Rapid, Sensitive, and Specific Detection of *Clostridium tetani* by Loop-Mediated Isothermal Amplification Assay

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Tetanus is a specific infectious disease, which is often associated with catastrophic events such as earthquakes, traumas, and war wounds. The obligate anaerobe *Clostridium tetani* is the pathogen that causes tetanus. Once the infection of tetanus progresses to an advanced stage within the wounds of limbs, the rates of amputation and mortality increase manifold. Therefore, it is necessary to devise a rapid and sensitive point-of-care detection method for *C. tetani* so as to ensure an early diagnosis and clinical treatment of tetanus. In this study, we developed a detection method for *C. tetani* using loop-mediated isothermal amplification (LAMP) assay, wherein the *C. tetani* tetanus toxin gene was used as the target gene. The method was highly specific and sensitive, with a detection limit of 10 colony forming units (CFU)/ml, and allowed quantitative analysis. While detecting *C. tetani* in clinical samples, it was found that the LAMP results completely agreed with those of the traditional API 20A anaerobic bacteria identification test. As compared with the traditional API test and PCR assay, LAMP detection of *C. tetani* is simple and rapid, and the results can be identified through naked-eye observation. Therefore, it is an ideal and rapid point-of-care testing method for tetanus.

**Key words:** Loop-mediated isothermal amplification (LAMP), *Clostridium tetani*, point-of-care testing (POCT)

Tetanus is a specific infectious disease, which is caused by the obligate anaerobe *Clostridium tetani*. It is often associated with earthquakes, traumas, and war wounds. The pathogenic bacteria proliferate extensively in deep wounds and produce the poisonous toxin *C. tetani* tetanus, which triggers violent spasms in victims of this disease. In acute cases, when the infection aggravates, the patient may suffer from respiratory paralysis or even death [11, 22]. In patients afflicted with war injuries, the contamination rates of tetanus vary between 25% and 80%. The incidence of tetanus is higher in cases where the wounds are co-infected with *C. tetani* and aerobes [10]. Although there is a low incidence of tetanus in peacetime, it often occurs in childbirth and wound infections of construction workers. Early diagnosis of *C. tetani* can effectively prevent the incidence of tetanus. However, the use of conventional detection methods is limited, given the lack of well-equipped laboratory facilities in the areas affected by earthquakes and other calamities. Therefore, it is necessary to develop a simple, rapid, and specific point-of-care detection method for *C. tetani*, which will help in the clinical treatment of tetanus.

Currently, the detection methods for *C. tetani* mainly include microscopic examination of wound exudates and anaerobic cultivation followed by biochemistry identification. The direct smear method is rapid and economical, but it has low specificity and detection rates. Thus, it is difficult to discriminate *C. tetani* from other *Clostridium* species by this method. Anaerobic cultivation [9] followed by API identification is currently the “gold standard” technique for detection of *C. tetani*. However, anaerobic cultivation is a time-consuming technique, which requires relatively high-standard laboratory facilities. As a result, this leads to a delay, which is beyond the optimal time window for treatment. Owing to these factors, the anaerobic cultivation method is only considered as a point-of-care testing technique, especially in cases of war wound and earthquake relief, which require a simple and rapid detection method. In previous studies, identification of *C. tetani* has been conducted through PCR assay and PCR based on fluorescent amplification-based specific hybridization (FLASH) [1, 20]. PCR detection is highly sensitive and a specific PCR assay is also recommended for reasonable quantitative analysis [3]. However, it is not suitable for point-of-care testing, as we need high-standard testing equipments and operators to conduct this assay.

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The loop-mediated isothermal amplification (LAMP) assay is a newly developed gene amplification method. To conduct the LAMP assay, we use two pairs of primers, which are specially designed for recognizing six regions within the target gene. Then, strand displacement DNA synthesis is initiated and self-cycled continuously using high-activity *Bacillus steatothermophilus* DNA polymerase. In this process, we obtain the stem-loop amplification product under the isothermal conditions of 63–67°C. An extensive and rapid amplification of the target gene can be achieved within 30–60 min. The reaction can be identified with the help of a turbidimeter, a device that detects the presence of white precipitates of magnesium dihydrogen phosphate, a by-product of the reaction. Alternatively, we can also identify the reaction through agarose gel electrophoresis [16, 27]. The reaction can be accelerated by the addition of loop primers [14, 26]. Furthermore, we can observe the reaction by the naked eye by applying appropriated chromogenic reagents (such as calcein [24] or hydroxynaphthol blue [25]). Thus, special testing equipment is not required. LAMP is extensively used in testing for detection of viruses [4, 18], bacteria [12, 23], and parasite [15], and food safety [5, 17] and aquaculture [7, 19]. LAMP is characterized as a rapid testing technique with high sensitivity and specificity. Regarding the identification of anaerobic bacteria, owing to the high dependence on anaerobic training requirements, a lot of small laboratories cannot carry out the detection of anaerobic bacteria. Therefore, the LAMP method has more advantages. In other words, LAMP could be used for point-of-care testing of *C. tetani*. A lot of detection methods based on LAMP of anaerobic bacteria (such as *C. perfringens* [8], *C. difficile* [2], and *C. botulinum* [21]) have been established in recent years. However, there is no literature that explains the rapid detection of *C. tetani* using the LAMP assay. In this study, to fulfill the requirements of point-of-care testing, we developed a rapid LAMP assay, which detected *C. tetani* using *C. tetani* tetanus toxin as the target gene.

**Material and Methods**

**Primer Design**

The nucleotide sequences of tetanus toxin genes expressed specifically by *C. tetani* were retrieved from GenBank, National Center for Biotechnology Information (NCBI). They were used as potential target genes in the assay. The primers were designed using PrimerExplorer 4.0 online software (Eiken Chemical Co., Ltd. Japan). The *C. tetani* tetanus toxin gene (GenBank Accession No. AF154828) was eventually selected as the target gene through the pilot experiment for screening. The LAMP primers were designed according to its conserved regions. The sequences of the primers are as follows (5' → 3'):

- F3: 5'-GATAAAAGATGCATTTTGAAGTT-3'
- B3: 5'-TCTTCTCTATTACACCCCAAC-3'
- FIP: 5'-AGTTGTGGCAATATATTATCCCTAGTGGTACCCATAATGTTCA-3'
- BIP: 5'-AACATGTTATTGTGACTTTGTAACCTATGTGCTATGTTGTGTT-3'

**Reaction Protocol for LAMP**

DNA of standard *C. tetani* strain (American Type Culture Collection; ATCC19406; USA) was extracted according to the instructions provided in the bacteria DNA extraction kit (Tiangen Biocchemical Technology (Beijing) Co., Ltd., China). In a specific LAMP reaction tube, the reaction was carried out using the following reaction ingredients: 2 µl of *C. tetani* DNA, 12.5 µl of reaction reagent, 1.0 µl of *Bst* DNA polymerase large fragment (Eiken Chemical Co., Ltd., Japan), 4 µl of primer mixture (containing 4 primers: 10 µmol/l F3, 10 µmol/l B3, 40 µmol/l FIP, and 40 µmol/l BIP), and 5.5 µl of double-distilled water. The mixture in the reaction tube was incubated isothermally at 65°C for 60 min in a LAC320C real-time turbidimeter (Eiken Chemical Co., Ltd., Japan). The resultant reaction products were analyzed by electrophoresis on 2% agarose gels (Biowest Co., Ltd, Spain) at a voltage of 100 V for 50 min. Then, it was visualized in a Kodak Gel Logic 212 PRO Imaging System (Eastman Kodak Co., Ltd, USA).

**Sensitivity and Specificity of the LAMP Assay**

A solution of standard *C. tetani* strain (ATCC19406) was diluted into eight serial solutions, whose concentrations varied between 1×10^6 and 1×10^9 CFU/ml. From every diluted solution, an aliquot of 1 ml was then subjected to DNA extraction and LAMP amplification. To determine the detection limit and evaluate the sensitivity of the method, the reaction products were analyzed by turbidimetry and electrophoresis on agarose gels, respectively.

To determine the changes in turbidity of the reaction tubes, we measured the time required by the reaction to reach the set turbidity threshold. This time-interval was recorded as CT values (min) (cycler threshold; i.e., the cycle number at which the sample fluorescence in a PCR reaction reaches the threshold level; here it is expressed as time). When the mean of the turbidity values in the first 5 min was set as a base line, the turbidity threshold was set as 0.1 NTU (nephelometric turbidity unit). The experiment was conducted in 10 replicates. To construct the standard curve, CT values were plotted against logarithms of *C. tetani* concentrations, and the sensitivity of the assay was calculated by regression analysis.

Fluorescent detection reagent (FDR, Eiken Chemical Co., Ltd., Japan) was added into the reaction system to investigate if the reaction results can be observed by the naked eye. DNA of 1 ml *C. tetani* solution that was an intermediate value between 1×10^6 and 1×10^9 CFU/ml (Note: This was an intermediate concentration that guaranteed a positive result.) was extracted and divided into two equal groups. FDR at 1 µl was added to one group, but FDR was not added to the other group. Then, both groups were subjected to LAMP reaction. The result of chromogenic reaction was observed only after performing the LAMP assay.

To investigate the specificity of the LAMP method, we used the following 16 strains of standard bacteria: *C. tetani* (ATCC19406), *C. perfringens* (ATCC13124), *C. difficile* (ATCC9689), *C. novyi* (ATCC19402), *C. histolyticum* (ATCC19401), *C. septicum* (ATCC12464), *A. baumannii* (ATCC19600), *E. faecalis* (ATCC14506), *H. influenza* (ATCC10211), *E. coli* (ATCC25922), *S. aureus* (ATCC25923), *P. aeruginosa* (ATCC27853), *S. pneumonia* (ATCC49619), *N. gonorrhoeae* (ATCC19424), *P. vulgaris* (ATCC33420), and *S. flexneri* (ATCC12022) from American Type Culture Collection (ATCC; USA). These 16
strains of standard bacteria were recovered, cultured, and passaged. The colonies of each strain were counted and diluted into a $1 \times 10^5$ CFU/ml solution. (Note: this was an intermediate concentration that guaranteed a positive result.) In these solutions, DNA was extracted by the above described method and subjected to LAMP amplification. The reaction products were analyzed by turbidimetry and electrophoresis on agarose gels, respectively.

**LAMP Detection of C. tetani in Clinical Samples and Comparison with API 20A Anaerobic Bacteria Identification Test Results**

In the period extending from January to December 2011, clinical samples were collected from 50 patients with open deep wounds (> 3 cm), who reported at the Department of General Surgery, Xinqiao Hospital, Chongqing City. Among these 50 patients, 25 were males, whose ages varied between 12 and 49 years. The average age of these male patients was 32 years. The 25 female patients were of ages between 10 and 47 years. The average age of these female patients was 31 years. Aseptic slips were used to collect the exudates from the infected deep wounds of these patients. These exudates were inoculated on high-concentration blood agar plates (Chongqing Pangtong Medical Equipment Company, China) and cultured in anaerobic bags (Bio-Merieux, France) at 37°C for 48 h. Single colonies were collected and subjected to DNA extraction and LAMP reaction. Then, they were tested with the API 20A identification card (bioMerieux, France). The results of the LAMP reaction and API 20A anaerobic bacteria identification test were compared to investigate the consistency of the two assays.

**RESULTS**

**Sensitivity of the LAMP Assay**

To determine the detection limit of the LAMP assay, the following process was conducted. LAMP amplification of DNA was performed. This DNA was extracted from seven serial 10-fold dilutions of the standard C. tetani strain, ranging from $1 \times 10^6$ to $1 \times 10^0$ CFU/ml. Then, the reaction products were analyzed through turbidimetry and electrophoresis on agarose gels. The real-time turbidity monitoring in LAC320C gave positive turbidity results in solutions of $1 \times 10^1$ – $1 \times 10^6$ CFU/ml (Fig. 1A), and the gradient bands of amplification products were obvious after visualization of the electrophoresis results (Fig. 1B). On the other hand, the turbidity test and electrophoresis of solution of $1 \times 10^0$ CFU/ml showed a negative amplification result. Thus, the detection limit of the LAMP assay was found to be $1 \times 10^0$ CFU/ml, which indicated that it was a high-sensitivity technique. Moreover, a linear relationship between the CT value of the LAMP reaction and C. tetani concentration ($n = 10$).

![Fig. 1. Sensitivity of the LAMP assay for detection of C. tetani.](image)

(A) Effect of C. tetani concentration on LAMP reaction [(-): negative control; (+): positive control; C. tetani concentration: $10^0$, $10^1$, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$ CFU/ml]. (B) Electrophoresis analysis of C. tetani DNA LAMP reaction [M: marker; (-): negative control; (+): positive control; C. tetani concentration: $10^0$, $10^1$, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$ CFU/ml]. (C) The linear relationship between the CT value of the LAMP reaction and C. tetani concentration ($n = 10$).

To investigate the results of naked-eye observation of LAMP assay, FDR was added into the reaction system. After the addition of FDR, a pale brown color appeared in the negative reaction tubes, whereas a yellowish-green color was found in the positive reaction tubes (Fig. 2A and 2B). With a detection limit of $1 \times 10^1$ CFU/ml, the sensitivity of the naked-eye observation test is comparable to that of the turbidity test conducted on a LAC320C turbidimeter. Therefore, the addition of FDR is suitable for naked-eye observation of LAMP reaction products of C. tetani DNA.

**Specificity of the LAMP Assay**

The LAMP method developed in this work was used in detecting 16 bacterial strains that were commonly found in wound infections. The results after LAMP reaction are shown in Fig. 3 (A: dynamic turbidimetry; and B: electrophoresis). Of all the tested bacteria, only the
amplification of C. tetani DNA yielded positive results. The amplification of DNA from other infectious bacteria rendered negative results. This indicated that the LAMP assay showed high specificity in the detection of C. tetani.

**LAMP Detection of C. tetani in Clinical Samples and Comparison with API 20A Anaerobic Bacteria Identification Test Results**

To validate the application of the LAMP assay in the clinical diagnosis of C. tetani, 50 clinical specimens were tested by the LAMP method developed in this study. The “gold standard” API 20A anaerobic bacteria identification test was performed simultaneously, with the purpose of comparing the consistency of the two methods. As shown in Table 1, the LAMP amplification identified 5 positive samples in the presence of C. tetani, which were consistent with the positive results of the API 20A anaerobic bacteria identification test. Using the API 20A anaerobic bacteria identification method, other strains of bacteria or no bacteria were found to be present in the negative samples determined by the LAMP assay. These consistent results indicate that the results of LAMP reaction were in complete agreement with those of the API 20A anaerobic bacteria identification test, with an accuracy rate of 100%.

**DISCUSSION**

In this study, a LAMP method was developed for the detection of C. tetani. C. tetani tetanus toxin was selected as the target gene. The primers were designed to target the relatively conserved regions of the gene, which were identified through sequence alignments. In this context, the established LAMP method is rapid, cost-efficient, and easy-to-operate. Furthermore, the results of LAMP assay conducted on clinical samples were in complete agreement with those of the API 20A anaerobic bacteria identification test conducted simultaneously. Therefore, it is an ideal and rapid detection method for C. tetani.

The rapid detection and identification of pathogens are critical to control infectious diseases. Tetanus is a potentially
lethal infectious disease that affects the central nervous system, which may occur in various wounds. Tetanus may also occur in newborn infants and their mothers, if the delivery is conducted under unhygienic conditions. Currently, the identification of the pathogen *C. tetani* is conducted by anaerobic culture of wound exudates, followed by API test or automatic microbial culture and identification analysis, or PCR assay. Anaerobic culture requires advanced equipments and is time-consuming (around 18–72 h). Thus, it often causes a delay beyond the optimal time window for treatment. Both the API test and automatic identification are based on anaerobic culture of the pathogen, so the detection process is time-consuming. On the other hand, the LAMP reaction gets completed within 1h. Therefore, it is saves valuable time in the diagnosis and treatment of tetanus.

We found that the established LAMP method was specific for *C. tetani* and had no amplified products for other bacteria commonly found in wounds, suggesting a high specificity of the assay. This also indicates that the primers designed for the reaction are highly specific to the *C. tetani* tetanus toxin. The high specificity has been attributed to the two pairs of the primers, which were able to recognize 6 independent regions within the target gene. On the other hand, only one pair of primer is used in the PCR assay. Practically, *C. tetani* tetanus toxin shows high specificity in the detection and identification of *C. tetani*. Conventional PCR assay is also highly specific. However, the current PCR method has limited applications in bedside and point-of care testing, as this technique requires high-level laboratory equipments, including PCR gene amplifier, water bath, centrifuge, and super-clean bench, and the complicated reaction protocol. Unlike the thermal cycles essential to PCR assay, the LAMP reaction can be performed under isothermal conditions. The LAMP method developed for the detection of *C. tetani* demonstrated a low detection limit of 1×10^1 CFU/ml. This result is similar to the reported detection limits for the identification of other pathogens, such as porcine circovirus type 2, yellow head virus (YHV), and *Riemerella anatipestifer* [6, 13, 28]. Quantification analysis of *C. tetani* is also possible when the experimental protocol is optimized and standardized.

The LAMP method have several limitations. For instance, it puts higher standards on primers (i.e., more than one pair of primers are needed for amplification), and the length of amplified target sequences should be lower than 300 bp. The electrophoresis results of LAMP reaction products are illustrated in the form of gradient bands, which makes it difficult to discriminate nonspecific amplification of other DNA. The reaction is highly vulnerable to contamination owing to its high sensitivity, and attention needs to be paid in the operation [27]. Earlier studies have reported that the procedure of DNA extraction can be omitted, and the serum samples can be used directly in the LAMP detection of clinical samples. As compared with the sensitivity of PCR assay, the sensitivity of LAMP is slightly affected by the components of clinical samples. This simplified the sample pretreatment procedure of the LAMP assay. Thus, the LAMP assay is a rapid and economical form of testing, as compared with the PCR assay. Therefore, the LAMP assay is recommended for modestly equipped hospitals and point-of-care testing, because it does not require expensive instruments. Moreover, the sample pretreatment protocol is relatively simple. In summary, the LAMP detection method of *C. tetani* established in our study is highly specific, highly sensitive, simple, and rapid. Therefore, it could be a potentially valuable tool for the clinical and point-of-care testing of the pathogen.

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**References**


