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Ultra-efficient multiple cross displacement amplification-lateral flow biosensor (MCDA-LFB) for serogroup identification of prevalent *Neisseria meningitidis*

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ABSTRACT

Meningococcal disease caused by *Neisseria meningitidis* remains a major global public health concern. Serogroup A, B, C and W135 were the major disease-causing serogroups. It is vital to timely and efficiently detect and differentiate these four serogroups. Herein, we developed multiple cross displacement amplification-lateral flow biosensor (MCDA-LFB) assays targeting *ctrA*, *sacB*, *siaD*, *siaD* and *synG* gene respectively for detection and subtyping of four *N. meningitidis* serogroups. This assay utilizes LFB to detect FITC and biotin-labeled target amplicons produced by MCDA through double antibody sandwich principle, to allow sensitive and specific detection under a constant temperature. The detection limit was as low as 10 fg or 100 fg genomic DNA in pure cultures and 5.5 CFUs or 36 CFUs in spiked cerebrospinal fluid (CSF) specimens, which were overall 100 to 1000-fold more sensitive than conventional PCR. High specificity of these assays was also validated through type strains and clinical isolates, with no cross-reactions. MCDA-LFB testing procedure can be finished within 1 h. In conclusion, the *N. meningitidis*- and serogroup-MCDA-LFB assays established in this study are simple, rapid and efficient, providing valuable molecular methods for diagnosis and surveillance of meningococcal disease, especially in resource-limited regions and when specimen culture fails.

1. Introduction

Meningococcal disease is a severe infection caused by *Neisseria meningitidis*, the most common form of which is meningococcal meningitis [1]. Meningococcal meningitis remains a major global public health challenge that can cause large-scale epidemics, which mainly affects young children, adolescents, and young adults [2]. The disease is devastating, which is fatal in 50% of patients if untreated and in 5%–10% of patients even with adequate treatment within 24–48 h after onset, and it may cause permanent disabilities in 10%–20% of survivors [1,3]. *N. meningitidis* is a Gram-negative, coffee-bean shaped diplococci that only infects humans and transmits through respiratory droplets or throat secretions from carriers, whereas the carrier rate of *N. meningitidis* among healthy population is about 5%–10% worldwide and 2.7% in China [1,4].

Twelve distinct serogroups of *N. meningitidis* have been identified to date, six of which (A, B, C, W, X and Y) can cause epidemics and cause most invasive meningococcal disease [2]. The serogroup distribution of invasive meningococcal disease varies by region and may change due to vaccine immunization. In African meningitis belt, meningococcal A meningitis has been the largest burden before 2010 [5]. At present, meningococcus serogroups C and W accounted for majority of

Abbreviations: MCDA, multiple cross displacement amplification; LFB, lateral flow biosensor; CSF, cerebrospinal fluid; CFU, colony forming unit; PCR, polymerase chain reaction; LAMP, loop-mediated isothermal amplification; FITC, fluorescein isothiocyanate; VDR, visual detection reagent.

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meningitis epidemics in Africa, while serogroup B was the most prevalent serogroup in Europe and Americas [6]. The predominant cause of meningococcal meningitis in China has been meningococcus serogroup A, whereas serogroup C was gradually prevalent since 2003, the proportion of serogroup A cases decreased significantly and serogroups B and W135 cases have increased since 2010 [7]. These four meningococcus serogroups (A, B, C and W135) are responsible for substantial proportions of meningococcal disease in China. Timely and effectively diagnosis, controlling and preventing the epidemic of these four serogroups represent a major challenge.

In general, culture, Gram stain and latex agglutination testing of clinical specimens (cerebrospinal fluid (CSF) or blood) from suspected cases of bacterial meningitis were used for detection and characterization of *N. meningitidis*, then followed by serogroup identification by serological testing [8]. Culture is considered the gold standard for confirmation of meningococcal meningitis, whereas it is complicated and time-consuming, and the positive rate is relatively low due to antibiotic treatment administered prior to specimen collection or inappropriate storage conditions [9,10]. Gram staining and latex agglutination testing can merely provide a presumptive diagnosis, but not for confirmatory identification. Serological testing is highly dependent on the successful isolation and pure culture of *N. meningitidis*, which is not feasible for specimens that failed to be cultured. To resolve these problems, conventional and real-time polymerase chain reaction (PCR) have been developed as complementary approaches for detection and subtyping of *N. meningitidis*, which can be used on DNA extracted from clinical specimens or bacterial isolates. The *ctrA*, *sacB* or *orf-2*, *siaD* or *synB*, *siaD* or *synE*, *siaD* or *synG* have been recommended as species-specific, serogroup A, B, C, and W135-specific gene targets to identify *N. meningitidis* and four major disease-causing serogroups [11–13]. However, the PCR detection system is expensive, complex to perform, requiring skilled operator, and susceptible to false positivity, making it difficult to expand in resource-limited regions. In the past several years, loop-mediated isothermal amplification (LAMP), a more rapid, simple, sensitive and specific method than classic culture and PCR, has been developed for detection and subtyping of *N. meningitidis* [14,15]. LAMP detects amplification products by using a colorimetric visual inspection dye, agarose gel electrophoretic, or monitoring the real-time turbidity. The results reading of LAMP by visual inspection of color change is subjective, and it is difficult to interpret when specimens containing the bacterial pathogens in low density. Gel electrophoretic-based detection increases the risk of contamination. Turbidity-based detection requires a matching real-time turbidimeter, thus complicating the procedure. In addition, LAMP is less sensitive than another isothermal amplification called multiple cross displacement amplification (MCDA) [16].

MCDA is a novel nucleic acid isothermal amplification technology developed by Wang et al. in recent years [17], which is based on strand displacement DNA synthesis under the action of *Bst* DNA polymerase. In the MCDA reaction, ten primers were designed, including two displacement primers (F1 and F2), two cross primers (CP1 and CP2) and four amplification primers (C1, C2, D1, D2, R1 and R2), to specifically recognize ten distinct sequences of target gene for maximum coverage of specific region, with higher specificity and sensitivity than PCR and LAMP [16]. The MCDA amplification principle is as described previously [17]. Moreover, MCDA assay does not require a thermocycler, which can be performed in a simple heater and completed within 1 h. In the result reading of MCDA, lateral flow biosensor (LFB) may be the preferred method because it is more simple, rapid and objective than current molecular methods. The LFB is based on the binding of antibody (e.g., *anti*-FITC antibody) embedded on the LFB and hapten (e.g., FITC) labeled on the amplicons. Then, the combination of streptavidin coated onto the LFB and biotin labeled on the amplicons results in a purplish red band on the test line of LFB. In recent years, this assay has been used for detection of many bacterial pathogens, such as *Klebsiella pneumoniae* [18], *Listeria monocytogenes* [19], and *Vibrio parahaemolyticus* [20]. Thus, MCDA-LFB assay provides a valuable complementary method to currently available molecular methods.

In this study, a set of rapid and efficient MCDA-LFB assays for detection and subtyping of *N. meningitidis* were developed. The *ctrA*, *sacB*, *siaD*, *siaD*, and *synG* genes are respectively targeted for *N. meningitidis*, serogroup A, B, C, and W135, four predominant meningococcus serogroups in China. The specificity, detection limit and analytical sensitivity were evaluated employing purified cultures and spiked CSF specimens.

2. Materials and methods

2.1. Bacteria strains

This study used four prevalent *N. meningitidis* strains, including meningococcus serogroup A, B, C, and W135 stored in Anhui Provincial Center for Disease Control and Prevention (AHCDC), and 35 non-meningococcus strains (Table 1).

For detection limit of *N. meningitidis* species- and serogroup-specific MCDA assays, genomic DNAs of meningococcus serogroup A, B, C, and W135 strains cultured on chocolate agar culture plate were extracted using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) and quantified by a NanoDrop ND-1000 (Calibre, China), and then which were serially diluted (1 ng/μL, 100 pg/μL, 10 pg/μL, 1 pg/μL, 100 fg/μL, 10 fg/μL, 1 fg/μL) and used as templates. For analytical sensitivity of *N. meningitidis* species- and serogroup-specific MCDA assays in clinical specimens, we used a simulated specimen method, artificially adding

Table 1
Bacterial strains used in this study.

ID	Bacterial strains	Sources
z1	*N.meningitidis* Serogroup A	Isolated strains (AHCDC)
2	*N.meningitidis* Serogroup B	Isolated strains (AHCDC)
3	*N.meningitidis* Serogroup C	Isolated strains (AHCDC)
4	*N.meningitidis* Serogroup W135	Isolated strains (AHCDC)
5	*Shigatoxin-producing E. coli*	*E. Coli* 933
6	*Enteroaggregative E. coli*	*E. Coli* 042
7	*Enteroinvasive E. coli*	*E. Coli* 44825
8	*Enteropathogen E. coli*	*E. Coli* 2348/69
9	*Enterotoxigenic E. coli*	*E. Coli* 10407
10	*Aeromonas caviae*	ATCC 15468
11	*Salmonella typhi*	Isolated strains
12	*Citrobacter freundii*	Isolated strains
13	*Listeria ivanovii*	BAA 678
14	*Klebsiella pneumoniae*	ATCC 2146
15	*Streptococcus pneumoniae*	ATCC 49619
16	*Shigella flexneri*	Isolated strains
17	*Acinetobacter baumannii*	Isolated strains
18	*Staphylococcus epidermidis*	Isolated strains
19	*Streptococcus suis*	Isolated strains
20	*Proteus mirabilis*	Isolated strains
21	*Corynebacterium ammoniagenes*	Isolated strains
22	*Providencia rettgeri*	Isolated strains
23	*Streptococcus salivarius*	Isolated strains
24	*Neisseria perflava*	Isolated strains
25	*Enterobacter cloacae*	ATCC 700323
26	*Serratia marcescens*	Isolated strains
27	*Staphylococcus aureus*	ATCC 25923
28	*Bacillus cereus*	Isolated strains
29	*Pseudomonas aeruginosa*	ATCC 27853
30	*Escherichia coli*	ATCC 35218
31	*Neisseria gonorrhoeae*	Isolated strains
32	*Streptococcus agalactiae*	Isolated strains
33	*Morganella morganii*	Isolated strains
34	*Candida albicans*	ATCC 14053
35	*Staphylococcus haemolyticus*	Isolated strains
36	*Streptococcus bovis II*	Isolated strains
37	*Vibrio parahaemolyticus*	Beijing quality control products
38	*Enterococcus faecium*	Beijing quality control products
39	*Shigella sonnei*	Beijing quality control products

AHCDC, Anhui Provincial Center for Disease Control and Prevention.

different amount of colony forming units (CFUs) of meningococcus serogroup A, B, C, and W135 strains into *N. meningitidis* negative CSF specimens. For specificity validation, genomic DNAs were extracted from 35 non-meningococcus culture strains using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany).

2.2. MCDA primer design

According to the genomic sequence of *N. meningitidis* species- and serogroup A, B, C, and W135-specific genes [13,14], *ctrA* (GenBank accession number AE002098.2), *sacB* (GenBank accession number FR774048.1), *siaD* (GenBank accession number CP002424.1), *siaD* (GenBank accession number AM421808.1), and *synG* (GenBank accession number AY234197.1) were selected as target gene for *N. meningitidis* and serogroup A, B, C, and W135, respectively. MCDA primer sets targeting ten distinct regions of target gene, including two displacement primers (F1 and F2), two cross primers (CP1 and CP2), and four amplification primers (C1, C2, D1, D2, R1 and R2), were designed using Primer 5.0 (Premier Biosoft, San Francisco, CA, USA) and PrimerExplorer V4 (Eiken Chemical CO., LTD, Tokyo, Japan) according to the principle of MCDA amplification [17]. The specificity of MCDA primer sets was confirmed by BLAST analysis (Basic Local Alignment Search Tool). The details of MCDA primer sets, including primer sequences and modifications, and their location and direction in target gene, were shown in Table S1 and Fig. S1. The 5′ ends of primers C1 and D1 were labeled with fluorescein isothiocyanate (FITC) and biotin, respectively. Primer synthesis and HPLC grade purification were completed by AoKeDingSheng Biotechnology Co., Ltd. (Beijing, China).

2.3. MCDA and PCR reactions

The MCDA reactions were carried out in a 25 μL reaction mixture, including 0.4 μM each of F1 and F2; 0.8 μM each of C1*, C2, D1*, D2, R1, and R2; 1.6 μM each of CP1 and CP2; 12.5 μL of 2 × reaction buffer; 1.25 μL of *Bst* DNA polymerase (*Bst* 2.0); 1uL visual detection reagent (VDR); and 1 μL of DNA template. The DNA Isothermal® Amplification Kit and visual detection reagent (VDR) were obtained from TianJin HuiDeXin Technology Development Co., Ltd. (Tianjin, China). The MCDA reactions for *N. meningitidis*, serogroup A, B, C, and W135 were isothermally amplified at 65 °C, 61 °C, 59 °C, 60 °C, and 56 °C for 60 min, respectively. *P. aeruginosa* and *K. pneumoniae* were used as negative control, distilled water was used as blank control.

In the study of detection limit and analytical sensitivity, conventional PCR recommended by Diagnosis Standard for Meningococcal meningitis of China (WS 295–2019) were performed for *N. meningitidis* and serogroup identification (Table S2), as a comparison of MCDA. TaKaRa Ex Taq® DNA polymerase applied for conventional PCR was obtained from Takara Biomedical Technology (Beijing) Co., Ltd.. For *N. meningitidis*, PCR was performed in a 50ul mixture containing 0.4 μM each of forward primer and reverse primer, 5 μL of 10 × Ex Taq Buffer, 4 μL of dNTP Mixture, 0.25 μL of TaKaRa Ex Taq (5 U/μL) and 1uL of DNA template. PCR conditions were as follows: 94 °C for 5 min; 30 cycles of 92 °C for 30 s, 55 °C for 40 s, and 72 °C for 30 s; followed by a final extension at 72 °C for 2 min. For serogroup identification, PCR was performed in a 50 μL reaction including 0.3 μM each of forward primer and reverse primer, 5 μL of 10 × Ex Taq Buffer, 4 μL of dNTP Mixture, 0.25 μL of TaKaRa Ex Taq (5 U/μL) and 1uL of DNA template. PCR conditions were as follows: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s; followed by a final extension at 72 °C for 5 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis.

2.4. Analysis of MCDA products

The *N. meningitidis* species- and serogroup-specific MCDA amplification products were detected by three methods, including disposable lateral flow biosensor (LFB), visual detection reagent (VDR) and 1.5% agarose gel electrophoresis. The LFB was purchased from TianJin HuiDeXin Technology Development Co., Ltd. (Tianjin, China) and used according to the manufacturer's instructions. In the positive reaction, two purplish red bands (test line band and control line band) appear on the biosensor within 2 min, whereas only one control line band appears in the negative reaction. For visual inspection by visual detection reagent (VDR), the color of the positive reaction remains to be lake green, while the negative reaction changes to colorless. MCDA products were separated by 1.5% agarose gel electrophoresis at 100 V for 40 min, the positive reaction products are shown as ladder-like bands under UV light, but do not appear for negative reaction products.

2.5. Optimization of the amplification temperature of N. meningitidis species- and serogroup-specific MCDA assays

To determine the optimal temperatures, the *N. meningitidis* species- and serogroup-specific MCDA mixtures (10 pg DNA template per reaction) were isothermally amplified at temperatures ranging from 62 °C to 67 °C and 54 °C–64 °C for 60 min, respectively. Of which, meningococcus serogroup A strain was used for *N. meningitidis* species-specific MCDA assay. The real-time turbidity was monitored at 650 nm using a turbidimeter (Loopamp Realtime Turbidimeter LA-320C, EIKEN CHEMICAL CO., LTD), turbidity >0.1 is defined as the positive threshold value. The temperature that promotes a high-efficiency amplification with high products, that is, high turbidity occurs earlier in the corresponding kinetic curve, was selected as the optimal amplification temperature. The *P. aeruginosa* and distilled water were used as negative control and blank control, respectively. The experiment was repeated in triplicate.

2.6. Specificity of the N. meningitidis species- and serogroup-specific MCDA assays

The specificity of the *N. meningitidis* species- and serogroup-specific MCDA assays were analyzed independently using genomic DNAs extracted from 39 bacterial strains including 4 *N. meningitidis* serogroup strains, 2 non-meningococcal *Neisseria* species and 33 other bacterial strains (Table 1), and were repeated three times. LFB was applied to read MCDA results rapidly and objectively.

2.7. Sensitivity of the N. meningitidis species- and serogroup-specific MCDA assays

The detection limit of *N. meningitidis* species- and serogroup-specific MCDA assays were determined by using serial dilutions (1 ng/μL, 100 pg/μL, 10 pg/μL, 1 pg/μL, 100 fg/μL, 10 fg/μL and 1 fg/μL) of genomic DNA extracted from *N. meningitidis* serogroup A, B, C, and W135 strains. The MCDA mixtures added with 1 μL of each dilution were amplified at optimal temperatures for 60 min in triplicate. The MCDA results were compared analyzed by three methods, LFB, visual detection reagent and 1.5% agarose gel electrophoresis, to confirm the detection limit. Distilled water was used as blank control.

2.8. Analytical sensitivity of N. meningitidis species- and serogroup-specific MCDA assays in CSF specimens

To evaluate the clinical feasibility of *N. meningitidis* species- and serogroup-specific MCDA assays, a simulated specimen method was adopted to determine the methods' analytical sensitivity in CSF specimens. *N. meningitidis* serogroup A, B, C, and W135 strains were incubated in 5% CO_2 at 37 °C for 24 h. Then, the bacterial suspensions with an OD (optical density) value of 0.6 were prepared, and which were subsequently 10-fold serial diluted (10^{-1}-10^{-7}) by adding 100uL of bacterial suspensions into 900uL of sterile saline. 100 μL of 10^{-6} bacterial dilution was inoculated on three chocolate agar culture plate and

the colony forming units (CFUs) were counted after 24 h at 37 °C. Meanwhile, 100 μL of each bacterial dilutions were artificially added into 900uL of *N. meningitidis* negative CSF specimens, and the order of magnitude of *N. meningitidis* was adjusted to 10^6-10^0 CFU/mL, and then these simulated contaminated CSF specimens were subjected to DNA extraction using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) and were eluted into 100uL of Buffer AE. 1uL of DNA templates was applied for *N. meningitidis* species- and serogroup-specific MCDA reactions, and the results were analyzed by visual detection reagent and 1.5% agarose gel electrophoresis, and were compared with conventional PCR. It was worth noting that the CSF specimens were derived from the remaining specimens of clinical testing and the supernatant was used for simulation experiment, and the uncontaminated CSF specimen was used as negative control.

3. Results

3.1. Establishment and validation of N. meningitidis species- and serogroup-specific MCDA-LFB assays

To confirm the accuracy of MCDA primer sets (Table S1 and Fig. S1) for detection and differentiation of four prevalent *N. meningitidis* serogroups (A, B, C and W135), the genomic DNAs of meningococcus serogroup A, B, C, and W135 were amplified by species- and four serogroup-specific MCDA reactions at 60 °C for 60 min. Our results showed that the positive reactions were consistent with the expected results. The genomic DNA of serogroup A, B, C, and W135 were all positive in *N. meningitidis* species-specific MCDA, whereas which were separately positive in serogroup A, B, C, and W135 specific-MCDA, and the results were validated by three methods including LFB, visual detection reagent and gel electrophoresis (Fig. 1, Fig. S2 and Fig. S3). For positive reactions, there were two purplish red bands on the LFB (Fig. 1), remain to be lake green in the tube (Fig. S2), and ladder-like bands appear on the gel (Fig. S3). For negative reactions, there was only one control line band on the LFB (Fig. 1), color changes to colorless in the tube (Fig. S2) and no ladder-like bands appear on the gel (Fig. S3). These results indicated that the MCDA primer sets were reliable and can achieve high accuracy in the detection and subtyping of four prevalent *N. meningitidis* serogroups. Therefore, these MCDA primer sets were applied to establish the *N. meningitidis* species- and four serogroup-specific MCDA-LFB assays.

3.2. Optimal temperatures for N. meningitidis species- and serogroup-specific MCDA assays

To optimize the amplification temperature, *N. meningitidis* species- and serogroup-specific MCDA reactions (10 pg DNA template per reaction) were isothermally amplified at different temperatures ranging from 62 °C to 67 °C and 54 °C–64 °C for 60 min, respectively, and the real-time turbidity was monitored at 650 nm. It was shown that 65 °C was the optimal temperature for *N. meningitidis* species-specific MCDA from the kinetic curves (Fig. 2D), owing to the earlier appearance of high turbidity. The optimal temperatures of serogroup A, B, C, and W135 specific-MCDA were 61 °C, 59 °C, 60 °C, and 56 °C, respectively (data not shown). Thus, these optimal temperatures were used for the next studies.

3.3. Specificity of N. meningitidis species- and serogroup-specific MCDA assays

To evaluate the specificity of *N. meningitidis* species- and serogroup-specific MCDA assays, genomic DNAs from 39 bacterial strains were tested (Table 1). For *N. meningitidis* species-specific MCDA, only the genomic DNA of serogroup A, B, C, and W135 generated positive results with two purplish red bands on the LFB, whereas the genomic DNA from 2 non-meningococcal *Neisseria* species and 33 other bacterial strains

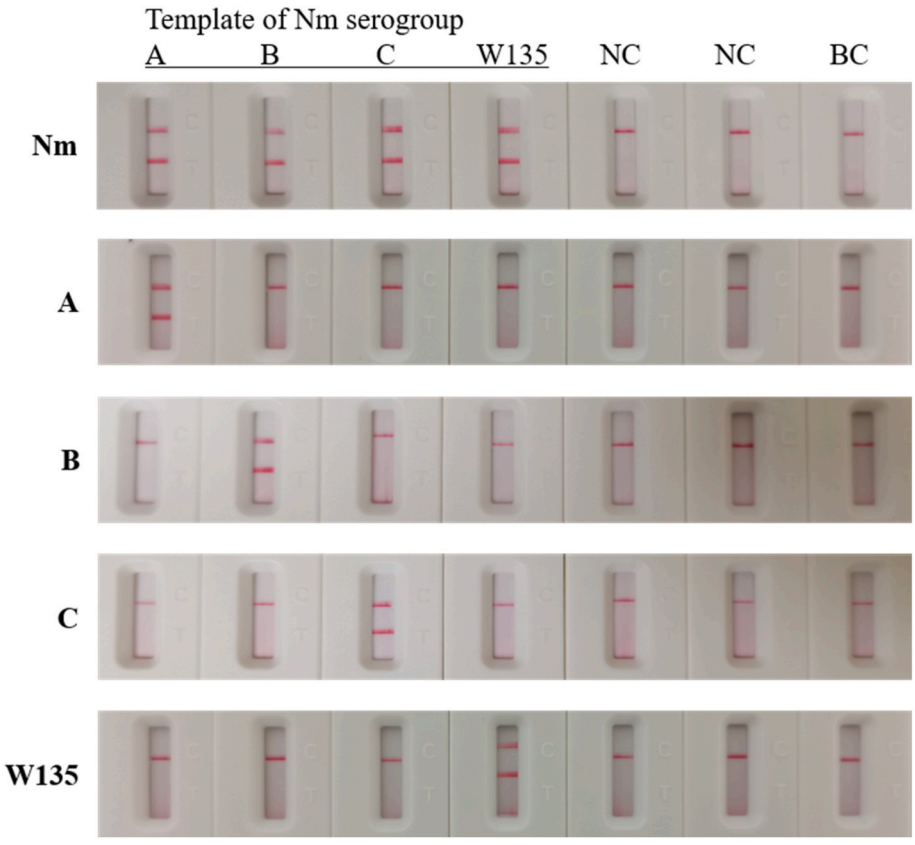

Figure 1. Establishment and validation of *N. meningitidis* species- and serogroup-specific MCDA-LFB assays. Lateral flow biosensor (LFB) was used to detect *N. meningitidis* species- and serogroup-specific MCDA products. Positive with two purplish red bands, a test line band and a control line band. **(Nm)** *N. meningitidis* species-specific MCDA. **(A)** serogroup A-specific MCDA. **(B)** serogroup B-specific MCDA. **(C)** serogroup C-specific MCDA. **(W135)** serogroup W135-specific MCDA. *P. aeruginosa* and *K. pneumoniae* were used as negative control, distilled water was used as blank control. Nm, *N. meningitidis*. NC, negative control. BC, blank control.

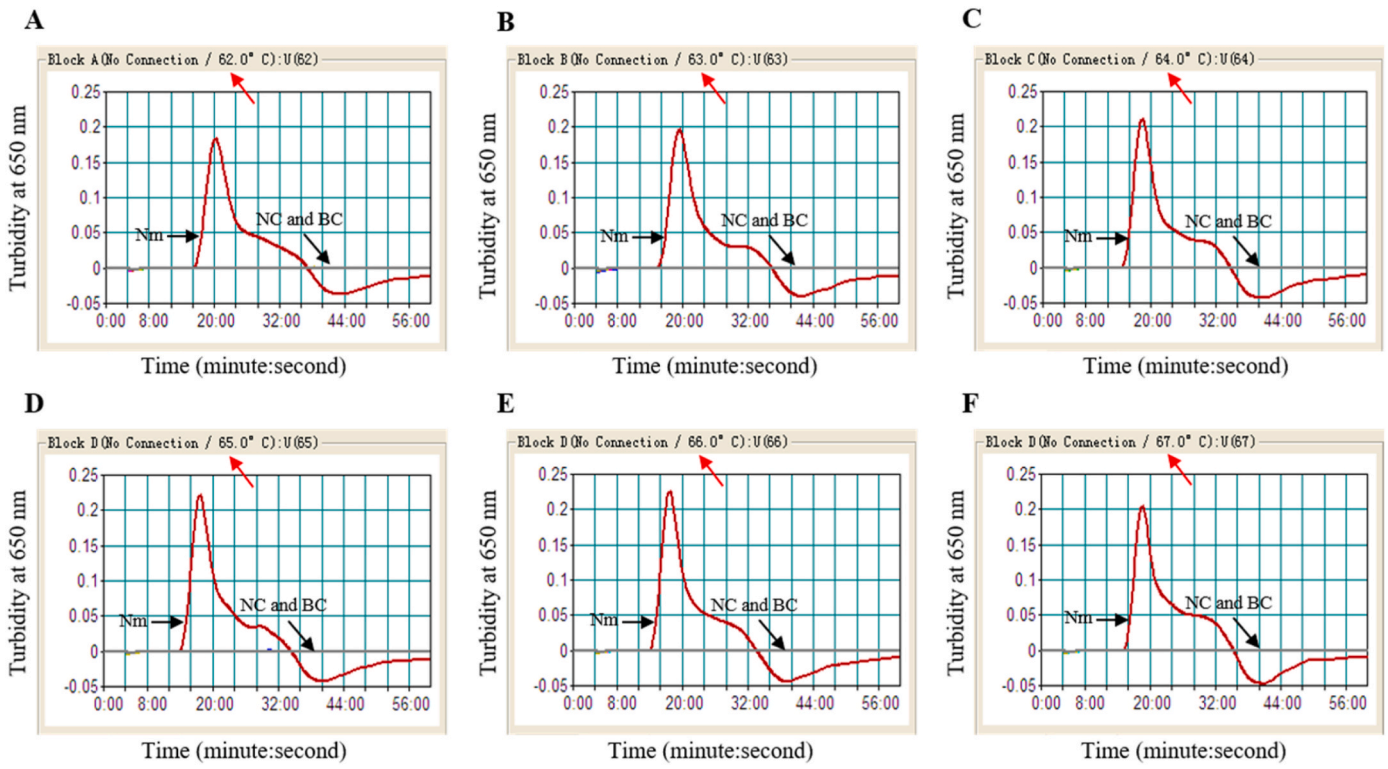

Figure 2. Optimal temperature for *N. meningitidis* species-specific MCDA detection system. The *N. meningitidis* species-specific MCDA reaction (10 pg DNA template per reaction) was amplified at different temperatures (62°C–67 °C), and the real-time turbidity was monitored at 650 nm using a turbidimeter. The corresponding kinetic curves were as shown in figure **(A–F)**. Turbidity >0.1 is considered positive. *P. aeruginosa* and distilled water were used as negative control and blank control, respectively.

showed negative results with only one control line band on the LFB (Fig. 3). For serogroup-specific MCDA, only the genomic DNA of corresponding serogroup strain generated positive result (Fig. S4). These results suggested that the *N. meningitidis* species- and serogroup-specific MCDA assays were highly specific for detection and subtyping of four prevalent *N. meningitidis* serogroups.

3.4. Detection limit of N. meningitidis species- and serogroup-specific MCDA and PCR

Serial dilutions of genomic DNA from meningococcus serogroup A, B, C, and W135 strains were used to assess the sensitivity of *N. meningitidis* species- and serogroup-specific MCDA assays. Our results showed that the detection limit of *N. meningitidis* species- and serogroup A, B, C, and W135-specific MCDA were as low as 10 fg, 10 fg, 100 fg, 100 fg, and 100 fg per reaction (Fig. 4, Fig. S5 and Fig. S6). The MCDA results analyzed by LFB (Fig. 4), visual detection reagent (Fig. S5), and gel electrophoresis (Fig. S6) were completely consistent. The detection limit of species- and serogroup A, B, C, and W135-specific PCR were 10 pg, 1 pg, 10 pg, 10 pg, and 10 pg per reaction (Fig. S7). The sensitivity of MCDA assays was overall 100-fold–1000-fold more sensitive than conventional PCR recommended by Diagnosis Standard for Meningococcal meningitis of China (WS 295–2019).

3.5. Analytical sensitivity of N. meningitidis species- and serogroup-specific MCDA assays in CSF specimens

In order to evaluate the analytical sensitivity of *N. meningitidis* species- and serogroup-specific MCDA assays in clinical application, their detection limit in CSF specimen detection were tested using a simulated specimen method. Our results showed that the *N. meningitidis* species- and serogroup A, B, C, and W135 specific-MCDA could achieve positive amplification when the number of meningococcus serogroup A, A, B, C, and W135 strains in CSF specimens were no less than 5.5×10^2 CFU/mL, 5.5×10^2 CFU/mL, 2.3×10^3 CFU/mL, 3.2×10^3 CFU/mL, and 3.6×10^3 CFU/mL, respectively (Table 2, Fig. S8 and Fig. S9). The MCDA results were 1000-fold more sensitive than conventional PCR recommended by Diagnosis Standard for Meningococcal meningitis of China (WS 295–2019) (Table 2 and Fig. S10).

4. Discussion

Meningococcal disease remains a major global public health concern due to its high fatality rate, disability rate and epidemic potential, and the destructive power and epidemic potential vary according to meningococcus serogroups [2]. For suspected cases of meningococcal disease, the confirmatory identification and serotyping of *N. meningitidis* is important to diagnosis and appropriate antibiotic treatment as soon as possible, whereas early diagnosis depends on rapid, specific and sensitive laboratory identification. In this study, MCDA-LFB assays, targeting *N. meningitidis* species- and serogroup A, B, C, and W135-specific genes, were developed for detection and subtyping of *N. meningitidis*. These assays have high specificity and sensitivity, and have been validated using DNA extracted from pure cultures and spiked CSF specimens. The strategy employed to detect and characterize *N. meningitidis* in suspected cases of meningococcal disease is to first run species-specific MCDA-LFB assay, the serogroup-specific assays were then performed on positive specimens. The testing procedure was simple, rapid, efficient and objective, providing valuable complementary approach to conventional culture and PCR methods, especially for specimens that do not yield any culture or contain the causative agent in low density.

Herein, high specificity of *N. meningitidis* species- and serogroup-specific MCDA-LFB assays were demonstrated through type strains and clinical isolates, with no cross-reaction with different meningococcus serogroups and other common bacterial pathogens (Figs. 1 and 3). Our results indicated that the selected gene targets and target

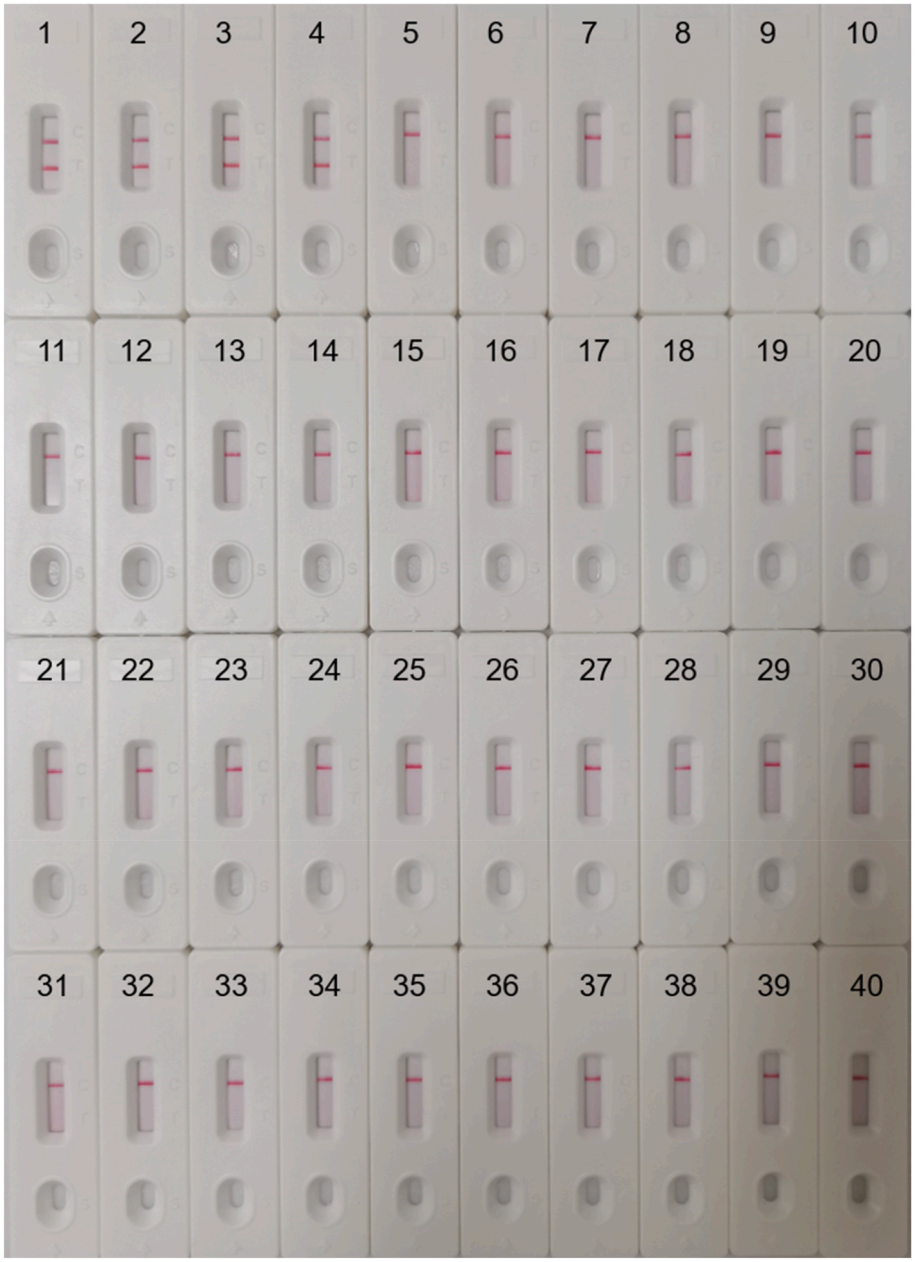

Figure 3. Specificity of *N. meningitidis* species-specific MCDA-LFB assay. Genomic DNA from different bacterial strains were used for *N. meningitidis* species-specific MCDA assay. Biosensors 1–4, *N. meningitidis* serogroup A, B, C, and W135; Biosensors 5–39, *Shigatoxin-producing E. coli*, *Enteroaggregative E. coli*, *Enteroinvasive Escherichia coli*, *Enteropathogen Escherichia coli*, *Enterotoxigenic Escherichia coli*, *Aeromonas caviae*, *Salmonella typhi*, *Citrobacter freundii*, *Listeria ivanovii*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Shigella flexneri*, *Acinetobacter baumannii*, *Staphylococcus epidermidis*, *Streptococcus suis*, *Proteus mirabilis*, *Corynebacterium ammoniagenes*, *Providencia rettgeri*, *Streptococcus salivarius*, *Neisseria perflava*, *Enterobacter cloacae*, *Serratia marcescens*, *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Streptococcus agalactiae*, *Morganella morganii*, *Candida albicans*, *Staphylococcus haemolyticus*, *Streptococcus bovis II*, *Vibrio parahaemolyticus*, *Enterococcus faecium*, *Shigella sonnei*; Biosensor 40, distilled water.

sequences for MCDA assays here are unique and can characteristically detect *N. meningitidis* and differentiate four major disease-causing serogroups (A, B, C and W135). Firstly, the capsule transport to cell surface gene, *ctrA*, was used as the species-specific gene target, which was exclusive to *N. meningitidis* and highly conserved within the major disease-causing serogroups [21–24], and has been used in both PCR and LAMP to identify *N. meningitidis* [11,25]. Furthermore, the capsule gene complex of *N. meningitidis* also has areas that are unique within each serogroup, providing serogroup-specific gene targets to differentiate each specific serogroup. The gene *sac* or *orf* for capsule biosynthesis, gene *sia* for sialic acid biosynthesis (also called *syn* for capsule biosynthesis), have been used for serotyping of serogroup A (*sacB* or *orf-2*), B (*siaD* or *synB*), C (*siaD* or *synE*), and W135 (*siaD* or *synG*), showing high specificity [11–14]. Through comprehensive comparison and sequence alignment, four serogroup-specific gene targets, *sacB*, *siaD*, *siaD*, and *synG*, were then selected for the establishment of MCDA assays targeting serogroup A, B, C, and W135, respectively (Table S1). In addition, the specific detection of MCDA also relies on ten different primers that specifically recognize different target sequences within a target gene, thus elevating the specificity of MCDA assay.

Moreover, as shown in Fig. 4 and Table 2, our results confirmed that species- and serogroup-specific MCDA were highly sensitive in detection and subtyping of *N. meningitidis*. The detection limit was 10 fg or 100 fg genomic DNA in pure cultures and 5.5 CFUs or 36 CFUs N. *meningitidis* in spiked CSF specimens. Traditional laboratory methods for detection and characterization of *N. meningitidis* mainly rely on bacterial culture, Gram stain, latex agglutination and serological tests [8]. However, culture-based methods are characterized by time-consuming and usually have low yield due to the empiric use of antibiotics before specimen collection, thus delaying clinical diagnosis and underestimating the true burden of *N. meningitidis* pathogens [9,10,26]. It has been reported that delayed diagnosis is closely related to high mortality of meningococcal disease [27]. Over the past dozen years, PCR and LAMP have been successively developed for detection and characterization of *N. meningitidis*. They have been proven to be valuable methods for diagnosis of *N. meningitidis* from clinical specimens regardless of the

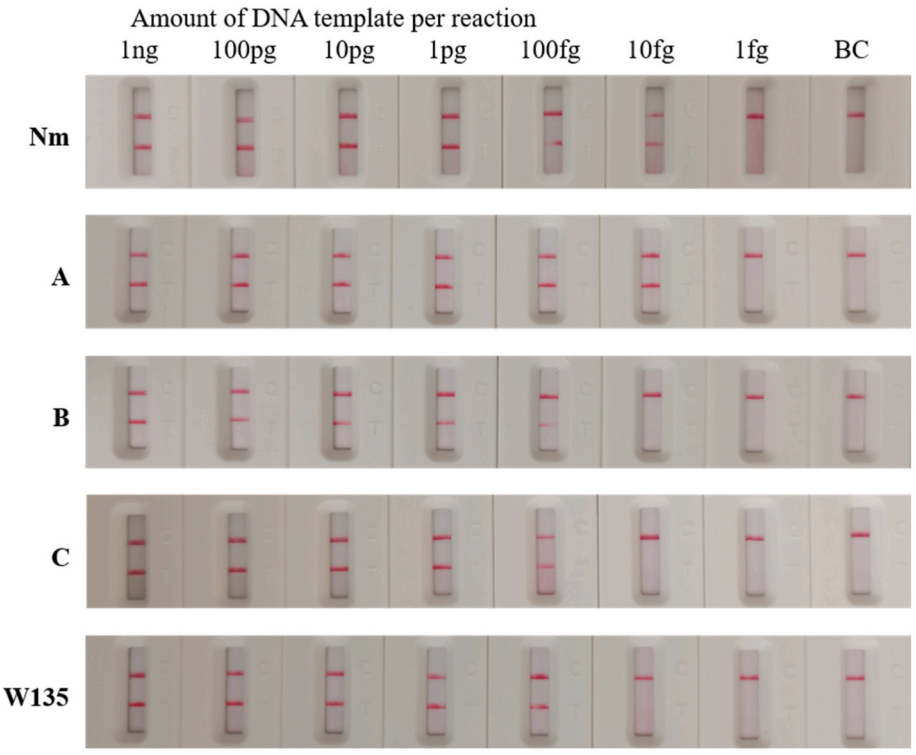

Figure 4. Sensitivity of *N. meningitidis* species- and serogroup-specific MCDA-LFB assays. Serial dilutions (1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg) of genomic DNA extracted from meningococcus serogroup A, B, C, and W135 strains were used to analyze the sensitivity of *N. meningitidis* species- and serogroup-specific MCDA assays. LFB was used to analyze MCDA products. There are two purplish red bands (a test line band and a control line band) on the LFB for positive reactions. **(Nm)** *N. meningitidis* species-specific MCDA. **(A)** serogroup A-specific MCDA. **(B)** serogroup B-specific MCDA. **(C)** serogroup C-specific MCDA. **(W135)** serogroup W135-specific MCDA. Distilled water was used as blank control. Nm, *N. meningitidis*. BC, blank control.

Table 2
Analytical sensitivity of *N. meningitidis* species- and serogroup-specific MCDA assays in CSF specimens.

Method	Detection limit of *N. meningitidis* and each serogroup				
	Nm	A	B	C	W135
MCDA	5.5×10^2 CFU/mL[a]	5.5×10^2	2.3×10^3	3.2×10^3	3.6×10^3
PCR[b]	5.5×10^5 CFU/mL	5.5×10^5	2.3×10^6	3.2×10^6	3.6×10^6

Nm, *N. meningitidis*.
[a] Colony forming units (CFUs) of *N. meningitidis* in CSF specimens.
[b] Conventional PCR, Diagnosis Standard for Meningococcal meningitis of China (WS 295–2019).

bacterial is live or not [25,28], but they are not sensitive enough to detect trace amount of genomic DNA. In this study, our results showed that the MCDA assays were 100–1000 times more sensitive than conventional PCR, which also has been confirmed in other bacterial pathogens [16,29]. Previous studies have shown that *N. meningitidis* and serogroup LAMPs can detect as low as 10 or 100 genome copies (equal to 25 fg or 250 fg) and 100 CFUs or 1000 CFUs [14,15], which were less sensitive than MCDA. In addition, PCR is also relative time-consuming and requires expensive precise equipment and skilled operators, limiting its application in resource-limited laboratories and on-site testing. In contrast, the MCDA testing procedure can be finished within 1 h, only requiring a simple heater, facilitating its application in resource-poor settings. Therefore, compared to bacterial culture, conventional PCR and LAMP, the species- and serogroup-specific MCDA-LFB assays established in this study is likely to be the preferred molecular methods for detection and characterization of *N. meningitidis* due to their advantages of simplicity, rapidity, specificity and sensitivity.

About result reading, visual detection reagent, real-time turbidity, agarose gel electrophoresis and LFB can be used. Visual inspection by visual detection reagent is based on color change, it is less objective and difficult to interpret, especially when the bacterial load is low. Real-time turbidity was monitored by a specialized turbidimeter, which complicating the testing procedure and making it inconvenient in on-site testing. Gel-based detection is time-consuming and easy to cause laboratory contamination. LFB was based on the combination of hapten labeled at the 5′ ends of the primer and antibody immobilized on the LFB. At the end point of MCDA amplification, the positive products were labeled with FITC and biotin. In the subsequent LFB detection, the positive amplicons specifically bind to *anti*-FITC antibody on the test line and streptavidin on the control line, respectively, and the result was displayed as two purplish red bands within 2 min. Thus, LFB was recommended as the optimal tool for MCDA result reading because it is convenient, time-saving and objective.

Our results suggested that the species- and serogroup-specific MCDA-LFB assays established in this study provide a valuable diagnostic tool for detection and subtyping of N. meningitidis. Given that the MCDA-LFB assays do not require expensive thermocyclers and skilled operators, and which can be finished in about 1 h, facilitating its application in resource-limited laboratories, point-of-care diagnosis and on-site testing. The MCDA-LFB assay can be finished using commercial kits including DNA Isothermal® Amplification Kit and Disposable Lateral Flow Biosensor. The cost of a MCDA-LFB test is around ¥130. Moreover, the equipment and labor costs are also significant reduced. Thus, the cost of MCDA-LFB was less than PCR.

5. Conclusion

In conclusion, one species and four serogroup MCDA-LFB assays targeting the species- and serogroup-specific genes were successfully developed for detection and subtyping of four major disease-causing *N. meningitidis* serogroups. These assays were validated through pure cultures and spiked CSF specimens, showing higher sensitivity and specificity than PCR and LAMP. Moreover, these assays are simple, time-saving, and do not require special equipment, making them suitable for a variety of conditions. Therefore, the *N. meningitidis* species- and serogroup-specific MCDA-LFB assays established in this study provide valuable molecular methods for the diagnosis and surveillance of

meningococcal disease.

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Ethical approval

This study was approved by the Ethics Committee of Peking University Shougang Hospital [grant number IRBK2018-024-01].

Declaration of competing interest

The author declares that there are no competing interests.

CRediT authorship contribution statement

Linlin Yan: Validation, Formal analysis, Data curation, Writing – original draft. **Chong Tang:** Validation, Formal analysis, Data curation. **Yu Cai:** Resources. **Jinqing Nong:** Resources, Data curation. **Ke Zhang:** Resources. **Linlin Zhu:** Resources. **Pengfei Wang:** Resources. **Lei Wu:** Resources. **Fan Zhao:** Conceptualization, Methodology, Supervision. **Shoukui Hu:** Conceptualization, Methodology, Supervision, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2022.114740.

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