeptogram (zg), (lanes 1–6), was tested. The second lane (CN) presents a control with unrelated target DNA. At the bottom: Concurrently, the same DNA dilutions were subjected to RT-PCR analysis. The agarose gel displays the RT-PCR results, providing a comparison of detection sensitivity with the LAMP reaction.

The "A" primers demonstrated specificity by selectively amplifying WhSMV. Fig. 3C illustrates the amplification of samples infected with the WhSMV virus.

To assess the relative sensitivity of the methods, RT-LAMP and RT-PCR were performed using a series of five dilutions of extracted RNA from WhSMV-infected wheat, covering a range of 1 to 1×10^{-15} μg per reaction. Positive RT-LAMP amplifications were detectable down to dilutions of 1×10^{-6} μg (Fig. 4D), while RT-PCR yielded positive results down to dilutions of 1×10^{-3} μg (Fig. 4D). Remarkably, assays conducted with total RNA at 1×10^{-9} μg or lower displayed inconsistent or indistinguishable product yields in both methods. Consequently, the RT-LAMP assay exhibited greater sensitivity than RT-PCR in detecting the WhSMV pathogen.

3.5. Specificity of the RT-LAMP assay for detecting WhSMV in the field

Conventional methods for DNA/RNA extraction tend to be expensive, time-consuming, and reliant on complex equipment, presenting challenges for the development of field-applicable techniques. However, the toothpick method has been explored as a simple and rapid alternative to access genetic material directly from plants.[21]

In our study, we initially attempted to extract RNA using the toothpick method. However, due to the porous nature of the wooden toothpick, the necessary reaction reagents were absorbed, rendering the procedure unfeasible. Consequently, we replaced the toothpick with a plastic pipette tip (10 μL) as a tool for extracting the genetic material. By making 10–15 punctures on leaves displaying typical symptoms caused by wheat mosaic virus, we obtained the desired RNA samples. After this step, the pipette tip was directly immersed in the RT-LAMP reaction mixture.

During the RT-LAMP procedure, the samples were incubated at 60 °C for 40 min, using 8 U of Bst DNA polymerase, 200 U of M−MLV reverse transcriptase, and primer "A" designed to amplify the target gene. Fig. 5 presents the specificity of the RT-LAMP technique, demonstrating the amplification of WhSMV RNA samples extracted in the field. Negative controls (NC) consisted of RNA samples obtained from non-infected plants using the Trizol method, while the positive control (2) contained RNA extracted from WhSMV-infected plants using the Trizol method. Notably, the RNA samples for lanes 3, 4, and 5 were extracted using the plastic pipette tip method to simulate field conditions.

4. Discussion

The rapid and accurate detection of viruses is crucial for agriculture, especially regarding diseases affecting important crops. WhSMV is a recently discovered virus in Brazil. Wheat stripe mosaic virus can cause outbreaks and economic losses of up to 50 % in yield, making developing diagnostic tools for proper WhSMV surveillance and control crucial.[2–3,29]

The LAMP and RT-LAMP assays have been the first isothermal techniques developed so far that successfully detected and distinguished WhSMV from RSNV and BYDV. Initially, two primer sets (Tables 1 and 2) were tested at different incubation times to amplify a conserved region of the WhSMV gene. According to Cryskely,[29] the use of Loop primers (LB and LF) accelerates the reaction, reducing the incubation time and, consequently, the time to obtain results.

While our LAMP specificity assay focused initially on RSNV and BYDV due to their substantial genetic similarity to WhSMV and the absence of suitable alternatives, it is imperative to acknowledge this as a limitation of our study, and future research may explore a broader spectrum of viruses for a comprehensive assessment.

Similarly, in this study, the Loop primer set was also used, resulting in a reduction of 30 min in the reaction time (Fig. 5. A).

Indeed, in previous studies[30] to detect *Salmonella enterica* by LAMP reactions, the absence of loop primers resulted in extended reaction times. It is important to note that an excessively long incubation period should be avoided as it may lead to false-positive results; thus, the ideal incubation time should be less than 120 min.

Therefore, Francois et al.[30] and Hao et al.,[31] working on the identification of wheat dwarf virus by LAMP, albeit without using Loop primers, demonstrated that 1 h of incubation was required for the amplification of the target gene. Similarly, as in the aforementioned studies, Zarzyńska-Nowak et al.[32] used the technique for the detection of barley stripe mosaic virus (BSMV) infecting barley.

Other commonly used diagnostic methods have been developed for WhSMV detection, including serological methods[6] and PCR-based methods.[1] However, no sensitivity testing has been performed among the methods used for wheat virus detection. Nevertheless, in a study conducted by Tiberini et al.[33] to detect the onion yellow dwarf virus (OYDV) infecting onion leaves and bulbs, it was shown that the performance of RT-LAMP is similar to RT-qPCR. The RT-LAMP technique detected a limit of 103 target RNA copies in samples with 1 fg of RNA, demonstrating a sensitivity 104 times higher than the ELISA technique and up to 100 times more sensitive than RT-PCR.

In the present study, the absolute quantification of the number of RNA molecules in infected samples allowed for a direct comparison between diagnostic methods based on RT-qPCR and RT-LAMP, as demonstrated by Ortega et al.[34] and Wilisiani et al.[21] Liu et al.[17] affirmed the effectiveness of the RT-LAMP technique compared to other diagnostic methods commonly applied in the detection of plant viruses.

In this study, the LAMP technique showed amplification results of the target gene from cDNA, allowing the detection of 202 viral copies

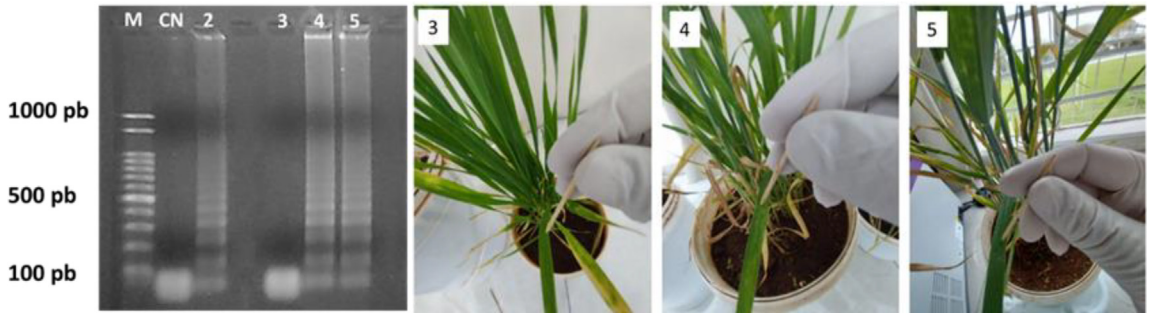

Fig. 5. RT-LAMP technique applied in the field to identify the WhSMV virus. Marker (M) of 100 bp molecular weight; well (1) negative control (NC) with non-infected wheat sample (extraction with Trizol); lane (2) positive control infected sample with wheat stripe mosaic virus (extraction with Trizol); lanes 3–5 field test pipette tip method, lane (3) Wheat plant (*Triticum aestivum L.*) infected with barley yellow dwarf virus (BYDV); (4) Wheat plant (*Triticum aestivum L.*) infected with WhSMV; (5) WhSMV-infected triticale plant (wheat-rye hybrid). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

at a dilution of 1:7 (see Fig. 3). However, when serial dilution of total RNA was performed, RT-LAMP was 10 times more sensitive than LAMP and RT-qPCR. The data reported by Tiberini et al.[33] using RT-qPCR with TaqMan allowed the identification of 103 copies of viral cDNA, specifically from OYDV. On the other hand, the LAMP-based assay demonstrated 10 times higher specificity and sensitivity. Furthermore, the assay allows for selectivity, repeatability, and reproducibility.

According to LIU et al.,[35] qPCR is considered the most commonly used and sensitive detection technique for plant viruses. These results highlight the feasibility of using RT-LAMP as a diagnostic tool in routine testing.

It is important to highlight that PCR-based methods are affected by various inhibitors.[33] However, easy sample preparation is crucial for field applications, without the need for expensive reagents and instruments. Two different nucleic acid extraction methods were used in this study for the RT-LAMP reaction: the conventional method (Trizol extraction method) and a method that could be easily used directly in the field (pipette tip method). Both extraction methods proved to be efficient. Data obtained in this study demonstrated that the LAMP technique may be less sensitive to inhibitors compared to the RT-qPCR technique. This detection method, in addition to being easier and more robust, allows for the use of extraction methods that reduce the cost of reagents and facilitate on-field application.

Therefore, it is important to emphasize that the RT-LAMP assay developed in this study showed excellent results when applied to naturally infected cultivars with WhSMV. Regardless of the extraction method, WhSMV was detected in samples extracted from the leaf and stem of wheat plants using Trizol. The same result was obtained when using the pipette tip method, reaffirming once again the efficacy of the RT-LAMP technique for rapid on-field analysis, enabling viral detection.

Furthermore, the technique allows for the detection of the target at low concentrations, simulating a natural infection condition. Additionally, the possibility of easily visualizing amplification products in agarose gel through direct SYBR Green staining in tubes or turbidity confirms the robustness of the assay without the need for advanced and expensive instruments.

5. Conclusion

In conclusion, this study successfully achieved its objectives of developing a new, rapid molecular test for the detection of wheat stripe mosaic virus (WhSMV) in wheat (*Triticum aestivum L.*) using the Loop-mediated isothermal amplification (LAMP) technique.

Specific oligonucleotides were designed for the isothermal amplification of WhSMV genetic material. The LAMP reaction was standardized, involving two separate steps: cDNA synthesis and amplification of the target gene. The RT-LAMP reaction for viral RNA detection was also successfully developed.

The sensitivity of RT-LAMP was determined by comparing it with the gold standard technique, Real-Time PCR. The results demonstrated that RT-LAMP exhibited comparable sensitivity to Real-Time PCR, providing reliable and accurate detection of WhSMV.

The specificity of RT-LAMP was confirmed by testing it against other viruses from the same family (Benyviridae). The assay specifically targeted WhSMV and did not cross-react with other related viruses, ensuring its specificity in diagnostic applications.

Field samples, specifically collected leaves, were used to test the diagnostic capability of RT-LAMP. The results confirmed the effectiveness of RT-LAMP in detecting WhSMV in field-collected material, highlighting its practical applicability for on-site diagnosis.

Various methods of result visualization were explored, including turbidity and agarose gel electrophoresis. These methods provided reliable means for result interpretation, offering flexibility in result visualization based on available resources and equipment.

In summary, the developed RT-LAMP assay for the detection of WhSMV in wheat demonstrated excellent performance in terms of sensitivity, specificity, and practical applicability. This new molecular test holds great promise as a rapid and reliable diagnostic tool for the timely detection of WhSMV, contributing to improved disease management and protection of wheat crops.

6. Availability of data and materials

Data supporting this study are included within the article and/or supporting materials.

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CRediT authorship contribution statement

Anderson Varela de Andrade: Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fernando Sartori Pereira:** Methodology, Formal analysis. **Fabio Nascimento da Silva:** Project administration. **Gustavo Felippe da Silva:** Formal analysis, Data curation, Conceptualization. **Maria de Lourdes Borba Magalhães:** Writing – original draft, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgeb.2024.100373.

References

1. Valente JB, Pereira FL, Stempkowski LA, et al. A novel putative member of the family *benyviridae* is associated with soilborne wheat mosaic disease in Brazil. *Plant Pathol.* 2019;68:588–600. https://doi.org/10.1111/ppa.12970.
2. Esquivel-Fariña A, Camelo-García VM, Kitajima EW, Alberto J, González-Segnana LR. First report of wheat stripe mosaic virus in paraguay, australasian plant disease. *Notes.* 2019;14. https://doi.org/10.1007/s13314-019-0355-4.
3. Terefe TG, Visser B, Botha W, et al. Detection and molecular characterization of wheat stripe mosaic virus on wheat in South Africa. *Crop Prot.* 2021;143. 105464. https://doi.org/10.1016/j.cropro.2020.105464.
4. Barbosa MM, Goulart LR, Prestes AM, Juliatti FC. Genetic control of resistance to soilborne wheat mosaic virus in Brazilian cultivars of *Triticum aestivum L. Euphytica.* 2001;122:417–422. https://doi.org/10.1023/a:1012937116394.
5. Lau D, Sartori F, Lucas P, Stempkowski A, Lima De Castro R. Caracterização da reação de cultivares de trigo do ensaio estadual do Rio Grande do Sul 2020 ao mosaico-comum. 2021. https://ainfo.cnptia.embrapa.br/digital/bitstream/item/232146/1/COT-385-Douglas-Lau.pdf (accessed August 22, 2023)..
6. Karoliny A, Echeverri L, Andersen B, et al. Production and characterization of chicken IgY for detecting wheat stripe mosaic virus. *Tropical Plant Pathol.* 2022;47:303–308. https://doi.org/10.1007/s40858-021-00481-x.

7. Kinnamon DS, Heggestad JT, Liu J, Chilkoti A. Technologies for Frugal and Sensitive Point-of-Care Immunoassays. *Annu Rev Anal Chem*. 2022;15:123–149. https://doi.org/10.1146/annurev-anchem-061020-123817.
8. Pfaffl MW. The ongoing evolution of qPCR. *Methods*. 2010;50:215–216. https://doi.org/10.1016/j.ymeth.2010.02.005.
9. Padzil F, Mariatulqabtiah AR, Tan W, et al. Loop-mediated isothermal amplification (LAMP) as a promising point-of-Care diagnostic strategy in avian virus research. *Animals*. 2021;12:76. https://doi.org/10.3390/ani12010076.
10. Notomi T, Okayama H, Masubuchi H, et al. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res*. 2000;28:63e63. https://doi.org/10.1093/nar/28.12.e63.
11. Notomi T, Mori Y, Tomita N, Kanda H. Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. *J Microbiol (Seoul, Korea)*. 2015;53:1–5. https://doi.org/10.1007/s12275-015-4656-9.
12. Nagamine K, Hase T, Notomi T. Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol Cell Probes*. 2002;16:223–229. https://doi.org/10.1006/mcpr.2002.0415.
13. Soroka M, Wasowicz B, Rymaszewska A. Loop-mediated isothermal amplification (LAMP): the better sibling of PCR?. *Cells*. 2021;10:1931. https://doi.org/10.3390/cells10081931.
14. Chaouch M. Loop-mediated isothermal amplification (LAMP): an effective molecular point-of-care technique for the rapid diagnosis of coronavirus SARS-CoV-2. *Rev Med Virol*. 2021;31. https://doi.org/10.1002/rmv.2215.
15. Somayyeh Sedaghatjoo M, Förster LK, Killermann NP, Maier B. Development of a loop-mediated isothermal amplification assay for the detection of Tilletia controversa based on genome comparison. *Sci Rep*. 2021;11. https://doi.org/10.1038/s41598-021-91098-2.
16. Vasoo S, Pritt BS. Molecular diagnostics and parasitic disease. *Clin Lab Med*. 2013;33:461–503. https://doi.org/10.1016/j.cll.2013.03.008.
17. Luo X, Zhang D, Zheng L, et al. Development of a reverse transcription loop-mediated isothermal amplification assay for the rapid detection of pepper mottle virus. *Can J Plant Pathol*. 2016;38. https://doi.org/10.1080/07060661.2016.1261371.
18. Kwok S, Higuchi R. Avoiding false positives with PCR. *Nature*. 1989;339:237–238. https://doi.org/10.1038/339237a0.
19. Bruña-Romero O, González-Aseguinolaza G, Hafalla JCR, Tsuji M, Nussenzweig RS. Complete, long-lasting protection against malaria of mice primed and boosted with two distinct viral vectors expressing the same plasmodial antigen. *Proc Natl Acad Sci*. 2001;98:11491–11496. https://doi.org/10.1073/pnas.191380898.
20. Aoki MN, de Oliveira CB, Góes LGB, et al. Colorimetric RT-LAMP SARS-CoV-2 diagnostic sensitivity relies on color interpretation and viral load. *Sci Rep*. 2021;11:9026. https://doi.org/10.1038/s41598-021-88506-y.
21. Wilisiani F, Tomiyama A, Hiroshi Katoh, Hartono S, Neriya Y, Nishigawa H, Natsuaki T. Development of a LAMP assay with a portable device for real-time detection of begomoviruses under field conditions, Journal of Virological Methods. 265 (2019) 71–76. https://doi.org/10.1016/j.jviromet.2018.10.005..
22. Fujino M, Yoshida N, Yamaguchi S, et al. A simple method for the detection of measles virus genome by loop-mediated isothermal amplification (LAMP). *J Med Virol*. 2005;76:406–413. https://doi.org/10.1002/jmv.20371.
23. Kaymaz SV, Elitas M. Optimization of loop-mediated isothermal amplification (LAMP) reaction mixture for biosensor applications. *MethodsX*. 2021;8. 101282. https://doi.org/10.1016/j.mex.2021.101282.
24. Nakao R, Stromdahl EY, Magona JW, et al. Development of loop-mediated isothermal amplification (LAMP) assays for rapid detection of ehrlichia ruminantium. *BMC Microbiol*. 2010;10. https://doi.org/10.1186/1471-2180-10-296.
25. Lai MY, Ooi CH, Lau YL. Validation of SYBR green I based closed-tube loop-mediated isothermal amplification (LAMP) assay for diagnosis of knowlesi malaria. Malaria Journal 2021;20. https://doi.org/10.1186/s12936-021-03707-0..
26. Sen B, Afrin S, Zahan S, Munshi SU, Zaman SU. Determination of chikungunya virus RNA by reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay. *Adv Microbiol*. 2022;12:398–414. https://doi.org/10.4236/aim.2022.127029.
27. Shpitalni M, Koren Y, Lo CC. Realtime curve interpolators. *Comput Aided Des*. 1994;26:832–838. https://doi.org/10.1016/0010-4485(94)90097-3.
28. Matute T, Nuñez I, Rivera M, et al. Homebrew reagents for low-cost RT-LAMP. *J Biomol Tech*. 2021;32:114–120. https://doi.org/10.7171/jbt.21-3203-006.
29. Cryskely M. Diagnóstico molecular comparativo da brucelose canina pela aplicação das técnicas de reação em cadeia pela polimerase (PCR) e amplificação isotérmica do DNA mediada por *loop* (LAMP). Universidade de São Paulo 2018;1. https://doi.org/10.11606/d.10.2017.tde-25052017-105756..
30. Francois P, Tangomo M, Hibbs J, et al. Robustness of a loop-mediated isothermal amplification reaction for diagnostic applications. *FEMS Immunol Med Microbiol*. 2011;62:41–48. https://doi.org/10.1111/j.1574-695x.2011.00785.x.
31. Hao X, Wang L, Zhang X, et al. A real-time loop-mediated isothermal amplification for detection of the wheat dwarf virus in wheat and the insect vector psammotettix alienus. *Plant Dis*. 2021;105. https://doi.org/10.1094/pdis-10-20-2279-re.
32. Zarzyńska-Nowak A, Hasiów-Jaroszewska B, Jeżewska M. Molecular analysis of barley stripe mosaic virus isolates differing in their biological properties and the development of reverse transcription loop-mediated isothermal amplification assays for their detection. *Arch Virol*. 2018;163:1163–1170. https://doi.org/10.1007/s00705-018-3725-x.
33. Tiberini A, Tomlinson J, Micali G, Fontana A, Albanese G, Tomassoli L. Development of a reverse transcription-loop- mediated isothermal amplification (LAMP) assay for the rapid detection of onion yellow dwarf virus. *J Virol Methods*. 2019;271. 113680. https://doi.org/10.1016/j.jviromet.2019.113680.
34. Ortega SF, Tomlinson J, Hodgetts J, Spadaro D, Gullino ML, Boonham N. Development of loop-mediated isothermal amplification assays for the detection of seedborne fungal pathogens fusarium fujikuroi and magnaporthe oryzae in rice seed. *Plant Dis*. 2018;102:1549–1558. https://doi.org/10.1094/pdis-08-17-1307-re.
35. Liu RD, Wang ZQ, Wang L, Long SR, Ren HJ, Cui J. Analysis of differentially expressed genes of trichinella spiralis larvae activated by bile and cultured with intestinal epithelial cells using real-time PCR. *Parasitol Res*. 2013;112:4113–4120. https://doi.org/10.1007/s00436-013-3602-1.