Development of a loop-mediated isothermal amplification (LAMP) assay for rapid, simple and sensitive detection of *Mycobacterium avium* subsp. *Paratuberculosis*

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**Key words:** Loop mediated isothermal amplification (LAMP), PCR, Mycobacterium avium subsp. paratuberculosis, Johne’s disease.

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**Abstract**

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) causes Johne’s disease, chronic progressive enteritis in ruminants. The organism has also been isolated from primates, including humans. The disease is economically important in the cattle industry but its control is hampered by the lack of accurate rapid diagnostic tests. Range of diagnostic tests is available, but all have inborn limitations. In the present study, a loop-mediated isothermal amplification (LAMP) assay for the rapid and simple detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was developed. Six primers were specially designed for recognizing eight distinct sequences of insertion sequence 900 (IS900). Optimization of LAMP reaction was performed. Time and temperature conditions for amplification of MAP were optimized as 60 min at 63°C. LAMP produces extremely large amounts of amplified products and enables simple detection methods such as visual judgment by the turbidity or fluorescence of the reaction mixture, which is kept in the reaction tube. The visual detection eliminates the need for time-consuming electrophoresis and costly specialized equipment. The performance of LAMP was compared to that of a highly sensitive Nested PCR. The sensitivity of LAMP assay for detection of MAP was 4 fg and the specificity was 100%. The sensitivity was 1000 times greater than the Nested-PCR. Furthermore, the LAMP assay described in this report is simple to use, inexpensive, highly sensitive, and particularly well suited for the early diagnosis of MAP in less well equipped laboratories and in rural settings where resources are limited.

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Introduction

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the etiological agent of Johne’s disease or paratuberculosis (Castellanos et al., 2012; Whittington, 2009). MAP is a hardy, slow-growing, gram- positive and acid- fast bacterium (Tiwari et al., 2006). A possible association between MAP infection and Crohn’s disease in humans has been also described (Autschbach et al., 2005). Although animals with clinical disease are often culled from the herd, animals with subclinical paratuberculosis may cause economic losses because of reduced milk production and poor reproductive performance. The level of infection in a herd increases over time and if the disease is left unmanaged, the economic effect becomes increasingly significant. Diarrhea and rapid weight loss are the two main symptoms of paratuberculosis. Johne’s disease is usually underreported due to difficulties in diagnosing preclinical cases (Sohal et al., 2007). Conventional diagnostic methods currently used for MAP detection include culture of fecal and tissues and enzyme-linked immunosorbent assay (ELISA). A disadvantage of the conventional culturing is the long incubation (12 to 16 weeks) and specific culture requirements. ELISA can be performed in a few hours, but its sensitivity is estimated at 45% since antibodies may not be detectable until late in infection (Bosward et al., 2010; Fang et al., 2002). Several PCR tests have been developed to detect MAP DNA, but all of these methods are remained to be complex, long and not reliable enough for use in clinical practice (Seyyedin et al., 2010; Thekisoe et al., 2005). Besides, they require the use of electrophoresis to detect amplified products, which is time consuming and tedious. Further, another real-time PCR assay is not routinely used due to the need for an expensive thermal cycler with a fluorescence detector (Aborodey et al. 2012; Alhassan et al. 2007; Yamazaki et al., 2014). Recently loop-mediated isothermal amplification (LAMP), a rapid technique for amplifying DNA has been reported (Jayawardena et al., 2007; Mori and Notom., 2009; Notomi et al., 2000). LAMP employs a minimum of four specially designed primers, including a forward outer primer (F3), a backward outer primer (B3), a forward inner primer (F1P, comprised of two binding domains, F1c and F2), and a backward inner primer (BIP, comprised of two binding domains, B1 and B2c) that, in combination, recognize six specific regions within the target genetic locus. Additional primers, including a forward loop primer (FLP) and a backward loop primer (BLP), are typically optional and may be used to accelerate or enhance the sensitivity of the LAMP assay (Njiru et al., 2008, Saleh et al., 2008; Yang et al., 2010). Predictably, LAMP assays tend to have high specificity, as the amplification occurs only when six specific regions of the target amplicon are recognized by the primers. In addition, LAMP assay does not require expensive equipment to acquire a high level of precision, and there are fewer preparation steps than with conventional PCR. Because of the high amplification efficiency, up to $10^9$ copies of a target can be accumulated in less than 1 h of incubation (Kim et al., 2010; Lin et al., 2012; Parida et al., 2008). The LAMP assay may be monitored by measuring turbidity of magnesium pyrophosphate, a by- product of LAMP, measuring fluorescence using a DNA intercalating dye such as SYBR green or a metal ion-binding fluorophore, such as calcein and/or by color change using a metal ion-binding indicator dye and agarose gel electrophoresis (Barkway et al., 2011; Goto et al., 2009; Lam et al. 2008; Ohtsuki et al; 2008; Hill et al., 2008).

In the present study we attempted to develop an affordable, rapid and reliable LAMP assay based on the amplification of the IS900 for diagnosis of paratuberculosis and evaluated the sensitivity and specificity of the assay, comparing it with Nested-PCR.

Material and methods

Bacterial strains

Bacterial strains used to standardize the LAMP method in the present study were MAP 316F which provided by tuberculosis department at Razi Vaccine & Serum Research Institute of Tehran, Iran. Additional strains including *Mycobacterium bovis* AN5, *Mycobacterium tuberculosis DT* and *Mycobacterium avium avium* were used to determine
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the LAMP specificity.

Clinical sample, Sample processing and DNA extraction
A total of 225 fecal samples were collected from 14 dairy cattle farms of Tehran province. The samples were directly taken from the cow's rectum. Samples were immediately placed in a container with ice packs and transported to the laboratory of tuberculosis, Razi Vaccine & Serum Research Institute, Tehran, Iran. The samples cultured in Harrold's egg yolk medium with and without mycobactin J at 37 °C for 8 to 16 week. DNA was extracted from all samples. About 100-200 mg of fecal samples was used for extraction of DNA by Stool-DNA extraction kit (AccuPrep Bioneer.) according to the manufacturer’s instructions and eluted with 50 ul of the kit-supplied elution buffer.

LAMP Primer designs
A total of four primers including 2 outer primers (F3 and B3) and 2 inner primers (FIP and BIP) were designed using the Lamp primer designing software.

Primer Explorer (http://primerexplorer.jp/elamp3.0.0/index.html) and 2 loop primers (LF and LB) were manually designed based on the insertion sequence of IS900 was obtained from Genbank (Accession number:AF416985), (Chamberlin et al., 2001; Bull et al., 2000). Nucleotide sequences of the functional LAMP primers are shown in table 1.

Optimization of reaction conditions for LAMP
The LAMP assays was carried out in a 25µl of final reaction mixture containing 60 pM each of F3, B3, FIP and BIP, 30 pM each of FLP and BLP, 7.5 mM MgSO4, 40 mM dNTP, 0.8 M Betaine (Sigma Aldrich), 8 U Bst DNA polymerase (New England Biolabs, USA),1µl nuclease-free water and 2.5 µl DNA sample. The LAMP reaction was performed in a thermal mixer (comfort, eppendrf,1.5 ml). For comparison, it was also performed using a conventional thermal cycler and both machines performed well. To find the optimum time and temperature, the reactions were performed at 58 to 68 °C for 15 to 120 min. Finally each reaction was incubated at 63 °C for 60 min. A positive and negative control was included in each run. LAMP products were directly detected with naked eye by forming of white precipitation and adding 2µl of SYBR Green I (Sigma-Aldrich) to the reaction tube and observing the color of the solution under UV transilluminator. Furthermore, the micro tube centrifugation were down to help the detection of primary turbidity by formation button precipitation. In addition, LAMP products were electrophoresed on 2% agarose gel (Invitrogen agarose in lx TBE buffer) with red gel staining and evaluated in UV gel doc.

Nested-PCR
To compare LAMP with PCR, Nested PCR assay was performed for IS900 amplification by 4 primers P90, P91 AV1 and AV2. The primers sequence are following in table1-2 (Burgelt and Williams,. 2004; Naser et al,. 2004). Amplification was carried out in a 25 µL of total volume containing PCR master mix, 5µL DNA sample, 1µl P90, 1 µl P91 and 18 µl nuclease free water for step 1. Then 5 µl of initial amplification products was transferred to new tubes containing PCR master mix, 1 µl AV1, 1 µl AV2 and 18 µl nuclease free water and then re-amplified. The second PCR products were analyzed on a 1% agarose gel containing ethidium bromide and visualized by UV gel doc.

Analytical sensitivity and specificity of the LAMP and Nested -PCR
To determine the sensitivity of the LAMP assay, 10-fold serial dilutions were made from 431 ng/µl MAP stock solution and compared with Nested-PCR results using similar templates at identical concentrations. Detection limit of the LAMP was defined as the last positive dilution and the reactions were performed four times to examine the reproducibility of the test. The specificity of the assay was evaluated by testing three gram-positive bacteria including Mycobacterium bevies AN5, Mycobacterium tuberculosis DT and Mycobacterium avium avium.

Results
**Primer design and optimization of the LAMP assay**

As described in Materials and Methods, the LAMP primers were designed targeting IS900 (GenBank, Accession number: AF416985). LAMP assays were performed with and without the loop primers (FLP and BLP). The results analyzed by agarose gel electrophoresis. In the absence of the loop primers, assays didn’t performed. Therefore, all LAMP assays reported afterwards used the loop primers. LAMP products were detected at 5 different temperatures (58, 60, 63, 65 and 68 °C) with and without heat denaturation. To determine the optimum duration of the LAMP assay, LAMP reactions were set up for 6 different incubation times including 15, 30, 45, 60, 90, 120 min. Then the results analyzed by agarose gel electrophoresis, turbidity and the color change methods. The best results were obtained at 63 °C for 60 min. The positive reaction turned fluorescent after addition of SYBR Green 1 whereas, the negative reaction remained unchanged (Fig. 1).

**Table 1** LAMP primers used for amplification of MAP.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer name</th>
<th>Primer sequence(5’-3’)</th>
<th>Length(nucleotids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS900</td>
<td>B3</td>
<td>CACCTCCGTAACCGTCATTG</td>
<td>20</td>
</tr>
<tr>
<td>IS900</td>
<td>F3</td>
<td>GACGTCGGGTATGGGCTTTCA</td>
<td>20</td>
</tr>
<tr>
<td>IS900</td>
<td>BIP</td>
<td>AGATGGGATCGTGTTGTTTTTCCAGATCAACC</td>
<td>46</td>
</tr>
<tr>
<td>IS900</td>
<td>FIP</td>
<td>ATTAGCGGTCGAGTCGCGTTTTGTGGTATTGTA</td>
<td>45</td>
</tr>
<tr>
<td>IS900</td>
<td>LF</td>
<td>CGCCGGGGCGGCAAATCTCC</td>
<td>19</td>
</tr>
<tr>
<td>IS900</td>
<td>LB</td>
<td>AAGGACACGGTCGGGTGTC</td>
<td>20</td>
</tr>
</tbody>
</table>

Electrophoresis of LAMP amplified products demonstrated typical ladder-like pattern and produced many bands of different sizes (Fig. 2). Furthermore, LAMP results could be evaluated with naked eye, under natural light showing an increase in turbidity which was visible as white turbidity (Fig. 3).

**Table 2.** Nested-PCR primers used for amplification of MAP.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer name</th>
<th>Primer sequence(5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS900</td>
<td>P90</td>
<td>GTT CGG GGC GTG CGT TTA G</td>
</tr>
<tr>
<td>IS900</td>
<td>P91</td>
<td>GAG GTC GAT CGC CCA CTG GA</td>
</tr>
<tr>
<td>IS900</td>
<td>AV1</td>
<td>ATG TGG TTG CTG TGT TGG ATG G</td>
</tr>
<tr>
<td>IS900</td>
<td>AV2</td>
<td>CCG CCG CAA TCA ACT CCA G</td>
</tr>
</tbody>
</table>

**Analytical specificity and sensitivity of the LAMP assay**

The analytical sensitivity and specificity of LAMP assay were tested and compared with the Nested-PCR. LAMP and Nested-PCR correctly detected MAP and so, the specificity of both PCR was 100% in this small study (fig. 4, 5). For determination of sensitivity or the limit of detection (LOD) of MAP, serial dilutions of DNA from MAP 316 F were provided. The detection limit of LAMP assay was 4fg of DNA compared with the detection limit of Nested-PCR 4 pg (fig. 6, 7). The sensitivity was 1000 times greater than the Nested-PCR.

**Diagnostic specificity and sensitivity of the LAMP assay**

The specimens used to determine diagnostic specificity and diagnostic sensitivity included feceses
obtained from 14 dairy cattle farms. The samples were directly taken from the cow's rectum. The samples cultured in Harrold's egg yolk medium with and without mycobactin J at 37 °C for 8 to 16 week. DNA was extracted from these samples and amplified by both LAMP and Nested-PCR. All the samples tested negative by fecal culture except 6 samples which all of them tested positive by lamp sensitivity 100% and only 3 of 6 tested positive by Nested-PCR (sensitivity 50%). Diagnostic specificity of LAMP and Nested-PCR was calculated 79% and 92%, respectively. DNA extracted from these samples was amplified by LAMP and Nested-PCR and the results are summarized in Table 3. These data revealed significant agreement of test results between LAMP and Nested-PCR (Table 4).

Table 3. The results of fecal culture, LAMP and Nested-PCR.

<table>
<thead>
<tr>
<th>Assay</th>
<th>LAMP</th>
<th>Nested-PCR</th>
<th>Fecal culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.pos</td>
<td>52</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>No.neg</td>
<td>173</td>
<td>205</td>
<td>219</td>
</tr>
</tbody>
</table>

Table 4. Agreement of test results between LAMP and Nested-PCR.

<table>
<thead>
<tr>
<th>Agreement</th>
<th>Expected Agreement</th>
<th>Kappa</th>
<th>Std. Err.</th>
<th>Z</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>80.44%</td>
<td>72.11%</td>
<td>0.2989</td>
<td>0.0573</td>
<td>5.21</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

**Discussion**

Effective control of paratuberculosis (an economically important disease in the cattle industry) has hampered due to lack of rapid and accurate diagnostic test (Enosawa et al., 2003). Range of diagnostic tests is available, but all have inborn limitations (shoaal et al., 2007). This necessitates the development of high-throughput, sensitive diagnostic methods for the detection of infected animals and animal products. Accurate diagnostic tests for johne's disease are important in enabling the culling or segregation of infected animals to maintain the economic viability of dairy farming. Bacterial isolation has to date been considered the most reliable test for detecting MAP infection in cattle. Fecal culturing for MAP is technically simple and semi quantitative, detecting as few as 10 CFU/g. in addition; fecal culturing requires little investment in equipment. However, because of the slow growth of MAP, it takes more than 8 weeks for colony formation. This prolonged test period favors the growth of bacterial and fungal contaminants that are able to survive the decontamination process. Several PCR protocols to rapidly detect MAP have been published. However, these methods require several operations, such as an electrophoresis step, and the risk of contamination is very real. recently, a real-time PCR method was develop but, real time requires a special apparatus to monitor the amplification of DNA. Therefore, a rapid diagnostic test for johne's disease has been anxiously awaited. In this study, a rapid and sensitive diagnostic system based on LAMP technology was developed to detect MAP. The LAMP assay requires only a simple heating block to incubate the reaction mixture at 63 °C for 1hr before the reaction products are visualized. So LAMP amplifies DNA under isothermal conditions, can be performed using simple laboratory equipment, such as a regular water bath or heating block for maintaining the temperature at 63 °C and make it more economical and practical than PCR.

**Fig 1.** Visual detection of LAMP reaction under UV light. C+, LAMP product of positive control. C-, negative control.
In addition, LAMP is more effective and rapid than conventional PCR. LAMP has been shown to be tolerant against naturally occurring inhibitors in biological samples (Lee et al., 2010; Gandelman et al., 2011; 15, 17). In Nested PCR, nearly 8hrs (3.5 hrs. for step 1, 3.5 hrs. for step 2 and about 1hr for electrophoresis) is required for the detection and post PCR analysis, while LAMP assay require about 1h. LAMP produces extremely large amounts of amplified products and enables simple detection methods such as visual judgment by the turbidity or fluorescence of the reaction mixture, which is kept in the reaction tube. Turbidity in LAMP reaction mixtures is caused by production of insoluble magnesium pyrophosphate, a by-product of DNA synthesis catalyzed by Bst DNA polymerase. Since the production of magnesium pyrophosphate directly correlates with the amount of DNA synthesized. The visual detection eliminates the need for time-consuming electrophoresis and costly specialized equipment. Owing to the fact that, LAMP assay requires 6 highly specific primers to distinguish regions on the target DNA (IS900), a gene sequence specific to MAP, Designing appropriate primers for LAMP is one of the most important key points in optimizing the LAMP reaction. While, therefore the design of LAMP primers is much more complex than that for conventional PCR, the use of primer designing support software enabled us to design primers. The LAMP primers used in this study were designed using the most commonly used software, Primer Explorer software. As described in Materials and Methods, this software failed to efficiently design all the LAMP primers, and loop primers were designed manually.

An early study of LAMP methods for detection of MAP from culture with for primers had been reported, that a total of 102 primers set amplify the IS900, HSPX and F57 gene sequence of MAP.
In that LAMP assay, more than 2 hrs is required for the detection and it doesn’t have loop primers while our assay requires nearly 1 hr and detect MAP in clinical samples. These observations show this our novel LAMP assay targeting the IS900 gene is more sensitive than the LAMP method used in a previous study. So, our study develops the LAMP assay with newly designed primers that showed specificity and sensitivity in comparison with the LAMP method used in previous study. In conclusion, this LAMP assay compares very favorably with culture and Nested PCR for direct detection of Mycobacterium avium subspecies paratuberculosis in bovine feces and would be useful for rapid diagnosis of paratuberculosis at an early stage of infection and will facilitate management of control program for the disease in cattle industry.

**Fig. 6.** Sensitivity of LAMP reaction. Detection limit (LOD) of the LAMP was 4 fg. Tubes 1 to 10 contained 431 ng, 43 ng, 4 ng, 431 pg, 43 pg, 4 pg, 431 fg, 43 fg and 4 fg, 0 fg and negative control, respectively.

**Fig. 7.** Sensitivity of Nested-PCR. Detection limit of the Nested-PCR was 4 pg. Tubes 1 to 10 contained 431 ng, 43 ng, 4 ng, 431 pg, 43 pg, 4 pg, 431 fg, 43 fg and 4 fg, and negative control, respectively.

**Acknowledgements**

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