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Scientific Research

The Development of a Loop-Mediated Isothermal Amplification (LAMP) Procedure for Plague Diagnostic

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Abstract

Plague caused by *Yersinia pestis* is one of the infectious diseases subject to the International Health Regulations (IHR). Permanent monitoring of the focal plague areas is mandatory in order to enable prompt control measures to prevent the spread of the disease. Therefore, the availability of efficient diagnosis tests is of paramount importance. Here, we describe a loop-mediated isothermal amplification (LAMP)-based procedure for rapid *Y. pestis* detection. We constructed a set of LAMP primers, which were used in assays to establish the reaction conditions that would lead to the quick visualization of the results by evaluating the test tube with the naked eye. The primers were specifically designed to target the *caf*1 gene located on pFra/Tox (pMT), a prototypical plasmid of *Y. pestis.* The LAMP procedure was performed at 65˚C for 45 min in a water bath and allowed for the detection of at least 10 pg of bacterial DNA. Due to its simplicity, specificity, sensitivity and rapidity, the LAMP technique is an additional tool that may be implemented in routine plague diagnoses, especially in emergencies.

Keywords

Plague, *Yersinia pestis*, Diagnosis Tests, Loop-Mediated Isothermal Amplification (LAMP)

1. Introduction

Yersinia pestis is a gram-negative bacterium of the Enterobacteriaceae family and is the causative agent of plague, a zoonotic disease of rodents (reservoirs) and their fleas (vectors), that affects humans and other mammals.

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Depending on the transmission route, the human disease presents in different clinical forms; the most common are bubonic, septicemia and pneumonic plague [1]. Pneumonic plague is a potential public health emergency of international concern (PHEIC); therefore, any pneumonic case must to be immediately reported to the World Health Organization (WHO) [2].

In spite of all the technological advances and research, plague is still not eradicated. Human cases of the disease are reported annually to the WHO in various countries in Africa, Asia, the former Soviet Union and the Americas. The infection remains in natural foci that are strongly associated with people living in poor living conditions, those in extreme poverty. Currently, the WHO considers plague a reemerging disease with a trend of an increasing number of cases with geographical dispersion [3]-[5]. Thus, permanent monitoring of the focal plague areas is mandatory in order to trigger rapid and effective measures to control and prevent the spread of the disease. This requires the availability of adequate laboratory infrastructure, trained staff, and adequate supplies, including efficient diagnostic tests, which are difficult to acquire and use in poor resources countries.

We have previously developed several PCR-based procedures for identifying *Y. pestis* in biological samples [6]-[9]. While effective, these techniques require a high level of technical expertise and the use of expensive equipment for the reactions and visualization of the results.

The loop-mediated isothermal amplification (LAMP) technology, a variation of a PCR reaction, occurs at a constant temperature using the enzyme Bst polymerase and a set of four to six primers: two outer, two inner and two loop primers. The primers are specifically designed to amplify six to eight regions in the targeted gene. The outer primers participate in strand displacement during a non-cyclic step. The internal primers participate in the formation of a loop. The loop primers are optional and serve to accelerate the amplification reaction by binding to additional sites that are not accessed by the internal primers [10] [11].

The LAMP technology is quite simple, fast and inexpensive. It can be carried out in a water bath, and the results can be visualized directly through the test tube with the naked eye by observing the change of the reaction mix's turbidity caused by magnesium pyrophosphate accumulation in proportion to the amplified products [12] [13]. Eventually, the addition of chromogenic products may be necessary if the LAMP products cannot be visualized under natural light [14].

The aim of this study was to test the potential of LAMP technology for detection of *Y. pestis* for subsequent implementation in the diagnosis of plague. In this work, we constructed a set of primers that were specifically designed to target the *caf*1 gene. The *caf*1 gene, which is specific to *Y. pestis*, is located on the pFra/Tox plasmid which is also called pMT and is a prototypical *Y. pestis* plasmid [15] [16]. Many of the plague diagnostic techniques target this plasmid gene or detect its products [17]. The primers were used in assays to establish the reaction conditions. Amplification at 65˚C for 45 min allowed for the detection of at least 10 pg of *Y. pestis* DNA. Due to its specificity, sensitivity, rapidity and low cost, this LAMP procedure is an additional tool that may be implemented in the routine of plague diagnoses, especially in emergencies.

2. Experimental Procedures

2.1. Bacterial Strains and Genomic DNA Extraction

This study used the *Y. pestis* reference strain A1122 and the Brazilian strains P.Exu 369, P.Exu 390 and P.CE 882, *Y. pseudotuberculosis* IP32953, and one strain each of *Vibrio cholerae* (#462), *Listeria monocytogenes* (#226) and *Pseudomonas aeruginosa* (#127) kindly provided by the Fiocruz-CYP, Institut Pasteur Paris and Laboratório de Zoonoses Bacterianas, Instituto Oswaldo Cruz (IOC/FIOCRUZ) collections. The culture conditions and genomic DNA extraction were performed as previously described [9] [17]-[19].

2.2. Construction of the LAMP Primers

The LAMP primers were built based on the analysis of sequences of the *caf*1 gene from the reference strain (*Y. pestis* A1122) and the three Brazilian strains from different geographical, temporal and source origins (P.Exu 369, P.Exu 390 and P.CE 882). The *caf*1 gene is located on the pFra/Tox plasmid (pMT) [15] [16]. Many of the plague diagnostic techniques target this plasmid gene or detect its products [17].

2.2.1. Amplification of the *caf*1 Gene from *Y. pestis* Strains by Conventional PCR (C-PCR)

Y. pestis DNA from the strains A1122, P.Exu 369, P.Exu 390 and P.CE 882 was used to amplify a 513 bp (base pair) segment of the *caf*1 gene [15] by C-PCR using the method outlined by Leal *et al.* [6]. The reactions were

made in 25 µL of the reaction mixture composed of 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 200 mM dNTP, 20 pmol of each primer (F1F: CAGGGATCCATGAAAAAAATCAGTTC and F1R: GGGCTCGAGTTGGTTAGATACGGTTA), 20 ng DNA, 1 U Taq DNA polymerase. The thermocycler conditions, which used a thermocycler (Biometra) included 3 min at 94˚C and 30 cycles for 1 min at 94˚C, 1 min at 55˚C, 1 min at 72˚C, and 7 min at 72˚C. Then, 5 µL of each of the PCR products was mixed with 5 µL of the sample buffer (0.25% bromophenol blue and 30% glycerol in water) and electrophoresed in 1% agarose gels plus 10 µL of SYBR® Safe (Invitrogen) in 0.5% TBE at room temperature (RT) at 100 V-150 mA for 1 hour. The gels were observed under ultraviolet (UV) light and digitalized using the Kodak 1D Image Analysis Software, Version 3.5 (Digital Kodak Science). Each PCR run included a negative control without DNA.

2.2.2. Purification of the C-PCR Products

To 40 µL aliquots of PCR products from each *Y. pestis* strain, 4 µL of 3 M NaCl and 100 µL of ethanol was added and incubated overnight at −20˚C. The tubes were centrifuged at 20,000 × g for 10 min, 420 mL of 70% ethanol was added to the precipitate, re-centrifuged at 20,000 × g for 10 min and the remaining pellet was suspended in 30 µL of milli-Q water.

2.2.3. Sequencing of the Purified C-PCR Products

Purified C-PCR products were sequenced in an ABI Prism 3100 Genetic Analyzer (Applied Biosytems®) by the Sanger method [20]. The sequences were analyzed by SeqMan™ and EditSeq™ (Lasergene®, Version 4.01, DNASTAR Inc., Madison, WI). A comparison with the *caf*1 sequence (accession number X61996.1) from the National Center for Biotechnology Information (NCBI) revealed a 100% identity between the database sequence and the products. The BLAST (Basic Local Alignment Search Tool) analysis confirmed a high similarity with the other 26 *caf*1 sequences available at the NCBI.

2.2.4. Design of the LAMP Primers

Based on the sequences from C-PCR products, a set of five primers [two outer: forward (F3), backward (B3); two inner: forward (FIP), backward (BPI), and one backward loop primer (BLP)] were designed for the LAMP reaction (**Table 1**). The LAMP primers were designed using the Primer Explorer V4 software (http://primerexplorer.jp/) (Eiken Chemical Co., Ltd.). The identification of the hybridizing sites was determined through MapDraw (Lasergene®, Version 4.01, DNASTAR Inc., Madison, WI). The critical parameters [10], the GC (guanine-cytosine) content, Tm (melting temperature), stability of the final sequence of the primers, secondary structure formation and distance between primers, were analyzed. Integrated DNA Technologies (IDT) synthetized the LAMP primers. The use of HPLC (High-Performance Liquid Chromatography) purified internal primers is crucial for the production of LAMP primers [21]. Two sets of internal primers (FIP, BPI, **Table 1**) were produced and purified by HPLC and desalination, respectively.

2.3. Quality Assessment of the Outer Primers (F3, B3)

The quality of the F3 and B3 primers (**Table 1**) was assessed by C-PCR as described in Section 2.2.1 using 20 ng DNA from two *Y. pestis* cultures. For further confirmation, the products were purified and sequenced as described in Sections 2.2.2 and 2.2.3, respectively.

Assessment of the Hybridization Temperature of the Outer Primers (F3, B3)

The hybridization temperature of the F3 and B3 primers (**Table 1**) was assessed by C-PCR as described in

Table 1. LAMP primers for the *caf*1 gene of *Yersinia pestis*.

Primers	Sequences
Forward outer primer (F3)	TCAGGATGGAAATAACCACCAA
Backward outer primer (B3)	GTTACGGTTACAGCATCAGTGTA
Forward inner primer (FIP)	CCACAAGGTTCTCACCGTTTACCTTCACTACAAAAGTGATTGGCAAGG
Backward inner primer (BIP)	GGATGACGTCGTCTTGGCTACGTGCAAGTTTACCGCCTTTGG
Backward loop primer (BLP)	GCAGCCAGGATTTCTTTGTTCGC

Section 2.2.1 using 20 ng of *Y. pestis* DNA at a temperature gradient of 55˚C to 65˚C (55˚C - 55.2˚C - 55.8˚C - 56.7˚C - 57.8˚C - 59.1˚C - 60.4˚C - 61.7˚C - 62.9˚C - 63.9˚C - 64.6˚C - 65˚C).

2.4. Assessment of the Inner Primers (FIP, BPI) Quality

The two sets of internal primers (FIP, BPI, **Table 1**) that were purified by either HPLC or desalination were used in comparative LAMP assays for efficacy at 65˚C for 90 min.

2.5. Determination of the LAMP Reaction Time

To determine the minimum incubation time for visualization of the amplified products, assays were carried out at increasing incubation times (15, 30, 45, 60, 75 and 90 min) at 65˚C using a thermocycler (Biometra) and a water bath in parallel. Reactions were performed with 20 ng of *Y. pestis* A1122 DNA, the outer primers (F3, B3), the inner primers (FIP, BPI) and with or without the backward loop primer (BLP).

2.6. Determination of Amplification Temperature for LAMP

The amplification temperature for the LAMP was determined with assays performed for 60˚C, 63˚C and 65˚C after 90 min using a thermocycler (Biometra) and a water bath in parallel. Reactions were performed with 20 ng of *Y. pestis* A1122 DNA, the outer primers (F3, B3), the inner primers (FIP, BPI) and with or without the backward loop primer (BLP).

2.7. Visualization of LAMP Products

To evaluate the best mode of visualization, the products were stained by addition of the fluorescent dyes (1 µL:10 µL). SYBR® Safe (Invitrogen) was used for the inspection with the naked eye, and SYBR® Green (Promega) was used for the inspection under UV. For comparison, the products were analyzed under UV light after electrophoresis was performed on 1% agarose gels and staining the gels with SYBR® Green (Promega) as described in Section 2.2.1.

2.8. Optimization of the LAMP Reaction Components

The components of the reaction mixture were settled following a protocol based on the procedure described by Parida, *et al.* [10] varying the primers and dNTPs concentrations. The reactions were performed at 65˚C for 90 min using a thermocycler (Biometra) and a water bath in parallel. Each assay included a negative control without DNA. The products were analyzed using electrophoresis and inspected using the naked eye and under UV light, as described in Section 2.7.

2.9. Assessment of LAMP Sensitivity and Specificity

The LAMP sensitivity was determined in parallel assays with C-PCR using serial dilutions (10 ng, 1 ng, 100 pg, 10 pg and 1 pg) of *Y. pestis* A1122 DNA. To determine the specificity of the reactions, the assays were performed in parallel with C-PCR using 20 ng DNA of the strains *Y. pestis* A1122, *Y. pseudotuberculosis* IP32953, *Vibrio cholerae* (#462), *Listeria monocytogenes* (#226) and *Pseudomonas aeruginosa* (#127). All of the reactions included the outer primers (F3, B3), the inner primers (FIP, BPI) and the backward loop primer (BLP) and were performed at 65˚C for 90 min using a thermocycler (Biometra) and a water bath in parallel. The C-PCR was performed as described in Section 2.2.1. The products were analyzed with electrophoresis and inspected by the naked eye and under UV light, as described in Section 2.7.

3. Results

3.1. Assessment of the Outer Primers' (F3, B3) Quality and Hybridizing Temperature

The segment of the expected size (207 bp) was amplified in the C-PCR reactions that were carried out in the listed temperatures between 55˚C and 65˚C (**Figure 1**). Aiming for a higher stringency, the 65˚C temperature was adopted for the LAMP procedure.

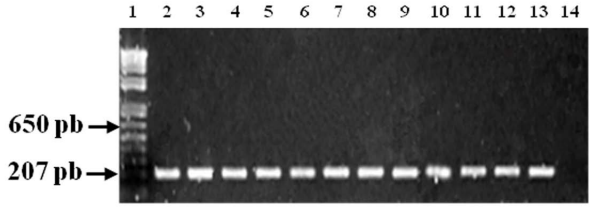

Figure 1. 1% agarose gel electrophoresis of the C-PCR-amplified 207 bp segment of the gene *caf*1 with the outer primers (F3 and B3) at a temperature gradient of 55˚C to 65˚C. Lane 1: 1 Kb plus DNA ladder; 2: 55˚C; 3: 55.2˚C; 4: 55.8˚C; 5: 56.7˚C; 6: 57.8˚C; 7: 59.1˚C; 8: 60.4˚C; 9: 61.7˚C; 10: 62.9˚C; 11: 63.9˚C; 12: 64.6˚C; 13: 65˚C; 14: negative control.

3.2. Assessment of the Inner Primers' (FIP, BPI) Quality

Identical results were obtained with *Y. pestis* DNA and the inner (FIP, BPI) desalinized and the HPLC purified primers at 65˚C after 90 min incubation. Because of this and the lower synthesis cost, desalinized primers were adopted.

3.3. Determination of the LAMP Reaction Time

In the reactions with *Y. pestis* DNA and the outer (F3, B3) and inner primers (FIP, BPI), the LAMP products were faintly detectable after 45 min of incubation, and after 60 min of incubation, the LAMP products were clearly detectable (**Figure 2(a)**, **Figure 2(b)**). When the backward loop primer (BLP) was included, the products were clearly detectable at 45 min (**Figure 2(c)**, **Figure 2(d)**). Identical results were obtained using a water bath or the thermocycler.

3.4. Determination of the Amplification Temperature for LAMP

Identical results were obtained with *Y. pestis* DNA and the outer (F3, B3), inner (FIP, BPI) and the backward loop primer (BLP) at the tested temperatures between 55˚C to 65˚C after a 90 min incubation (**Figure 3(a)**, **Figure 3(b)**). Identical results were obtained using the water bath or the thermocycler. Aiming for a higher stringency, the 65˚C temperature was adopted for the LAMP procedure.

3.5. Standardization of LAMP Reaction Conditions

The following conditions were established for the LAMP reactions: 25 µl of reaction mixture was comprised of 40 pmol of the inner primers (FIP, BPI), 10 pmol of the outer primers (F3, B3), 20 pmol of the backward loop primer (BLP), Tris-HCl (pH 8.8) 20 mM, $(NH4)_2SO_4$ 10 mM, $MgSO_4$ 8 mM, KCl 10 mM, dNTP 5.6 mM, Betaine 0.8 M, Tween-20 0.1%, 8 U Bst DNA polymerase (New England Biolabs) and 20 ng of *Y. pestis* DNA. The mixture was incubated for 45 min at 65˚C using either a water bath or the thermocycler. The DNA must be denatured at 94˚C for 5 min prior to using it in this mixture.

These conditions allowed for visualization of the amplified products under UV light upon the addition of SYBR® Safe (Invitrogen) (**Figure 4(a)**) or visualizing the products by the naked eye by observing the color change from orange to green upon the addition of SYBR® Green (Promega) (**Figure 4(b)**). The amplification was confirmed by visualizing the products on 1% agarose gels stained with SYBR® Safe (Invitrogen) (**Figure 4(c)**).

3.6. Sensitivity of the LAMP Technique

In parallel assays using five dilutions containing 10 ng to 1 pg of purified *Y. pestis* A1122 DNA, the detection limit was 10 pg of DNA per LAMP or C-PCR reactions (**Figures 5(a)-(c)**).

3.7. Specificity of the LAMP Technique

In parallel assays with the LAMP and the C-PCR, only the DNA from the *Y. pestis* strain A1122 was amplified. No amplification was observed with the *Y. pseudotuberculosis* IP32953, *V. cholerae* (#462), *L. monocytogenes*

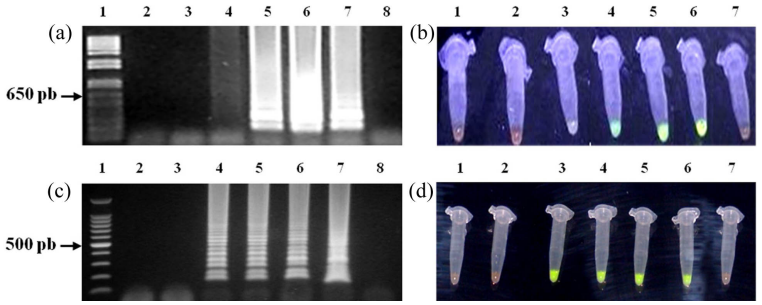

Figure 2. Inspection of LAMP products at the 15 - 90 min incubation times: (a) 1% agarose gel electrophoresis. Lane 1: 1 Kb plus DNA ladder; 2: 15 min; 3: 30 min; 4: 45 min; 5: 60 min; 6: 75 min; 7: 90 min; 8: negative control; (b) Visualization of LAMP products stained with SYBR® Safe and inspected under UV light. Tubes: 1: 15 min; 2: 30 min; 3: 45 min; 4: 60 min; 5: 75 min; 6: 90 min; 7: negative control; (c) 1% agarose gel electrophoresis of the LAMP products including the BLP primer. Lanes 1: 1 Kb plus DNA ladder; 2: 15 min; 3: 30 min; 4: 45 min; 5: 60 min; 6: 75 min; 7: 90 min; 8: negative control; (d) Visualization of LAMP products including BLP primer stained with SYBR® Safe and inspected under UV light. Tubes: 1: 15 min; 2: 30 min; 3: 45 min; 4: 60 min; 5: 75 min; 6: 90 min; 7: negative control.

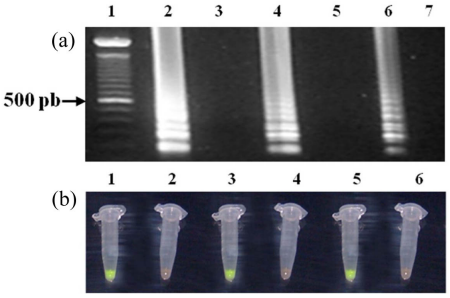

Figure 3. Inspection of LAMP products at the 60, 63, 65 min incubation times: (a) 1% agarose gel electrophoresis. Lanes 1: 100 pb DNA ladder; 2: 60˚C; 3: negative control; 4: 63˚C; 5: negative control; 6: 65˚C; 7: negative control; (b) Visualization of LAMP products stained with SYBR® Safe and inspected under UV light. Tubes 1: 60˚C; 2: negative control; 3: 63˚C; 4: negative control; 5: 65˚C; 6: negative control.

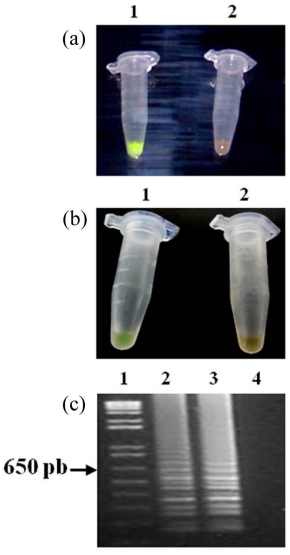

Figure 4. Inspection of LAMP products: (a) Visualization of LAMP products stained with SYBR® Safe and inspected under UV light. Tube 1 positive reaction, tube 2 negative reaction; (b) Visualization of LAMP products stained with SYBR® Green and inspected by the naked eye. Tube 1 positive reaction, tube 2 negative reaction; (c) 1% agarose gel electrophoresis of the LAMP products. Lanes 1: 1 Kb plus DNA ladder; 2-3: *Y. pestis* A1122; 4: negative control.

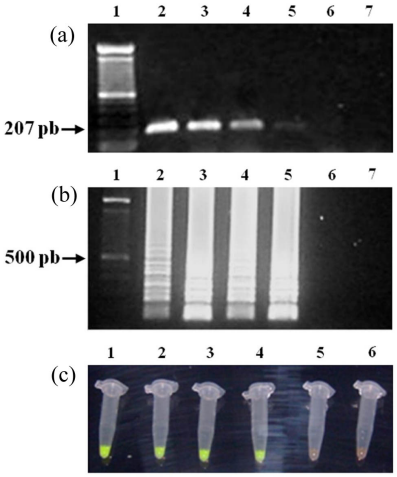

Figure 5. Assessment of LAMP sensitivity: (a) 1% agarose gel electrophoresis of C-PCR, Lanes 1: 100 bp DNA ladder; 2: 10 ng; 3: 1 ng; 4: 100 pg; 5: 10 pg; 6: 1 pg; 7: negative control; (b) 1% agarose gel electrophoresis of LAMP products. Lanes 1: 100 bp DNA ladder; 2: 10 ng; 3: 1 ng; 4: 100 pg; 5: 10 pg; 6: 1 pg; 7: negative control; (c) Visualization of LAMP products stained with SYBR® Safe and inspected under UV. Tubes 1: 10 ng; 2: 1 ng; 3: 100 pg; 4: 10 pg; 5: 1 pg; 6: negative control.

(#226) or *P. aeruginosa* (#127) strains (**Figure 6(a)**, **Figure 6(b)**).

4. Discussion

One of the most important features of the LAMP technology is its simplicity. The only required equipment for DNA amplification is a water bath, which eliminates the need for a thermocycler, a complex and expensive piece of equipment. Furthermore, the visualization of the results is also equipment-free, which simplifies and reduces the cost of this technique. These characteristics make LAMP very attractive for use in resource-limited areas in developing countries and very important for the plague surveillance laboratories network [13].

Plague diagnosis employs bacteriological analyses to identify and isolate the bacteria, serological tests to detect anti-plague antibodies and molecular techniques to identify specific *Y. pestis* sequences [17]. The bacteriological diagnosis of plague may be hampered by the low quality of the samples collected in remote areas and improper shipping to the diagnosis laboratories [8] [22]. Molecular diagnostic techniques can occasionally replace bacterial culture and are feasible even when the bacteria are not viable or are from multi-contaminated samples. However, while effective, these techniques require a high level of technical expertise, the use of expensive equipment for the reactions and visualization of the results.

Considering the LAMP advantages, we developed a procedure for detecting the *Y. pestis caf*1 gene that was both specific and sensitive. At least 10 pg of *Y. pestis* DNA were be detected in parallel reactions by LAMP and C-PCR, and no amplification was observed with other species tested than *Y. pestis*. The diagnostic rapidity is extremely important for plague surveillance and control. When the LAMP reaction was carried out at 65˚C in a water bath, the reaction took 60 min using a set of four primers (two outer and two internal primers). The reaction time was reduced to 45 min by using the optional backward loop primer. Although the use of HPLC purified internal primers is emphasized by Tomita *et al.* [21], our results with desalinized- and HPLC-purified inner primers were similar, hence desalinized primers were adopted for our LAMP procedure, due to a lower synthesis cost.

The inspection of the LAMP products was improved by adding chromogenic products [14]. In this study, the LAMP products were visualized with the naked eye and under UV light with the addition of SYBR® Safe (Invitrogen) (**Figure 4(a)**) or using only visible light by observing a color change from orange to green with the addition of SYBR® Green (Promega) (**Figure 4(b)**). The amplification was confirmed by an agarose gel electrophoresis (**Figure 4(c)**).

In spite of all of the LAMP advantages, the risk of laboratory contamination by amplicons remains, as it does for other molecular techniques [21]. Therefore, for technical safety, the LAMP kits can be prepared in a central or reference laboratory and distributed to other laboratories with lower capabilities [13]. The use of the

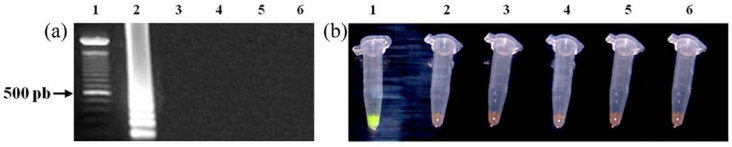

Figure 6. Assessment of LAMP specificity: (a) 1% agarose gel electrophoresis of LAMP products. Lanes 1: 100 bp DNA ladder; 2: *Y. pestis* A1122; 3: *Y. pseudotuberculosis* IP32953; 4: *L. monocytogenes* (#226); 5: *P. aeruginosa* (#127); 6: *V. cholerae* (#462); 7: negative control; (b) Visualization of LAMP products stained with SYBR® Safe and inspected under UV. Tubes 1: *Y. pestis* A1122 2: *Y. pseudotuberculosis* IP32953; 3: *L. monocytogenes* (#226); 4: *P. aeruginosa* (#127); 5: *V. cholerae* (#462); 6: negative control.

pre-formulated test tubes could reduce errors in the reaction preparation and improve the efficiency of plague monitoring and control programs [8].

5. Conclusion

The LAMP technique is a simple, quick and inexpensive procedure that uses only a water bath. The result of this technique is visualized directly in the test tube by naked eye, thus dispensing the need for a thermocycler and electrophoresis. Due to its specificity, sensitivity, rapidity and low cost, this LAMP procedure represents an additional tool to use in routine plague diagnosis, especially in emergencies.

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