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Original Article

LAMP Using a Disposable Pocket Warmer for Anthrax Detection, a Highly Mobile and Reliable Method for Anti-Bioterrorism

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SUMMARY: A quick, reliable detection system is necessary to deal with bioterrorism. Loop-mediated isothermal amplification (LAMP) is a DNA amplification method that can amplify specific DNA fragments in isothermal conditions. We developed a new highly mobile and practical LAMP anthrax detection system that uses a disposable pocket warmer without the need for electricity (pocket-warmer LAMP). In our tests, the detection limit of the pocket-warmer LAMP was 1,000 copies of *Bacillus anthracis pag* and *capB* gene fragments per tube. The pocket-warmer LAMP also detected *B. anthracis* genes from DNA extracted from 0.1 volume of a *B. anthracis* colony. The lower detection limit of the pocket-warmer LAMP was not significantly different from that of a conventional LAMP using a heat block, and was not changed under cold (4°C) or warm (37°C) conditions in a Styrofoam box. The pocket-warmer LAMP could be useful against bioterrorism, and as a sensitive, reliable detection tool in areas with undependable electricity infrastructures.

INTRODUCTION

Anthrax, a lethal disease in humans, is caused by *Bacillus anthracis*. Although natural cases of anthrax are rare in humans, the threat of bioterrorism using *B. anthracis* has increased (1). To deal with this possibility, a rapid, reliable detection system is necessary. Several detection systems, such as PCR, have been developed, but almost all of them require heavy, stationary equipment (2). Highly mobile detection systems for anthrax have been developed that rely on immunochromatography methods, such as the Sensitive Membrane Antigen Rapid Test (SMART) and the Antibody-based Lateral Flow Economical Recognition Ticket (ALERT) (2). However, their sensitivity is not sufficient for reliable detection (2). Here, we describe a sensitive and highly mobile anthrax detection system, combining the loop-mediated isothermal amplification (LAMP) method with a disposable pocket warmer, designated as "pocket-warmer LAMP." LAMP is a recently developed DNA amplification method with high specificity, efficiency, and speed under isothermal conditions (3,4). LAMP requires a set of four primers (B3, F3, BIP, and FIP) that recognize six distinct sequences (B1, B2, B3, F1, F2, and F3) in the target DNA. The use of the four primers enhances the specificity of DNA amplification. The most significant advantage of LAMP is that the reaction proceeds under isothermal conditions. Since the amplification requires a constant temperature range of 60–65°C for 1 h, LAMP requires only a heat blocker as equipment. This is a big advantage in developing countries or laboratories equipped with no thermal cycler. Disposable pocket warmers are a well-known winter commodity in Japan. They cost less than 100 yen each and are available anywhere in Japan. In this study, we used a disposable pocket warmer as a heat source for the LAMP reaction. We evaluated the lower detection limit of the pocket-warmer LAMP for anthrax detection and also investigated the influences of environmental temperature and the differences among pocket warmers made by different manufacturers.

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MATERIALS AND METHODS

Disposable pocket warmers: Disposable pocket warmers were purchased at drugstores in Japan. Four different pocket warmers made by different companies (A–D) were used. According to the manufacturers' information, these pocket warmers reach 68°C at maximum, and the average temperature during a 12-h period is 53°C.

Bacterial strains: Three strains of *B. anthracis*—BA101, BA103, and BA104—were used (5–7). BA101 was previously used as a vaccine strain for cattle and horses in Japan. BA103 was isolated from dairy cattle in Miyagi Prefecture in 1991. BA104 was isolated from swine in Shizuoka Prefecture in 1982. *B. thuringiensis* GTC2847, *B. cereus* GTC419, *B. cereus* GTC1777, and *B. cereus* GTC2826 provided by the National Bio Resource Project, Gifu University Graduate School of Medicine, and *B. subtilis* NIID-1 by the National Institute of Infectious Diseases were used as controls.

Culture and DNA extraction: One colony of *B. anthracis* was cultured in 2 mL of trypticase soy broth at 37°C overnight (8). DNA was extracted from 1 ml of the overnight-cultured *B. anthracis* using the phenol and chloroform method. Another 1 ml was serially diluted and plated on a trypticase soy agar plate. After overnight culture, colonies were counted to determine colony formation units (CFUs).

Preparation of control DNA: To determine the lower detection limit of LAMP, *pag* and *capB* genes from *B. anthracis* were amplified from BA101 using F3 and B3 primers (9).

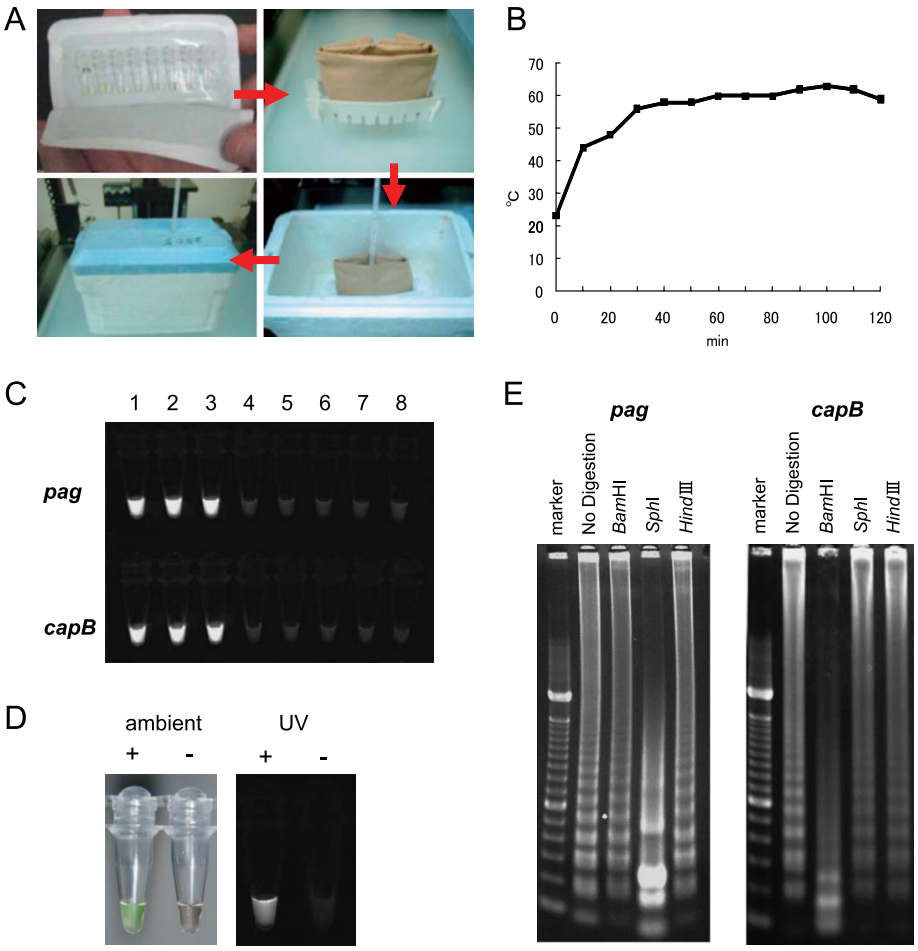

Fig. 1. Establishment of pocket-warmer LAMP. (A) The procedure of pocket-warmer LAMP. Reaction tubes were placed on a pocket warmer (upper left panel), folded and surrounded by a paper towel (upper right panel), and put into Styrofoam box with a thermometer (lower panels). The tubes were incubated for 90 min. (B) Temperature of pocket warmers in Styrofoam box. The temperature reached to 60°C in about 30 min and stayed at about 60°C for 90 min. (C) Fluorescence image of pocket-warmer LAMP products in tubes using UV light exposure. Ten ng of DNA was applied per tube. 1, *B. anthracis* BA101; 2, *B. anthracis* BA103; 3, *B. anthracis* BA104; 4, *B. thuringiensis* GTC2847; 5, *B. cereus* GTC419; 6, *B. cereus* GTC1777; 7, *B. cereus* GTC2826; 8, *B. subtilis* NIID-1. (D) Color image of pocket-warmer LAMP products under ambient light (left) and UV light (right). LAMP product showed green color under ambient light. (+) and (−) indicate a positive and negative sample, respectively. (E) Agarose gels electrophoresis of LAMP products digested with restriction enzymes. The LAMP products of *pag* or *capB* were digested with *Bam*HI, *Sph*I, and *Hind*III. The left lane in each gel is a 100-bp ladder molecular weight marker.

The reaction mixture for the PCR consisted of 1 μl *B. anthracis* genomic DNA, 1 μmol/L of each primer (F3, B3), 12.5 μl 2 × high-fidelity PCR master mix (Roche Diagnostics, Boehringer Mannheim, Mannheim, Germany) and enough water for a final volume of 25 μl. Primary amplification conditions were 94°C for 2 min; 35 cycles at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s; and final extension at 70°C for 10 min. The PCR products were purified with a gel extraction kit (Qiagen, Hilden, Germany) and their copy numbers calculated based on their molecular weights. The PCR product was then used to make standard dilutions (10^0–10^8 copies/μl) to evaluate the lower detection limit of the LAMP process.

LAMP: The *pag* and *capB* genes of *B. anthracis* were amplified with a loopamp DNA amplification kit (Eiken Chemical, Tochigi, Japan) using primers published previously (9). Each 25-μl reaction mixture contained 1.6 μM each of FIP and BIP, 0.2 μM each of F3 and B3, 0.8 μM each of LF and LB, 2 × reaction mixture (12.5 μl), *Bst* DNA polymerase (1 μl), fluorescence detection reagent (Eiken Chemical, 1 μl), 3.5 μl distilled water, and 1 μl sample. Reaction tubes were incubated in a heat blocker (GeneAmp 9700; Applied Biosystems, Foster City, Calif., USA) or a pocket warmer. In the heat blocker, tubes were incubated at 60°C for 60 min. For the pocket-warmer LAMP, tubes were sandwiched in a twofold pocket warmer surrounded by a paper towel and put in a Styrofoam box for 90 min (Fig. 1A). To study the influence of environmental temperature, we operated the pocket-warmer LAMP in cold conditions (4°C) or hot conditions (37°C), with or without a Styrofoam box. The positive LAMP reactions were checked under both ultraviolet (UV) light and ambient light. LAMP products were subjected to electrophoresis on a 2% agarose gel. The gels were visualized under UV light after ethidium bromide staining. In some experiments, LAMP products of *pag* and *capB* were purified with a PCR purification kit (Qiagen), digested with *Bam*HI, *Sph*I, and *Hind*III (New England Biolabs, Ipswich, Mass., USA), and subjected to electrophoresis on a 2% agarose gel.

RESULTS

Establishment of pocket-warmer LAMP: The LAMP method requires constancy of temperature at about 60°C for 60 min in order to amplify DNA. To know if a commercially

available disposable pocket warmer is able to maintain that temperature for the appropriate period, we monitored the temperature of a pocket warmer in a Styrofoam box first. It reached 58°C in 30 min and stayed around 60°C for more than 60 min (Figs. 1A and 1B). We then conducted the LAMP reaction with a pocket warmer. Observation under UV light revealed that the pocket-warmer LAMP amplified the *pag* and *capB* gene fragments in DNA samples from three strains of *B*. *anthracis*—BA101, BA103, and BA104—but not from strains of *B*. *thuringiensis*, *B*. *cereus*, and *B*. *subtilis* for 90 min (Fig. 1C). In addition, the amplification for *B*. *anthracis* was also observed as a change of color under ambient light (Fig. 1D). To confirm the specificity of the pocket-warmer LAMP, the LAMP products of *B*. *anthracis* were digested with restriction enzymes. Each target gene of *pag* and *capB* LAMP has a restriction enzyme site for *Sph*I and *Bam*HI, respectively. A few bands sized around 200 bp were observed in the lane of *Sph*I (*pag*) and *Bam*HI (*capB*)-digested LAMP products, while smear and ladder bands were seen in the other lanes (Fig. 1E). Similar results were observed in enzyme-digested LAMP products with both the heat block and pocket warmer (data not shown). These data indicate specific amplification for *B*. *anthracis* by the pocket-warmer LAMP.

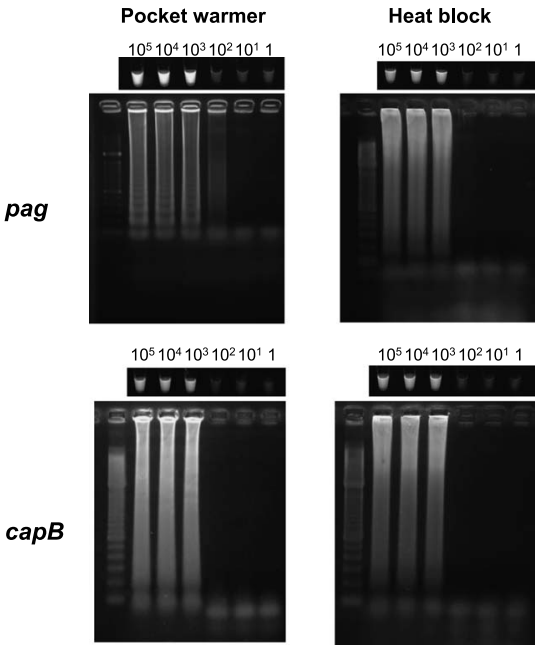

Fig. 2. Lower detection limit of LAMP using pocket warmer or heat block. Serial dilutions of *pag* and *capB* gene fragments were examined to determine the lower detection limit of the assay. The numbers in the top of upper panels are copy numbers of the target gene. Fluorescence image of tubes using UV light exposure (upper panels) and agarose gels electrophoresis (lower panels) are shown. The left lane on each gel is a 100-bp laddered molecular weight marker.

Comparison of lower detection limit with conventional LAMP: To compare the lower detection limit and quality of the pocket warmer LAMP with the conventional LAMP using a heat block, diluted DNA fragments of *pag* and *capB* were examined by both methods. Exposure to UV light showed that the detection limit of both methods was 1,000 copies of the *pag* and *capB* genes per tube (Fig. 2). Gel electrophoresis demonstrated that the pocket-warmer LAMP produced a pattern of electrophoresis similar to that of the conventional LAMP. We then investigated whether or not the pocket-warmer LAMP could amplify the *pag* and *capB* genes from DNA samples extracted from *B*. *anthracis* at a lower detection limit similar to that of conventional LAMP. The pocket-warmer LAMP amplified the *pag* and *capB* genes from DNA extracted from 0.1 and 1.0 volume of CFU, while conventional LAMP amplified them from 0.1 CFU (Fig. 3). These data suggest no significant difference in the lower detection limit between the conventional and pocket-warmer methods.

Conditions of the pocket-warmer LAMP: To evaluate the effects of environmental temperature on this method's performance, we used the pocket-warmer LAMP under cold (4°C) or warm (37°C) conditions, with or without a Styrofoam box (Table 1). With a Styrofoam box, the pocket-warmer LAMP consistently amplified the *pag* and *capB* genes from DNA containing 1,000 copies of the genes under both cold and warm conditions. However, without the Styrofoam box, the pocket-warmer LAMP did not amplify the genes from DNA containing 10^8 copies of the *pag* and *capB* genes. When we put these LAMP reaction mixtures with a pocket warmer into a pocket of pants worn by one of the experimenters, the

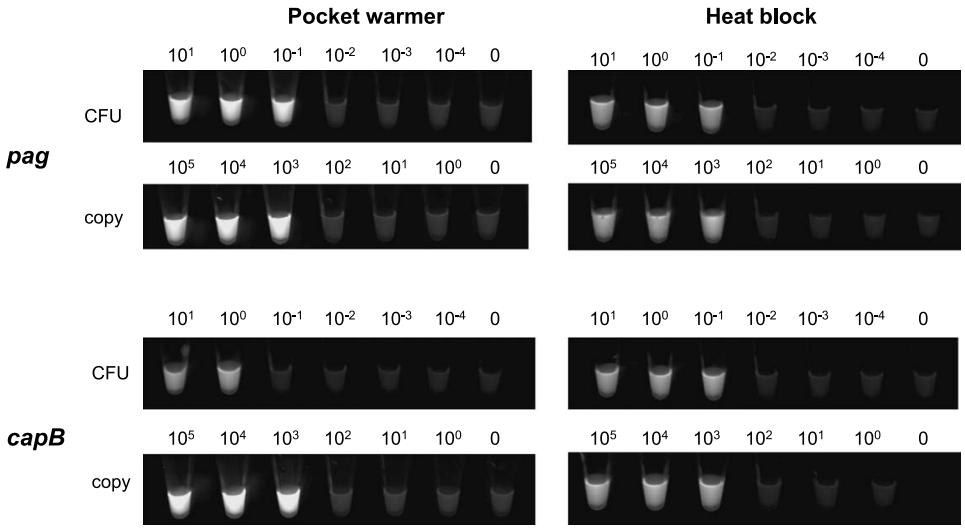

Fig. 3. Comparison of CFUs and copy numbers in pocket-warmer LAMP (left panels) and conventional LAMP using a heat block (right panels). Serial dilutions of *pag* and *capB* gene fragments and DNA extracted from *B*. *anthracis* were examined to determine the lower detection limit of the assay. Fluorescence images using UV light exposure are shown.

Table 1. Condition of pocket-warmer LAMP

	Pocket warmer							
	4°C		RT		37°C		In pocket	Heat block
Styrofoam box	+	−	+	−	+	−	−	
pag	10^3	$>10^8$	10^3	10^3	10^3	10^3	$>10^8$	10^3
capB	10^2	$>10^8$	10^3	10^3	10^3	10^3	$>10^8$	10^3

10^1–10^8 copies of the *pag* and *capB* genes were examined. Copy numbers detected in LAMP are shown.
RT, room temperature.

Table 2. Comparison of pocket warmers

	Pocket warmer				Heat block
Company	A	B	C	D	
pag	10^3	10^3	10^3	10^3	10^3
capB	10^3	10^3	10^3	10^3	10^3

10^1–10^8 copies of *pag* and *capB* genes were examined. Copy numbers detected in LAMP are shown.

pag and *capB* genes were not amplified even from DNA containing 10^8 copies. These data suggest that the pocket-warmer LAMP should be used in a Styrofoam box. To verify the differences among commercially available pocket warmers from different manufacturers, we tested the performance of pocket warmers from four different companies. All of them had similar abilities and showed no significant differences in lower detection limit (Table 2).

DISCUSSION

In the present study, we established the pocket-warmer LAMP, a new, highly mobile and sensitive method for anthrax detection. This system is able to amplify the *pag* and *capB* genes of *B. anthracis* from DNA containing 1,000 copies corresponding to 0.1 volume of a CFU. It takes less than 90 min and can be detected with ambient light.

The most significant advantage of this pocket-warmer LAMP is its high mobility. For bioterrorism or similar emergencies, rapid and accurate detection are necessary. So far, several early detection systems for bioterrorism agents have been developed (2). However, because almost all of them require at least heat blocks and a centrifuge, it is difficult to detect bioterrorism or other disease agents immediately at the scene of a suspected outbreak. If the detection system requires any equipment, samples must be shipped from the outbreak site to the laboratory where the equipment is. The pocket-warmer LAMP does not require any heavy equipment such as heat blockers or thermal cyclers. Moreover, it does not need any electric power. The pocket-warmer LAMP, therefore, can reduce transit time and produce a rapid detection. Anti-bioterrorism methods developed thus far are not especially applicable to events happening in undeveloped areas or disaster sites in which electricity infrastructures are inadequate or destroyed. In addition to its mobility, it is also inexpensive. Although a DNA amplification kit and specific primers are required, pocket warmers are disposable and typically cost less than 100 yen each. Such low cost is a big advantage, especially in developing countries.

LAMP's specificity and sensitivity are similar to those of PCR (3). Because the four specific primers and two loop-primers used in LAMP recognize six different regions of the target genes, these primers enhance the specificity of the reaction. The specificity and sensitivity of conventional LAMP using the primers that we used in the present study have already been examined by a previous study (9). It detected 10 spores of *B. anthracis* per tube (9). This lower detection limit is higher than conventional PCR (at about 100 spores per tube). Our results showed the pocket-warmer LAMP's lower detection limit is similar to that of conventional LAMP, suggesting that its lower detection limit might be 10 spores per tube.

Condition experiments revealed that the pocket-warmer LAMP should be used only under certain conditions. To obtain the target temperature of the pocket-warmer LAMP, it is important to insulate it from environmental temperatures. We used a Styrofoam box to isolate the pocket warmers from the environment, which worked very well. It should be noted that the LAMP reaction did not work under cold conditions without a Styrofoam box or in a pants pocket.

To establish an entire mobile detection system for anthrax, we have to think about other steps. The procedure for DNA extraction usually requires a heat block and a centrifuge. However, a previous study demonstrated that DNA samples extracted from *B. anthracis* with the boiling method (95–100°C for 30 min in sterile water) were sufficient for LAMP (9). Moreover, DNA extraction kits using magnetic beads do not require any centrifuge or heat block. In addition, DNA amplification reagents for LAMP should be shipped under cold conditions to the bioterrorism or outbreak site. The method of collecting samples from the environment is another concern for the establishment of the entire system. Thus, although further experimentation and development are necessary to resolve these problems, the pocket-warmer LAMP will contribute to rapid, reliable anti-bioterrorism responses. This technique also has the potential to provide detection tools for infectious diseases in areas that do not have functional electricity infrastructures.

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