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Research Article

Rapid detection of bacterial seed-borne disease *Xanthomonas translucene* in wheat using loop-mediated isothermal amplification (LAMP) assay in Iran

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Abstract: Bacterial leaf streak (BLS) is one of the most important seed borne diseases of cereals, especially in wheat and barley. This disease can cause yield loss of up to 40% by producing dark brown spots on the glumes, called black chaff. Although this disease has spread widely in Iran recently, a quick and economical method for molecular and accurate detection of the disease agent, *Xanthomonas translucene,* has not been identified in the country. Quick and accurate detection of the bacteria in seed masses plays an important role in disease management. In this research, the development of a diagnostic kit for the causal agent of wheat bacterial leaf streak disease based on LAMP (Loop Mediated Isothermal Amplification) was performed by designing specific primers based on two new housekeeping genes *rpoD* and *dnaK*, which have not been used as LAMP targets previously). The serial dilution method of the bacterium was used to determine the sensitivity of the designed primers. The efficiency of the diagnostic kit was evaluated by collecting commercial wheat seed masses from different regions of the country and using the pure culture of the bacteria. The results showed that the primers designed based on the *rpoD* gene were the most effective. These primers detected up to 3.5 x 10^2 bacteria cells in one milliliter. The specificity of the primers was proved by performing the LAMP reaction on different genera of wheat pathogenic bacteria. The results of this project can be used for the rapid detection of *X. translucense* in wheat seeds and can be used as one of the management strategies for preparing healthy seeds.

Keywords: Bacterial leaf streak disease, kit, LAMP

Introduction

Bacterial leaf streak disease of cereals was first reported in 1902 on wheat from the United States (Indiana) (Tubajika *et al*., 1998). There were also other reports of the disease in wheat, rye, and barley (Jones *et al*., 1916; Reddy *et al*., 1924). The spread of the disease has been reported on all continents, and different strains of bacteria show great genetic diversity (Curland *et al*., 2018). The pathogen can spread from one region to another through contaminated seeds, wind and rain, crop residues, secondary hosts, insects such as trips

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and aphids, and soil, and can persist from year to year (Boosalis 1952; Wiese 1987).

In Iran, the disease was first reported in some farms in Kerman (Alizadeh and Rahimian 1989).

It is difficult to estimate the damage caused by this disease; however, some researchers have reported that a yield reduction of up to 40% has occurred in infected fields (Tillman 1994). In Idaho, USA, in infected fields with rainfed irrigation, damage to 30-40% has also been reported (Schaad and Forster 1985). During a study on the estimation of yield reduction caused by bacterial damage, it was found that whenever the severity of the disease increases, the weight and number of seeds in the cluster would decrease. In two wheat varieties, Savanah and Florida 3041, for every 10% increase in disease severity, the average grain weight reduction in each spike was 7% and 9%, respectively (Tillman *et al*., 1999).

The LAMP (Loop Mediated Isothermal Amplification) technique is one of the most accurate and cost-effective methods used in recent years to diagnose plant diseases. In 2000, Notomi and colleagues invented a technique called loop-mediated isothermal amplification, which is a method of isothermal amplification. This technique did not require a thermocycler and had high accuracy and sensitivity (Notomi *et al*., 2000; Saharan *et al*., 2014).

In this technique, the template is made in a reaction without needing DNA polymerase with the ability to displace the strand. In addition, six special primers known as internal primers (FIP) and (BIP), external primers (F3 and B3), and specific loop primers (LF and LB) are used with very high specificity (Mori *et al*., 2001; Parida *et al*., 2008). LAMP is a simple diagnostic tool where the reaction occurs in a single tube. For this purpose, the buffer, primer, and DNA polymerase are mixed; then, the mixture is placed at a constant temperature.

The technique is simple but simultaneously very accurate and sensitive. The primer design stage is very important, and choosing the best primer among the designed primers is important according to various factors. The primers used in this method are simultaneously attached to eight regions in the template DNA. The final product of the reaction is a mixture of stem-loop DNA with inverted repeats of template DNA and cauliflower-like structures with abundant loops (Nagamine *et al*., 2001).

Due to the specific nature of these primers, the amount of DNA produced in the LAMP method is significantly higher than in the PCR method (Nagamine *et al*., 2002). Although these reactions are considered isothermal at the beginning of the reaction, an annealing step is needed (Gotoh *et al*., 2012). Therefore, this method has higher sensitivity and accuracy than conventional PCR due to the type of primer design and can be a suitable alternative to the PCR technique (Bahrami *et al*., 2019).

The LAMP method has been widely used to detect RNA/DNA viruses. This method was used to detect Tomato Yellow Leaf Complex Virus (TYLCV) by Fukuta *et al*. (2003). Recently, LAMP has been effectively used to diagnose the pathogenic bacteria *Mycobacterium bovis* (Zhang *et al*., 2010) and *Vibrio cholera* (Okada *et al*., 2010), the agents of tuberculosis and cholera. In addition, Langlois *et al*. used this technique in 2017 to identify different *Xanthomonas translucens* bacteria. Using primers designed for different protected genomic regions, they could separate *Xanthomonas translucens* pathogens that caused disease in cereals and legumes (Langlois *et al*., 2017). In Iran, this technique has been used to identify Salmonella (Moradi *et al*., 2009), cucumber mosaic virus (Ghasemi *et al*., 2011), *Verticillium dahliae* (Aslani *et al*., 2017), and potato leaf curl virus (Almasi *et al*., 2013).

Until now, no quick and accurate diagnostic method in seed masses has been provided for this wheat bacterial seed disease in Iran. This study aims to design a kit and carry out rapid detection of *X. translucens* bacterium by the LAMP method, which can be helpful in imported samples, quarantine sites, centers, and research stations at the lowest cost and without the need for expensive equipment such as thermocycler. In this research, two new housekeeping gene regions, *rpoD*, and *dnaK*, have been used to design specific primers for Iranian isolates.

Materials and Methods

Seed lot sampling

Sampling was done from infected seed masses of different wheat cultivars in Lorestan (Falat, Mihan, and Bolani) and Kurdistan (Pishgam) provinces of Iran in 2021.

Isolation and detection of bacteria from seed lots

For isolation of the bacterium, the seed washing method of Schaad and Donaldson (1980) was used, in which 40 grams of seed samples were mixed with 40 ml of cold saline solution for 3 to 5 minutes. Then, 200 µl of the above suspension was cultured on NAY medium.

Phenotypic and Biochemical characteristics

The phenotypic and biochemical characteristics of the isolates, such as aerobic/anaerobic growth tests, levan, phosphatase, and catalase production, were investigated according to the Schaad method (Schaad *et al*., 2001). Tests of arginine dihydrolase, oxidase, and H_2S gas production from cysteine, the production of ketolactose and lecithinase was evaluated by the Dye method (Dye, 1962) and the ability to use several carbon and nitrogen sources according to the identification keys of plant pathogenic bacteria (Schaad *et al*., 2001).

Fast extraction of DNA from bacteria

To ensure the contamination of the seed mass with the above bacteria, genomic DNA extraction of the isolated bacteria was performed using the modified method of Rademaker and De Bruijn (Rademaker and De Bruijn 1997). In this method, bacteria were cultured on NA medium for 48 hours at 28°C. Four hundred microliters of bacterial suspension were prepared (10^9 dissolved in sterile distilled water) and centrifuged for three minutes (13000 rpm) in 1.5 microliter tubes, and the supernatant was discarded. Four hundred microliters of 1M NaCl were added to the above bacterial pellets and, after vortexing, centrifuged for three minutes (13000 rpm), and the supernatant was discarded. One hundred and fifty microliters of distilled sterile water and 50 microliters of 0.05 M NaOH were added to the pellets, and after vortexing, they were placed in boiling water for 10 minutes. Then, the samples were centrifuged for 2 minutes at 13000 rpm. Finally, the supernatant phase (containing DNA) was transferred to new tubes and used for polymerase chain reaction (PCR).

Molecular detection

The diagnostic PCR test used the *X. translucens*-specific primer pair designed based on *rpoD* gene sequence (Curland *et al*., 2018). PCR products were electrophoresed on a 1.5% Agarose gel. For further confirmation, the PCR products were sent for sequencing.

Direct extraction of DNA from seed lots

Schaad and Donaldson (Schaad and Donaldson 1980) used the method to extract DNA from seed samples. One milliliter of the seed suspension prepared in the previous step was centrifuged at 13,000 rpm for 1 minute. Then 100 microliters of the supernatant were heated at 95 °C for 15 minutes. This suspension contains DNA that is used in the LAMP-based diagnostic kit.

Primer design

Primer Explorer V4 software is used for primer design. The housekeeping genes *rpoD* and *dnaK* are used to design primers. The primers were synthesized by Metabion, Germany. The primers introduced by Langlois *et al.* (2017) were used to compare the efficiency of primers in this study.

LAMP reaction for detecting the bacteria

The reaction was done in a total volume of 25 microliters containing primers F3 and B3 0.2 µM each (metabion), primers FIP and BIP 1.6 µM each(metabion), primers loop LF and LB 0.8 µM each(metabion), enzyme buffer 10x (Isothermal Amplification Buffer, New England Biolabs), betaine 0.8 M, dNTPs 1.4 mM (Sigma-Aldrich), MgSO4 8 mM and finally 8 units of Bst enzyme (Bst DNA Polymerase 2.0, New England Biolabs) in isothermal temperature conditions of 65 °C for 70 minutes. The results were evaluated using 2% agarose gel and ethidium bromide dye (Langlois *et al*., 2017).

Specificity of designed primers

To evaluate the specificity of designed primers, genomic DNA of different genera of wheat pathogenic bacteria were used including; *Xanthomonas translucens*; *Pseudomonas syringe* pv. *syringe; Pseudomonas syringe* pv. *atrophaciens*; *Clavibacter tessellarius* (bacteriological lab collection of Iranian research institute).

DNA extraction from the bacteria was performed by the modified method of Rademaker and De Bruijn (Rademaker and De Bruijn 1997). Finally, the supernatant phase (containing DNA) was transferred to the new tubes kept at -20 °C and used for the LAMP reaction.

Sensitivity of the LAMP assay

To determine the sensitivity of the designed primers, a serial dilution of *X. translucens* bacteria was prepared. For evaluating the number of bacterial cells in each dilution, 20 microliters of different dilutions were cultured on NAY (Nutrient Agar Yest) medium, and incubated for 48 hours at 28 °C, then the population of bacteria (CFU) /ml) evaluated (Pruss, *et al*., 2008). In addition, DNA was extracted from different dilutions of bacterial suspension and used for LAMP reaction.

Results

Bacterial isolates and detection

Seed suspension was prepared from seed masses and cultured on NAY medium. After performing biochemical tests, for molecular identification, PCR reaction is done using *rpoD* gene primers and sent for sequencing. After aligning the obtained sequences with NCBI, isolated strains were identified as *X. translucens*, confirming the seed masses' contamination.

LAMP assay reaction

DNA extracted from infected seed masses was used to evaluate the function of the designed primers (Table 1) in performing LAMP reactions. The gene locations used to design the primers are shown in Fig. 1 and Fig. 2. In addition, to investigate the possible difference between the efficiency of the primers designed in this study and the ones designed by Langlois *et al*. in 2017, both of them examined on the Iranian strains Fig. 3 and Fig. 4, respectively.

Table 1 Sequences of the primers used in the LAMP assay.

Gene	[a]Primers	Sequence (5'-3')	Reference
Xt-CLS	FIP	AGCCAGATTGGCTTGCCTGCGATGAGGTGGCGCATTGG	Langlois *et al*. 2017
	BIP	TGCAAGACAAATCTTCGTGCGCGTAGACAACTGCGCTTCCG	
	F3	AACGAGCGAAGCCGTATG	
	B3	GCATCCAACTTGGCTACAGT	
rpoD	FIP	GTGGCTTCCTGCTCGGAGACGCACTGAAGCGCAAGCAGAA	This study
	BIP	GACCTACCTGAGCCTGACCGACCTTGGCTTCGCCATAGG	
	F3	CCAACGTGGCCTGGGT	
	B3	GGCCTCGACCATTTCCTTCT	
	LF	TCTGATCCTTGACGTCGCG	
	LB	GCTGAAGGAAATCAGCCGC	
dnaK	FIP	TATAGCGGGCCTGCTCGCGTTCAACCGCCGAAGACAACC	This study
	BIP	ACAAGTCGCTGGCCAAGTTCGCGAAGGACACCTCCACCT	
	F3	AGGCCTCGCAGACCTT	
	B3	GCCGTTGGCGTCGATGT	
	LF	TGCAGCACGTGCACGGT	

[a] FIP = forward inner primer, BIP = backward inner primer, F3 = forward outer primer, B3 = backward outer primer, LF= Loop forward outer, and LB= Loop backward outer.

Figure 1 Position of the primers used for LAMP on *rpoD* gene.

Figure 2 Position of the Oligonucleotide primers used for LAMP on *dnaK* gene.

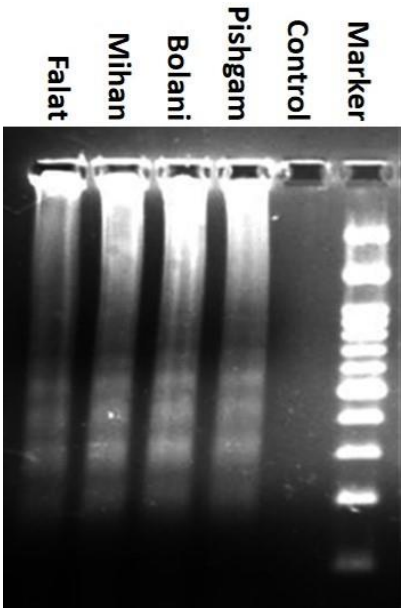

Figure 3 Amplification of LAMP in infected seed masses of Lorestan (Falat, Mihan, and Bolani cultivars) and Kurdistan provinces (Pishgam variety), using primers designed based on the *rpoD* gene. Control: negative control (healthy seed mass), Marker: 100 bp Plus DNA ladder (BioFACTTM). Positive and negative samples on 2% agarose gel.

LAMP assay specificity

The specificity evaluation of the designed primers (based on the *rpoD* gene) showed 100% matches with *X. translucens* DNA, but they did not react with any of the *Pseudomonas syringe* pv. *syringe; Pseudomonas syringe* pv. *atrophaciens*; *Clavibacter tessellarius* bacteria (Fig. 5).

LAMP assay sensitivity

The sensitivity assay was performed based on serial dilution. The lowest detection limit by the LAMP reaction was 10^{-7} in the serial dilution, which contained 3.5 x 10^{2} bacterial cells of *X. translucens* in one milliliter (Fig. 6 a, b).

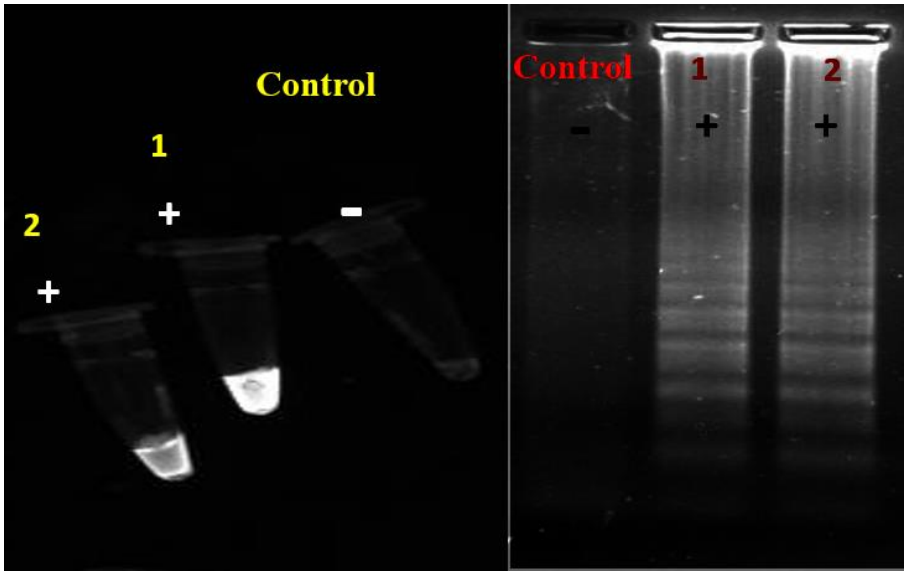

Figure 4 LAMP reaction in infected seed masses. Samples one (Cultivar of Flat in Lorestan) and two (Cultivar of Pishgam in Kurdistan) using primers designed by Langlois *et al*. a) Positive and negative samples in 2% agarose gel. b) Positive and negative samples stained with ethidium bromide under UV light.

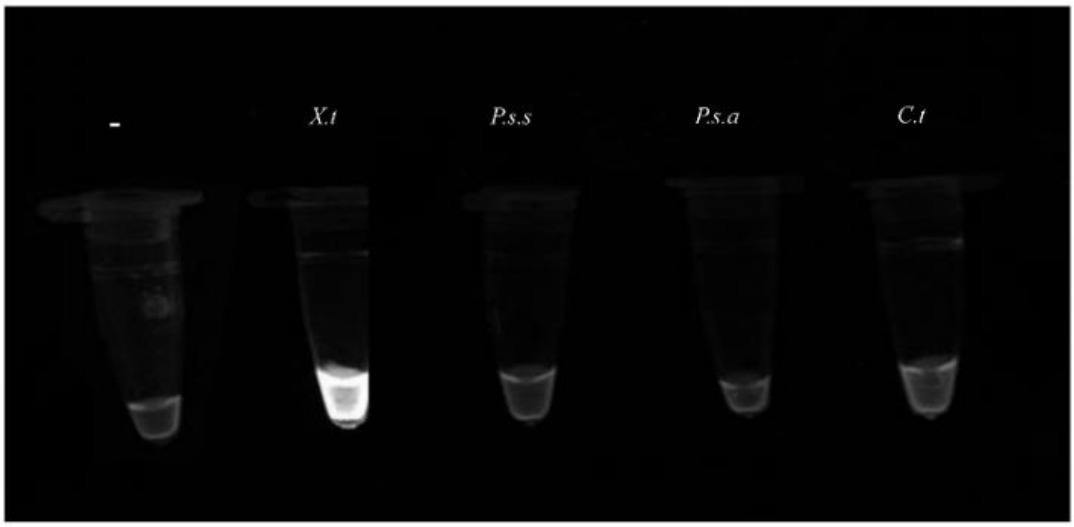

Figure 5 Specificity reaction of designed primers (based on *rpoD* gene) using different genera of wheat pathogenic bacteria (*X. translucens* (*X.t*); *P. syringe* pv. *syringe* (*P.s.s*); *P. syringe* pv. *atrofaciens* (*P.s.a*); *C. tessellarius* (*C.t*)). Positive and negative samples were stained with ethidium bromide under UV light.

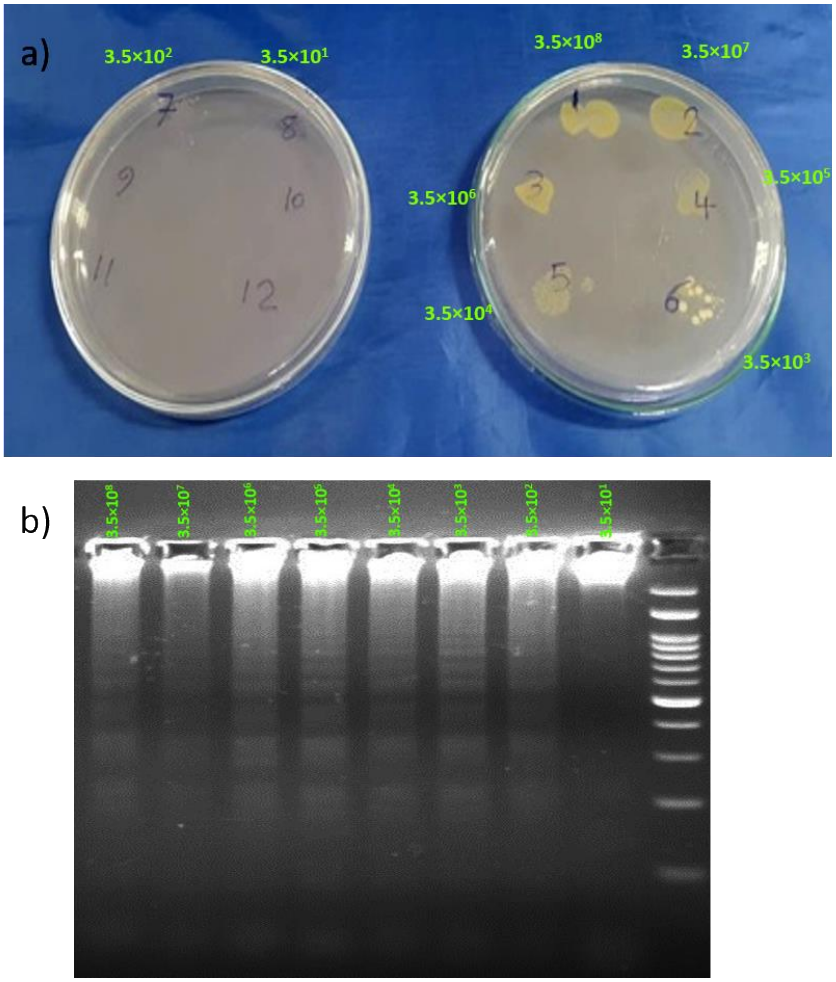

Figure 6 LAMP sensitivity assay. a) Serial dilution of *X. translucens* cultured on NAY medium. b) LAMP reaction of different dilutions of *X. translucens* detected by LAMP assay.

Discussion

Preparing healthy wheat seeds is one of the most important management solutions for seed-borne diseases. Common methods of detecting infected and healthy seeds are very time-consuming and lack the efficiency to quickly and accurately detect on a large scale. LAMP is one of the sensitive molecular techniques for detecting plant pathogens with minimal requirements. In 2017, researchers were able to use primers designed for different protected regions of the genome, all of which could reproduce different *X. translucens* pathovars using the LAMP method. The genes included *in the ina-Xt* gene reproduce all pathovars except graminis. *The gyrB-Xt* gene replicated all the pathovars. *Xt-CLS* gene that reproduces undulosa, translucens (hordei), and secalis pathovars. The *Xt-Poae* gene propagated the pathovar of poae and finally, the *Xt-Cerealis* gene reproduced the cerealis pathovar (Langlois *et al*., 2017).

In Iran, diagnostic kits (based on LAMP) have not been used to detect and manage wheat bacterial leaf streak. In this research, due to the lack of effective chemical solutions to control the disease and the necessity of preparing healthy seeds, the lamp-based diagnostic kit (LAMP) was used to rapidly detect *X. translucens* causal agent of wheat bacterial leaf streak disease.

This research used phenotypic, biochemical, and molecular diagnostic methods based on PCR and LAMP. Among the LAMP primers designed based on the housekeeping gene sequences of two new genes, *rpoD* and *dnaK*, the primers designed using the *rpoD* gene were the most effective, which is probably due to the lack of LB (Backward-side Loop) primer in *dnaK* gene. Therefore, *rpoD* gene primers were selected to perform experiments. The efficiency of primers was designed based on *the rpoD* gene compared with the primers used by Langlois in 2017. The results showed that primers designed in this study have good efficiency and sensitivity. Therefore, based on the results, the diagnostic kit can detect up to 3.5×10^2 bacterial cells of *X. translucens* in one milliliter.

The comparison of conventional methods with LAMP showed that using those methodes takes a long time (Notomi *et al*., 2000; Saharan *et al*., 2014) and needs to have a large number of bacteria in one milliliter of the sample. In addition, laboratory equipment is expensive and needs experts, but the LAMP method has minimized the disadvantages of those methods. LAMP is also very effective in identifying other human and plant pathogenic bacteria (Karami *et al*., 2012). Aslani *et al*, 2017 used this method in olive tree screening against fungal wilt (Verticillium) disease. In other research, detection of Potato Leafroll Virus by reverse transcription- LAMP assay was used (Almasi *et al*., 2013).

Providing the above diagnostic kit in research institutions and stations, wheat seed purchase centers, farms, and quarantine sites at the lowest cost and without the need for expensive equipment such as thermalcycler (PCR machine) can significantly prevent the spread of the bacteria in the country and can help prepare a healthy seed mass to cultivate by screening of seeds against possible seed-borne pathogens.

Conflict of Interests

The authors declare that they have no conflict of interest.

Authors' contribution

This research paper was accomplished with the collaboration of all authors. Fahimeh Nazari performed the experiments, designed the study, and wrote the manuscript. Abolghasem Ghasemi performed the analysis. Hossein Jafary was the advisor and provided consultation.

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تشخیص سریع باکتری لکه‌نواری در بذور گندم به روش تکثیر هم‌دما به‌واسطه حلقه (LAMP)

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چکیده: بیماري نواري باکتریایي از مهم‌ترین بیماري‌هاي بذرزاد غلات به‌خصوص گندم و جو می‌باشد. اگرچه این بیماری در سال‌های اخیر در کشور گسترش زیادی داشته اما تاکنون روشی سریع و مقرون به‌صرفه از لحاظ اقتصادی برای شناسایی مولکولی و دقیق عامل بیماری در کشور معرفی نشده است. شناسایی سریع و دقیق باکتری عامل بیماری در توده‌های بذری نقش مهمی در مدیریت بیماری دارد. در این تحقیق، توسعه کیت تشخیصی باکتری عامل بیماری نواری گندم مبتنی بر LAMP (Loop Mediated Isothermal Amplification) با طراحی آغازگرهای اختصاصی براساس دو ژن جدید خانه‌داری *rpoD* و *dnaK* (قبلاً ژن‌های فوق در لمپ استفاده نشدند) انجام گرفت. به‌منظور تعیین حد تشخیص پرایمرهای طراحی شده از روش سریال رقت استفاده شد. اعتبارسنجی کارایی کیت تشخیصی با جمع‌آوری توده‌های بذری تجاری گندم از مناطق مختلف کشور و هم‌چنین استفاده از کشت خالص باکتری فوق مورد ارزیابی قرار گرفت. نتایج نشان داد آغازگرهای طراحی شده براساس ژن *rpoD* بیش‌ترین کارایی را داشتند. این آغازگرها قادر به ردیابی و تشخیص 3.5×10^2 سلول باکتری در یک میلی‌لیتر بودند. تعیین اختصاصیت پرایمرها از طریق انجام واکنش LAMP روی جنس‌های مختلف باکتری‌های بیماری‌زای گندم اثبات گردید. نتایج این پروژه کمک زیادی به ردیابی و شناسایی سریع عامل بیماری لکه‌نواری باکتریایی در بذور گندم نموده و می‌تواند به‌عنوان یکی از راهکارهای مدیریتی در تهیه بذور سالم مورد استفاده قرار گیرد.

واژگان کلیدي: بیماری باکتریایی نواری گندم، کیت، LAMP