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ORIGINAL RESEARCH PAPER

Loop-mediated isothermal amplification for rapid detection of *Bacillus anthracis* spores

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Abstract A loop-mediated isothermal amplification (LAMP) assay system was employed for detecting *Bacillus anthracis* spores in pure cultures as well as in various simulated powder samples. The specificity of the designed LAMP primer sets was validated by assaying 13 *B. anthracis* strains and 33 non-*B. anthracis* species. The detection limits of the LAMP assay were 10 spores/tube for pure cultures and 100 spores/2 mg powder for simulated powder samples. The results show that the LAMP protocol is a promising method for detecting *B. anthracis.*

Keywords *Bacillus anthracis* · Detection · Isothermal amplification · LAMP

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Introduction

Bacillus anthracis is a Gram-positive, spore-forming bacteria that causes the fatal disease of anthrax in animals and humans (Edwards et al. 2006) through the means of two major toxins (lethal and edema factors) in presence of protective antigen, and an anti-phagocytic capsule. The genes encoding these toxins and capsule exist in two virulence plasmids, pXO1 and pXO2, respectively. Although natural cases of human inhalational anthrax infection are now rare, there is a growing threat of biological weapons using *B. anthracis* spores due to their high lethality and the ease of production and dissemination (Wang and Roehrl 2005). Therefore, rapid and sensitive detection of *B. anthracis* spores is important.

Conventional culture-based methods, including colony morphology, penicillin susceptibility, gamma phage susceptibility, lack of hemolysis and motility, are considered as the 'gold' standards for identification of *B. anthracis* (Redmond et al. 1998). These methods, however, are very time-consuming and hence not used for rapid tests. ELISA and PCR are recommended by the World Health Organization (WHO 2003). ELISA is mainly for detection of the protective antigen of *B. anthracis*, while PCR is for detection of the specific plasmid DNA coding for the virulence factors or chromosome sequence. Both methods are now widely used in laboratories around the world. Sensitivity of the methods can be

evaluated by their detection limits of spore numbers. According to the literature data, the detection limits are 10,000 spores for immunofluorescence (Phillips and Martin 1983), 1,000 spores for ELISA, immunoradiometric assays (Phillips et al. 1984) and flow cytometry (Stopa 2000), and 5–10 spores for real-time PCR (Hoffmaster et al. 2002).

Recently, a novel nucleic acid amplification method, loop-mediated isothermal amplification (LAMP), has been developed (Notomi et al. 2000). The LAMP reaction is an auto-cycling, strand-displacement DNA synthesis carried out by a DNA polymerase with high strand displacement activity. The amplification products are stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops. Since its publication, many LAMP methods have been developed for identification of viruses, bacteria, protozoa and fungus (Endo et al. 2004; Okafuji et al. 2005). In the present study, a LAMP assay was developed for detection of *B. anthracis* spores. The sensitivity, specificity and applicability of the method for direct detection of *B. anthracis* in pure cultures and simulated powder samples were evaluated.

Materials and methods

Bacterial strains

Bacillus anthracis isolates listed in Table 1 were provided by Professor Ruifu Yang (Beijing, China). Other strains were from Wuhan Institute of Virology, Chinese Academy of Sciences (Wuhan, China). *B. anthracis* cultures and treatment were performed in the P3 laboratory of the Institute of Microbiology and Epidemiology (IME).

Table 1 Identifying the target DNA from *B. anthracis* isolates and other organisms using LAMP assay

Bacillus strains[a]	Plasmid contained		LAMP assay result		
	pXO1	pXO2	Ba813	PA	Cap B
Bacillus anthracis A16	+	+	+	+	+
Bacillus anthracis 17003-10	+	+	+	+	+
Bacillus anthracis 17003-11	+	+	+	+	+
Bacillus anthracis 17003-18	+	+	+	+	+
Bacillus anthracis 17003-19	+	+	+	+	+
Bacillus anthracis 17003-28	+	+	+	+	+
Bacillus anthracis 17003-32	+	+	+	+	+
Bacillus anthracis 17003-54	+	+	+	+	+
Bacillus anthracis 17003-25	+	–	+	+	–
Bacillus anthracis 17003-42	+	–	+	+	–
Bacillus anthracis 170044	+	–	+	+	–
Bacillus anthracis 170045	+	–	+	+	–
Bacillus anthracis 170046	–	+	+	–	+
Bacillus cereus F3502/73	–	–	+	–	–
Bacillus cereus 421-4	–	–	+	–	–
Bacillus mycoides	–	–	+	–	–

[a] Negative results of LAMP assays were observed from other *Bacillus* species: *B. cereus* 421-3, *B. cereus* F4433/73, *B. cereus* MADM 1279, *B. cereus* ENSP5(ATCC 33018), *B. cereus* CIP 5832, *B. mycoides* DSMZ 2048, *B. thuringiensis* subsp. aleti, *B. thuringiensis* subsp. dakoda, *B. thuringiensis* subsp. finifimus, *B. thuringiensis* subsp. galleriae, *B. thuringiensis* subsp. kumamotoensis, *B. thuringiensis* subsp. ostriniae, *B. thuringiensis* subsp. pacificus, *B. thuringiensis* subsp. sotto, *B. thuringiensis* subsp. tochigiensis, *B. thuringiensis* subsp. tohokuensis, *B. thuringiensis* subsp. toumanoffi, *B. thuringiensis* var. tienmensis, *B. subtilis, B. pumilus, B. coagulans and B. Circulans.* In addition, the following also gave negative results. *Yersinia pestis, Pseudomonas sp.* WBC-3, *P. putita, P. aeruginosa, Oceanimonas doudoroffii* GB6, *Alcaligenes sp.* HCC128, *Corynebacterium sp.* MB1 and *Agrobacterium radiobacter*

Spores preparation

Spores of *B. anthracis* A16 were obtained by seeding vegetative cells onto new sporulation medium and incubating the preparation at 37°C for 2 weeks. Microscopic examination revealed that approximately 90% sporulation had occurred by this time. The spores were washed off the agar surface with distilled water and held at 60°C for 1 h to kill vegetative cells. The spores were then washed three times with sterile phosphate-buffered saline (PBS, 0.01 M, pH 7.4) and stored at 4°C. Concentrations of the spores were determined by direct colony counting on nutrient agar.

Preparation of DNA samples for LAMP

Target DNA for LAMP assay was prepared as described previously (Drago et al. 2002; Makino and Cheun 2003). Briefly, the *B. anthracis* A16 spores suspension were centrifuged at 8,000*g* for 10 min and resuspended in 25 µl sterile water. The spores were lysed by heating at 95∼100°C for 30 min and then centrifuged at 15,000*g* for 10 min at 4°C. Finally, 2 µl of the supernatant was used directly for LAMP.

LAMP assay

A six-primer manner was adopted for the LAMP assay, including two inner primers (FIP, BIP), two outer primers (F3, B3) and two loop primers (LF, LB) for one target gene. These primers (Table 2) were designed from the Ba813, *pag*, and *capB* sequences, respectively, using Primer Explorer software, version 3.0 (http://primerexplorer.jp/elamp3.0.0/index.html). The LAMP reaction was conducted as described previously (Notomi et al. 2000). Briefly, the reaction was carried out in 40 µl containing 1.6 µM each FIP and BIP, 0.2 µM each F3 and B3, 0.8 µM each LF and LB, 1.4 mM each deoxynucleoside triphosphate (dNTP), 20 mM Tris/HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Tween 20, 8 U of the *Bst* DNA polymerase large fragment (New England Biolabs), and 2 µl target DNA. The mixture was incubated at 60°C then heated to 80°C for 2 min to terminate the reaction. The LAMP products were digested with the appropriate restriction enzymes (*Mbo* II for Ba813 amplicons, *Eco*R I for *pag* amplicons and *Eco*T22 I for *capB* amplicons) and electrophoresed on 2% (w/v) agarose gels containing 0.5 µg ethidium bromide/ml. The Mg^{2+} concentrations, reaction temperature and reaction time were optimized by checking

Table 2 Primers for LAMP assay

Target	Primer	Sequence 5′–3′
Ba813	Ba813-F3	TGGGATTTCTTTCTGACTTGG
	Ba813-B3	GGGATCCGACAAAACTAACG
	Ba813-FIP	ACAGCAAACACAGAATTTGAAGCATCTTGCTGATACGGTATAGAACC
	Ba813-BIP	TCCAAATGTAGGAGCTATCGTTTGTATTTAGCGAAGATCCAGTGC
	Ba813-LF	GTTACTCAATGAGTCTTTTAATGCCA
	Ba813-LB	CCCTGGGAAATTCTCTGTATAACG
pag	Pag-F3	ATACTTCTACAAGTAGGACACAT
	Pag-B3	ATTGGCATTTAATCTTGCTGTA
	Pag-FIP	CCTGCAGATACACTACCACCAATATGAAGTACATGGAAATGCAGAA
	Pag-BIP	CGGTCGCAATTGATCATTCACTGTATTTAAACCCATTGTTTCAGC
	Pag-LF	CAAAGAACGACGCATGCA
	Pag-LB	ATCTCTAGCAGGGGAAAGAACT
capB	CapB-F3	GCGGATAATTCTAGAATTTCAGAAG
	CapB-B3	ATGTTGATGAGGGATCATTCG
	CapB-FIP	GCTGTTTCCTCATCAATCCCAAGATGATTACATGGTCTTCCCAGA
	CapB-BIP	CGGATCCAGGAGCAATGAGAATACCATTTACGAAGAACGCAG
	CapB-LF	ACCGCTAAAGCAAGCGATG
	CapB-LB	CGTTTTGCTGACCAATCTAAGC

the LAMP products of *pag* gene on agarose gels. The sensitivity of the assay was confirmed using serially diluted *B. anthracis* A16 spores.

Visual inspection of the LAMP amplicons in the reaction tube were performed by adding three fluorescent dyes SYBR Gold (Invitrogen), EB (Sigma) and EvaGreen (Biotium), labeling the Ba813, *pag* and *capB* LAMP amplicons, respectively. The fluorescent signals of the solutions were observed under a UV transilluminator (UVP Inc).

Multiplex PCR

Multiplex PCR of *pag*, *capB* and Ba813 were performed using the corresponding primers (Reif et al. 1994; Belgrader et al. 1999). The reaction mixture (25 μl) contained 2 mM $MgCl_2$, 200 μM each dNTP, and 0.4 μM of each primer. The thermal cycler (T1 Thermocycler, Biometra) was programmed as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, and finally 72°C for 5 min. The PCR products were electrophoresed on agarose gels.

Detection of spores in simulated powder samples by LAMP

A group of white powders including flour, baking soda, dried milk and tryptone were used as representative powders. *B. anthracis* A16 spores were serially diluted in sterile PBS to the desired concentration to prepare simulated test samples. The samples contained 1–10^4 spores and 2 mg powder in 500 μl PBS. Before test, the samples were centrifuged at 8,000*g* for 10 min and washed with distilled water, and then resuspended in 25 μl sterile water. The suspensions were disrupted by heat treatment as described above. LAMP reactions were performed with 2 μl spore lysis as template. The amplification products were analyzed on agarose gels.

Results

Optimization of LAMP reaction condition

To optimize the LAMP assay for detection of *B. anthracis* spores, the primers for *pag* gene (Table 2) and the DNA template from the heating lysis of *B. anthracis* A16 spores were applied. Spores DNA extracted by boiling gave rise to a typical ladder pattern as shown in all figures in this paper. There were many bands with different sizes up to the loading wells. Effects of the tested factors on the reaction were evaluated by electrophoresis. Because free Mg^{2+} availability affects primer annealing and DNA polymerase activity, the effect of Mg^{2+} concentrations ranging from 2 to 12 mM on the LAMP reaction was determined. As shown in Fig. 1A, Mg^{2+} concentration at 6 mM gave the optimal amplification. Several reports showed that *Bst* DNA polymerase could effectively amplify DNA templates at temperatures from 60 to 65°C in the LAMP reaction (Endo et al. 2004). The temperature condition in this study was optimized with the results that the reaction at 60°C produced better amplification than those at 63 and 65°C (Fig. 1B). Reaction time is another important consideration. In a previous study, less than 60 min has been used for the reaction (Iwamoto et al. 2003). However, as shown in Fig. 1C, no amplification of the templates was found after 15 or 30 min; 60 min amplification produced the best result. Thus the optimal reaction condition was determined to be: 6 mM Mg^{2+}, 60°C and 60 min. This reaction condition was used in the subsequent experiments.

Specificity of *B. anthracis* LAMP assay

The primers for the *pag* and *capB* genes were used to confirm the presence of plasmids pXO1 and pXO2, respectively, and another primer set was used for identification of the chromosome sequence Ba813 (Table 2). These sequences are possessed specifically by *B. anthracis* (Wang et al. 2004). For rapid detection, a one-step three-tube LAMP assay manner was adopted. Each of the three tubes contained one set of the primers and targeted one of the three sequences, i.e., *pag*, *capB* genes and Ba813 sequence, individually. Since the LAMP products consist of several inverted-repeat structures, the positive amplification generated the ladder-like pattern of bands on agarose gel. No amplification was observed in the tube containing distilled water only. To confirm that the amplification products had the corresponding DNA structures, the products were digested with restriction enzymes and the sizes of the fragments were analyzed by electrophoresis. As shown in Fig. 2, the electropherogram was in good agreement

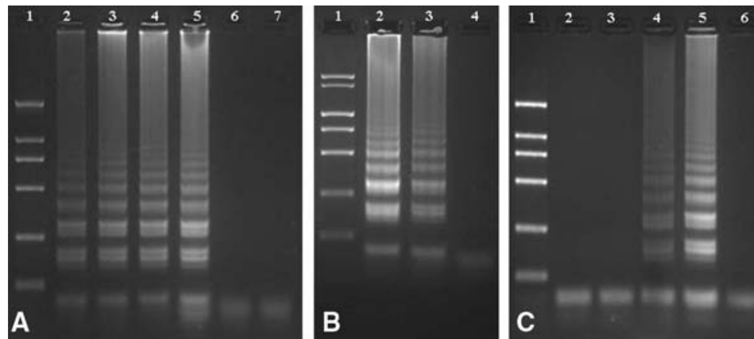

Fig. 1 Optimization of the LAMP reaction detection of *B. anthracis* A16 *pag* gene. The LAMP products were electrophoresed on 2% (w/v) agarose gels containing 0.5 µg EB/ml. (**A**) Effect of $MgCl_2$ concentrations on the LAMP reaction at 60°C. Lanes 1: DNA marker (DL2000) with 2000, 1000, 750, 500, 250 and 100 bp. Lanes 2–7: LAMP amplicons with 12, 10, 8, 6, 4 and 2 mM $MgCl_2$, respectively. (**B**) Effect of temperature on the LAMP reaction with 6 mM $MgCl_2$. Lanes 1: DNA marker (DL2000). Lanes 2–4: LAMP amplicons at 60°C, 63°C and 65°C, respectively. (**C**) Effect of reaction time on the LAMP reaction with 6 mM $MgCl_2$ at 60°C. Lanes 1: DNA marker (DL2000). Lanes 2–7: LAMP amplicons for 15, 30, 45 and 60 min, respectively. Lane 6: LAMP reaction without DNA template

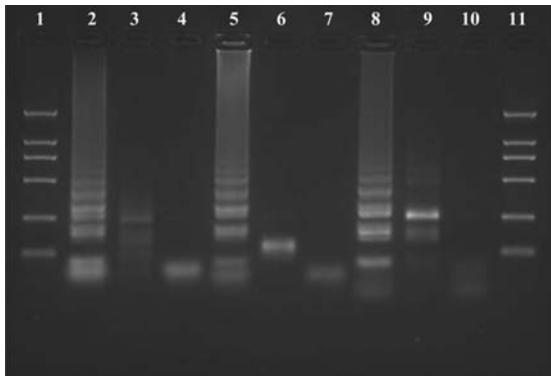

Fig. 2 Agarose gel electrophoresis and restriction analysis of *B. anthracis* A16. Lane 1 and 11: DNA marker (DL2000) with 2,000, 1,000, 750, 500, 250 and 100 bp. Lanes 2: LAMP product carried out with Ba813 LAMP primers in the presence of *B. anthracis* DNA. Lane 3: LAMP product from lane 2 after digestion with *Mbo* II (260 bp). Lanes 5: LAMP product carried out with *pag* LAMP primers in the presence of *B. anthracis* DNA. Lane 6: LAMP product from lane 5 after digestion with *Eco*R I (205 bp). Lanes 8: LAMP product carried out with *capB* LAMP primers in the presence of *B. anthracis* DNA. Lane 9: LAMP product from lane 8 after digestion with *Eco*T22 I (270 bp). Lanes 4, 7 and 10: LAMP carried out in the absence of template DNA with Ba813, *pag, capB* LAMP primers, respectively

with the band sizes predicted theoretically from the expected DNA structures.

To evaluate the species specificity of the method, 13 *B. anthracis* isolates, 25 other *Bacillus* strains and eight non-*Bacillus* strains were examined by the test (Table 1). Significant amplification of the DNAs isolated from the targeted organisms was observed after a 60-min reaction. By contrast, DNAs of the non-targeted strains were not amplified even after a 60-min reaction with exceptions of one *B. mycoides* strain and two *B. cereus* strains (F3502/73 and 421-4), which the exceptions produced positive amplification of Ba813. The existence of Ba813 sequence in these strains was further confirmed by multiple PCR (data not shown). Although the Ba813 sequence happens to be found in other Bacillus strains (Ramisse et al. 1999), it was widely used as one of the marker sequences for detection of *B. anthracis* because it presents in all *B. anthracis* strains. Hence, samples are considered positive if two or three of the targets for *B. anthracis* were amplified. As shown in Table 1, all the positive strains were identified correctly. In this regard, the LAMP protocol is not only specific for identification of *B. anthracis* from other species, but also usable for differentiation of virulent and avirulent strains.

Sensitivity of the LAMP reaction in detection of *B. anthracis*

Ten-fold serial dilutions of *B. anthracis* A16 spores were used to evaluate the sensitivity of the method. As shown in Fig. 3A and B, the LAMP products of *pag* and *capB* gene from the tubes containing 10, 10^2, 10^3 and 10^4 spores, respectively, exhibited obvious amplification. These results indicated that the sensitivity can be down to 10 spores per tube. While the sensitivity for Ba813 LAMP assay was 100 spores per

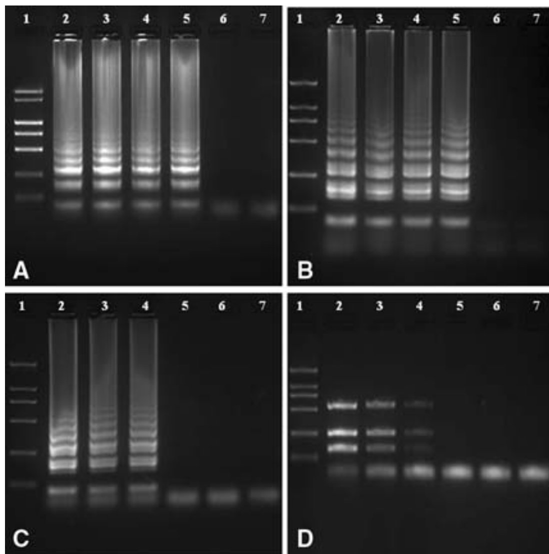

Fig. 3 Sensitivities of *B. anthracis* A16 LAMP and multiplex PCR methods. The amplified products were electrophoresed on 2% (w/v) agarose gels containing 0.5 μg EB/ml. (**A**, **B**, **C**) Sensitivities of electrophoretic analysis of LAMP amplified products from *pag* gene, *capB* gene and Ba813 sequence, respectively. Lanes 1: DNA marker (DL2000) with 2,000, 1,000, 750, 500, 250 and 100 bp. Lane 2–7: LAMP reactions products with 10^4, 10^3, 10^2, 10, 1 spores and distilled water, respectively. (**D**) Sensitivities of electrophoretic analysis of multiplex PCR amplified products. Lanes 1: DNA marker (DL2000). Lane 2–7, multiplex PCR products with 10^4, 10^3, 10^2, 10, 1 spores and distilled water, respectively

tube (Fig. 3C). The multiplex PCR resulted in about 10-fold less sensitivity for the same targets (Fig. 3D).

Detection of *B. anthracis* spore in simulated powder samples

Simulated powder samples consisted of the *B. anthracis* A16 spores (1–10^4) and various powders (2 mg) were subjected to the LAMP assay. The results are summarized in Table 3. In *pag* and *capB* LAMP assays, 100 spores could be detected in baking soda, dried milk and tryptone powder, respectively. While in Ba813 LAMP assay, 1,000 spores could be detected in the same simulated samples. However, when flour powder was employed to prepare the simulated samples, the detection limits increased one fold in all LAMP assays. No amplification product was found in the negative control samples containing *B. thuringiensis* or *B. cereus* DNA. Meanwhile, the multiplex PCR produced the detection limit of 1,000 spores/2 mg powder, which was about 10 times higher than the LAMP assays.

Table 3 Detection limit of the LAMP assay for simulated powder samples[a]

Powder	Detection limit[b]		
	Ba813	*pag*	*CapB*
Flour	2,000	200	200
Baking soda	1,000	100	100
Dried milk	1,000	100	100
Tryptone	1,000	100	100

[a] The LAMP amplification products of simulated powder samples (2 mg used in each case) were analyzed on 2% (w/v) agarose gels containing 0.5 μg ethidium bromide/ml

[b] Counted as the minimal number of the spores

Detection of LAMP products with multiplex fluorescent dyes

Fluorescent signals were noted on visual inspection of LAMP reaction tubes after addition of diluted fluorescent dyes. As shown in Fig. 4, yellow-green, red and green fluorescence were observed with naked eyes for positive reactions of Ba813, *pag* and *capB* LAMP assay upon addition of fluorescent dyes SYBR Gold, EB and EvaGreen, respectively. No fluorescence was observed for the negative control and no-template control reactions. These observations agreed with gel electrophoresis results.

Discussions

Detection of *B. anthracis* spores using the LAMP protocol has showed a number of advantages. First, its specificity is very high. Because of the high similarity, identification of *B. anthracis* from other *Bacillus* strains is ever challenging. In the LAMP assay, positive detection is judged by parallel amplification of two unique virulence genes and one chromosome target. In addition, each sequence is targeted by six primers at eight distinct internal regions, which further secures the specific assay. As result, 13 *B. anthracis* strains were successfully distinguished from other thirty-three non-*B. anthracis* strains. Isothermal amplification may also be performed at 40°C in nucleic acid sequence-based amplification (NASBA) experiment (Baeumner et al.

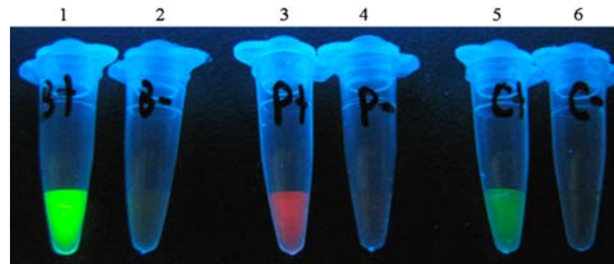

Fig. 4 Detection of LAMP products with multiplex fluorescent dyes. 0.5 μl LAMP products were added to 50 μl of diluted dyes solution (1:3000 SYBR Gold, 0.5 μg ethidium bromide/ml, 1:20 EvaGreen). The fluorescent signals of the solution were observed under a UV transilluminator (302 nm). Tubes 1 and 2: fluorescent detection of Ba813 LAMP products with SYBR Gold for *B. anthracis* A16 and negative control, respectively. Tubes 3 and 4: fluorescent detection of *pag* LAMP products with ethidium bromide for *B. anthracis* A16 and negative control, respectively. Tubes 5 and 6: fluorescent detection of *capB* LAMP products with EvaGreen for *B. anthracis* A16 and negative control, respectively. All the negative controls were LAMP assays without DNA template

2004), but lowering the isothermal amplification temperature would reduce specificity in detection of *B. anthracis* genes.

Second, the assay time is very short, which is the main feature of the LAMP assay. There are two reasons: (1) the amplification is carried out under isothermal condition of about 60°C, the thermal cycling adopted in PCR is completely avoided, and (2) the inhibition reaction that usually exists at the later stage of PCR is unlikely to occur in isothermal amplification. Introduction of fluorescence not only further reduced the assay time, but also alleviate the need for gel electrophoresis, and thus make the method adept to field tests.

Third, the sensitivity is extremely high. Under the optimal condition for detection of two virulence genes, 10 spores/tube could produce the visible signal in the LAMP assay, while it required 100 spores/tube to produce the visible signal in the multiplex PCR required 100 spores/tube. Compared with the assay of plasmid genes *pag* and *capB*, assay of the chromosome sequence Ba813 by LAMP had 10 times less sensitivity. Recent study demonstrated that *B. anthracis* may carry multiple copies of the plasmids (Coker et al. 2003). The ratio of plasmid copies to chromosomes varies greatly among the *B. anthracis* isolates, even many as 40.5 for pXO1 and 5.4 for pXO2. Although the ratio of plasmid copies to chromosomes in *B. anthracis* A16 is not sure, this maybe the main reason for the difference between the sensitivity of *pag*, *capB* and Ba813 LAMP assays. Besides the influence of sequence copy numbers, nucleic acid amplification from chromosome is more difficult than that from plasmid because of the length effect of DNA. For instance, the length of chromosome DNA is 29 and 56 folds longer than that of plasmid pXO1 and pXO2, respectively, making the collision between target DNA and primers more difficult.

In conclusion, a LAMP method is firstly used for *B. anthracis* spores assay. The experiment protocol and the optimized condition resulted in rapid and super sensitive detection of spores either in pure cultures or in simulated powder samples. The method could further be exploited for field test with inexpensive equipments because the LAMP assay can be carried out under isothermal conditions at 60°C. The results presented suggest that LAMP method constitute a powerful tool for the detection of *B. anthracis*.

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