

Rapid visual detection of binary toxin producing *Clostridium difficile* by loop-mediated isothermal amplification

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Abstract. The binary toxin *Clostridium difficile* transferase (CDT) is frequently observed in *C. difficile* strains and is associated with an increased severity of *C. difficile* infection. CDT-producing *C. difficile* infections cause higher fatality rates than infections with CDT negative isolates. Thus, the rapid and accurate identification of a CDT positive *C. difficile* infection is critical for effective treatment. The present study demonstrates how loop-mediated isothermal amplification (LAMP) can be used to detect CDT-producing *C. difficile* based on visual observation. This is a low complexity, rapid molecular method that has the potential to be used within a point of care setting. The specificity and sensitivity of the primers in the LAMP reactions for CDT detection were determined using two different methods, a real-time turbidity monitor and visual detection after the addition of calcein to the reaction tube. The results revealed that target DNA was amplified and visualized by these two detection methods within 60 min at a temperature of 60°C. The sensitivity of the LAMP assay was identified to be 10-fold greater than that of polymerase chain reaction analysis. When 25 alternative bacterial strains lacking CDT were tested, the results of the amplification were negative, confirming the specificity of the primers. In conclusion, the visual LAMP method established in the present study may be a rapid, reliable and cost-effective tool for detecting CDT-producing *C. difficile* strains at the point of care.

Introduction

Clostridium difficile is a gram-positive toxin-producing bacillus that causes intestinal infections in humans and animals (1). *C. difficile* presents an emerging threat in hospital environments (2), causing a range of digestive disorders, including inflammation of the bowel, abdominal pain, fever and diarrhea (1). *C. difficile* infection has increased in prevalence and severity over the last decade (2,3). Its pathogenesis comes from its ability to release two different toxins; toxins A and B. These toxins glucosylate and inactivate the Rho factor proteins of host cells. In addition, certain *C. difficile* isolates produce the binary toxin *C. difficile* transferase (CDT) (1,4,5). CDT belongs to the family of binary adenosine diphosphate (ADP)-ribosylating toxins and is made up of two separate toxin components: i) The enzymatic ADP-ribosyltransferase that modifies actin (CDTa); and ii) CDTb that binds to host cells and translocates CDTa into the cytosol (6). These two independent, unlinked protein chains are encoded by two separate genes, designated *cdtA* and *cdtB* (1,5,7). CDT serves an important role in the immediate colonization of *C. difficile*; it can create thin microtubule protrusions on the surface of intestinal epithelial cells, which increase the adherence of *C. difficile* onto the surface of these cells (4). CDT positive strains were previously reported as an infrequent cause of *C. difficile* infection in human populations but have become increasingly prevalent over the past decade (6). CDT is an important contributing factor towards the pathogenesis of *C. difficile*, and CDT-producing *C. difficile* infections are known to be more severe and have higher fatality rates than those caused by CDT-negative isolates (4). This has led to CDT being viewed as a serious health threat (1).

The accurate and rapid diagnostic testing for CDT-producing *C. difficile* is essential for patient management and the timely implementation of infection control measures; however, sensitive and specific diagnostic tests for CDT detection are lacking (3). Advances in molecular methods for the detection of pathogens may offer increased sensitivity and specificity. For example, the binary toxin CDT encoding genes *cdtA* and *cdtB* are potential biomarkers for diagnostic purposes (3).

In the present study, a low-complexity method for the detection of binary toxin-producing *C. difficile*, that makes use of loop-mediated isothermal amplification (LAMP) was used.

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LAMP is a nucleic acid detection technique first described in 2,000, which commands high specificity as the amplification of DNA is conducted by two to three pairs of primers recognizing six independent regions on a target gene (8). LAMP was developed to amplify target DNA without the temperature shifts normally required for denaturing, annealing, and extension during the polymerase chain reaction (PCR) (9). As this method proceeds at a constant temperature, requiring only a thermostat, it can be effectively performed in the field. In the present study, the CDT encoding genes *cdtA* and *cdtB* were used for the diagnosis of CDT-producing *C. difficile* using LAMP (10). Although LAMP has previously been applied to detect the *C. difficile* enterotoxin A encoding gene *tcdA* and the cytotoxin B encoding gene *tcdB* (2,3,11,12), electrophoresis or other sophisticated apparatus, such as a real-time turbidimeter, were still required for the determination of the amplified products. The present study describes a visual detection method based on a color change in the calcein/Mn²⁺ mixture, allowing immediate interpretation of the LAMP results by the naked eye. This newly developed visual LAMP assay is simple, fast and only requires thermostatic equipment.

Materials and methods

Collection of clinical samples. A total of 10 stool samples were collected from hospitalized patients in the Infection Control and Hospital Epidemiology (Beijing, China). Written informed consent was obtained from all patients prior to sample collection. Of the 10 *C. difficile* strains that tested positive for binary toxin genes, only 1 strain that was isolated from a patient with Crohn's disease was identified to be positive for the *cdtA* and *cdtB* genes by LAMP detection. This was consistent with the results of polymerase chain reaction (PCR) analysis.

Pathogen strains and DNA extraction. A total of 25 strains used in the present study are from our microorganism center (Institute of Disease Control and Prevention, Academy of Military Medical Sciences; Beijing, China). These strains included *Bacillus megaterium*, *Vibrio piscium*, *Pseudomonas maltophilia*, *Mycobacterium tuberculosis* 4368, *Vibrio cholera* O139, *Bacillus anthracis*, enterohemorrhagic *Escherichia coli*, *Yersinia enterocolitica*, *Vibrio parahaemolyticus* (5474), Enteropathic *Escherichia coli*, enteroadherent *Escherichia coli*, Enteroinvasive *Escherichia coli*, Enterotoxigenic *Escherichia coli*, *Yersinia pestis*, *Streptococcus pneumoniae*, *Neisseria meningitidis* group B CMCC29022, *Burkholderia pseudomallei*, Methicillin resistant *Staphylococcus aureus*, *Acinetobacter baumannii*, *Escherichia coli*, *Bordetella pertussis* ATCC18530, *Haemophilus influenzae*, *Corynebacterium diphtheriae* CMCC38001, *Mycobacterium tuberculosis* 4368 and *Neisseria meningitidis* (NM29019). In addition, *C. difficile* strains were cultured in a cycloserine cefoxitin fructose agar base (BD Biosciences, Franklin Lakes, NJ, USA); *Mycobacterium tuberculosis*, *Vibrio parahaemolyticus* (5474), *Neisseria meningitidis* and *Haemophilus influenzae* were cultured in Chocolate Agar Plates (Beijing Land Bridge Biotechnology Co., Ltd., Beijing, China); and other strains were cultured in Brain Heart Infusion (BD Biosciences). The genomic DNA of all strains was extracted using the TIANamp

Bacteria DNA kit (Tiangen Biotech Co., Ltd., Beijing, China) following the manufacturer's instructions.

Primer design. The sequences of the *cdtA* and *cdtB* genes, with the accession numbers HQ639673.1 and HQ639677.1 respectively, were retrieved from the NCBI GenBank database (<https://www.ncbi.nlm.nih.gov/genbank>). Primer Explorer Version 4 (primerexplorer.jp/elamp4.0.0/index.html; Fujitsu Ltd., Tokyo, Japan) was used to analyze the sequences, and design 8 primers for *cdtA* and 6 for *cdtB*. The primers were synthesized commercially by Sangon Biotech Co., Ltd. (Shanghai, China) and their sequences are presented in Table I.

LAMP assay. A LAMP assay was performed using a Loopamp DNA Amplification kit (Eiken Chemical Co., Ltd., Tochigi, Japan) according to the manufacturer's protocol.

LAMP assay product detection. The amplification products were detected using two methods; a real-time turbidimeter (La-320C; Eiken Chemical Co., Ltd.) at 650 nm and visual observation. Pyrophosphate ions are released during the LAMP reaction process and they form a white magnesium phosphate precipitate. This can be monitored using a real-time turbidimeter, drawing reaction curves every 6 sec with Mg²⁺ ions in the reaction buffer (13). For visual detection, 1 μ l of Loopamp Fluorescent Detection reagent (Eiken Chemical Co., Ltd.), containing a metal indicator, was added into the reaction system prior to amplification. The reaction buffer initially turned orange because the calcein was quenched by Mn²⁺ ions. Then, during amplification the calcein was displaced by pyrophosphate ions from the calcein/Mn²⁺ complex, and the color changed from orange to green. By contrast, if no amplification occurred, no color change was observed.

PCR analysis. To assess the detection limit of LAMP compared with traditional PCR, genomic DNA extracted from *C. difficile* was serially diluted at a ratio of 1:10 (from 24.8 ng/ μ l to 0.000248 pg/ μ l) and tested using the real-time turbidity monitor, the visual method and using the GeneAmp[®] PCR system 9700 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The reaction mixture for PCR contained 12.5 μ l 2X Taq PCR Master Mix (Tiangen Biotech Co., Ltd.), 1 μ l of each primer, 1 μ l genomic DNA as a template and ddH₂O to a final volume of 25 μ l. The thermocycling conditions for PCR were as follows: 95°C for 5 min; followed by 30 cycles of 95°C for 30 sec 60°C for 5 min and 95°C for 5 min; and then a final extension step at 72°C for 7 min. For detection of the PCR products, a 1% agarose gel containing ethidium bromide was used. The marker used was DL2000 (Takara Biotechnology, Dalian, China). *C. difficile* was used as a positive control, ddH₂O as the negative control and 25 different strains of pathogen (listed above) were used as test strains.

Results

Optimal conditions for the simultaneous detection of *cdtA* and *cdtB*. To the best of our knowledge, the present study was the first to use the *cdtA* and *cdtB* genes as the target sequences for a LAMP assay detecting *C. difficile*. For this reason it was necessary to establish the optimal conditions for the reaction.

Table I. Sequences of the primers used for the specific amplification of *cdtA* and *cdtB*.

A, <i>cdtA</i> primers		
Primer type	Reaction used in	Sequence (5'-3')
F3	LAMP	TCTGGTCCTCAAGAATTTGG
B3	LAMP	AATAGCTGATAGATAAGCTCCA
FIP	LAMP	GCTTGTCTTCCCATTTTGATTTAATTTTAACTCTTACTTCCCCTGA
BIP	LAMP	ATTGGTAGTGTGAATATGAGTGCATTACCTTTAGGTATAGTTATACGTAGT
LF	LAMP	AAATCATATTCAGGGGAA
LB	LAMP	TTTGCTAAAAGAAAAATAGTACTAC
F	PCR	TGAACCTGGAAAAGGTGATG
B	PCR	AGGATTATTTACTGGACCATTTG
B, <i>cdtB</i> primers		
Primer type	Reaction used in	Sequence (5'-3')
F3	LAMP	GAGTCAAATACTGCTGGAGA
B3	LAMP	TAGTAGCTCTGGAAACAGTT
FIP	LAMP	CGGATCTCTTGCTTCAGTCTTTTTTCAGATTATGAAAAAGCTTCAGGTT
BIP	LAMP	AGTTGCAGCATATCCAATTGTTGGTTTCCTTGATCAGTAGAGGCATG
F	PCR	CTTAATGCAAGTAAATACTGAG
B	PCR	AACGGATCTCTTGCTTCAGTC

cdt, *Clostridium difficile* transferase; LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction; F, forward; B, backwards; 3, outer; L, loop; I, inner; P, primer.

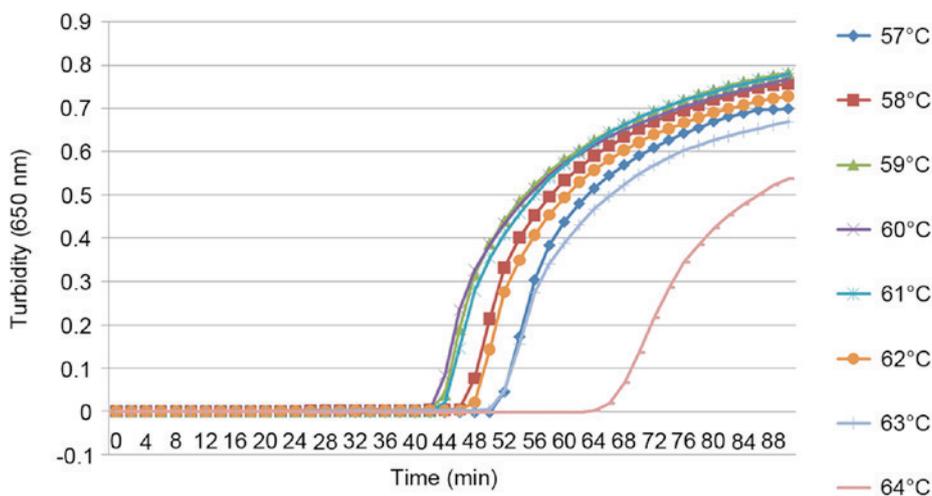


Figure 1. LAMP specific to *cdtA*, performed at increasing temperatures. A LAMP reaction specific to *cdtA* was tested using a real time turbidimeter at 650 nm at increasing temperatures in order to determine the optimal conditions. LAMP, loop-mediated isothermal amplification; *cdt*, *Clostridium difficile* transferase.

Six and four primers were designed for the detection of the *cdtA* and *cdtB* genes, respectively, using the same reaction conditions. Using a real-time turbidimeter, turbidity curves at 650 nm were constructed according to the amplification data. To optimize the reaction conditions for the LAMP assay, different reaction temperatures from 57-68°C were analyzed using the real-time turbidimeter (Figs. 1 and 2). When detecting *cdtA*, the optimal conditions were 59, 60 and 61°C for 60 min;

however, for the detection of *cdtB*, the optimal conditions were 60°C for 60 min. Therefore, for the detection of *cdtA* and *cdtB*, the optimal conditions were set at 60°C for 60 min.

Specificity of the LAMP assay to C. difficile. To evaluate the specificity of LAMP detection for *cdtA* and *cdtB*, genomic DNA extracted from *C. difficile* was used as a positive control, ddH₂O as the negative control and 25 different

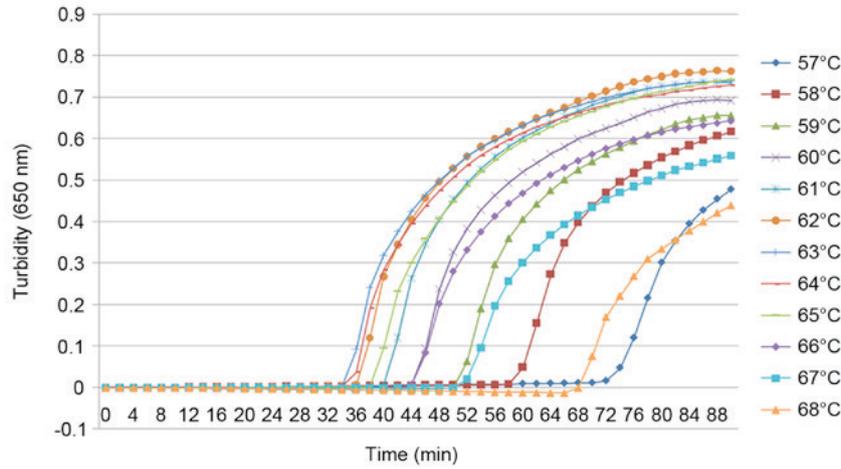


Figure 2. LAMP specific to *cdtB*, performed at increasing temperatures. A LAMP reaction specific to *cdtB* was tested using a real time turbidimeter at 650 nm at increasing temperatures in order to determine the optimal conditions. LAMP, loop-mediated isothermal amplification; *cdt*, *Clostridium difficile* transferase.

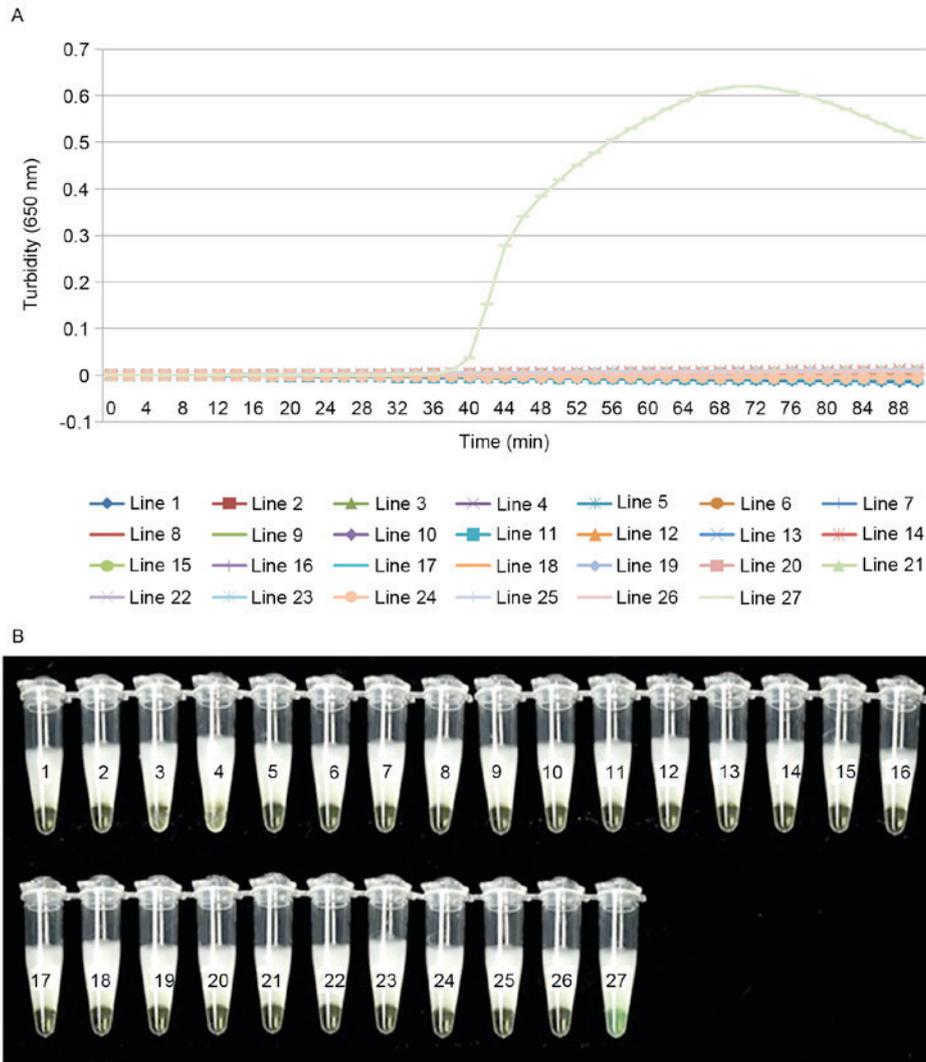


Figure 3. LAMP of *cdtA* has a high degree of specificity. Determination of the specificity of *cdtA* detection of the LAMP assay by (A) real-time turbidimeter or (B) visual detection after the addition of calcein to the reaction system. Amplification was performed at 60°C for 60 min. 1, *Bacillus megaterium*; 2, *Vibrio piscium*; 3, *Pseudomonas maltophilia*; 4, *Mycobacterium tuberculosis* 4368; 5, *Vibrio cholera* O139; 6, *Bacillus anthracis*; 7, enterohemorrhagic *Escherichia coli*; 8, *Yersinia enterocolitica*; 9, *Vibrio parahaemolyticus* (5474); 10, Enteropathic *Escherichia coli*; 11, enteroadherent *Escherichia coli*; 12, Enteroinvasive *Escherichia coli*; 13, Enterotoxigenic *Escherichia coli*; 14, *Yersinia pestis*; 15, *Streptococcus pneumoniae*; 16, *Neisseria meningitidis* group B CMCC29022; 17, *Burkholderia pseudomallei*; 18, Methicillin resistant *Staphylococcus aureus*; 19, *Acinetobacter baumannii*; 20, *Escherichia coli*; 21, *Bordetella pertussis* ATCC18530; 22, *Haemophilus influenzae*; 23, *Corynebacterium diphtheriae* CMCC38001; 24, *Mycobacterium tuberculosis* 4368; 25, *Neisseria meningitidis* (NM29019); 26, negative control; 27, positive control; LAMP, loop-mediated isothermal amplification; *cdt*, *Clostridium difficile* transferase.

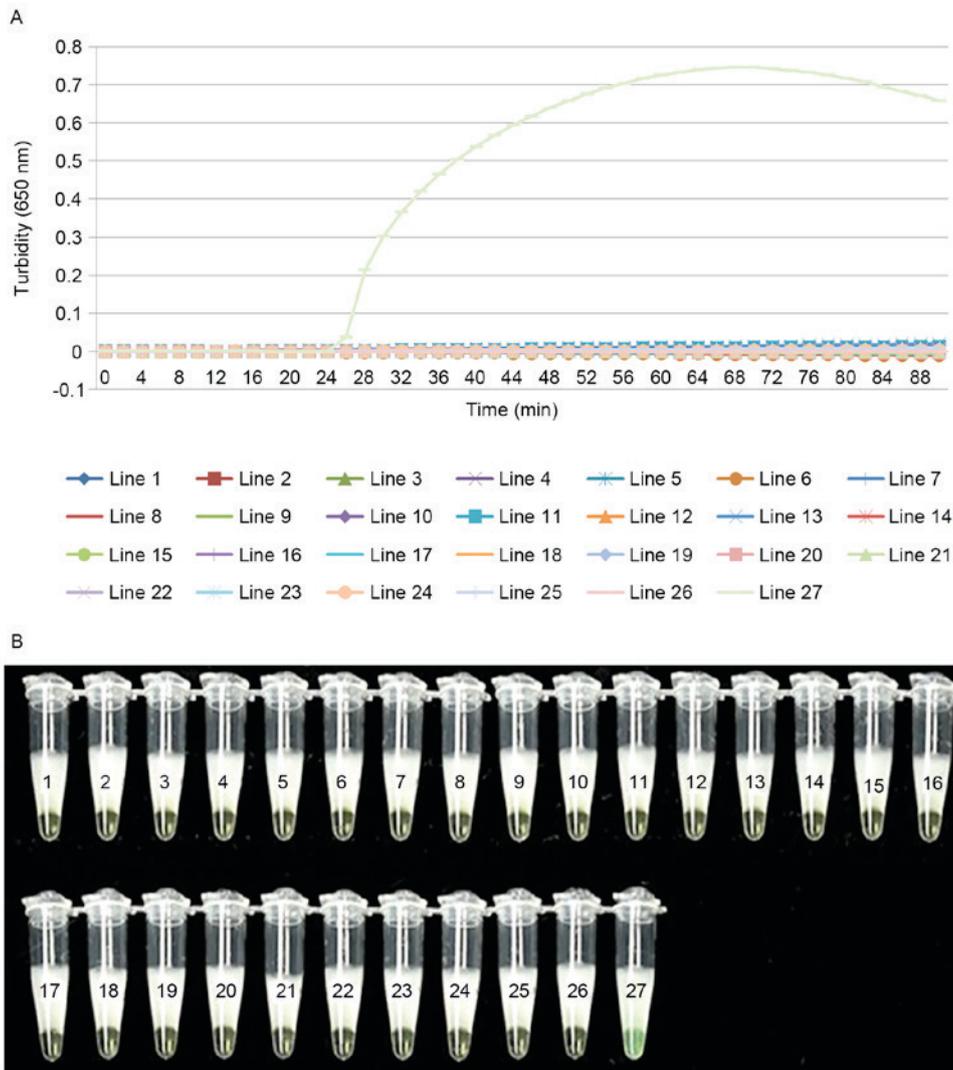


Figure 4. LAMP of *cdtB* has a high degree of specificity. Determination of the specificity of *cdtB* detection of the LAMP assay by (A) real-time turbidimeter or (B) visual detection after the addition of calcein to the reaction system. Amplification was performed at 60°C for 60 min. 1, *Bacillus megaterium*; 2, *Vibrio piscium*; 3, *Pseudomonas maltophilia*; 4, *Mycobacterium tuberculosis* 4368; 5, *Vibrio cholera* O139; 6, *Bacillus anthracis*; 7, enterohemorrhagic *Escherichia coli*; 8, *Yersinia enterocolitica*; 9, *Vibrio parahaemolyticus* (5474); 10, enteropathic *Escherichia coli*; 11, enteroadherent *Escherichia coli*; 12, enteroinvasive *Escherichia coli*; 13, enterotoxigenic *Escherichia coli*; 14, *Yersinia pestis*; 15, *Streptococcus pneumoniae*; 16, *Neisseria meningitidis* group B CMCC29022; 17, *Burkholderia pseudomallei*; 18, Methicillin resistant *Staphylococcus aureus*; 19, *Acinetobacter baumannii*; 20, *Escherichia coli*; 21, *Bordetella pertussis* ATCC18530; 22, *Haemophilus influenzae*; 23, *Corynebacterium diphtheriae* CMCC38001; 24, *Mycobacterium tuberculosis* 4368; 25, *Neisseria meningitidis* (NM29019); 26, negative control; 27, positive control; LAMP, loop-mediated isothermal amplification; *cdt*, *Clostridium difficile* transferase.

strains of pathogen were used as test strains. The two detection methods gave the same results and no reactivity was recorded with any other pathogenic bacterial strains or the negative control (Figs. 3 and 4). This demonstrates that the LAMP assay used in the present study is specific to *C. difficile*.

LAMP has a higher sensitivity in the detection of *C. difficile* compared with PCR. Serial dilutions of genomic DNA were tested using the real-time turbidity monitor, the visual method and PCR. The limit of detection for *cdtA* and *cdtB* was 24.8 pg/ μ l using the visual method and the real-time turbidimeter, which was 10-fold higher than that of the traditional PCR assay (Figs. 5 and 6). This suggests that the LAMP assay is more sensitive than traditional PCR for the detection of *C. difficile*. In addition, the minimum detectable concentration reaction time was within 60 min.

To avoid non-specific amplification, the response time was limited to 60 min.

Discussion

C. difficile is the most prevalent cause of healthcare-associated infectious diarrhea (14). Infection due to CDT-producing strains of *C. difficile* has been correlated with higher mortality rates compared to CDT negative strain infections (4). A previous study has suggested an association between the presence of CDT in infectious *C. difficile* strains and the increased mortality of patients (6). In infections due to CDT positive strains, abdominal pain and diarrhea were also reported to be more severe (4). As such, rapid and sensitive laboratory diagnostic testing for CDT positive strains of *C. difficile* is highly desirable. Diagnostic testing for toxigenic *C. difficile* has traditionally been accomplished by time-consuming

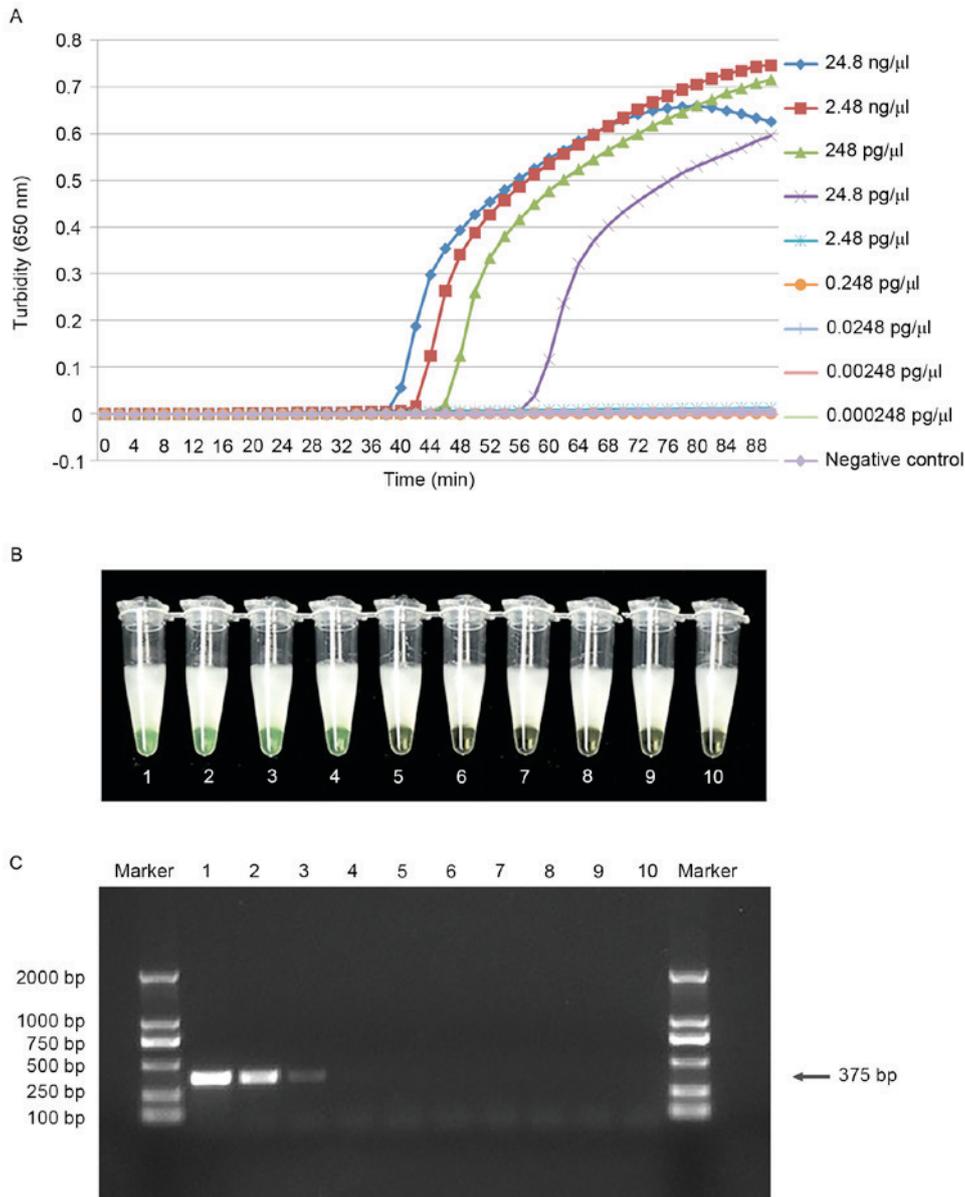


Figure 5. LAMP is more sensitive in the detection of *cdtA* than PCR amplification. Comparative sensitivities of *cdtA* detection by (A) real-time turbidimeter observations of the LAMP assay, (B) visual observation of the LAMP assay and (C) traditional PCR. Tubes 1-9 and lanes 1-9 contain a 10-fold serial dilution of pure genomic DNA extracted from *C. difficile* from (24.8 ng/μl to 0.000248 pg/μl); tube 10 and lane 10 contain ddH₂O as a negative control. Marker lane, DL-2000. LAMP, loop-mediated isothermal amplification; *cdt*, *Clostridium difficile* transferase; PCR, polymerase chain reaction.

culture methods and by immunoassays, which are faster but in general do not have sufficient sensitivity. Immunoassays that detect the glutamate dehydrogenase antigen display high sensitivity but poor specificity for *C. difficile* (15). By comparison, molecular tests have increased sensitivity and specificity (16). To the best of our knowledge, there have been no studies on the detection of CDT encoded genes. The present study investigated the efficacy of LAMP to directly detect the *cdtA* and *cdtB* genes in order to aid in the diagnosis of binary toxin-associated *C. difficile* infection. The *cdtA* and *cdtB* genes were targeted by specific primer sets in this molecular-based assay with rapid turnaround times. Although this method has been applied for the detection and identification of other microbial pathogens (17), to the best of our knowledge this is the first time this technique has been applied to detect CDT positive *C. difficile*. The LAMP

assay does not require skilled operators or precise instruments, and is therefore practically applicable within point of care settings with poor resources. Since its introduction by Notomi *et al* (8) in 2000, it has been widely applied to a variety of fields, including clinical diagnosis, food safety, livestock breeding and determination of sex (18,19). This is largely due to its rapid and simple methodology. Numerous reports have demonstrated that LAMP offers high sensitivity and specificity for the detection of pathogens (20,21).

The present study investigated a visual LAMP method for detecting the *cdtA* and *cdtB* genes of *C. difficile*. The specificity of this assay was confirmed by testing strains of 26 bacterial species, including *C. difficile*, using a real-time turbidimeter and visual methods. A positive result was only obtained for *C. difficile*, all other species gave a negative result. This confirmed the specificity of the assay for

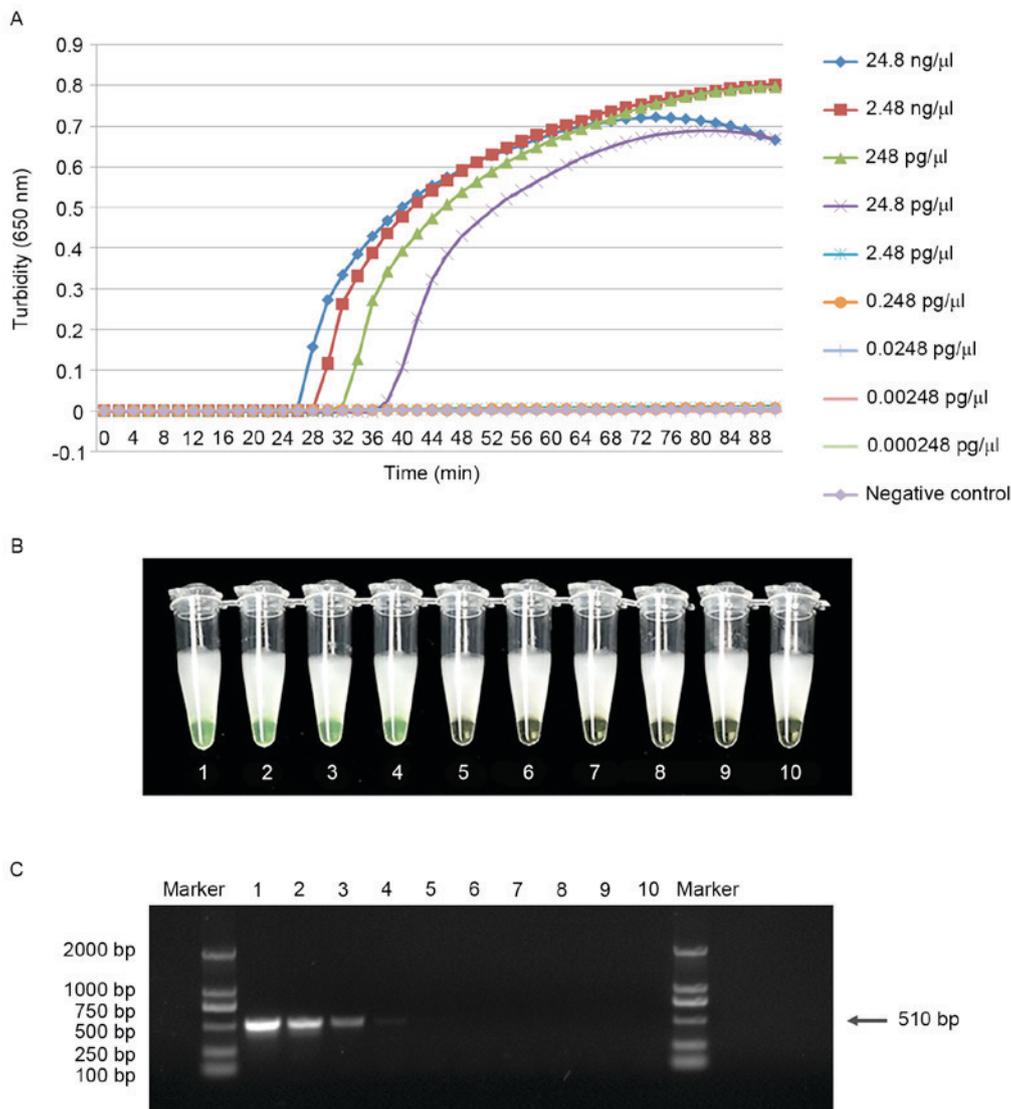


Figure 6. LAMP is more sensitive in the detection of *cdtB* than PCR amplification. Comparative sensitivities of *cdtB* detection by (A) real-time turbidimeter observations of the LAMP assay, (B) visual observation of the LAMP assay and (C) traditional PCR. Tubes 1-9 and lanes 1-9 contain a 10-fold serial dilution of pure genomic DNA extracted from *C. difficile* (from 24.8 ng/μl to 0.000248 pg/μl); tube 10 and lane 10 contain ddH₂O as a negative control. Marker lane, DL-2000. LAMP, loop-mediated isothermal amplification; cdt, *Clostridium difficile* transferase; PCR, polymerase chain reaction.

detecting *C. difficile*, and indicated that the turbidimeter and visual detection methods exhibited the same level of specificity. The sensitivity of the LAMP assay for detecting the *cdtA* and *cdtB* genes was tested using a 10-fold serial dilution of pure genomic DNA extracted from *C. difficile*. Consistent with previous reports, LAMP was identified to be 10-fold more sensitive than PCR analysis, with a detection limit of 24.8 pg/μl for the real-time turbidimeter and visual methods. Furthermore, the LAMP assay was completed within 1.5 h using a simple thermostat, whereas PCR took 3 h and required a specialized thermocycler. The LAMP assay offers a simpler method and a faster result, and is therefore preferential for urgent diagnosis in the field.

In conclusion, the visual LAMP assay developed for detecting CDT positive *C. difficile* strains in the present study has a number of advantages over regular PCR amplification, such as the ability for results to be observed with the naked eye immediately after amplification is complete. The LAMP assay is also more sensitive than PCR in detecting CDT positive

C. difficile. The results obtained with this visual method were consistent with the real-time turbidimeter observations. Consequently, this procedure offers a faster, simpler, more sensitive and lower cost method for diagnosing CDT-producing *C. difficile*, which may be particularly useful within point of care settings. No rapid diagnostic tool is available for detecting CDT-producing *C. difficile* at present, and this assay could be a useful and reliable diagnostic tool for infection control in clinical settings, even those lacking a clinical microbiology laboratory. Rapid diagnosis can be crucial during an infection outbreak and may potentially decrease the nosocomial transmission, morbidity and mortality associated with *C. difficile* infection.

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