ORIGINAL RESEARCH

Development of a multiple cross displacement amplification combined with nanoparticles-based biosensor assay to detect *Neisseria meningitidis*

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Background: *Neisseria meningitidis* is a leading pathogen of meningococcal disease in humans worldwide. Multiple cross displacement mplification (MCDA) combined with nanoparticles-based lateral flow biosensor (MCDA-LFB) has been reported for the rapid detection of several bacterial pathogens in recent years. Here, therefore we developed an MCDA-LFB assay for the rapid detection of *N. meningitis*.

Methods: A set of 10 primers specifically to recognize 10 different regions of the *ctrA* gene of *N. meningitidis* were designed. MCDA was developed and combined with a LFB to detect the *ctrA* gene of *N. meningitidis*. The reaction time and temperature condition for the MCDA-LFB were optimized and then the MCDA-LFB was applied to detect the DNA from clinical samples.

Results: MCDA-LFB assay was successfully established for the detection of *N. meningitidis* based on the *ctrA* gene. The MCDA assay was optimized at 64°C for only 35 mins and the products of amplification were directly sensed by LFB. The whole operation, including DNA template preparation (~20 mins), MCDA reaction (35 mins) and results interpretation (~2 mins) could be finished in no more than 60 mins. The detection limit was as low as 10 fg/reaction (around 3 CFUs/reaction) of pure *N. meningitidis* DNA, with no cross-reaction with other bacterial DNA.

Conclusion: The MCDA-LFB techniques developed in the present study are an effective tool for the rapid detection of *N. meningitidis*, especially in resource-poor countries in meningococcal disease epidemic period.

Keywords: *Neisseria meningitidis*, multiple cross displacement amplification, lateral flow biosensor, MCDA-LFB, limit of detection

Introduction

Meningococcal disease has been reported for more than 200 years, which recognized as a worldwide public health problem due to its global distribution, high-mortality rates and significant morbidity.[1] Furthermore, meningococcal disease is associated with high-financial costs both in patient treatment and rehabilitation.[1–3] Acute meningitis is, globally, a major threat to the health of infants, children, and adults.[4] *Neisseria meningitidis* is a leading causative agent of bacterial meningitis in humans,[5] which leads to around 1.2 million cases and 135,000 deaths every year.[6,7] *N. meningitidis*, a Gram-negative β-proteobacterium, belongs to the member of the bacterial family Neisseriaceae.[8,9] A total of 12 different *N. meningitidis* serogroups, including A, B, C, E, H, I, K, L, W, X, Y and Z, have been identified to date based on antigenic differences in the capsular polysaccharide.[10,11] The distribution of epidemic

serogroup of *N. meningitidis* may vary geographically,[8] but serogroups A, B, C, W, X, and Y are responsible for the majority of the disease.[9,10,12] For example, serogroup B is the dominant serogroup of *N. meningitidis* as the major cause of meningococcal disease in Thailand and Taiwan,[13] and serogroups A, C, X, and Y have been identified in these and other Asian countries,[9,13,14] while serogroups B and C are major causes of invasive meningococcal disease in Europe and other industrialized countries.[13] In the Americas, the majority of meningococcal disease is caused by serogroups B and C,[9] with the emergence of serogroups W and Y in some countries.[15] The incidence of meningococcal meningitis in sub-Saharan Africa greatly exceeds other regions in the world,[16,17] and the predominant serotype associated with outbreaks in the meningitis belt has been serogroup A from 1990 to 2010,[16,17] with the emergence of serogroups W and X in some countries in recent years.[18,19]

Invasive disease caused by *N. meningitidis* is traditionally diagnosed using bacterial culture, followed with identification by using Gram staining, immunochromatographic antigen detection.[8,20,21] However, the diagnosis is complicated and requires well-equipped laboratory with appropriate biosafety equipment and procedures.[22] Another disadvantage of these assays is the relatively low-diagnostic sensitivity.[23] Overuse of antibiotics reduces the utility of culture-based methods due to an inhibition of bacterial growth.[24] Several PCR-based methods have been developed for the identification of the *N. meningitidis* based on the polysialyltransferase *siaD* gene or other genes including *crgA*, 16S rRNA and *porA*.[25] Additionally, real-time and multiplex PCR assays for identification of *N. meningitidis* have also been reported.[5,26,27] However, these methods are expensive due to the requirement of specific equipment and they are complicated to perform in resource-poor laboratories in many developing countries.[8]

Apart from the PCR-based methods, the isothermal amplification technique, termed as loop-mediated isothermal amplification (LAMP), has also been developed to detect *N. meningitidis*.[4,8,28] LAMP assay offers an alternative to bacterial cultivation and PCR due to its greater specificity and sensitivity than that of conventional PCR and it requires simpler equipment or machines. Although LAMP assays displayed high-amplification efficiency comparable to that of the conventional PCR method, the marginal amounts of nucleic acid sequences were still difficult to analyze in various samples.[29,30] In particular, the results of *N. meningitidis*-LAMP techniques were determined using color indicator (such as SYBR green I and calcein dyes), agarose gel electrophoresis and real-time turbidity equipment. The analysis of *N. meningitidis*-LAMP amplifications by color with the naked eyes is potentially subjective, and it is possibility that a clinical sample may be somewhat ambiguous to the unaided eye when the concentration of *N. meningitidis* templates is low. The use of gel electrophoresis to analyze products is a complex process and increases the risk of degradation and carryover contamination. The real-time turbidity detection of *N. meningitidis*-LAMP results requires an additional optical instrument and easily suffers from background interference.

More recently, multiple cross displacement amplification (MCDA) was developed to overcome the technical barriers posed by current isothermal amplification strategies. MCDA assay was conducted under isothermal conditions, and simple heater or water bath that maintained a uniform temperature was sufficient and the amplicons can be sensed by disposable lateral flow biosensors (LFBs).[31] It is likely the preferred method as reading the results is less subjective and does not require instrumentation. MCDA has been applied for the rapid detection of serious bacterial pathogens such as Listeria *monocytogenes, Vibrio parahaemolyticus, Vibrio cholerae* and Klebsiella*pneumoniae* in the recent years.[30–34]

The *ctrA* gene, encoding an outer membrane protein involved in capsule transport, has been depicted specific for the identification of *N. meningitidis*.[25] In the current study, an MCDA-LFB assay, based on the *ctrA* gene, was developed to provide a simple, rapid, highly specific and sensitive method for *N. meningitidis* screening. The analytical sensitivity, specificity and availability were successfully evaluated using pure cultures and clinical samples.

Materials and methods
Reagents and instruments
Both visual detection reagent (malachite green, MG) and Isothermal Amplification Kits were obtained from BeiJingHaiTaiZhengYuan Technology Co., Ltd. (Beijing, China). A DNA Extraction Kit was obtained from SBS Genetech Co., Ltd. (Beijing, China). Biotin-BSA (biotinylated BSA) and anti-fluoresceinisothiocyanate (FITC) Ab (rabbit anti-fluorescein antibody) were purchased from the Abcam Co., Ltd. (Shanghai, China). Sample pads, membrane backing materials, nitrocellulose (NC) membranes, conjugate pads and absorbent pads were purchased from the Jie Yi Biotechnology Co., Ltd. (Shanghai, China). Streptavidin-immobilized 40-nm gold nanoparticles (SA-Gs) were obtained from BeiJingHaiTaiZhengYuan Technology Co., Ltd.

Preparation of a gold nanoparticle-based dipstick biosensor

A LFB (4 mm × 60 mm) was prepared based on previous reports with some modifications.[35,36] Briefly, the sample pad, conjugate pad, NC membrane and absorbent pad were laminated onto a plastic adhesive backing card. In order to form the test line (TL) and control line (CL), the anti-FITC Ab (0.2 mg/mL) and biotin-BSA (2.5 mg/mL) were sprayed onto the NC membrane. The TL and CL were separated by 5 mm. SA-Gs diluted in 0.01 M PBS (pH 7.4) were deposited on the conjugate pad of the strip. The assembled cards were cut into 4-mm wide strips (Deli No. 8012) and packaged in a plastic box with desiccant gel and kept at room temperature.

Bacterial strains and DNA template preparation

The *N. meningitidis* reference strains for serogroups A, B, C, D,W-135, 29-E, X, Y, Z and C were obtained from ATCC (Table 1). The *N. meningitidis* isolates and non-*N. meningitidis* strains used for specific validation were isolated in Guizhou Province and stored in Laboratory of Guizhou Provincial Center for Disease Control and Prevention (GZCDC) (Table 1). The *N. meningitidis* strains were cultured on chocolate agar culture plate (Shanghai Laibo Bio-chemical Co. Ltd, Shanghai, China) and harvested as described previously.[8] The bacterial cultures were collected in 1 mL PBS and the CFUs were determined by serial dilutions of culture suspensions inoculated on chocolate agar plate.[8] DNA was extracted from cultures of isolated strains using the DNA Extraction Kit (SBS Genetech Co., Ltd.) according to the manufacturer's directions, followed with quantification using a NanoDrop ND-1000 instrument (Calibre, Beijing, China). Genomic DNA of *N. meningitidis* strain 13007 was serially diluted (1 ng/μL, 100 pg/μL, 10 pg/μL, 1 pg/μL, 100 fg/μL, 10 fg/μL and 1 fg/μL) for sensitivity analysis of *N. meningitidis*-MCDA-LFB detection. Other bacterial strains (non-*N. meningitidis* strain) were stored in 10% (w/v) glycerol broth at −70°C and all samples were extracted from all culture strains using the QIAamp DNA Mini Kit (Qiagen, Germantown, MD, USA).

MCDA assay primers design and synthesis

The MCDA primers set, including F1, F2, CP1, CP2, C1, C1*, C2, D1, D1*, D2, R1 and R2, was designed using primer software PREMIER 5.0 (Canada). Hairpin structures and hybrids were analyzed using the Integrated DNA Technologies design tools. Blast analysis was applied to verify the specificity of the MCDA primers for *N.*

meningitidis. The 5′ ends of C1 and D1 primers were labeled with biotin and FITC, respectively. The details of primers are displayed in Figure 1 and Table 2, which were synthesized and purified by Tianyi-Huiyuan Biotech Co., Ltd. (Beijing, China) at HPLC purification grade.

The standard MCDA assay

MCDA reactions were carried out in 25-μL reaction system as described in previous reports.[33,34] Briefly, each reaction contained 0.4 μM each of displacement primers F1 and F2, 0.8 μM each of amplification primers C1*, C2, R1, R2, D1* and D2, 1.6 μM each of cross primers CP1 and CP2, 12.5 μL of 2× reaction mix (Isothermal Amplification® kit), 1 μL of *Bst* DNA polymerase (8 U) and 1 μL of DNA template. Three detection methods, including colorimetric indicator (MG), real-time turbidimeter (LA-320C) and LFB, were applied to monitor the MCDA amplification products.

The amplified products should cause a color change from colorless to light green when using the MG method, with no changes in color for negative controls and blank control. Applying the LFB, both the CL and TL should be visible in positive reactions, and only the CL was observed in negative and blank controls. The optimal temperature of reaction was determined in the range of 60–67°C for 60 mins. Mixtures with 1 μL of genomic DNA of *Staphylococcus aureus* (*S. aureus*, GZCDC isolate) and *Streptococcus pneumoniae* (*S. pneumoniae*, GZCDC isolate) strains were used as negative controls, and mixtures with 1 μL of double-distilled water (DW) were chosen as blank controls.

Sensitivity and specificity of the *N. meningitidis*-MCDA-LFB assay

The genomic templates were applied to determine the MCDA limit of detection (LoD) by using serial dilutions as described above. Performances of *N. meningitidis*-MCDA-LFB, colorimetric indicator (MG) and turbidity analysis were analytically compared. Three replicates of each dilution were examined. The specificity of *N. meningitidis*-MCDA-LFB was analyzed using DNA templates from other 44 bacterial strains (Table 1). The examinations were repeated at least two times.

Optimization of the amplification time of the *N. meningitidis*-MCDA-LFB assay

The MCDA mixture was incubated at the optimal temperature for different lengths of time (15, 25, 35 and 45 mins). The amplification products were detected using an

Table 1 Bacterial strains used in this study

| No.	Bacteria	Serogroup/species	Strain name.(source of strain)	No. of strains
1	*N. meningitidis*	A	ATCC13077	1
2	*N. meningitidis*	B	ATCC 13090	1
3	*N. meningitidis*	C	ATCC 13102	1
4	*N. meningitidis*	D	ATCC 13113	1
5	*N. meningitidis*	W-135	ATCC 35559	1
6	*N. meningitidis*	29-E	ATCC 35558	1
7	*N. meningitidis*	X	ATCC 35560	1
8	*N. meningitidis*	Y	ATCC 35561	1
9	*N. meningitidis*	Z	ATCC 35562	1
10	*N. meningitidis*	C	Isolated strains (GZCDC002)	1
11	*N. meningitidis*	C	Isolated strains (GZCDC002)	1
12	*Bordetella pertussis*	U	Isolated strains (GZDC)	1
13	*Bordetella parapertussis*	U	Isolated strains (GZDC)	1
14	*Hemophililus parainfluenza*	U	Isolated strains (GZDC)	1
15	*Streptococcus pneumoniae*	U	Isolated strains (GZDC)	1
16	*Klebsiella pneumoniae*	U	Isolated strains (GZDC)	1
17	*Pseudomonas aeruginosa*	U	Isolated strains (GZDC)	1
18	*Mycoplasma pneumoniae*	U	Isolated strains (GZDC)	1
19	*Legionellae bacillus*	U	Isolated strains (GZDC)	1
20	*Acinetobacter baumannii*	U	Isolated strains (GZDC)	1
21	*Staphylococcus aureus*	U	Isolated strains (GZDC)	1
22	*Staphylococcus saprophyticus*	U	Isolated strains (GZDC)	1
23	*Salmonella*	Typhimurium	Isolated strains (GZDC)	1
24	Enteropathogenic *E. coli*	U	Isolated strains (GZDC)	1
25	Enterotoxigenic *E. coli*	U	Isolated strains (GZDC)	1
26	Invasive *E.coli*	U	Isolated strains (GZDC)	1
27	Enterohemorrhagic *E. coli*	U	EDL933	1
28	Enteroaggregative *E. coli*	U	Isolated strains (GZDC)	1
29	*Streptococcus suis*	U	Isolated strains (GZDC)	1
30	*Vibrio cholerae*	U	Isolated strains (GZDC)	1
31	*Vibrio parahemolyticu*	U	ATCC17802	1
32	*Enterococcus faecalis*	U	ATCC35667	1
33	*Enterococcus faecium*	U	Isolated strains (GZDC)	1
34	*Bacillus cereus*	U	Isolated strains (GZDC)	1
35	Bacillus proteus,	U	Isolated strains (GZDC)	1
36	*Enterobacter cloacae*	U	Isolated strains (GZDC)	1
37	*Listeria monocytogenes*	4a	ATCC19114	1
38	*Shigella flexneri*	F1a	Isolated strains (GZDC)	1
39	*Shigella boydii*	U	Isolated strains (GZDC)	1

Abbreviations: U, unidentified serotype; ATCC, American type culture collection; GZCDC, Guizhou Provincial Center for Disease Control and Prevention.

LFB, and two replicates of each amplification time were tested.

Application of *N. meningitidis*-MCDA-LFB detection in clinical samples

N. meningitidis-MCDA-LFB was used to detect *N. meningitidis* DNA from the total DNA of clinical samples. Fifty-six samples, including 2 cerebrospinal fluids samples, 5 blood samples and 39 nasal swab samples, were collected from patients distributed in prefecture Guiyang, Anshun, Zunyi, Tongren, Qiannan, Qiandongnan Qianxinan and Bijie of Guizhou Province. All the clinical samples used in this study were not specifically isolated for this research. They were part of the routine CDC (Center for Disease Control and Prevention) laboratory procedure in China. The National Health and Family Planning Commission of China determined that the collection of data from human cases of infectious disease was part of continuing public
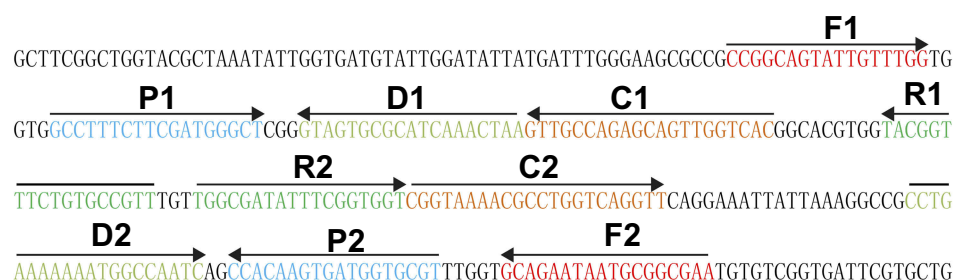
Figure 1 Location and sequence of the *ctrA* gene *of* Neisseria *meningitidis* applied for the MCDA primer design. The diagram displays the sequence and design of MCDA primers. The nucleotide sequence of the sense strand of the *ctrA* gene of *N. meningitidis* was shown. Right arrows and left arrows indicate sense and complementary sequences which were used in the present study, respectively.
Abbreviation: MCDA, multiple cross displacement amplification.

Table 2 The primers used in this study

Primers name[a]	Sequences and modifications	Length	Gene
F1	5′-CCGGCAGTATTGTTTGG-3′	17 nt	*CtrA*
F2	5′-TTCGCCGCATTATTCTGC-3′	18 nt	
CP1	5′-GTGACCAACTGCTCTGGCAACGCCTTTCTTCGATGGGCT-3′	39 mer	
CP2	5′-CGGTAAAACGCCTGGTCAGGTTACGCACCATCACTTGTGG -3′	40 mer	
C1	5′-GTGACCAACTGCTCTGGCAAC-3′	21 nt	
C1*	5′-Biotin-GTGACCAACTGCTCTGGCAAC-3′	21 nt	
C2	5′-CGGTAAAACGCCTGGTCAGGTT-3′	22 nt	
D1	5′-TTAGTTTGATGCGCACTAC-3′	19 nt	
D1*	5′-FITC-TTAGTTTGATGCGCACTAC-3′	19 nt	
D2	5′-CCTGAAAAAAATGGCCAATC-3′	20 nt	
R1	5′-AACGGCACAGAAACCGTA-3′	18 nt	
R2	5′-TGGCGATATTTCGGTGGT-3′	18 nt	

Notes: [a]C1*, 5′-labeled with biotin when used in MCDA-LFB assay; D1*, 5′-labeled with FITC when used in MCDA-LFB assay.
Abbreviations: FITC, fluorescein isothiocyanate; mer, monomeric unit; nt, nucleotide.

health surveillance of a notifiable infectious disease and was exempt from institutional review board assessment. All data were supplied and analyzed in an anonymous format, without access to personal identifying information. The clinical samples have been detected by using traditional cultivation and isolation methods. Simultaneously, traditional PCR targeting *crgA* gene recommended by the Diagnosis Standard for Meningococcal meningitis of China (WS 295—2019) was applied for the detection of the above clinic samples. Briefly, each 20 μL reaction system contained approximately 2 μL of DNA, 10 μL of 2x Taq PCR Mix (TaKaRa, Otsu, Japan), 1 μL of forward primer (GCTGGCGCCGCTGGCAACAAAATTC) and reverse primer (TTCTGCAGATTGCGGCGTGCCGT) and 7 μL of deionized water. Amplification was performed as per the following conditions: 94°C for 5 mins, followed by 30 cycles of 92°C for 30 s, 55°C for 40 s, and 72°C for 30 s, with a final single extension of 72°C for 2 mins, and then held at 4°C. Amplified products were characterized by

electrophoresis of 1 μL of each reaction on a 1.2% agarose gel for 30 mins at 85 V. The specificity and sensitivity of *N. meningitidis*-MCDA-LFB, traditional PCR and the conventional cultivation methods detection of *N. meningitidis* were compared.

Results
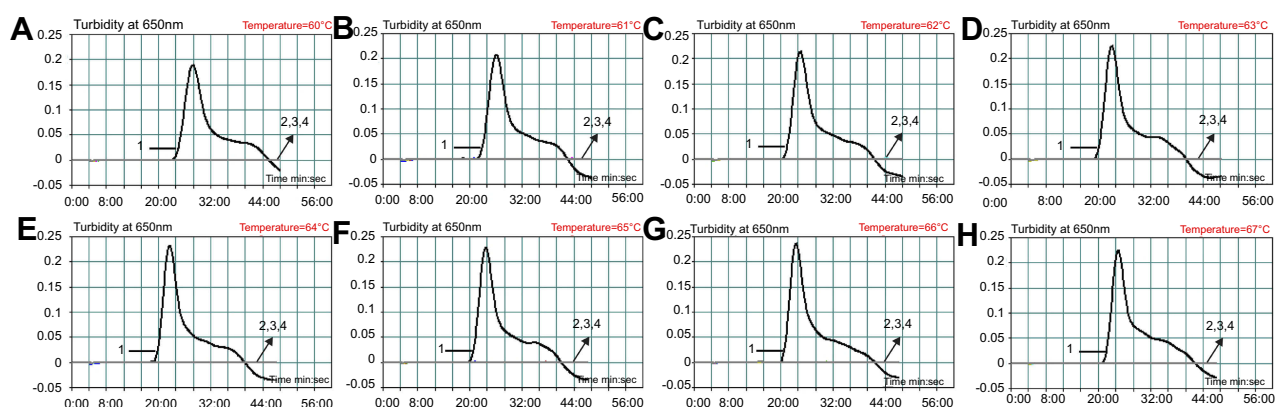## Confirmation and detection of *N. meningitidis* MCDA products
In order to confirm the effectiveness of primers for *N. meningitidis*-MCDA detection (Table 2), the DNA for pure cultures of *N. meningitidis* was detected using MCDA at 64°C for 1 hr. DNA from *N. meningitidis* strain 13007 was effectively amplified, but no amplification was observed with DNA from *S. aureus* (GZCDC isolate) and *S. pneumoniae* (GZCDC isolate) and the blank control (DW) (Figure 2). Therefore, the *N. meningitidis*-MCDA primer set was chosen to develop the MCDA-LFB assay for *N. meningitidis* detection.
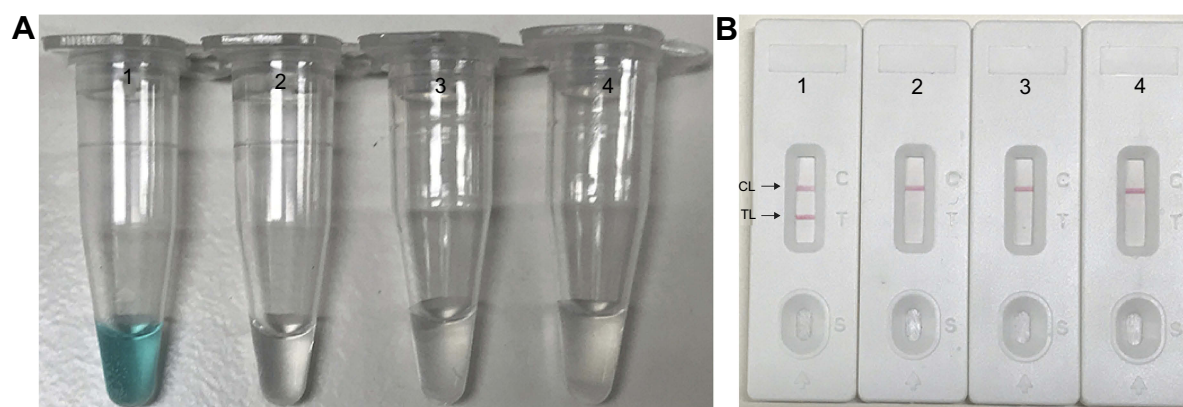
Figure 2 Confirmation and detection of Neisseria *meningitidis*-MCDA products. **(A)** By the MG method, amplification products of the *N. meningitidis*-MCDA assay were visually analyzed by observation of the color change. **(B)** A lateral flow biosensor was applied for visual detection of *N. meningitidis*-MCDA products. Tube 1/Biosensor 1: positive amplification of *N. meningitidis* strain 13007 Tube 2/Biosensor 2: negative control of *Staphylococcusaureus* (GZCDC isolate); Tube 3/Biosensor 3: negative control of Streptococcus*pneumoniae* (GZCDC isolate); Tube 4/Biosensor 4: blank control (DW).
Abbreviations: TL, test line; CL, control line; MCDA, multiple cross displacement amplification; MG, malachite green; GZCDC, Guizhou Provincial Center for Disease Control and Prevention; DW, double-distilled water.

Figure 3 Reaction temperature optimization for *N. meningitidis*-MCDA primers. The standard MCDA reactions for detection of *N. meningitidis* were monitored by the determination of real-time turbidity, and the DNA concentrations were displayed with corresponding curves marked in the figures. The threshold value was 0.1 and a turbidity >0.1 was set as positive. A total of 8 kinetic graphs (**A–H**) were produced at different temperatures points (60–67°C, 1°C intervals) with target DNA at the level of 10 pg per reaction. The graphs from (**B–H**) show strong amplification.
**Abbreviation:** MCDA, multiple cross displacement amplification.

Optimum temperature determined for the *N. meningitidis*-MCDA-LFB

To verify the optimum amplification temperature, *N. meningitidis* strain 13007 was used as the positive control at a level of 10 pg per reaction and the reactions were monitored by the real-time turbidity method. All tested temperatures (60–67°C with 1°C increments) generated typical kinetics graphs, with faster amplification achieved at assay temperatures of 64°C (Figure 3). Thus, the amplification temperature of 64°C was applied to perform the remaining experiments performed in the current report.

Sensitivity of MCDA-LFB for *N. meningitidis* detection

Serial dilutions of *N. meningitidis* genomic DNA were used to examine the LoD of the MCDA-LFB assay. Our results demonstrated that the analytical sensitivity of the *N. meningitidis*-MCDA-LFB assay was 10 fg per reaction (around 3 CFU per reaction) (Figure 4A). Furthermore, the analytical sensitivity of the MCDA assay was examined by real-time turbidity detection and colorimetric monitoring of amplification products with MG reagents (Figure 4B and C). By MG reagents and real-time turbidity, the LoD of the MCDA assay was also 10 fg per reaction, which was completely consistent with LFB detection.
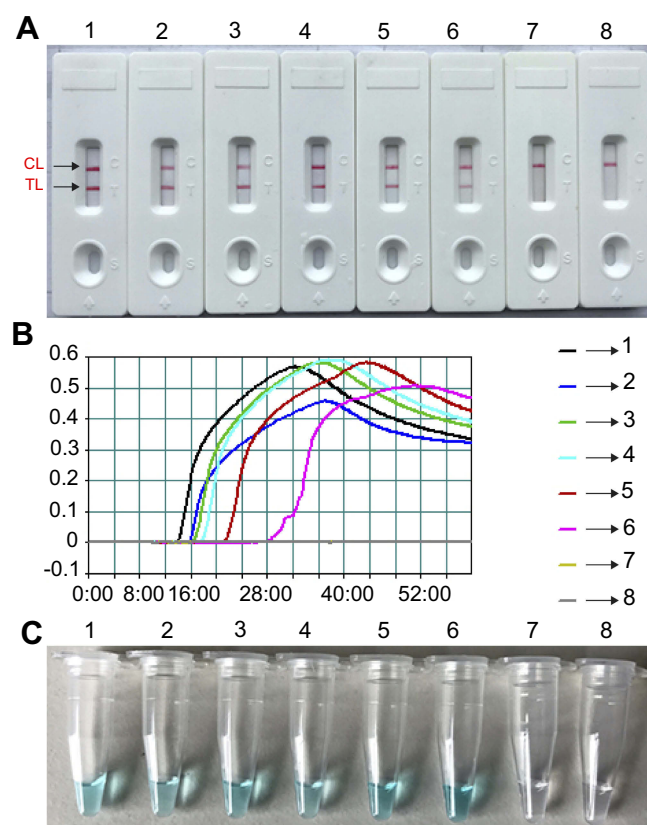
Figure 4 Sensitivity analysis of the MCDA-LFB assay using serial dilutions of genomic DNA extracted from *Neisseriameningitidis* strain 13007. A total of three monitoring techniques, including the lateral flow biosensor (**A**), real-time turbidity (**B**) and colorimetric indicator (MG; **C**) methods, were applied to analyze the amplification products. Serial dilutions of target templates were subjected to standard MCDA reactions. Biosensors (**A**)/Tubes (**B**)/Turbidity signals (**C**) 1–8 represent the DNA levels of 1 ng per reaction, 100 pg per reaction, 10 pg per reaction, 1 pg per reaction, 100 fg per reaction, 10 fg per reaction, 1 fg per reaction of target templates and the blank control (DW), respectively. The genomic DNA levels of 1 ng per reaction, 100 pg per reaction, 10 pg per reaction, 1 pg per reaction, 100 fg per reaction, 10 fg per reaction produced positive reactions.
Abbreviations: TL, test line; CL, control line; MCDA-LFB, multiple cross displacement amplification with lateral flow biosensor; MG, malachite green; DW, double-distilled water.

Optimized the reaction time for *N. meningitidis*-MCDA-LFB assay

To evaluate the optimum time required for the *N. meningitidi*s-MCDA-LFB assay during reaction stage, a total of four reaction times (15, 25, 35 and 45 mins) were compared at 64°C according to the standard MCDA conditions. The lowest DNA level (10 fg of *N. meningitidi*s templates per reaction) displayed two visible-red lines (TL and CL) when the amplification only proceeded for 35 mins at 64°C (Figure 5). So, the reaction time of 35 mins was recommended as an optimal reaction time for *N. meningitidi*s-MCDA-LFB assay.

Specificity of MCDA-LFB for *N. meningitidis* detection

When DNA from the bacteria listed in Table 1 was used in the MCDA-LFB assay, only the DNA from the *N. meningitidis* strains provided positive results. DNA from non-pathogenic *N. meningitidis* strains and from all non-*N. meningitidis* isolates did not generate detectable amplification products (Figure 6). Two red lines, including the TL and CL, appeared on the strips for the positive tests, and only one red line (the CL) appeared on the biosensors, suggesting negative results for non-*N. meningitidis* bacterial isolates and the blank control.

Application of MCDA-LFB for detection of *N. meningitidis* in clinical samples

A total of 56 samples were used for the application of *N. meningitidis*-MCDA-LFB developed in the present study. Among the 56 samples, 16 samples had been confirmed as *N. meningitidi*s positive using the traditional PCR method, and the other 40 samples were negative. The results of MCDA-LFB detection showed 19 samples were positive, which covered all the 16 positive samples using traditional PCR. Consistent detection results were observed when using MCDA-LFB and traditional cultivation and isolation methods, both of the two methods confirmed 19/56 positive samples (Table 3).

Discussion

In the present study, MCDA-LFB assay was established for the detection of *N. meningitidis* targeting on the *ctrA* gene with excellent specificity and sensitivity. The assay was optimized at 64°C for only 35 mins, and the products were directly sensed by LFB. The whole operation could be finished within 60 mins. The LoD was 10 fg/reaction of pure *N. meningitidis* DNA. The protocol was convenient, simple and does not require costly specialized equipment. Therefore, the MCDA-LFB assay presented in this study will offer an effective strategy for the rapid detection of *N. meningitidis*, which is of potential application value in the field and resource-deficient situations.

The high specificity of this method is likely due to using the *ctrA* as target gene, encoding an outer membrane protein involved in capsule transport, which has been depicted specific for the identification of *N. meningitidis*.[25] Besides, the 10 primer sequences designed based on different regions of the target gene sequence also enhanced the specificity of MCDA technique, which has been
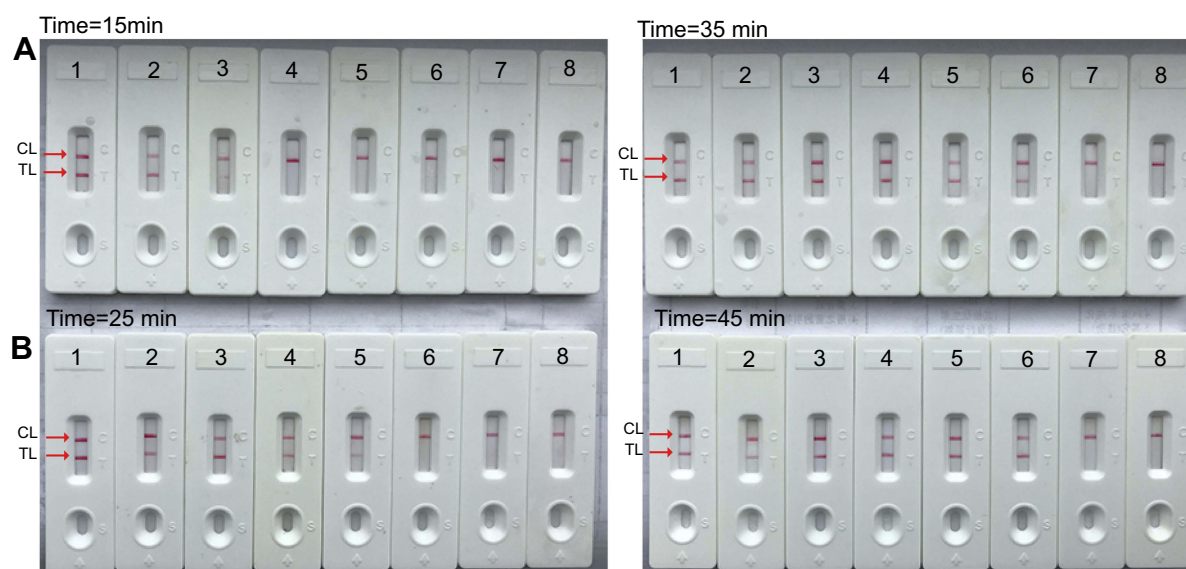
Figure 5 The optimal duration of time required for Neisseria *meningitidis*-MCDA-LFB method. Four distinct reaction times (**A**, 15 mins; **B**, 25 mins; **C**, 35 mins; **D**, 45 mins) were examined and compared at 64°C. Biosensors 1, 2, 3, 4, 5, 6, 7 and 8 represent DNA levels of 1 ng of templates, 100 pg of *N. meningitidis* templates, 10 pg of *N. meningitidis* templates, 1 pg of *N. meningitidis* templates, 100 fg *N. meningitidis* templates, 10 fg *N. meningitidis* template, 1 fg *N. meningitidis* template and blank control (DW), respectively. The best sensitivity was observed when the amplification lasted for 35 mins (C).
Abbreviations: TL, test line; CL, control line; MCDA-LFB, multiple cross displacement amplification with lateral flow biosensor; DW, double-distilled water.

proven in previous studies.[29,37] The specificity of the assay was successfully verified using pure cultures. The test was positive for all *N. meningitidis* isolates but negative for other non-*N. meningitidis* strains (Figure 6). Apart from the excellent specificity, the *N. meningitidis*-MCDA-LFB method developed in the present study showed sufficient sensitivity in the detection of *N. meningitidis*. The LoD was 10 fg DNA per reaction (about 3 CFU/reaction) extracted from *N. meningitidis* pure cultures (Figure 4). Although the traditional detection methods, including bacterial isolation and Gram staining, immunochromatographic antigen detection testing for *N. meningitidis*, are the most common methods for the *N. meningitis* detection,[8,20,21] the shortage of sensitivity for these assays has also been previously described.[23] The more recent PCR and LAMP techniques have been developed, but the marginal amounts of nucleic acid sequences were still hard to be detected in various samples.[29,30] The application of the newly established MCDA-LFB in the detection of *N. meningitidis* in clinical samples showed higher positive rates than traditional PCR (Table 3). Although the positive results were equal to that of traditional cultivation and isolation methods, MCDA-LFB is likely the preferred technique as the result reading is less subjective and needs only minimal instrumentation. Therefore, the newly developed MCDA-LFB is able to make up for the shortages of these methods. Rapid identification of target

pathogens was valuable for the accurate clinical diagnosis and choosing rational therapy for the patients. For example, the detection results are helpful for the selection of therapeutic drug for killing specific bacteria or other pathogens. The MCDA-LFB established in the current study offers a rapid tool to detect the DNA of *N. meningitidis*, which can be finished in 65 mins. Therefore, the MCDA-LFB developed in this report is a time-saving and convenient molecular technique for *N. meningitidis* detection compared with the traditional bacterial isolation and conventional PCR or LAMP methods. Compared to PCR-based methods, MCDA-LFB assay can be finished without the thermal denature and temperature changes during the reaction, avoiding the requirement of complicated and precise equipment. Moreover, it only needs a simple incubation at 63°C, and thus facilitates its application in resource-poor laboratories.[37] MCDA can be performed using commercial kit for isothermal amplification kits such as NEB Warmstart kits and Isothermal Amplification kits. The cost for an MCDA reaction is around US$3.5, and the cost of the LFB per test costs about US$2, which is cheaper than PCR methods. In addition, labor costs are decreased because the operation of MCDA-LFB does not require certified technical personnel in a laboratory. However, although MCDA-LFB targeting *ctrA* gene of *N. meningitides* has strength for the rapid detection with advantages in specificity,

Figure 6 Specificity analysis of the *Neisseriameningitidis*-MCDA-LFB assay for different bacterial strains. MCDA reactions were carried out using different genomic DNA templates and were analyzed visually. Biosensors 1, 2, 3, 4, 5, 6, 7, 8, 9 used *N. meningitidis* serogroup A, *N. meningitidis* serogroup B, *N. meningitidis* serogroup C, *N. meningitidis* serogroup D, *N. meningitidis* serogroup W-135, *N. meningitidis* serogroup 29-E, *N. meningitidis* serogroup X, *N. meningitidis* serogroup Y, *N. meningitidis* serogroup Z; 10–11, Biosensors 10–11 used *N. meningitidis* isolate GZCDC001, *N. meningitidis* isolate GZCDC002, respectively; Biosensors 12–39 used *Bordetella pertussis*, *Bordetella parapertussis*, *Hemophilus parainfluenza*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Mycoplasma pneumoniae*, *Legionellae bacillus*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Salmonella*, enteropathogenic *E. coli*, enterotoxigenic *E. coli*, invasive *E.coli*, enterohemorrhagic *E. coli*, enteroaggregative *E. coli*, *Streptococcus suis*, *Vibrio cholerae*, *Vibrio parahemolyticus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Bacillus cereus*, *Bacillus proteus*, *Enterobacter cloacae*; *Listeria monocytogenes*, *Shigella flexneri*, *Shigella boydii*, respectively; Biosensor 40 used the blank control (DW).
Abbreviations: MCDA-LFB, multiple cross displacement amplification with lateral flow biosensor; DW, double-distilled water; GZCDC, Guizhou Provincial Center for Disease Control and Prevention.

Table 3 Comparison of conventional PCR, culture-biotechnical and MCDA-LFB for the detection of *N. meningitidis* in clinical samples

Detection method	Samples (n=56)	
	Positive	Negative
PCR	16	40
Culture	19	37
MCDA-LFB	19	37

sensitivity, time-saving, detection cost and so on, it also possesses limitation when compared with methods which can be used for *N. meningitides* serogroup identification and antimicrobial resistance detection.

In conclusion, we successfully developed an MCDA-LFB assay for the detection of *N. meningitidis* based on the *ctrA* gene. This method displayed high degree of specificity for the detection of *N. meningitidis*, with sensitivity of 10 fg per reaction with pure culture (around 3 CFUs/reaction). The whole process of detection can be completed in 65 mins and the protocol was convenient, simple and does not require costly specialized equipment. Moreover, the detection cost of this method is cheaper than other methods such as PCR. Thus, the MCDA-LFB assay presented in this study will offer an effective strategy for the rapid detection of *N. meningitidis*, which is especially of potential application value in the field and resource-deficient situations.

Conclusion

The MCDA-LFB techniques developed in the present study are an effective tool for the rapid detection of *N. meningitidis*, especially in resource-poor countries in meningococcal disease epidemic period.

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Disclosure

The authors report no conflicts of interest in this work.

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