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# DNA detection of *Clostridium difficile* infection based on real-time resistance measurement

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**ABSTRACT.** We used a newly developed electrochemical method, real-time resistance measurement, based on loop-mediated isothermal amplification (LAMP), with real-time resistance monitoring and derivative analysis. DNA extracted from specimens was amplified through LAMP reaction. The 2 products of LAMP, DNA and pyrophosphate, both are negative ions; they combine with positive dye (crystal violet) and positive ions ($Mg^{2+}$), which leads to an increase in the resistivity of the reaction liquid. The changes of resistivity were measured in real-time with a specially designed resistance electrode, to detect *Clostridium difficile* DNA. We found that electrochemical detection of *C. difficile* could be completed in 0.5-1 h, with a detection limit of $10^2$ CFU/mL, with high accuracy (95.0%), sensitivity (91.1%), and specificity (97.3%) compared to PCR methods. *C. difficile* is commonly associated with antibiotic-induced diarrhea. Due to the difficulty in performing anaerobic culture and cytotoxicity neutralization assays, a simple, rapid, sensitive, and accurate method is preferred. We conclude that real-time resistance measurement is a rapid, sensitive, and stable method

 

for the diagnosis of *C. difficile* infection that could be applied to gene chips and pocket instruments.

**Key words:** DNA detection; Real-time resistance measurement; Loop-mediated isothermal amplification; *Clostridium difficile*

## INTRODUCTION

Recently, polymerase chain reaction (PCR) has become the common method of DNA detection. On the basis of PCR, scientists have developed a series of technologies, such as quantitative real-time PCR (Demidenko and Penin, 2012), multiplex PCR (Becker et al., 2012), chip PCR (Wu et al., 2012), microfluidic PCR (Nagatani et al., 2012), and isothermal PCR (Paris et al., 2011). Loop-mediated isothermal amplification (LAMP) was invented by Notomi et al. (2000), which is characterized as rapid and of high specificity and sensitivity. It uses Bst DNA polymerase at a constant temperature (65°C) and completes DNA amplification in 20-60 min. In recent years, there has been progress in LAMP in the detection of viruses (Wang et al., 2012), bacteria (Han et al., 2011) and parasites (Abdul-Ghani et al., 2012), food safety (Sowmya et al., 2012), and animal embryo sex identification (Nogami et al., 2008). LAMP is relatively simple and can amplify DNA with high specificity (Mori et al., 2004). With the development of gene chips, more attention has been paid to PCR or LAMP on microfluidic gene chips (Tjong et al., 2011).

There are several determination methods using LAMP. Electrophoresis with the fluorescent dye method is a common technique (Le Roux et al., 2009). Rapid electrophoresis and microchip electrophoresis technology have promoted the level of electrophoresis (Iseki et al., 2007). However, the application of electrophoresis has been restricted because of slow response and strong toxicity of dyes (Paris et al., 2007). The turbidimetric method is another common technique (Tomita et al., 2008). However, it requires optical-electrical devices to transform and output the results, which are expensive and bulky. It is therefore not suitable with gene chips and pocket instruments (Le Roux et al., 2009).

*Clostridium difficile* is an anaerobic bacterium. It releases toxins that can cause *C. difficile*-associated disease (CDAD), such as antibiotic-associated diarrhea and vaginal infections (Chen and Shih, 2011). Rapid and sensitive laboratory diagnostic testing is highly desirable for appropriate treatment of CDAD (Wilkins and Lyerly, 2003). The cytotoxicity assay and toxigenic culture has been considered as the "gold standard" for the diagnosis of CDAD (Cohen et al., 2010). However, they are time-consuming, cost-ineffective, and highly standardized. Other commercial detection methods used are immunoassays, but they are not sensitive enough (Planche et al., 2008; Eastwood et al., 2009). Therefore, a simple, rapid, accurate method for *C. difficile* detection is required.

In the present study, we developed a new electrochemical method for real-time resistance measurement (Jiang et al., 2012), based on LAMP, for the identification of *C. difficile*. This assay consists of DNA extraction from stool specimens, followed by LAMP reaction to detect the *C. difficile* tcdA gene. The 2 LAMP products, DNA and pyrophosphate, 2 kinds of negative ions, were combined with a positively charged dye (crystal violet) and the positive ions ($Mg^{2+}$), leading to an increase in the resistance of the reaction medium. The resistance was real-time monitored by a specially designed resistance elec-

trode to detect the products of DNA amplification on gene chips and pocket instruments, which need a simple and real-time method of measuring. The scheme of the experiment is shown in Figure 1.

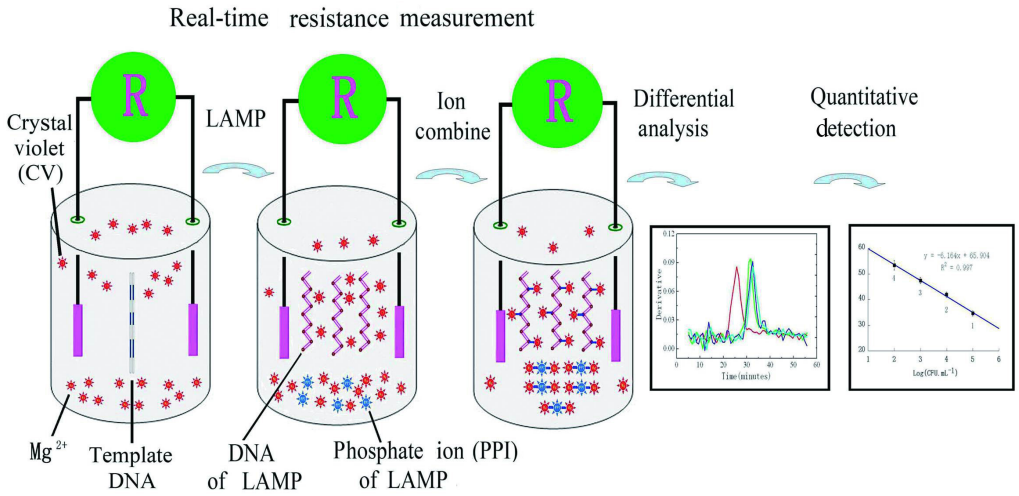


**Figure 1.** Scheme of the real-time resistance measurement.

## MATERIAL AND METHODS

### Reagents

The LAMP reagents were purchased from Eiken Chemical (Japan). The DNA extraction reagents were provided by Tiangeng (China). Crystal violet was obtained from Sigma-Aldrich (USA), dissolved in distilled water at a concentration of 100 mg/L, which was diluted to appropriate concentrations before use.

### Bacterial strains and growth conditions

*C. difficile* (ATCC 43255) and 8 kinds of other bacteria, namely *Campylobacter jejuni* (ATCC 33291), *Salmonella typhimurium* (ATCC 14028), *Escherichia coli* (ATCC 25922), *Vibrio parahaemolyticus* (ATCC 17802), *Shigella dysenteriae* (ATCC 13313), *Staphylococcus aureus* (ATCC 25923), *Yersinia enterocolitica* (ATCC 23715), and *Klebsiella pneumoniae* (ATCC 10031), were obtained from the American Type Culture Collection (ATCC, USA), grown on corresponding medium (Pangtong, China), and preserved in normal saline at -20°C until further use.

### Collection of CDAD specimens

The stool samples of patients with CDAD were collected on time and submitted to the

laboratory for *C. difficile* testing. A total of 120 stool samples were collected from May 2011 to May 2012 from the inpatients of Xinqiao Hospital. The samples were stored at -80°C for later testing.

## Primer design and synthesis

The nucleotide sequences expressed specifically in *C. difficile* were retrieved from the National Center for Biotechnology Information (NCBI) as target genes. The primers were designed using PrimerExplorer 4.0 (Eiken, Japan) online. The primers were synthesized by Sangong (Shanghai, China).

## DNA detection

The DNA was extracted by a bacterial DNA extraction kit (Tiangeng). The amplification was performed in a specific PCR tube containing 2 µL DNA extract, 12.5 µL reaction reagent, 1 µL bst DNA polymerase, 4 µL primer mixture (10 µM F3 and B3, 40 µM FIP and BIP; Table 1), 1 µL 100 mg/L crystal violet, and 4.5 µL water. The mixture was incubated isothermally at 65°C for 60 min in a Thermo bath ALB64 (Finepcr, Korea). A special electrode was designed for real-time resistance monitoring. The surface of electrode was electroplated with gold to increase its electrochemical stability. The measurements were performed on a VICTOR 86°C digital multimeter (Victor, China) with an Ag/AgCl electrode as reference, and a resistance electrode as working electrode. The reaction was real-time monitored by the DMM software (Victor). The results were analyzed by the OriginPro 7.5 software (OriginLab, USA).

**Table 1.** Primers of real-time resistance measurement and PCR.

| Target gene | GenBank | Method | Primer (5'→3') |
|---|---|---|---|
| tcdA gene | FN545816 | Real-time resistance measurement | F3: AGTTTGTTTACAGAACAAGAGTT<br>B3: ATCATTTCCCAACGGTCTA<br>FIP: CCGCCAAAATTTTTTAGGGCTAATATTTATAGTCAGGA GTTGTTAAATCG<br>BIP: AGATGTTGATATGCTTCCAGGTATTCCAATAGAGCTAG GTCTAGG |
| | | PCR | F: AGTTTGTTTACAGAACAAGAGTT<br>B: ATTTTATCATTTCCCAACGGTCTA |

## Specificity analysis

*C. difficile* and 8 bacteria that are diarrhea-associated (*C. jejuni*, *S. typhimurium*, *E. coli*, *V. parahaemolyticus*, *S. dysenteriae*, *S. aureus*, *Y. enterocolitica*, *K. pneumoniae*) were cultured, counted, diluted to the same concentration [$10^5$ colony forming units (CFU)/mL], and submitted to DNA extraction and real-time resistance measurements in the same way.

## Sensitivity and regression analysis

*C. difficile* was grown at 37°C for 48 h. The counts were performed by plating 100 µL dilutions in sterile phosphate-buffered saline and incubating for 24 h at 37°C. CFU were then

calculated. The suspension of *C. difficile* was diluted into serial concentrations (from 1 x $10^5$ to 1 x $10^0$ CFU/mL), DNA extraction and real-time resistance measurements were performed as previously described. The derivative peak times of real-time resistance measurement were monitored by the OriginPro 7.5 software. The semi-logarithmic linear regression was analyzed by Excel 2003 (Microsoft, USA).

## Comparative analysis with PCR

The samples were divided into 2 parts. One part was identified by real-time resistance, and the other part was amplified by PCR. In the PCR assay, the 25-µL multiplex-PCR mixture contained 2.0 µM primer F and B (Table 1), 12.5 µL 2X Fast qPCR MasterMix (Eurogentec Deutschland GmBH, Germany), and 2.5 µL template DNA. Thermal cycling conditions comprised a uracil-N-glycosylase step at 50°C for 2 min, hot start DNA polymerase activation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 3 s, annealing at 64°C for 30 s, and extension at 72°C for 10 s. The products were analyzed by electrophoresis on 2% agarose gels (Biowest, Spain) at 100 V for 50 min, and visualized in a Kodak Gel Logic 212 PRO Imaging System (Eastman Kodak, USA). The results were compared with regard to difference (chi-squared test), accuracy [PCR(+) real-time resistance (+) and PCR(-) real-time resistance (-) out of all samples], sensitivity [real-time resistance (+) out of PCR(+)] and specificity [real-time resistance (-) out of PCR(-)].

# RESULTS

## Primer design

The tcdA gene (GenBank: FN545816) of *C. difficile* was chosen to design the primers for real-time resistance measurement of *C. difficile* by exploring the GenBank database of NCBI. The primers of real-time resistance measurement and PCR are shown in Table 1. The specificity of primers had been positively tested by BLAST of NCBI (http://www.ncbi. nlm. nih.gov/blast).

## Specificity analysis

The results of real-time resistance measurement (Figure 2A) and derivative analysis (Figure 2B) showed that *C. difficile* (ATCC43255) was positive and other bacteria were negative. The test suggested a high specificity of LAMP assay for detection of *C. difficile*.

## Sensitivity analysis

The results of real-time curves (Figure 3A) and derivative analysis (Figure 3B) showed that the real-time resistance measurement could be completed in 1 h with the lowest bacteria level of $10^2$ CFU/mL. It displayed a good performance in the regression analysis (Figure 3C). There was a good relationship between *C. difficile* concentration (logarithm) and the derivative peak time over a range of 1 x $10^2$ to 1 x $10^5$ CFU/mL (y = 60-0.52x, R = 0.995). Furthermore, the concentration of *C. difficile* in fecal samples could be quantified.

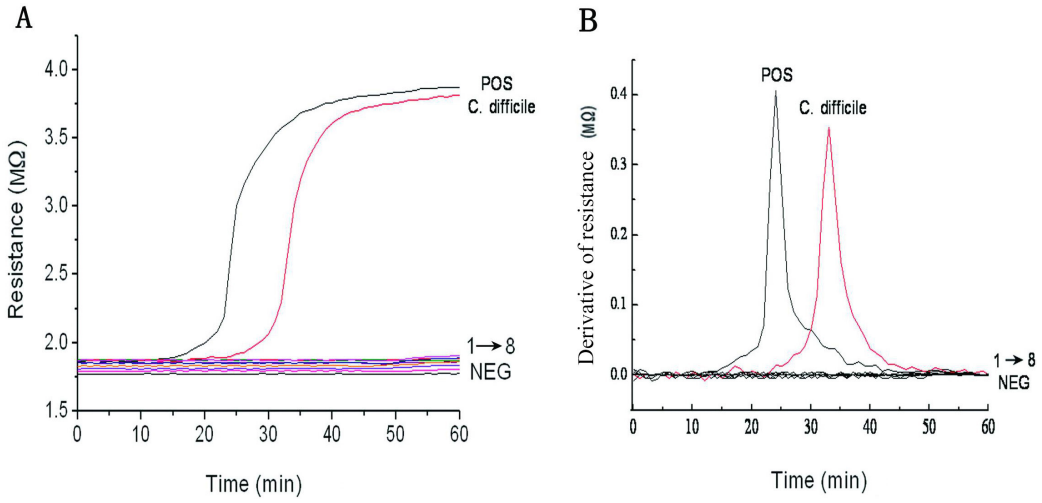


**Figure 2.** Real-time resistance curves (**A**) and derivative analysis (**B**) of the specificity analysis. POS = positive control; NEG = negative control; *Clostridium difficile*; 1→8: *Campylobacter jejuni*, *Salmonella typhimurium*, *Escherichia coli*, *Vibrio parahaemolyticus*, *Shigella dysenteriae*, *Staphylococcus aureus*, *Yersinia enterocolitica*, and *Klebsiella pneumoniae*.

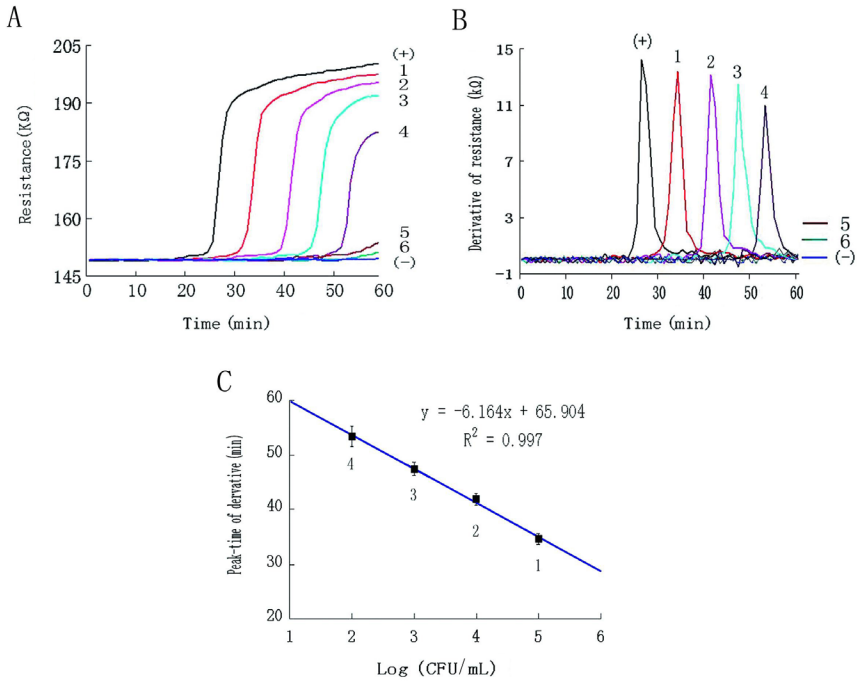


**Figure 3.** Real-time resistance curves (**A**), derivative analysis (**B**), and regression analysis (**C**) of the real-time resistance measurement. 1→5: *Clostridium difficile*; 1: $10^5$ CFU/mL; 2: $10^4$ CFU/mL; 3: $10^3$ CFU/mL; 4: $10^2$ CFU/mL; 5: 10 CFU/mL.

## Comparison with PCR

The results of real-time resistance measurement compared with PCR (Table 2) showed that there was no statistical difference between the two methods (chi-squared test, $P > 0.05$). The real-time resistance measurement displayed a high accuracy (95.8%), sensitivity (91.1%), and specificity (97.3%) compared to PCR.

**Table 2.** The comparison of real-time resistance measurement and PCR.

| | PCR | |
|---|---|---|
| | (+) | (-) |
| Real-time resistance measurement | | |
| (+) | 41 | 2 |
| (-) | 4 | 73 |
| P[a] | 0.167 | |
| $\chi^2$ | 0.683 | |
| Accuracy | 95.0% | [(41+73)/120] |
| Sensitivity | 91.1% | (41/45) |
| Specificity | 97.3% | (73/75) |

[a]There was no statistical difference between the two methods ($P > 0.05$).

## DISCUSSION

PCR has become the common method of DNA detection. On the basis of PCR, scientists have developed a series of technologies, such as chip PCR, microfluidic PCR, and LAMP. With the development of the gene chip, more attention has been paid to PCR or LAMP on microfluidic gene chips. Rapid electrophoresis and microchip electrophoresis technology have promoted the level of electrophoresis. However, slow response and the strong toxicity of dyes have restricted their application. The turbidimetric method is another common determination technique. However, it requires optical-electrical devices to transform and output the results, which are expensive and bulky. It is therefore not suitable for construction on gene chips and pocket instruments.

*C. difficile*, a spore-forming Gram-positive anaerobic bacillus, is a major cause of healthcare- and antibiotic-associated diarrhea. The most common symptoms of infection include diarrhea, abdominal cramping, and peripheral leukocytosis (Cohen et al., 2010). *C. difficile* produces toxin A and B, which cause mucosal damage and fluid secretion leading to diarrhea. A third toxin, binary toxin, has been suggested that binary toxin may improve the adhesion of bacteria to target cells (Schwan et al., 2009). *C. difficile* infection can range in severity from asymptomatic to severe and life-threatening. Epidemiological trends in the last decade have shown marked increases in incidence, severity, persistence, and mortality of CDAD (Tenover et al., 2010). Unfortunately, the lack of a rapid, accurate, and inexpensive diagnostic test for CDAD remains an important barrier to clinical and epidemiological containment of the disease.

In this study, we developed an electrochemical detection, real-time resistance method for the amplification and detection of *C. difficile*. First, the *C. difficile* tcdA gene (GenBank: FN545816) was chosen as the target gene, and the primers were designed using PrimerExplorer 4.0 (Eiken) online. DNA extracted from specimens was then amplified by the LAMP reaction. The two amplification products, DNA and pyrophosphate, are 2 kinds of negative ions.

They were combined with the positively charged dye (crystal violet) and positive ions ($Mg^{2+}$), leading to an increase in the resistivity of the reaction medium. The changes of resistivity were real-time monitored by special designed resistance electrode. The results indicated that it had a good specificity. Furthermore, it was demonstrated that this electrochemical detection can be completed in 1-2 h with a detection limit of $10^2$ CFU/mL. The real-time resistance measurement had a high accuracy (95.0%), sensitivity (91.1%) and specificity (97.3%) compared to PCR. Thus, it is a rapid, sensitive and stable method that can be used with gene chips and pocket instruments, which need a simple and real-time method of measuring.

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