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Original Research

Development of a loop-mediated isothermal amplification assay for rapid detection of *Burkholderia mallei*

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Abstract: The present study was conducted to establish a Loop-mediated isothermal amplification (LAMP) technique for the rapid detection of *B. mallei* the etiologic agent of glanders, a highly contagious disease of equines. A set of six specific primers targeting integrase gene cluster were designed for the LAMP test. The reaction was optimized using different temperatures and time intervals. The specificity of the assay was evaluated using DNA from *B.pseudomallei* and *Pseudomonas aeruginosa.* The LAMP products were analyzed both visually and under UV light after electrophoresis. The optimized conditions were found to be at 63ºC for 60 min. The assay showed high specificity and sensitivity. It was concluded that the established LAMP assay is a rapid, sensitive and practical tool for detection of *B. mallei* and early diagnosis of glanders.

Key words: Glanders, identification, Burkholderia mallei, LAMP.

Introduction

The genus *Burkholderia* is composed of more than 20 valid species, most of which are environmental bacteria. Three important pathogens of this group are *B. mallei*, *B. pseudomallei* and *B. cepacia*. *B.mallei* is a Gram-negative, non-motile aerobic rod-shaped bacterium. It is an etiologic agent of glanders, a highly contagious disease of equines (1). Glanders is considered as an important endemic disease of animals in Africa, Asia, the Middle East and Central and South America (2). Other animals such as goats, sheep, camelids, cats, dogs, and various zoo carnivores are likely to be affected (3). There is no environmental reservoir for bacteria outside natural hosts. Infection is mainly occurs through ingestion of contaminated feed or water from infected animals (4). *B.mallei* presents in nasal discharge, granulomas and ulcers in the upper and lower respiratory tract (glanders) and skin lesions (farcy) due to lymphangitis and lymphadenitis (5). Although human infection is rare, certain groups such as veterinarians, horse handlers and laboratory workers are at risk of exposure (6). Transmission occurs through skin abrasions and mucosal contact. Untreated cases lead to a life-threatening stage with mortality rate of 95% (7). There are reports of intentional use of both *B. mallei* and *B. pseudomallei* as bioterrorism agents. They are classified as Category B bio threat agents by CDC (8).

The diagnosis of *B. mallei* infection is based on cultural, serological and molecular assays. Growth on standard blood culture occurs in less than 5 days (9). The *B. mallei* and *B. pseudomallei* genomes show high homology, making their differentiation very difficult. Fortunately, molecular assays with high clinical accuracy based on *Burkholderia* DNA extraction for specific detection of *B. mallei* and *B. pseudomallei* (10, 11, and 12) are developed in the recent years. However, these methods have some limitations such as high cost and need of specialized equipment especially in developing countries where the disease remains endemic.

Loop-mediated isothermal amplification (LAMP) assay, a technique that amplifies specific sequences of DNA under isothermal conditions, is considered as a simple and low-cost alternative for molecular diagnosis (13). The technique employs a set of four to six primers to anneal several regions of genome as target genes providing high specificity and efficiency (14). Bst and Bsm DNA polymerase are the required enzymes for LAMP that doesn't possess 5'-3' exonuclease activity (15). In less than one hour, high amount of stem-loop DNA with several inverted repeats of the target and cauliflower-like structures are produced at the end (16). The results could be interpreted visually by naked eye or using spectrophotometer after accumulation of by-products. The technique has been used successfully for identification of several pathogenic bacteria (17, 18, and 19). The purpose of the present study was to develop a LAMP assay for detection of *B. mallei* that amplifies a region in the integrase gene.

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Materials and Methods

Bacterial strains and DNA extraction

LAMP was initially developed using *B. mallei* standard strain (ATCC 23344) and one clinical isolate obtained from a case of equine strangles and validated in the laboratory using standard strain *B. pseudomallei* and *P. aeroginosa* obtained from Razi Institute of Iran. DNA extraction was performed using the method described previously (20). A loopful of culture was dissolved in a microtube containing 400 µl TE buffer (10 mM Tris/HCL (pH 8.0), 1mM EDTA). The mixture was heated at 95ºC for 10 min., centrifuged at 12000 ×g for 15 min. and the supernatant was used as DNA template.

Purity of extracted DNA was investigated based on measurement of OD260/280 nm using NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., USA). The quality of extracted DNA was determined using agarose (Sigma-Aldrich, Germany) gel electrophoresis (1% w/v in TBE buffer) stained with ethidiume bromide (Sigma, Germany) and analyzed under UV.

Primer design

The forward outer primer (F3), backward outer primer (B3), forward internal primer (FIP), backward internal primer (BIP) and loop backward primers (LB and LF) listed in Table 1 for the LAMP test were designed by targeting the integrase gene (Gene Bank accession number:gb|CP008732.2, Locus:748799-749038). All of primers were designed using the Primer Explorer (http://PrimerExplorer.jp/e/v3_manual/In. Primers were synthesized by CinnaGen (Tehran, Iran).

Polymerase chain reaction

A gradient PCR assay was carried out using F3 and B3 primers at different annealing temperatures (54-62ºC) to determine the optimum temperature for primers annealing. The reactions were conducted with a total volume of 25 µl, containing 12.5 µl of 10 X PCR master mix, 11 µl of distilled water, 0.25 µl of each B3 and F3 and 1µl of template DNA extracted from standard strain of *B.mallei*. Amplification was performed using a thermocycler (Eppendorf, Germany) with the following conditions: 5 min at 94ºC, 34 cycles of 30s at 94 ºC, 30 Sec at (54-62ºC), 30 Sec at 72ºC and final extension at 72 ºC for 5 min. The PCR products were electrophoresed on 2% agarose gel.

Optimization of LAMP assay and LAMP reaction

The optimization of LAMP reaction was performed using different concentrations of the template DNA (22, 54, 82, 146, 204, 221, 262, 316 and 367 ng/µl), different amplification temperatures (63ºC and 65ºC) and time intervals (30, 60, 90, 120, 150, 180 and 240 min). Optimal reaction mix with final volume of 25µl was prepared as follows: 0.5 µl of F3 and B3 20 pmol (each), 1 µl of FIP and BIP 20 pmol (each), 0.25 µl of each loop primers 20pmol, 0.25 µl of Betaine 5M, 1.5 µl $MgSo_4$50 mM, 2 U Bst DNA polymerase large fragment (New England Biolabs Inc., USA), 2.5 µl of Thermobuffer 10x, 13.75 µl distilled water and 1.5 µl of dNTP 10 mM. Final mixture was incubated at63ºCin a thermoblock for 60 min. Color change of LAMP products were directly observed visually and also after adding 2.0 µl of 10 fold diluted SYBER Green I (Sigma-Aldrich) stain in the reaction tube by naked eye. The product was also run on 2% agarose gel (21).

Analysis of LAMP products and conventional PCR assays

Color change of lamp products were directly observed visually and also after adding 2.0 µl of 10 fold diluted SYBER Green I (Sigma-Aldrich) stain in the reaction tube by naked eye. The solution turned green in the presence of LAMP amplicon, while it remained orange with no amplification. For further confirmation, some of the amplified products were also detected by 2% agarose gel electrophoresis (21).

Two sets of conventional PCR assays were performed to verify the extracted DNA of *B. mallei* using F3/B3 and Bma-IS407 primers (Table1). Thermocycler conditions for both reactions consisted of: 94ºC for 5min, 35 cycles at 94ºC for 30s, 65ºC for 30s, 72ºC for 60s and final extension at 72ºC for 7min (22). The PCR products were subjected to electrophoresis on 2% agarose gel.

Specificity of LAMP primers

In order to evaluate the specificity of LAMP assay, standard strains of *B. pseudomallei* and *P. aeruginosa* from Razi Institute of Iran were used.

Results

Quality and purity of the extracted DNA

The results of DNA electrophoresis is shown in figure 1. The DNA was intact without any structural fracture.

Gradient polymerase chain reaction

To determine the optimum temperature a PCR using F3 and B3 was conducted at six different temperatures (54, 55, 56, 58, 60 and 62ºC). Maximum amplification was obtained at 58 ºC; therefore, the optimal temperature for PCR assay was established at 58°C and applied for all the subsequent runs (Figure 2). The obtained am-

Table1. Primers used in the LAMP assay and Bma-IS407 primers.

Primer	Sequence (5'->3')	gene
B3	GCG TAC TGG AAT ACG GCG	integrase
F3	TTC GTT CGA GGC AGT TGC	Integrase
BIP	AGC ATC GAA GCA CCG GAA ATC CTT TTC AGT CGC TTGGCG GTT TC	integrase
FIP	TGC ATC GAT CTC CAG CGA GTG TTT TGA CAA TCA GCG CGT AGG ATG	integrase
LF	GAC TTT CTC AGC GTA GGT C	integrase
LB	CCG CGC AAT CGA AGC ACG GG	integrase
IS407 F	5'-TCA-GGT-TTG-TAT-GTC-GCT-CGG-3'	Is407
IS407 R	5'-CTA-GGT-GAA-GCT-CTG-CGC-GAG-3'	Is407

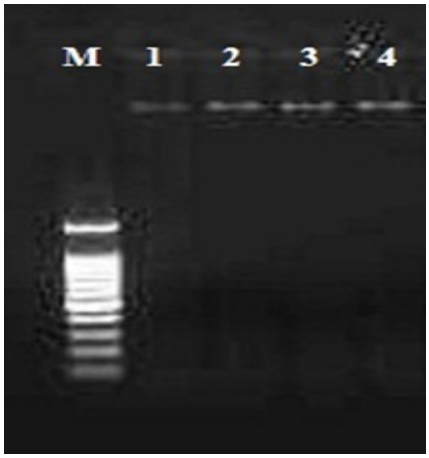

Figure 1. Electrophoresis of extracted DNA on 1% agarose gel. Lane M, 100 bp DNA marker (CinnaGen, Iran); Lanes 1-4, extracted DNA.

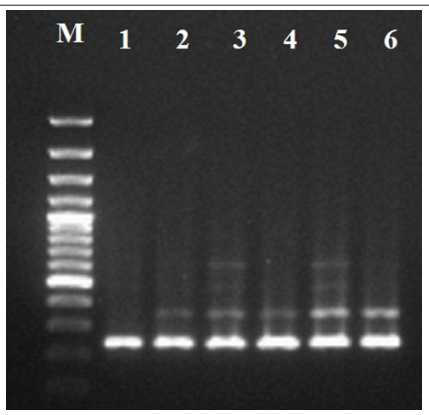

Figure 2. Electrophoresis of amplified product of gradient PCR assay at different temperatures on 2% agarose gel. Lane M, 100bp DNA marker; Lane 1: 54°C, Lane 2: 55°C; Lane 3: 56°C; Lane 4: 58°C; Lane 5: 60°C; Lane 6: 62°C.

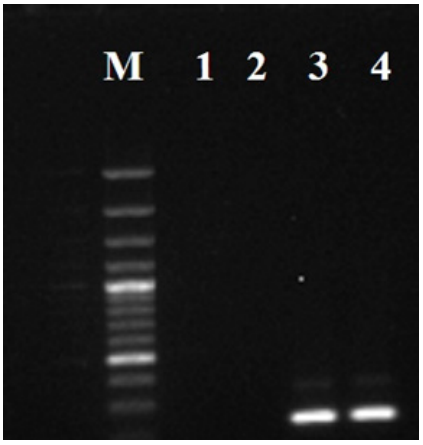

Figure 3. Detection *of B. mallei* strains using LAMP outer primers. Lane M, 100 bp DNA marker; Lane 1:*B. pseudomallei*; Lane 2: *P. aeruginosa*; Lane 3: *B. mallei*, clinical isolates; Lane 4: *B. mallei* ATCC 23344 , Lane 5: negative control.

plicon was at 240 bp.

Specificity of LAMP assay

The specificity of the LAMP assay was evaluated using DNAs from *B. pseudomallei* and *P.aeruginosa.* There was no amplification using the above mentioned bacteria (Figure 3).

Optimization of LAMP assay

Different incubation times (30, 60, 90, 120, 150, 180, and 240) were evaluated to optimize the assay. Although amplification was observed in all the above-mentioned times, the best result was obtained at 60 min (Figure 4). Different concentrations of template DNA were tested to obtain the best result (Figure 5). For LAMP assay the best result was obtained at 22 ng/µl.

Turbidity evaluation in the reaction tube for the control and B.mallei samples was performed by the naked eye (Figure 6 and 7).

Discussion

In the present study, loop-mediated isothermal am-

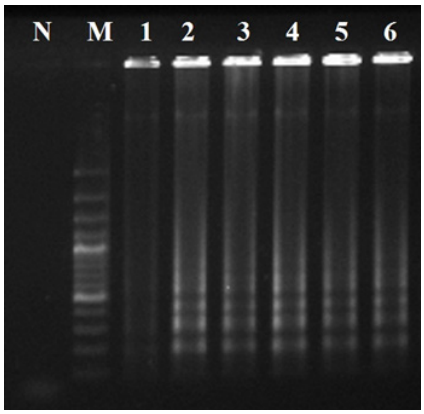

Figure 4. Electrophoresis of amplified product of LAMP assay at different temperatures on 2% agarose gel. Lane M: 100 bp DNA marker; Lane 1: LAMP reaction within 30 min; Lane 2: LAMP reaction within 60 min; Lane 3: LAMP reaction within 90 min; Lane 4: LAMP reaction within 120 min; Lane5: LAMP reaction within 150 min; Lane 6: LAMP reaction within 180 min; Lane N: Negative control.

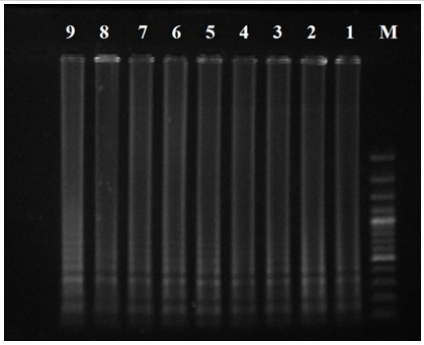

Figure 5. Agarose gel electrophoresis of LAMP amplified product at different DNA concentrations. Lane M: DNA marker; Lane 1-9 concentration of DNA is 367, 316, 262, 221, 204, 146, 82, 54 and 22 ng/µl, respectively.

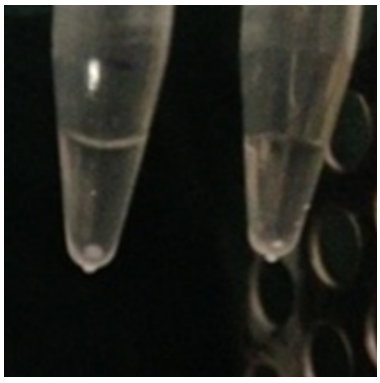

Figure 6. LAMP products detected by turbidity change. Left tube: positive reaction and right tube: negative reaction.

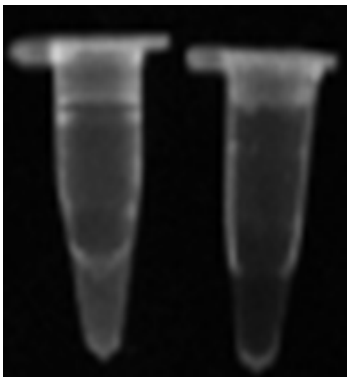

Figure 6. LAMP products detected by fluorescence. Left tube: positive reaction and right tube: negative reaction.

plification (LAMP) assay was developed for the detection of *B. mallei*. Glanders is a severe zoonotic disease, posing a potential risk for laboratory personnel who handle *B.mallei*. Working with *B. mallei* requires level 3 biosafety facilities and skilled technicians. Furthermore the disease has economic impact on the international trade of animals and their by-products (23).

Isolation and identification of bacteria from the clinical samples remains the gold standard for diagnosis of glanders.However, the method is time consuming and not applicable for large-scale screening. Serological tests are limited in sensitivity and there are cross-reactions with *B. pseudomallei*. Most molecular methods are sensitive but require specialized equipment (24).

Loop-mediated isothermal amplification is a feasible cost-effective alternative for detection of many pathogens. The amplification products are stem-loop DNA structure with several inverted repeats and cauliflower-like structure with multiple loops (14). It is applicable to laboratory and field analysis of samples. The major challenges of LAMP method are complexity of the primer designing and using specialized software (25).

In the present study the specificity of the LAMP assay was investigated using *B. pseudomallei* and *P.aeruginosa*. No amplification product was observed using *non-B.mallei* strains. Although the disease is eradicated in many countries, but the outbreaks still occurs. It is known that the presence of pre-symptomatic or carrier animals in different countries is one of the predisposing factors for endemicity and outbreaks (24).Therefore the LAMP assay can be a suitable candidate to be used for monitoring the disease at the herd level. Beside bacteria, the technique has been used successfully to detect viral (26, 27) and protozoal (28, 29) diseases of human and animals.

Loop primers are believed to increase the performance and rapidity of LAMP assays through binding to the additional sites that are not accessed by internal primers. In the present study the loop primers were used and the amplification period was decreased to 60 min. The comparison between Real time-LAMP and RT-PCR for detection of hepatitis C virus revealed that RT-LAMP method is 10-fold more sensitive (30). Moradi et al. (31) used LAMP and PCR techniques for detection of *Salmonella spp*. The result revealed that the LAMP method was 100 fold more sensitive than the PCR. The amplification period decreased from 3 hours in PCR to 90 min in the LAMP assay.

Due to high specificity of LAMP assay, in order to prevent cross contamination and aerosol formation leading to false-positive result (14, 23), the samples should be handled with caution. Color change can be observed at the end of reaction due to accumulation of magnesium pyrophosphate which can be visualized by the naked eye. To facilitate the visualization, adding different reagents such as ethidium bromide, SYBR Green I, and Calcein have been proposed (32). In the present study, naked eye and SYBER green stain were used for visualization. The latter is rapid and more applicable.

The diagnosis of *B. mallei* is difficult especially outside the endemic areas. The results of the present study indicate that our proposed LAMP assay could be implemented as an accurate and cost-effective alternative for the detection of *B. mallei* with high specificity and sensitivity. Considering the advantages of rapid amplification, simple operation and easy detection, LAMP has potential applications for clinical diagnosis as well as surveillance of infectious diseases in developing countries without requiring sophisticated equipment or skilled personnel.

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