Development and evaluation of a rapid visual loop-mediated isothermal amplification assay for the *tcdA* gene in *Clostridioides difficile* detection

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Abstract

Background. The *tcdA* gene ~~is of great significance to the~~codes for an important toxin ~~carrying status of~~produced by *Clostridioides difficile* (*C. difficile*), but there is currently no simple and cost-effective method of detecting it. This paper establishes and validates a rapid and visual loop-mediated isothermal amplification (LAMP) assay for the detection of the *tcdA* gene ~~of *C. difficile*~~.

Methods. Three sets of primers were designed and optimized to ~~target~~ amplify the *tcdA* gene in *C. difficile* using a LAMP assay. The sensitivity and specificity of LAMP primers were detected by ____. The *tcdA* gene of *C. difficile* was detected in 164 stool specimens using both LAMP and polymerase chain reaction (PCR). Positive and negative results were distinguished using real-time monitoring of turbidity and chromogenic reaction.

Results. At a temperature of 66 °C, the target DNA was successfully amplified with all three sets of LAMP primers, and visualized within 60 minutes, for a *C. difficile strain that carries the tcdA gene*. Under the same conditions, ~~The results of~~ the target DNA was not amplified with the all/two/one set(s) of LAMP primers ~~detection of~~for 26 pathogenic bacterial strains ~~without the~~that do not carry the *tcdA* gene ~~were negative, indicating that the primers were extremely specific. Moreover, t~~The detection limit of LAMP was ~~20.7~~21 pg/µl, which was 10 times more sensitive than that of conventional PCR. ~~Finally, the~~The detection rate of *tcdA* in 164 stool specimens, using the LAMP method, was 17% (28/164), significantly higher than the 10% (16/164) detection rate of the PCR method (X^2=47, p < 0.01).

Conclusion. ~~In this study, the~~ LAMP method ~~proved to be~~ is an effective technique for the rapid and visual detection of the *tcdA* gene of *C. difficile*~~, demonstrating~~and shows potential advantages over PCR in terms of speed, simplicity, and sensitivity. The *tcdA*-LAMP assay is particularly suitable for medical diagnostic environments with limited resources and is a promising diagnostic strategy for the screening and detection of *C. difficile* infection in populations at high risk. ~~Future research will need to further validate the applicability of the LAMP technique across various experimental and clinical settings and address any technical challenges that may arise during its broader implementation.~~

Introduction

Clostridioides difficile (*C. difficile*) is ~~a serious problem in health care institutions~~ the major cause of antibiotic-associated diarrhea worldwide (Rodríguez et al., 2020). Although the estimated national burden of *C. difficile* infection (CDI), and associated hospitalizations, decreased from 2011 through 2017 in 10 USA states ~~(California, Colorado, Connecticut, Georgia, Maryland, Minnesota, New Mexico, New York, Oregon, and Tennessee)~~, the number of *C. difficile* cases in the USA remained as high as 15,512 in 2017, with an estimated total national burden of 462,100 cases and an estimated incidence of 144 cases per 100,000 population (Guh et al., 2020). In Shandong and Zhejiang provinces, China, tertiary hospitals reported a consistent 14% incidence of CDI (72/504) among hospitalized patients with suspected CDI in Shandong and 14% (115/804) among acute gastroenteritis outpatients in a Zhejiang pediatric hospital (Luo et al., 2018; Shuai et al., 2020). These findings are consistent with other studies from mainland China, which report a 14% crude incidence of toxigenic *C. difficile* in diarrheal patients (Tang et al., 2016).

With the global prevalence of CDI, specific and sensitive methods for identifying *C. difficile* are needed. The primary virulence factors of *C. difficile* are two structurally similar toxins ~~, denoted as~~ toxin A and toxin B (~~*tcdA and tcdB,*~~ which are encoded by the *tcdA* and *tcdB* genes respectively ~~;~~(Kuehne et al.,

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2011). Most cases of CDI are attributed to strains expressing both toxins A and B (A+B+) (ref). Although the toxin A-B+ strain is uncommon, it can also cause disease and has been relevant to previous outbreaks of CDI (Alfa et al., 2000; Kuijper et al., 2001; Drudy et al., 2007). ~~It was previously believed that the~~Earlier reports indicated that toxin A+B- strain only rarely caused human diseases (Rupnik, 2008). As a result, numerous studies have emphasized the importance of toxin B in the pathogenesis of CDI, while downplaying the importance of toxin A. However, a study recently reported the discovery of clinical pathogenic *C. difficile* strains that produce high levels of toxin A but minimal or no toxin B, indicating that toxin A alone can cause CDI (Lin et al., 2020). This pattern of toxin production, observed in more than 5% of isolates, is consistently found both *in vitro* and *in vivo* in humans and mice (Lin et al., 2020). Furthermore, the production of either toxin A or toxin B by these isolates is sufficient to induce the full spectrum of CDI symptoms (Drudy et al., 2007; Freeman et al., 2010). Additionally, ~~it has been demonstrated in studies that~~ both toxins A and B can independently cause disease in animal models (Kuehne et al., 2011; Kuehne et al., 2010). ~~These studies indicate that greater attention should be paid to toxin A because of its pathogenicity.~~

Current laboratory tests for the diagnosis of toxin A in *C. difficile* strains include the *C. difficile* cytotoxin neutralization assay (CCNA), toxigenic culture (TC), toxin A enzyme immunoassay (EIA), glutamate dehydrogenase (GDH) assay, and nucleic acid amplification test (NAAT).

Although CCNA and TC remain the current gold standards, their use for routine clinical detection is challenging due to their requirements for harsh culture conditions, involvement of highly technical and complex operations, and time-consuming nature (Shah et al., 2020; Liu et al., 2021). Enzyme immunoassays are specific and rapid, but not sensitive (Nicholson & Donskey, 2023). The glutamate dehydrogenase assay is sensitive and rapid, but it has some disadvantages such as ~~cross-reactivity~~cross-reactivity, poor specificity, and a high false positive rate (Bartlett, 2010; Crobach et al., 2016). ~~Previous studies have developed and evaluated ways of~~ Assays for~~detecting the~~ *tcdA* gene ~~encoding toxin A~~ by NAAT ~~including~~ include Polymerase Chain Reaction (PCR; Kim et al., 2022), multiplex-PCR (Moosavian et al., 2022), quantitative real-time PCR (Brennhofer et al., 2022), and multiplex real-time PCR (Novakova et al., 2021). Despite the specificity and sensitivity of these diagnostic methods, their suitability for rapid detection in primary hospitals and on-site detection is limited due to their time-consuming and complex nature, as well as the requirement for expensive equipment. Thus, a rapid, simple, and cost-effective assay is needed to complement current PCR methods for detecting the *tcdA* gene.

Loop-mediated isothermal amplification (LAMP) is a powerful molecular technique for nucleic acid amplification. LAMP~~,~~ leverages~~ing~~ the strand displacement activity of *Bst* DNA polymerase, which facilitates DNA amplification under isothermal conditions (Notomi et al., 2000; Ushikubo, 2004). The high amplification efficiency of this technique~~,~~ - capable of generating up to 10^9 copies of target DNA within an hour~~,~~ - underscores its potential in rapid diagnostic applications. The excellent specificity of LAMP is attributed to its use of four (or six) primers, which can identify six (or eight) distinct regions on the target DNA or RNA (Notomi et al., 2000; Parida et al., 2008). Additionally, the detection limit of LAMP surpasses that of PCR, and the results can be visually interpreted without the need for sophisticated equipment. With its high sensitivity and specificity, LAMP has been effectively used to detect various pathogens, including bacteria (Hong-Min et al., 2023), viruses (Nawab et al., 2024), parasites (Chen et al., 2023), and fungi (Badparva et al., 2022), as well as different toxin types (Norén et al., 2011; Pancholi et al., 2012), binary toxin genes (Yu et al., 2017), and resistant genes (Lin et al., 2015; Lin et al., 2022) of *C.difficile*. This study designed three novel sets of LAMP primers and optimized

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LAMP for *tcdA* detection. To ascertain the specificity of the *tcdA* primer within the LAMP assay, 26 distinct pathogenic bacterial strains devoid of the *tcdA* gene were analyzed as negative controls. Primer sensitivity was assessed by conducting serial dilutions of *C. difficile* VPI10463 DNA. Finally, the study compared the consistency of LAMP and PCR methods in detecting the *tcdA* gene of *C. difficile* in 164 stool specimens.

Materials & Methods

Bacterial strains

A total of 26 pathogenic bacterial strains were selected ~~for~~ to evaluate the specificity of the LAMP ~~detection~~ assay (Table 1). *C. difficile* VPI10463, which ~~carrying~~ carries the *tcdA* gene, was used as the positive control. The *tcdA* gene of VPI10463 ~~the strain~~ showed 100% identity with those of the tcdA gene in ~~previously reported genes~~????, which was confirmed by PCR-based sequencing (Fig. S1). ~~To evaluate the specificity of the LAMP assay, 26 pathogenic bacterial strains lacking the *tcdA* gene were tested.~~

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Clinical stool specimens

Fresh stool specimens of suspected CDI inpatients with diarrhea were ~~continuously~~ collected from August 1, 2013 to February 28, 2014 in Nanfang Hospital of Southern Medical University, Guangzhou, China. Inpatients over 18 years old who had received antibiotic or chemotherapy treatments within the past 60 days were included. Stool samples were included of patients who experienced diarrhea within 48 hours of hospitalization, with no less than three episodes of diarrhea within a 24-hour period, and with shapeless stool classified as Bristol types 5-7. The exclusion criteria for this study were as follows: patients who were under the age of 18; patients who had previously been sampled; patients with chronic diarrhea; patients who had used laxatives; patients with various types of infectious diarrhea, such as bacillary dysentery, typhoid fever, food poisoning, and amebic dysentery; patients with intestinal functional diseases, such as irritable bowel syndrome; patients with other types of diarrhea with clear causes unrelated to antibiotics, such as lactose intolerance; and patient samples that did not complete the entire testing process due to instrument or human errors. Of the 197 fresh stool specimens collected from inpatients with suspected CDI presenting diarrhea, 33 were excluded due to duplication (n=23) or inadequate volume and freshness (n=10). All stool specimens were frozen at 80° C until detection. This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Fudan University Affiliated Huashan Hospital (committee ethic number: FDEC-2012-014). As a research member unit, Nanfang Hospital was successfully granted an ethics exemption by the Ethics Committee of Southern Medical University.

DNA extraction

To determine the specificity and sensitivity of the LAMP reactions under real conditions, genomic DNA was extracted from *C. difficile* VPI10463 and purified by the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA). The purified genomic DNA was serially diluted in distilled water by a factor of 10, from 207 ng/ul to 0.000207 pg/ul. The concentration of pure genomic DNA before and after dilution was measured using the ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA). Genomic DNA of 26 pathogenic bacterial strains was extracted using a bacterial genomic DNA extraction kit (Tiangeng, Ningbo, China) according to the manufacturer's

instructions. Additionally, a stool genome extraction kit (Tiangeng, Ningbo, China) was employed to extract genomic DNA from stool samples. Genomic DNA was stored immediately at -20 °C until use.

Primer design

Based on the *tcdA* gene sequence of *C. difficile* obtained from NCBI GenBank database (GenBank accession number: X92982.1), three sets of LAMP primers were designed (Table 2). Further analysis of the sequences with the Primer Explorer V4 software [http:primerexplorer.jp/lamp] yielded the outer forward primer (F3), outer backward primer (B3), forward inner primer (FIP), and backward inner primer (BIP). The FIP and BIP primers recognized both sense and antisense strands and were linked by a four-thymidine spacer (TTTT). The two loop primers (LF and LB) were designed to accelerate the amplification reaction. To compare the sensitivity and specificity of LAMP and PCR, conventional PCR was performed with the *NK1* and *NK2* primers (Table 2; Kato et al., 1991). All the primers were synthesized commercially (Sangon Biotech Co., Ltd., Shanghai, China).

LAMP reaction

The LAMP reactions were performed in a 25 µl reaction mixture (DNA amplification kit; Eiken Chemical Co., Ltd., Tochigi, Japan) containing the following reagents in the final concentration: 20 mM Tris–HCl (pH 8.8), 10 mM (NH4)2SO4, 10 mM KCl, 0.1% Tween-20, 0.8 M betaine, 8 mM MgSO4, 1.4 mM deoxynucleoside triphosphate and 8 U Bst DNA polymerase. Each LAMP reaction, using a real-time turbidimeter, was composed of 40 pmol FIP and BIP, 20 pmol LB and LF, 5 pmol F3 and B3 primers, and 2 µl DNA template. An additional 1 µl of calcein/Mn^{2+} complex (Fluorescent Detection Reagent; Eiken Chemical Co., Ltd., Tochigi, Japan) was added if direct visual inspection was required. The reaction was conducted in reaction tubes (Eiken Chemical Co., Ltd., Tochigi, Japan) within 60 minutes at an isothermal temperature of 66 °C.

Detection of LAMP products

Data were collected as previously described in Liu et al. (2022). Specifically, two different methods, chromogenic reaction with calcein/Mn^{2+} complex and real-time monitoring of turbidity, were applied to detect LAMP products. For direct visual inspection, 1 µl of calcein (fluorescent detection reagent; Eiken Chemical Co., Ltd., Tochigi, Japan) was added to 25 µl of reaction mixture in a LAMP tube before the LAMP reaction. For a positive reaction, the color changed from orange to green, while a negative reaction failed to turn green and remained orange. The color change could be observed by naked eye observation under natural light or 365 nm ultraviolet light. For assessing turbidity (Mori et al., 2001), real-time amplification was monitored through spectrophotometric analysis by measuring the optical density (λ650 nm) at 400 nm every 6 s with the aid of a Loopamp real-time turbidimeter (LA-230; Eiken Chemical Co., Ltd., Tochigi, Japan).

PCR detection

The PCR conditions used for amplification were described previously (Kato et al., 1991). Electrophoresis using a 2% agarose gel (Amresco, Solon, Ohio, USA) containing ethidium bromide was applied to analyze the PCR-amplified products. Images were captured using a Bio-Rad Gel Doc EQ Imaging System (Bio-Rad, Hercules, CA, USA).

Statistical analysis

The required sample size was estimated using Buderer's method (Buderer, 1996), setting the Z-value at 1.96 for the normal distribution and constraining the width of the 95% confidence interval to a maximum of 10%. Previous similar research reported a specificity and sensitivity of 95% for the LAMP assay (Soroka et al., 2021). Given the prevalence of CDI in China is 11% (Wen et al., 2023), the sample size of the study had to be at least 160 to ensure statistical validity. The McNemar test was used to analyze count data, and the Cohen's kappa (κ) statistic was employed to evaluate the agreement between the LAMP and PCR methodologies. A κ correlation value of 0.40 or below signifies a weak level of agreement, a value ranging from 0.41 to 0.60 reflects moderate agreement, and a value exceeding 0.60 denotes a strong agreement between observations. The specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) of the LAMP and PCR methods were calculated and compared to gene sequencing, respectively. All statistical analyses were performed using SPSS software version 26.0 (IBM Corp., Armonk, NY, USA). A *p*-value of <0.05 was considered statistically significant.

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Results

Optimal primers for rapid detection of *tcdA*

All three sets of primers designed herein (Table 2) produced turbidity after 26 minutes (Fig. 1). Primer set *tcdA12* showed the fastest amplification (Fig. 1), so *tcdA12* primers were selected for optimization. The reaction time of *tcdA12* primers with additional loop primers (LB and LF) was less than one-half that of the *tcdA8* primers without loop primers.

Appropriate temperature for *tcdA* LAMP reaction

The *tcdA12* primer set was evaluated across a temperature range of 58 °C to 69 °C at intervals of 1 °C. The ideal temperature range for the *tcdA12* primer set was determined to be 60 °C to 67 °C, with peak amplification efficiency identified at 66 °C (Fig. 2).

Specificity of *tcdA* LAMP reaction

To estimate the specificity of the LAMP reaction for the *tcdA* gene, the *C. difficile* VPI10463 strain with the *tcdA* gene was used as the positive control. All 26 pathogenic bacterial strains of different species without the *tcdA* gene and distilled water were designed as the negative controls. As exhibited in Fig. 3A, the increased turbidity curve appeared only when the *C. difficile* VPI10463 strain with the *tcdA* gene was used as the template instead of distilled water or other bacterial species. The results were also monitored using a direct visual method (Fig. 3B). Prior to the LAMP reaction, 1 µl of calcein/Mn^{2+} complex was added to 25 µl of the LAMP reaction mixture. When the reaction was complete, the result was interpreted as positive if the color changed from orange to green, and negative if it did not. The results of the chromogenic reaction did not differ from the results of the real-time monitoring of turbidity.

Sensitivity of *tcdA* LAMP reaction

To measure the sensitivity of the primers in the LAMP detection of the *tcdA* gene, both real-time turbidity measurements and visual detection of LAMP were compared to traditional PCR. Pure genomic DNA was

extracted from *C. difficile* VPI10463 using a Wizard Genomic DNA Purification kit, and then serially diluted 10-fold from 207 ng/μl to 0.000207 pg/μl. Distilled water was used as a negative control. As demonstrated in Fig. 4A and Fig. 4B, the detection limit of real-time turbidity was 20.7 pg/μl, which was identical to that of visual detection. The *NK1* and *NK2* primers with the same concentration of *C.difficile* VPI10463 DNA were also evaluated using PCR. The detection limit for PCR was 207 pg/μl (Fig. 4C), which was 10-fold lower than that of the LAMP reaction.

Evaluation of the assay with stool specimens

A total of 164 stool specimens were considered eligible and suitable for this study. The subjects all showed ~~As a common clinical presentation of CDI,~~ pseudomembranous colitis, which is a common symptom of CDI ~~was investigated in our inpatients and the results are shown in~~ (Fig. 5A-B). Electronic colonoscopy revealed numerous scattered pale yellow pseudomembranes and areas of congested, brittle mucosa (Fig. 5A). Histopathological imaging (Fig. 5B) revealed an infiltration of inflammatory cells into the mucosal lamina propria. ~~The results of LAMP and PCR detection of the *tcdA* gene in the stool specimens are compared in Table 3.~~

The detection rate of the *tcdA* gene using the LAMP method was 17% (28/164), significantly higher than the 10% (16/164) detection rate of the PCR method (X^2=47, *df*=1, *P*<0.01). The consistency between the LAMP and PCR methods was moderate (Kappa=0.533, $p < 0.01$). Notably, 15 of the stool specimens that were negative in PCR but positive in LAMP were found to be positive in *tcdA* gene sequencing. Of the three specimens that were negative in LAMP but positive in PCR, only one was found to be positive using *tcdA* gene sequencing ~~results, while the other two specimens were negative~~. Using sequencing as the reference standard, the LAMP assay outperformed the PCR assay with a sensitivity of 97% compared to 48%, and a specificity of 100%, nearly identical to PCR's 99%. The LAMP assay achieved a PPV of 100% and an NPV of 99%, whereas the PCR assay had a PPV of 88% and an NPV of 90% (Table 4 and Table 5).

Discussion

This study marks a significant advancement in the rapid detection of *C. difficile*, ~~, with the~~ The optimized LAMP assay reducing the time to detect the *tcdA* gene to under 60 minutes. Incorporating loop primers (LB and LF), which are complementary to the dumbbell-shaped DNA structures, substantially reduced LAMP reaction times by over 50%, underscoring their utility in expediting the amplification process. Including loop primers also enhanced the LAMP reaction's efficiency and sensitivity by multiplying the initiation sites for amplification. Moreover, the reaction's selectivity was markedly improved as these loop primers are designed to be activated singularly during the synthesis of the artificial template (Nagamine et al., 2002). LAMP-based detection yielded negative results for ~~all~~ 26 pathogenic bacterial strains ~~lacking the *tcdA* gene~~from different genera than *C. difficile*, demonstrating the primers' exceptional specificity. The 10-fold higher sensitivity of the LAMP method compared to conventional PCR corroborates findings from prior studies (Kim et al., 2022; Carvajal-Gamez et al., 2023) ~~reinforcing the method's superiority and potential to supplant conventional PCR in clinical diagnostics. These results advocate for the~~and supports integration of ~~the~~ this LAMP assay into routine diagnostic workflows~~, promising significant improvements in the speed and accuracy of pathogen detection~~.

~~This study demonstrated that t~~The LAMP method yielded a detection rate of 17% for the *tcdA* gene of *C. difficile* in stool specimens, ~~a figure that~~which is similar to ~~the~~ rates observed in diarrheal stool samples of

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diverse populations across China. For instance, a multicenter study in Shanghai, China, reported a detection rate of 18% (93/531) for the *tcdA* gene (Mi et al., 2020), while an independent cross-sectional study in Southwest China noted a detection rate of 14% (125/978; Liao et al., 2018). These findings suggest that the LAMP method's efficacy in detecting the *tcdA* gene is comparable to the methods used in previous studies. Moreover, this study also revealed a significant discrepancy in the detection rates of the *tcdA* gene in stool specimens between LAMP and PCR~~, with.~~ LAMP ~~exhibiting~~ showed a superior detection rate. This was further evidenced by the fact that 15 of the stool specimens that were negative in PCR but positive in LAMP were subsequently confirmed to be true positives through sequencing of the *tcdA* gene. In our previous study of 300 cultured *C. difficile* strains from Southern Medical University Nanfang Hospital, a perfect concordance (kappa = 1) was observed between LAMP and PCR detections of the *tcdA* gene (Lin et al., 2022), supporting the notion that LAMP is equally reliable when bacterial DNA is present in higher quantities. The difference in consistency between LAMP and PCR in detecting *C. difficile* strains in stool specimens in this study may be attributed to the variable concentration of the *C. difficile* DNA present. In stool specimens where *C. difficile* DNA may be at relatively low concentrations, LAMP's lower detection limit allows for a higher detection rate. Conversely, the increased DNA content in cultured *C. difficile* strains following enrichment culture may mask the differences in detection limits between the two methods, leading to a high degree of consistency in detecting the *tcdA* gene in these strains. These findings collectively advocate for the integration of LAMP in clinical diagnostics of CDI, particularly for cases where PCR may not provide sufficient sensitivity.

~~Building on the empirical evidence, the further statistical analysis performed in this study strengthens the case for the clinical application of LAMP.~~ In the analysis of ~~164~~ stool samples, the LAMP assay~~, with its~~ showed high sensitivity (97%) and perfect specificity (100%), along with a PPV of 100% and an NPV of 99%,~~, demonstrated superior diagnostic accuracy~~ when using sequencing as the gold standard. ~~The data~~This suggests that the LAMP assay offers a high degree of reliability in both detecting the presence of the target pathogen and verifying its absence. In contrast, the PCR assay, despite its high specificity (99%), demonstrated significantly reduced sensitivity (48%), potentially limiting its effectiveness as a standalone diagnostic tool. The PCR's PPV of 88% and NPV of 90% suggest that while positive results are likely accurate, the risk of false negatives is increased, which could contribute to under-diagnosis. Consequently, the LAMP assay could represent a more robust approach for precise detection of CDI within the examined population.

The LAMP method exhibits considerable advantages in the detection of the *tcdA* gene for *C. difficile*. First, eliminating the DNA denaturation step simplifies the operational workflow, as reactions are carried out at an isothermal condition of 60-65 °C using the strand displacement activity of *Bst* DNA polymerase, thus obviating the need for the precise thermal cycling equipment required for PCR (Soroka et al., 2021). This significantly lowers the technical requirements of detection, allowing molecular diagnostic techniques to be performed in resource-limited settings. Second, the LAMP reactions can be directly visualized through color changes in the calcium-magnesium complex when exposed to UV light, circumventing the need for the complex gel electrophoresis step that is requisite in PCR. This advancement not only expedites detection speed, but also minimizes the dependency on carcinogenic dyes, thereby enhancing the safety profile of the procedure. Third, the sensitivity of LAMP is 10-100 times higher than that of conventional PCR (Kim et al., 2022; Ashmi et al., 2023; Carvajal-Gamez et al., 2023). This significant enhancement in sensitivity ensures accurate detection of pathogens even at low concentrations, which is vital for early intervention and for controlling the spread of infection. Finally, the specificity of LAMP is notably enhanced through the employment of 4-6 primers, which identify up to

eight specific locations on the DNA template. This significantly mitigates the likelihood of false positives, a potential risk associated with the two-primer system in PCR (Soroka et al., 2021). In conclusion, the LAMP technique, characterized by its increased specificity and heightened sensitivity, along with a streamlined operational procedure and safe visual detection mechanism, stands out as an optimal choice for the swift and accurate detection of the *tcdA* gene. In regions constrained by economic resources and advanced diagnostic infrastructure, the benefits of the LAMP technique become even more salient. It holds potential to catalyze the broader implementation of CDI diagnostic technology, thereby contributing to an improvement in global health outcomes.

While the LAMP method stands out for its advantages, it is not without its limitations. Cross-contamination arising from multiple pipetting steps is a notable concern, particularly with material present in the aerosol. This study mitigated this issue by employing low-melting-point paraffin to seal the reaction mixtures, thereby preventing the spread of amplification products. Rigorous sample handling protocols and enhanced ventilation should be conducted to minimize contamination risks. Another limitation is the suitability of LAMP products for downstream applications, such as sequencing or cloning (Sahoo et al., 2016). This drawback underscores the need for further methodological refinements to expand the utility of LAMP products beyond mere detection. Additionally, the sensitivity of LAMP ~~method's ability~~ to ~~detect~~ inhibitors ~~within samples~~ remains a complex challenge (Dong et al., 2014)~~, as it involves simultaneous inhibitor detection and material amplification~~. This highlights a potential area for the development of more sophisticated controls and detection metrics within the LAMP protocol. Finally, ~~the propensity for~~ primer-dimer formation in LAMP could lead to false positives~~. This underscores the importance of careful primer design and validation to ensure the reliability of the LAMP assay. Despite its transformative potential for resource-limited settings, the LAMP method requires careful consideration of its limitations. Addressing these challenges is essential for harnessing the full potential of LAMP in clinical diagnostics and ensuring its role in advancing global health diagnostics.~~

A limitation of this study is the exclusive use of the *C. difficile* VPI10463 strain as a positive control, which may not represent the diversity of *C. difficile* strains. To address this limitation and to understand its potential impact on the conclusions drawn from the results, a more comprehensive analysis was performed. The subsequent sequencing analysis of 15 stool specimens that tested positive with the LAMP assay but negative with PCR provided additional insights. Not only did this analysis confirm the presence of *C. difficile* VPI10463 in two cases, but it also identified other strains in the remaining samples: seven cases of GZ14, two cases of ZR48, and one case each of ZR80, ZR50, GZ5, and SH8. These findings suggest that the LAMP assay developed in this study may have the capacity to detect a broader spectrum of *C. difficile* strains than initially anticipated, which is a significant consideration for the validity of this method. This understanding of the assay's limitations and their possible impact on the conclusions drawn in this study is critical for interpreting the study's results and for guiding future research directions.

Conclusions

In this study, the LAMP method proved to be an effective technique for the rapid and visual detection of the *tcdA* gene of *C. difficile*, demonstrating potential advantages over PCR in terms of speed, simplicity, and sensitivity. The *tcdA*-LAMP assay is particularly suitable for medical diagnostic environments with limited resources and represents a promising diagnostic strategy for the screening and detection of CDI in populations at high risk. ~~Future research will need to further validate the applicability of the LAMP technique across various experimental and clinical settings and address any technical challenges that may arise during its broader implementation.~~

Acknowledgements
We would like to thank the Institute of Disease Control and Prevention and the Academy of Military Medical Sciences for their technical assistance.

Informed Consent Statement
Because this study was based on the clinical examination of existing stool specimens for clinical research, it did not require patients to provide additional samples. In the clinical analysis, all patient identification information was expressed in code or pinyin to maintain the personal privacy of the patients and prevent individual health information from being exposed. The test results were only used for clinical research, and no test report was issued that would affect the diagnosis and treatment of the subjects. No patient risk was involved in this study. Therefore, an informed consent waiver was obtained from Nanfang Hospital of Southern Medical University for this study.

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