

A Novel Detection of *Enterococcus faecalis* Using Multiple Cross Displacement Amplification Linked with Gold Nanoparticle Lateral Flow Biosensor

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Xu Chen^{1,2}

Kai Ma³

Xu Yi¹

Ziyu Xiao⁴

Lijuan Xiong³

Yu Wang⁵

Shijun Li²

¹Central Laboratory of the Second Affiliated Hospital, Guizhou University of Traditional Chinese Medicine, Guiyang, Guizhou 550003, People's Republic of China; ²Laboratory of Bacterial Infectious Disease of Experimental Centre, Guizhou Provincial Centre for Disease Control and Prevention, Guiyang, Guizhou 550004, People's Republic of China; ³Clinical Laboratory Centre of the Second Affiliated Hospital, Guizhou University of Traditional Chinese Medicine, Guiyang, Guizhou 550003, People's Republic of China; ⁴School of Public Health, Guizhou Medical University, Guiyang, Guizhou 550025, People's Republic of China; ⁵Department of Clinical Laboratory Centre, The First People's Hospital of Guiyang, Guiyang, Guizhou 550004, People's Republic of China

Background: *Enterococcus faecalis*, an opportunistic bacterial pathogen, is one of the most frequently isolated bacterial species and cause of serious nosocomial infections in recent decades. A reliable and rapid assay for *E. faecalis* detection is significant for the diagnosis and follow-up treatment.

Methods: A novel assay method, named multiple cross displacement amplification linked with nanoparticle-based lateral flow biosensor (MCDA-LFB), was applied for detecting *E. faecalis* strains. A set of special 10 primers was designed according to *E. faecalis*-specific gene *Ej0027*. The MCDA amplification conditions, including the target DNA concentration, reaction temperature and time, were optimized. The sensitivity and specificity of MCDA method were tested in the current study, and then, the MCDA-LFB technology was applied to detect the *E. faecalis* strain from clinical samples.

Results: The *E. faecalis* specific primers were valid for the establishment of MCDA-LFB technology for the detection of *E. faecalis* based on the *Ej0027* gene. The MCDA amplification condition was optimized at 62°C for 35 min. The MCDA products were directly sensed and displayed with a biosensor. The full process, comprising genomic DNA template preparation (approximately 30 mins), amplification of MCDA (35 mins), and the product identification (approximately 2 mins), could be achieved in 70 mins. The MCDA technique could detect as little as 10 fg per reaction system of pure *E. faecalis* genomic DNA. The specificity of *E. faecalis*-MCDA-LFB method is 100%, with no cross-reactions to non-*E. faecalis* strains.

Conclusion: The MCDA-LFB technique established in the present study is a reliable, simple, rapid, sensitive and specific method to assay *E. faecalis* and can be applied for the detection of clinical samples.

Keywords: *Enterococcus faecalis*, limit of detection, multiple cross displacement amplification, gold nanoparticle, lateral flow biosensor, MCDA-LFB

Introduction

Enterococcus faecalis (*E. faecalis*), a Gram-positive enterococci, is widely distributed in the gastrointestinal tracts of humans, animals, and insects and natural environment.^{1,2} *E. faecalis* has emerged as a major opportunistic pathogen cause of outbreaks of nosocomial infections affecting various tissues, including human urinary tract infections, intraabdominal infections, infective endocarditis, neonatal sepsis, and bacteremia.³ Thus, to develop a reliable and rapid assay for *E. faecalis*, detection is significant for the diagnosis and follow-up treatment.

Correspondence: Shijun Li
Experimental Centre, Guizhou Provincial
Centre for Disease Control and Prevention,
115 Bageyan Road, Guiyang, Guizhou
550004, People's Republic of China
Tel/Fax +86 851 86824290
Email zjumedjun@163.com

The traditional and gold standard for the detection of *E. faecalis* is cultivation on agar plates, while this detection method took more than 48 h of growth.^{4,5} Moreover, the sensitivity of cultivation-based techniques was significantly decreased if the clinical samples were collected after antimicrobial therapy.⁵ Polymerase chain reaction (PCR) and PCR-based technologies (real-time PCR and multiplex PCR) were applied to detect clinical pathogens because these detection methods are specific, rapid, and sensitive.^{6–9} Nevertheless, PCR-based methods need special experimental instruments and skilled personnel that may not be readily available in many resource-poor settings. Herein, advanced assays are urgently required for low-cost, reliable, sensitive, specific, and simple detection of target pathogens to ensure prompt treatment.

Multiple cross displacement amplification (MCDA) technique has been applied to detect a range of pathogens such as *Shigella* spp, *Listeria monocytogenes*, and *Neisseria meningitides*.^{10–12} MCDA method can amplify target gene sensitively and efficiently, using a water bath or sample heater at a constant temperature.^{13,14} In the MCDA amplification system, six primers for amplification (C1, D1, C2, D2, R1, and R2), two displacement primers (F1 and F2), and two cross primers (CP1 and CP2), designed according to target DNA sequence, were used for the amplification.^{15,16} MCDA method is able to yield amplicons from as little as three microbes. These amplification products can be rapidly, reliably, and visually detected with disposable gold nanoparticles-based lateral flow biosensors (LFB).¹⁷

In the present study, the MCDA-LFB detection was developed for the visual, rapid, highly specific, and sensitive detection of *E. faecalis* carrying the *Ef0027* gene, which appeared to be uniquely present in *E. faecalis* as it showed no homology with other microbial genomes at GenBank by BLAST searches. The detection performance was analyzed with pure cultures and clinical samples.

Materials and Methods

Ethics Statement

The study was approved by the Human Ethics Committee of the Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine and complied with the Declaration of Helsinki. All data/isolates were analyzed anonymously.

Reagents and Materials

Reagents including DNA extraction kits (Qiagen, Hilden, Germany), visual detection reagent (Malachite Green, MG) and isothermal amplification kits (Beijing HaiTaiZhengYuan technology Co., Ltd., Beijing, China), streptavidin-immobilized 30-nm gold nanoparticles (SA-Gs) (Resenbio Co., Ltd., Xian, China), Antarctic thermal-sensitive uracil-DNA-glycosylase (AUDG), dNTP ((New England Biolabs, Inc, Beijing, China) rabbit anti-fluorescein antibody (anti-FITC Ab), and biotinylated bovine serum albumin (biotin-BSA) (Abcam Co., Ltd., Shanghai, China) were used in this study. Materials including the sample pad, membrane backing card, nitrocellulose membrane (NC), conjugate pad, and absorbent pad were acquired from the Jieyi Biotechnology Co., Ltd. (Shanghai, China).

Design of MCDA Assay Primers

Based on the reaction mechanism of MCDA, 5 pairs of special primers according to the *Ef0027* gene (Genbank accession no. 1198935) of *E. faecalis* were devised by PRIMER PREMIER 5.0 and Primer Explorer V4 (Eiken Chemical, Japan).

Hybrids and hairpin structures of the primers were analyzed by the Integrated DNA Technologies design tools. The specificity of *E. faecalis*-MCDA primers was verified by Blast analysis tool. The C1 and D1 primers were labeled by biotin and fluorescein isothiocyanate (FITC) at their 5' end, respectively. All of the primers were synthesized by TsingKe Biotech Co., Ltd (Beijing, China) with HPLC purification grade. The primer sequences are shown in Table 1, and the primer positions of the *E. faecalis* gene are displayed in Figure 1.

Bacterial Strains and Genomic DNA Template Preparation

In the present study, forty-nine bacterial strains, including standard strain of *E. faecalis* (ATCC 29212), twelve clinical *E. faecalis*-positive isolates, and thirty-six non-*E. faecalis* strains, were used in this study (Table 2). The *E. faecalis* standard strain (ATCC 29212) was applied for the optimization of the target method. The DNA templates were prepared by extracting the genomic DNA from the bacterial isolates using QIAamp DNA Mini Kit in accordance with the instructions from the manufacturer and measured with a Nanodrop ND-2000 (Beijing, China) at A260/280.

Table 2 Bacterial Strains Used in the Current Study

No.	Bacteria	Strain No. (Source of Strains)	No. of Strains	MCDA-LFB Result
1	<i>E. faecalis</i>	ATCC 29212	1	P
2	<i>E. faecalis</i>	Isolated strains (2nd GZUTCM)	12	P
3	<i>Enterococcus faecium</i>	Isolated strains (2nd GZUTCM)	9	N
4	<i>Enterococcus avium</i>	Isolated strains (2nd GZUTCM)	1	N
5	<i>Enterococcus raffinosus</i>	Isolated strains (2nd GZUTCM)	1	N
6	<i>Staphylococcus aureus</i>	ATCC 25923	1	N
7	<i>Pseudomonas aeruginosa</i>	ATCC27853	1	N
8	<i>Legionella bacillus</i>	Isolated strains (2nd GZUTCM)	1	N
9	<i>Hemophilus parainfluenza</i>	Isolated strains (2th GZUTCM)	1	N
10	<i>Bordetella parapertussis</i>	Isolated strains (2nd GZUTCM)	1	N
11	<i>Klebsiella pneumoniae</i>	Isolated strains (2nd GZUTCM)	1	N
12	<i>Shigella boydii</i>	Isolated strains (2nd GZUTCM)	1	N
13	<i>Mycoplasma pneumoniae</i>	Isolated strains (2nd GZUTCM)	1	N
14	Enteropathogenic <i>Escherichia coli</i>	Isolated strains (GZCDC)	1	N
15	<i>Streptococcus pneumoniae</i>	Isolated strains (2nd GZUTCM)	1	N
16	<i>Staphylococcus saprophyticus</i>	Isolated strains (GZCDC)	1	N
17	Enterotoxigenic <i>Escherichia coli</i>	Isolated strains (2nd GZUTCM)	1	N
18	Invasive <i>Escherichia coli</i>	Isolated strains (2nd GZUTCM)	1	N
19	Enterohemorrhagic <i>Escherichia coli</i>	Isolated strains (2nd GZUTCM)	1	N
20	Enterotoxigenic <i>Escherichia coli</i>	Isolated strains (2nd GZUTCM)	1	N
21	<i>Streptococcus suis</i>	Isolated strains (GZCDC)	1	N

(Continued)

Table 2 (Continued).

No.	Bacteria	Strain No. (Source of Strains)	No. of Strains	MCDA-LFB Result
22	<i>Vibrio cholerae</i>	Isolated strains (GZCDC)	1	N
23	<i>Acinetobacter baumannii</i>	Isolated strains (2nd GZUTCM)	1	N
24	<i>Bacillus cereus</i>	Isolated strains (GZCDC)	1	N
25	<i>Vibrio parahemolyticus</i>	Isolated strains (GZCDC)	1	N
26	<i>Shigella flexneri</i>	Isolated strains (2nd GZUTCM)	1	N
27	<i>Listeria monocytogenes</i>	Isolated strains (2nd GZUTCM)	1	N
28	<i>Mycobacterium tuberculosis</i>	Isolated strains (GZCDC)	1	N
29	<i>Leptospira interrogans</i>	Isolated strains (GZCDC)	1	N
30	<i>Brucella suis</i>	Isolated strains (GZCDC)	1	N

Notes: 2nd GZUTCM, the Second Affiliated Hospital, Guizhou University of Traditional Chinese Medicine.

Abbreviations: ATCC, American type culture collection; GZCDC, Guizhou Provincial Center for Disease Control and Prevention; P, positive; N, negative.

Analytical Sensitivity of *E. faecalis*-MCDA-LFB Assay

The sensitivity of *E. faecalis*-MCDA-LFB method was determined using 1 µL of the serial dilution (10 ng, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg and 100 atto gram per micro-liter) of the genomic DNA extracted from *E. faecalis*. The amplification was carried out at the optimized temperature to measure the limit of detection (LoD) of *E. faecalis*-MCDA-LFB. Three replicates of each dilution were tested.

Optimization of the Amplification Time for *E. faecalis*-MCDA-LFB Assay

The *E. faecalis*-MCDA reaction system was amplified at the optimized temperature for diverse amplification times (from 15 to 45 mins, 10 mins of the interval). And then, the products were determined using LFB, and each amplification time was tested at least three times.

Specificity Analysis of *E. faecalis*-MCDA-LFB Detection

Genomic DNA templates from 49 different bacterial strains (Table 2) were used to verify the specificity of *E. faecalis*-

MCDA-LFB method. The 49 bacterial strains included the standard strain of *E. faecalis* (ATCC 29212), 12 *E. faecalis* isolates, and 36 non-*E. faecalis* strains. DW was used as a blank control. All of the MCDA results were tested with LFB and confirmed at least three times.

Application of *E. faecalis*-MCDA-LFB Method for the Detection of Clinical Samples

Fifty-three urine samples of patients, 30 hospital surface samples, and 35 urine samples of healthy volunteers were collected from the Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine. All of the samples were detected for *E. faecalis* using traditional culture, PCR, and DCDA-LFB techniques, respectively. Traditional culture methods including colony morphology, Gram stain, and biochemical identification were used for identification of the *E. faecalis* isolates. PCR detections of all the genomic DNA templates were conducted by using *E. faecalis* specific primers.⁸ The MCDA-LFB detection results were compared with that of traditional culture and PCR assay.

Results

Verification of *E. faecalis*-MCDA Productions

In order to confirm the validity of primers (Table 1) for the *E. faecalis*-MCDA assay, genomic DNA from pure *E. faecalis* cultures were amplified at 64°C for 60 mins. The products were identified using MG and LFB methods. The results showed that amplification appeared with genomic DNA from *E. faecalis* (ATCC 29212), but not with *E. faecium*, *S. aureus*, and the blank control (Figure 2A and B). Therefore, the *E. faecalis*-MCDA primers used in the current study were valid for the development of *E. faecalis*-MCDA-LFB.

Optimal Temperature for *E. faecalis*-MCDA-LFB Detection

To certify the optimum amplification temperature, 10 pg of genomic DNA of *E. faecalis* strain was utilized as the target template in each reaction mixture. The results showed that 62°C was the faster amplification as indicated by the kinetics graphs of real-time turbidity (Figure 3).

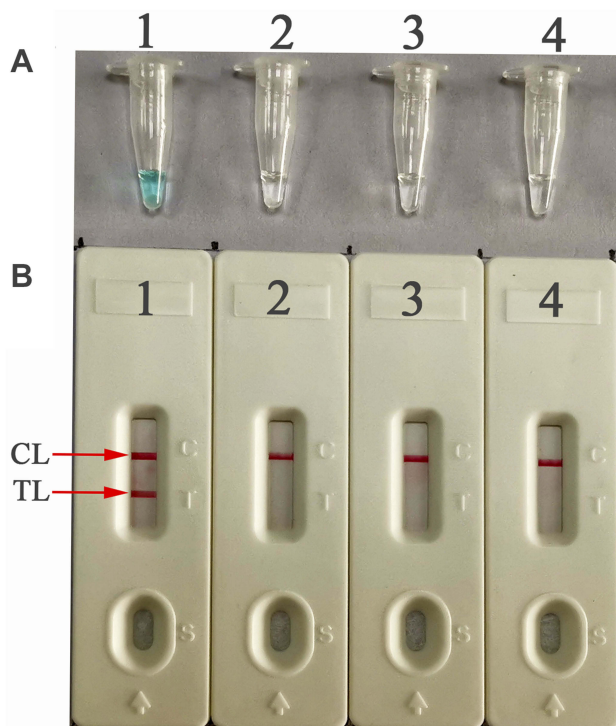


Figure 2 Confirmation and verification of *E. faecalis*-MCDA products. (A) The *E. faecalis*-MCDA amplification products were detected by the MG method through visual observation of the color change. (B) LFB was applied for the visual detection of *E. faecalis*-MCDA products. Tube 1/Biosensor 1: positive amplification of *E. faecalis* strain 29212; Tube 2/Biosensor 2: negative amplification of *Enterococcus faecium* (2nd GZUTCM); Tube 3/Biosensor 3: negative amplification of *Staphylococcus aureus* (ATCC25923); Tube 4/Biosensor 4: blank control (DW).

Therefore, the most suitable temperature for *E. faecalis*-MCDA-LFB was determined as 62°C, which was applied to the rest of the assays in the current study.

Sensitivity of MCDA-LFB Method for *E. faecalis* Assay

The sensitivity of *E. faecalis*-MCDA-LFB was tested by serially diluted DNA sample from *E. faecalis* strain (ATCC29212) and determined by visual inspection of reaction productions with MG reagents and lateral flow biosensors, respectively. Both of the two methods demonstrated that the LoD of *E. faecalis*-MCDA-LFB was 10 fg DNA template (Figure 4A and B).

Optimized Amplification Time for *E. faecalis*-MCDA-LFB Detection

In order to confirm the optimum amplification time for the *E. faecalis*-MCDA-LFB detection during amplification phases, 15-, 25-, 35-, and 45-min amplification times were selected and compared at 62°C, respectively. The detection limit level of DNA (10 fg of *E. faecalis* genomic templates per reaction) showed both TL and CL when the reaction proceeded for 35 and 45 mins (Figure 5). Therefore, 35 mins was deemed as an appropriate amplification time for *E. faecalis*-MCDA-LFB detection.

Specificity of MCDA-LFB Method for *E. faecalis* Assay

The genomic DNA from all of the bacterial strains (Table 1) were used for *E. faecalis*-MCDA-LFB specificity determination. The results showed that only the genomic DNA from *E. faecalis* strains showed positive results, while other non-*E. faecalis* isolates and blank control showed negative results (Figure 6).

Detection Results of *E. faecalis*-MCDA-LFB for Clinical Samples

In order to demonstrate that the MCDA-LFB method is a reliable and rapid tool for *E. faecalis* detection in clinical samples, 53 urine samples of patients, 30 hospital surface samples, and 35 urine samples of healthy volunteers collected from the Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine were used for the application of *E. faecalis*-MCDA-LFB established in the current study. The results showed that 23 of 118 samples had been verified as *E. faecalis*-positive results through traditional culture technique, and the other 95

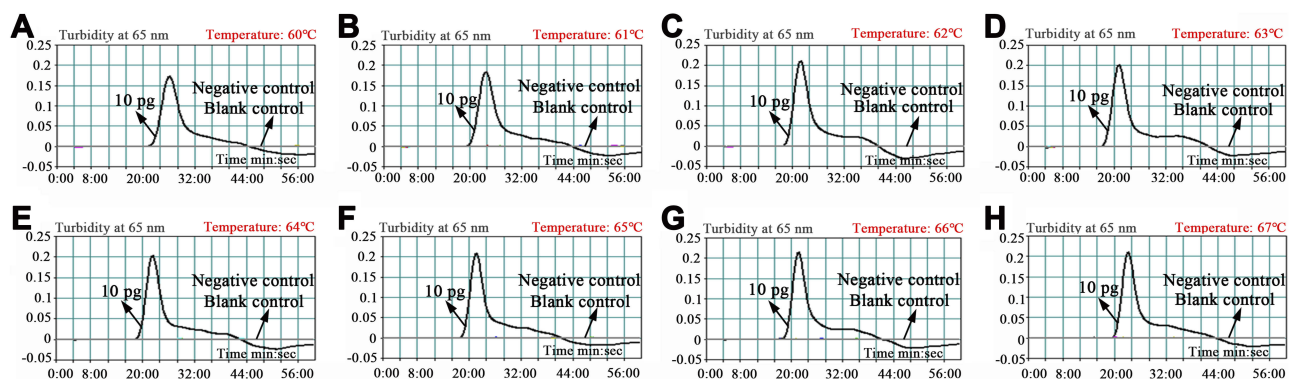


Figure 3 (A–H) Optimization of reaction temperature for *E. faecalis*-MCDA primers. The MCDA amplifications for the detection of *E. faecalis* were monitored through real-time turbidity, and the corresponding curves of DNA concentrations were displayed in the graph. The threshold value was 0.1 and a turbidity >0.1 was considered as positive. Mixtures with 10-pg genomic templates of *Enterococcus faecium* (isolated strain) and *Staphylococcus aureus* (ATCC 25923) were used as negative controls (NCs), and 1 μ L of double-distilled water (DW) was used as a blank control (BC). Eight kinetic graphs were obtained at different temperatures (60–67°C, 1°C intervals) with 10-pg target genomic DNA per reaction. The graphs from C to H showed robust amplification.

samples showed *E. faecalis*-negative outcome. The MCDA-LFB assay results were consistent with traditional cultivation detection results. However, only 15 urine samples showed positive results when using conventional *Ef0027* gene-based PCR, and all of the 15 positive urine samples were covered by the *E. faecalis* positive samples detected by traditional culture and MCDA-LFB methods (Table 3).

Discussion

E. faecalis is becoming increasingly important for causing bacteremia, endocarditis, and other nosocomial infections in recent decades.^{21,22} Moreover, more and more *E.*

faecalis isolates build up antibiotic resistance.^{2,23,24} Thus, developing a reliable, rapid, specific, and sensitive detection method for *E. faecalis* is essential for the disease diagnosis and its therapy. Our study showed that the newly established *E. faecalis*-MCDA-LFB is a rapid, sensitive, and specific method to detect *E. faecalis*.

The specificity of MCDA was powerfully confirmed with genomic DNA from *E. faecalis* pure cultures. All of the *E. faecalis* isolates showed positive results, but all the non-*E. faecalis* strains displayed a negative outcome (Figure 6). Thus, the *E. faecalis*-MCDA-LFB method has a high level of specificity for the identification of *E. faecalis* species. The high degree of specificity of the MCDA-LFB established in the present study seems due to using the *Ef0027* gene, which encodes a putative phosphosugar-binding transcriptional regulator. This gene appeared to be uniquely present in *E. faecalis* as it showed no homology with other microbial genomes at GenBank by BLAST searches, and the *Ef0027* gene also applied to develop some advanced methods for detecting *E. faecalis* species.^{8,25} Another reason to explain the high degree of specificity of *E. faecalis*-MCDA-LFB is because we designed ten primers targeting the different regions of the *Ef0027* gene sequence.

Compared with conventional culture and PCR-based methods, the MCDA-LFB developed in this study is more time-saving and economical.^{10,12,26} The *E. faecalis*-MCDA-LFB reaction just requires a simple incubation at 62°C for 35 mins. There are various portable user-friendly instruments for MCDA reaction. For example, the dry block heater (Labnet, USA) is one of the suitable instruments, which is a portable, battery-powered equipment supporting 96-channel MCDA reactions per detection.

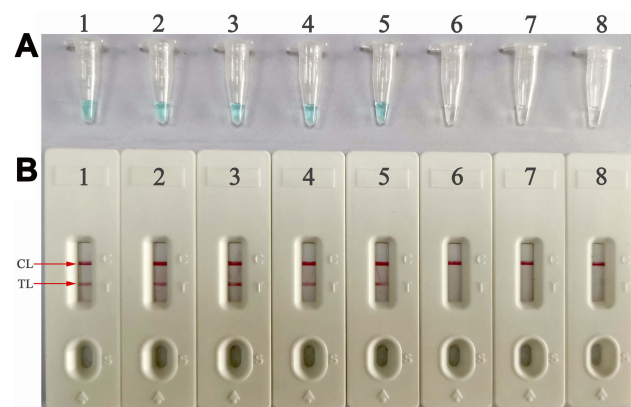


Figure 4 Sensitivity analysis of the MCDA-LFB detection by serial dilutions of genomic DNA extracted from *E. faecalis* strain. Two detection methods, colorimetric indicator (MG; **A**) and lateral flow biosensor (**B**), were used to analyze the amplification productions. The serial dilutions of *E. faecalis* genomic DNA (10 ng, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and 100 atg per microliter) were subjected to standard MCDA amplifications. Tubes (**A**)/Biosensors (**B**) 1–8 represent the DNA levels of 10 ng, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and 100 atg per reaction of target templates and blank control (DW), respectively. The target gene levels of 10 ng, 10 pg, 1 pg, 100 fg, 10 fg per reaction showed positive amplifications.

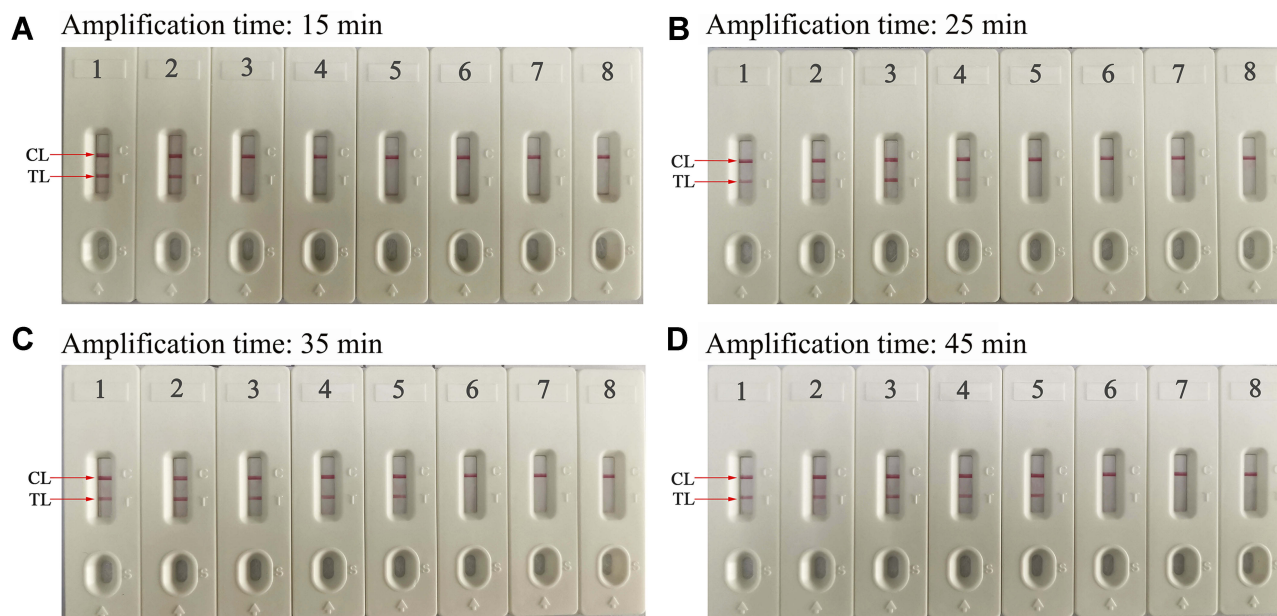


Figure 5 Optimization of amplification time for *E. faecalis*-MCDA-LFB detection method. Different amplification times (**A**, 15mins; **B**, 25 mins; **C**, 35 mins; **D**, 45 mins) were tested at 62°C, respectively. Biosensors 1, 2, 3, 4, 5, 6, 7, and 8 represent genomic DNA levels of 10 ng, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and 100 atto gram per reaction of target templates and blank control (DW). The best sensitivity was observed when the amplification lasted for 35 mins (**C**).

Moreover, the universal isothermal amplification kits such as NEB Warmstart and Eiken Loopamp kits can be commercially obtained and applied for MCDA amplification, and the cost of MCDA reaction is estimated to be \$3.5 USD, the LFB detection merely costs approximately \$2 USD, which is cheaper than the common PCR-based methods.^{15,16} Additionally, this method can decrease labor costs because performing the MCDA-LFB assay does not need skilled technical personnel. The entire detection process, including template preparation (approximately 30 mins), isothermal amplification (35 mins), and LFB reading (approximately 2 mins), could be accomplished within 70 mins. Thus, this method can save precious time and cost of detection compared with the traditional methods.

In addition, the advanced *E. faecalis*-MCDA-LFB technique was more sensitive than normal PCR and loop-mediated isothermal amplification (LAMP) methods. The newly developed *E. faecalis*-MCDA-LFB method was capable of assaying 10 fg of *E. faecalis* genomic DNA (Figure 4), which was more sensitive than *E. faecalis*-LAMP-LFB detection (250 fg per reaction) reported by the previous study.²⁵ Although the amplification products could be detected equally with the MG method used in the present study, the LFB was deemed as the preferred method as observing the amplification results is more objective and does not need any apparatus.^{19,27} In

conclusion, the newly *E. faecalis*-MCDA-LFB assay in this study is a valuable method for the convenient, rapid, sensitive, specific, visual, reliable, and low-cost detection of *E. faecalis* in clinical and environmental samples, especially in resource-constrained settings. However, the *E. faecalis*-MCDA-LFB method has some limitations when compared with some methods which can be applied for *E. faecalis* antimicrobial resistance detection. Later, we will study on the rapid and reliable assay of drug resistance genes of *E. faecalis* based on the *E. faecalis* MCDA-LFB method created in the current study.

Conclusion

In this study, a reliable and rapid *E. faecalis*-MCDA-LFB method was developed for detecting *E. faecalis*, causing nosocomial infection, including urinary tract infection, bloodstream infection, and surgical site infection in human. This method could rapidly, sensitively, specifically, and reliably detect the *E. faecalis* agent in the urine sample and did not rely on expensive instruments and reagents. Using LFB detection could help to obtain a rapid, objective, and easily interpretable result. Hence, the *E. faecalis*-MCDA-LFB assay could be considered as a useful method for reliable and rapid detection of *E. faecalis* in clinical and environmental samples, particularly in resource-limited regions of the world.

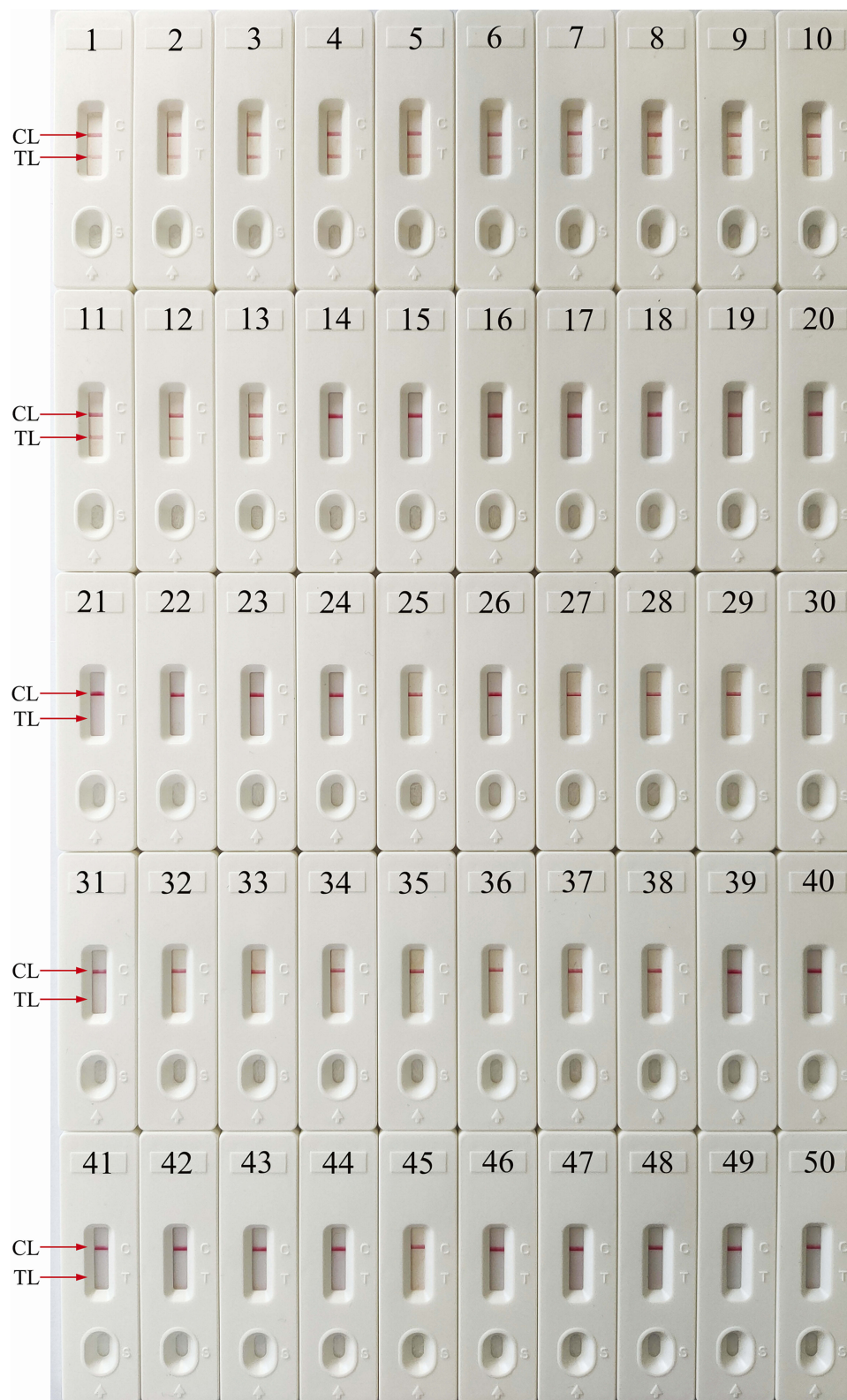


Figure 6 Specificity analysis of the *E. faecalis*-MCDA-LFB detection for different strains. The MCDA amplifications were carried out using different strain genomic DNA as templates and determined by means of visual LFB method. Biosensor 1, *E. faecalis* strain (ATCC 29212); biosensor 2–13, twelve isolated strain of *E. faecalis* from the Second Affiliated Hospital, Guizhou University of Traditional Chinese Medicine; biosensor 14–22, nine isolated *Enterococcus faecium* strains, biosensor 23–49, *Enterococcus avium*, *Enterococcus raffinosus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Legionella bacillus*, *Hemophilus parainfluenza*, *Bordetella parapertussis*, *Klebsiella pneumonia*, *Shigella boydii*, *Mycoplasma pneumonia*, Enteropathogenic *Escherichia coli*, *Streptococcus pneumonia*, *Staphylococcus saprophyticus*, Enterotoxigenic *Escherichia coli*, Invasive *Escherichia coli*, Enterohemorrhagic *Escherichia coli*, Enteroaggregative *Escherichia coli*, *Streptococcus suis*, *Vibrio cholera*, *Acinetobacter baumannii*, *Bacillus cereus*, *Vibrio parahemolyticus*, *Shigella flexneri*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Leptospira interrogans*, *Brucella suis*; biosensor 50, blank control (DW).

Table 3 Comparison of Conventional Culture, PCR, and MCDA-LFB Methods for the Assay of *E. Faecalis* in Clinical Samples

Detection Methods	Clinical Samples (n=53)		Hospital Surfaces Samples (n=30)		Healthy Volunteers Samples (n=35)	
	Positive	Negative	Positive	Negative	Positive	Negative
Culture	18	35	2	28	3	32
PCR	14	39	0	30	1	34
MCDA-LFB	18	35	2	28	3	32

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Disclosure

All of the authors declared that there are no competing interests in this article.

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