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Simultaneous detection of *Streptococcus pneumoniae* and prevention of carryover contamination using multiple cross displacement amplification with Antarctic thermal sensitive uracil-DNA-glycosylase and a lateral flow biosensor

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One sentence summary: Simultaneous *Streptococcus pneumoniae* detection and elimination of carryover contamination with Antarctic thermal sensitive uracil-DNA-glycosylase-supplemented multiple cross displacement amplification in a lateral flow biosensor.

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ABSTRACT

Streptococcus pneumoniae is an important clinical pathogenic bacterium that is the primary cause of meningitis, septicemia and community-acquired pneumonia. The mortality rate of pneumococcal disease is high, especially in children younger than 5-years-old. Rapid and accurate detection of *S. pneumoniae* is critical for clinical diagnosis. A *ply* gene-based multiple cross displacement amplification (MCDA) assay, amplifying DNA under 65°C for 40 min, was established to detect *S. pneumoniae*. Antarctic thermal sensitive uracil-DNA-glycosylase (AUDG) was applied to prevent carryover contamination. A lateral flow biosensor (LFB) was used to indicate the MCDA results. The *ply*-MCDA assay could detect as low as 10 fg of *S. pneumoniae* DNA and 447 colony forming units (CFU)/mL of spiked sputum samples. The analytical sensitivity of the *ply*-MCDA assay to detect clinical specimens was 100 times higher than that of PCR. The specificity of the *ply*-MCDA assay was evaluated using 15 *S. pneumoniae* strains and 25 non-*S. pneumoniae* strains, which confirmed the high selectivity of the *ply*-MCDA assay for *S. pneumoniae*. The AUDG enzyme could effectively eliminate carryover contamination and thus prevented false-positive results. In conclusion, *ply*-AUDG-MCDA-LFB is a simple, rapid and accurate method to detect *S. pneumoniae*.

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Keywords: *Streptococcus pneumoniae*; multiple cross displacement amplification; Antarctic thermal sensitive uracil-DNA glycosylase (AUDG); carryover contamination; lateral flow biosensor

INTRODUCTION

Streptococcus pneumoniae is a Gram-positive bacterium that regularly colonizes in the upper respiratory tract, and is the most common pathogen of human meningitis, septicemia, community-acquired pneumonia, otitis media and sinusitis (Brown and Lerner 1998; Yao and Yang 2008). According to data from the World Health Organization (WHO) published in 2005, *S. pneumoniae* infection causes 1.6 million deaths worldwide yearly, of which between 700 000 and 1 million are children under 5-years-old, mostly in developing countries (Organization 2007). Currently, two pneumococcal vaccines, the 13-valent pneumococcal conjugate vaccine (Prevnar 13) and the 23-valent pneumococcal polysaccharide vaccine (Pneumovax 23), are licensed for use in children and adults to prevent infection caused by pneumococcal bacteria, thus reducing the incidence of pneumococcal disease (Berical *et al.* 2016; Matanock *et al.* 2019). Nevertheless, rapid and accurate detection of *S. pneumoniae* remains an extremely important step to prevent the spread of this pathogen and reduce the risk of pneumococcal disease.

The traditional detection technique for *S. pneumoniae* is bacterial culture-colonial morphology-biochemical identification, which is time-consuming, usually requiring three or more days. Fast identification of *S. pneumoniae* is essential for physicians to put patients on the appropriate treatment. Polymerase chain reaction (PCR)-based molecular biology techniques have been used to detect *S. pneumoniae* using primers specific to the pneumolysin (*ply*) gene (Rudolph *et al.* 1993; Salo, Ortqvist and Leinonen 1995; Wheeler *et al.* 1999). Pneumolysin, a well-characterized virulence factor of *S. pneumoniae* that is involved in its pathogenesis (Berry *et al.* 1989; Rossjohn *et al.* 1998), represents an ideal diagnostic target for the specific detection of *S. pneumoniae*. Moreover, the commercially available BioFire System panels including the FilmArray Pneumonia (PN) panel, the FilmArray Meningitis/Encephalitis (ME) panel and the FilmArray Blood Culture Identification 2 (BCID2) panel also test for *S. pneumoniae* and deliver accurate results quickly. However, the PCR system is complicated to perform and requires expensive thermal cycle apparatus and professional technicians (Gillespie 1999). The BioFire System is simple, fast and comprehensive, but requires the use of matching instruments. Therefore, PCR-based methods or the above-mentioned commercially available methods are not suitable for resource-limited clinical laboratories and 'on-site' testing.

In recent years, isothermal amplification techniques have been developed to detect infectious pathogens, including loop-mediated isothermal amplification (LAMP) and helicase-dependent amplification (HDA). However, when the concentration of the pathogen is low, it is difficult to get objective and accurate results by color change read using the naked eye. Multiple cross displacement amplification (MCDA), a novel DNA isothermal amplification technique, was devised by Wang *et al.* (2015), which amplifies DNA under an isothermal temperature (60–69°C) with higher sensitivity and specificity, using only a simple heat block or water bath, and can be completed within 40 min. The MCDA reaction system requires ten primers that specifically recognize ten different regions of the target gene, and the results can be indicated more objectively using a lateral flow biosensor (LFB). This method has been used to detect *Klebsiella pneumoniae* (Wang *et al.* 2018b), *Vibrio parahaemolyticus* (Wang *et al.* 2016a) and *Listeria monocytogenes* (Wang *et al.* 2017). Therefore, the MCDA-LFB assay might be a preferred method to rapidly and accurately detect *S. pneumoniae*, especially when resources are limited.

In the present study, a *ply* gene-based MCDA assay was established to rapidly and accurately detect *S. pneumoniae*, and its performance was evaluated. Furthermore, Antarctic thermal sensitive uracil-DNA glycosylase (AUDG) was applied in the *ply*-MCDA reaction system to eliminate carryover contamination and prevent false-positive results.

MATERIALS AND METHODS

Primer design

Pneumolysin, a virulence factor, encoded by the *ply* gene, was chosen as the specific target for *S. Pneumoniae* detection. Ten primers were designed targeting the *ply* gene of *S. pneumoniae* according to the primer design principle of MCDA (Wang *et al.* 2017). Primer 5.0 (Premier Biosoft, San Francisco, CA) and PrimerExplorer V4 (EIKEN CHEMICAL CO., LTD, Tokyo, Japan) were used. The *ply* primer sets are listed in Table 1. BLAST analysis was performed to confirm the specificity of the primer sets. The 5'-end of primer C1 was labeled with fluorescein isothiocyanate (FITC). The 5'-end of primer D1 was labeled with biotin. Primer synthesis was performed by Aoke Dingsheng Biotechnology Co., Ltd. (Beijing, China).

Bacteria strains

In this study, 15 *S. pneumoniae* strains and 25 non-*S. pneumoniae* strains were used (Table 2). The identities of the isolated strains listed in this table were confirmed using traditional culture method. The serotypes of the *S. pneumoniae* isolates were determined using the latex agglutination test with Pneumotest kits and the quellung reaction using type-specific antisera (Statens Serum Institut, Copenhagen, Denmark), as previously described (Sørensen 1993). The QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from the bacterial strains. All protocols were carried out according to the manufacturer's instructions. *S. pneumoniae* ATCC49619 was used for performance confirmation, optimal temperature determination and sensitivity analysis. Serial dilutions of *S. pneumoniae* ATCC49619 genomic DNA (10 ng, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg and 0.1 fg/μL) were used to analyze the sensitivity of *ply*-MCDA assay.

S. pneumoniae ply-MCDA assay

The *S. pneumoniae ply*-MCDA assay was conducted according to the previously published methods of Wang *et al.* (2017). An isothermal amplification kit (Haitaizhengyuan, Beijing, China) was used for the MCDA assay. The *S. pneumoniae ply*-MCDA assay was performed in a 25-μL reaction system, including 12.5 μL of 2 × Reaction buffer; 0.4 μM of F1 and F2; 1.6 μM of CP1 and CP2; 0.8 μM of C1 (C1*), R1, D1 (D1*), C2, R2 and D2; 1 μL of *Bst* DNA polymerase (10U); and 1 μL of DNA template. Distilled water was used as the blank control. *Staphylococcus aureus* DNA and *Salmonella typhi* DNA were used as negative controls. The reactions were isothermally amplified at 65°C for 40 min.

In this study, four method: a colorimetric indicator (Malachite Green, MG), a turbidimeter (Loopamp Realtime Turbidimeter LA-320C, EIKEN CHEMICAL CO., LTD), a lateral flow biosensor (LFB) and 2% agarose gel electrophoresis were used to

Table 1. Primers used in this study.

Primer[a]	Sequence and modification (5′-3′)	Length[b]
F1	AGGATTTAAAACAGAGAGGAATT	23nt
F2	CGCCCCCTAAAATAACCG	18nt
CP1	GAGATAGACTTGGCGCCCATTCTGCAGAGCGTCCTTTG	38mer
C1	GAGATAGACTTGGCGCCCAT	20nt
C1*	5′-FITC-GAGATAGACTTGGCGCCCAT-3′	20nt
CP2	AAGGAGTCAAGGTAGCTCCTCCCTTCACTTCTGTATTGTCCAA	43mer
C2	AAGGAGTCAAGGTAGCTCCTC	21nt
D1	GCAACACTCGAAATATAGAC	20nt
D1*	5′-Biotin-GCAACACTCGAAATATAGAC-3′	20nt
D2	AGACAGAGTGGAAGCAGA	18nt
R1	ATCACTCTTACTCGTGGT	18nt
R2	AGTAGAGGCTGCTTTTGAAG	20nt

[a]C1*, 5′-labeled with FITC; D1*, 5′-labeled with Biotin.
[b]nt, nucleotide; mer, monomeric.

Table 2. Bacteria strains used in this study.

ID	Strains	Sources	Serotype	MCDA result
1	*Streptococcus pneumoniae*	ATCC49619	19F	Positive
2	*Streptococcus pneumoniae*	Isolated strains	3	Positive
3	*Streptococcus pneumoniae*	Isolated strains	5	Positive
4	*Streptococcus pneumoniae*	Isolated strains	6A	Positive
5	*Streptococcus pneumoniae*	Isolated strains	6B	Positive
6	*Streptococcus pneumoniae*	Isolated strains	9	Positive
7	*Streptococcus pneumoniae*	Isolated strains	14	Positive
8	*Streptococcus pneumoniae*	Isolated strains	15	Positive
9–10	*Streptococcus pneumoniae*	Isolated strains	19A	Positive
11–12	*Streptococcus pneumoniae*	Isolated strains	19F	Positive
13–14	*Streptococcus pneumoniae*	Isolated strains	23F	Positive
15	*Streptococcus pneumoniae*	Isolated strains	Non-typable	Positive
16	*Shigatoxin-producing E. coli (STEC)*	*E. Coli* 933	/	Negative
17	*Enteroaggregative E. coli (EAEC)*	*E. Coli* 042	/	Negative
18	*Enteroinvasive Escherichia coli (EIEC)*	*E. Coli* 44 825	/	Negative
19	*Enteropathogen Escherichia coli (EPEC)*	*E. Coli* 2348/69	/	Negative
20	*Enterotoxigenic Escherichia coli (ETEC)*	*E. Coli* 10 407	/	Negative
21	*Salmonella typhi*	Isolated strains	/	Negative
22	*Citrobacter freundii*	Isolated strains	/	Negative
23	*Listeria ivanovii*	BAA678	/	Negative
24	*Listeria monocytogenes*	Isolated strains	/	Negative
25	*Klebsiella pneumoniae*	ATCC2146	/	Negative
26	*Streptococcus suis*	Isolated strains	/	Negative
27	*Proteus mirabilis*	Isolated strains	/	Negative
28	*Streptococcus salivarius*	Isolated strains	/	Negative
29	*Neisseria perflava*	Isolated strains	/	Negative
30	*Staphylococcus cohnii*	Isolated strains	/	Negative
31	*Pseudomonas aeruginosa*	Isolated strains	/	Negative
32	*Enterobacter cloacae*	ATCC700323	/	Negative
33	*Serratia marcescens*	Isolated strains	/	Negative
34	*Candida krusei*	Isolated strains	/	Negative
35	*Bacillus cereus*	Isolated strains	/	Negative
36	*Escherichia coli*	ATCC25922	/	Negative
37	*Acinetobacter baumannii*	Isolated strains	/	Negative
38	*Pseudomonas aeruginosa*	ATCC27853	/	Negative
39	*Escherichia coli*	ATCC35218	/	Negative
40	*Streptococcus agalactiae*	Isolated strains	/	Negative

analyze the amplification results. The above four methods were performed as previously described (Wang *et al.* 2016b). When synthesizing DNA, MCDA produces large amounts of pyrophosphate ions, which can capture the manganese ions bound to calcein and restore the free state of calcein to fluoresce. The luminescent mixture can combine with the magnesium ions generated in the reaction to enhance fluorescence. The result can be interpreted by the color change of fluorescence, the positive reaction changes from colorless to lake green, while the negative reaction remains colorless.

Optimal temperature of *ply*-MCDA assay

The optimal temperature of *S. pneumoniae ply*-MCDA assay was measured at an isothermal temperature ranging from 62 to 67°C at intervals of 1°C. Distilled water was used as the blank control. *Staphylococcus aureus* DNA and *Salmonella typhi* DNA were used as negative controls. The temperature that produced more amplification products and turbidity that occurs earlier was considered as the optimal amplification temperature. The experiment was performed in triplicate.

Sensitivity of the *ply*-MCDA assay

The sensitivity of the *ply*-MCDA assay was determined using serial dilutions of *S. pneumoniae* ATCC49619 genomic DNA. Seven different concentrations of DNA templates (10 ng, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg and 0.1 fg/μL) were used. A total of 1 μL of each dilution was added to the mixture system and then amplified at the optimal temperature for 40 min. The colorimetric indicator, real-time turbidity, LFB and gel electrophoresis were used to analyze the *ply*-MCDA products and determine the detection limit of *S. pneumoniae ply*-MCDA assay. Distilled water was used as a blank control. The experiment was conducted in triplicate.

Use of the AUDG enzyme to eliminate carryover contamination

The *S. pneumoniae ply*-MCDA products without AUDG enzyme digestion were quantified using an ultraviolet spectrophotometer (NanoDrop ND-1000, Calibre, China) at 260 nm, and then serially diluted (ranging from 1×10^{-10} g/μL to 1×10^{-20} g/μL). These dilutions were used as simulated carryover contamination and served as templates in the MCDA reaction system. To demonstrate that the simulated carryover contamination (dUTP-incorporated *ply*-MCDA products) could contaminate the *ply*-MCDA reaction and to confirm that the AUDG enzyme could eliminate the carryover contamination, the sensitivity of *ply*-AUDG-MCDA and *ply*-MCDA were compared using the above serial dilutions of *S. pneumoniae ply*-MCDA products (1 μL).

Specificity of *ply*-AUDG-MCDA assay

To determine the specificity of the *S. pneumoniae ply*-AUDG-MCDA assay, 40 bacterial strains including 15 *S. pneumoniae* and 25 non-*S. pneumoniae* were used (Table 2). The *ply*-AUDG-MCDA products were analyzed using the colorimetric indicator and LFB. The experiment was repeated three times.

Analytical sensitivity of the *ply*-AUDG-MCDA assay in clinical specimens

The analytical sensitivity of *S. pneumoniae ply*-AUDG-MCDA assay in clinical specimens was evaluated by artificially adding different numbers of colony forming units (CFU) of *S. pneumoniae* ATCC49619 into sputum samples identified as negative for *S. pneumoniae* by the culture method. A single colony of ATCC49619 was enriched and cultured. Then, the CFU of ATCC49619 was counted and the culture suspension was added to *S. pneumoniae* negative sputum samples. The concentration of ATCC49619 in the sputum samples was adjusted to 4.47×10^0, 4.47×10^1, 4.47×10^2, 4.47×10^3, 4.47×10^4, 4.47×10^5, 4.47×10^6 CFU/mL. A total of 100 μL of each spiked sputum sample was taken for genomic DNA extraction and was eluted in 10 μL of elution buffer (Qiagen). Next, 1 μL of DNA template was used in the *ply*-AUDG-MCDA reaction system. In addition, conventional PCR targeting the *ply* gene was also performed. The experiment was independently repeated three times.

RESULTS

Confirmation of *ply*-MCDA products

To confirm the feasibility of *ply* gene-targeted MCDA to detect *S. pneumoniae*, the *ply*-MCDA assay, with and without target DNA, were conducted at 65°C for 40 min. The *ply*-MCDA products of *S. pneumoniae* ATCC49619 were detected as a lake green color in the tube (Fig. 1A), two red lines (control line and test line) on the LFB (Fig. 1B), and as ladder-like bands on the gel (Fig. 1C), which indicating successful amplification of the target sequence. However, the *ply*-MCDA products of negative controls and blank control were colorless (Fig. 1A), showed only one red line (control line; Fig. 1B) and had no ladder-like bands (Fig. 1C). Our results suggested that the *ply* gene-targeted MCDA assay was suitable to detect *S. pneumoniae*.

Optimal temperature of the *ply*-MCDA assay

To measure the optimal temperature of the *ply*-MCDA assay, the genomic DNA of *S. pneumoniae* ATCC49619 (1 pg per reaction) was amplified at intervals of 1°C in the range of 62–67°C, respectively. The real-time turbidity at different temperatures was monitored. From the kinetic curves shown in Fig. 2, we found that the optimal amplification temperature was 65°C, which was indicated by the early emergence of turbidity and the increase level *ply*-MCDA products compared with that at other temperatures (Fig. 2D). Therefore, 65°C was used in subsequent studies.

Sensitivity of the *ply*-MCDA assay

To identify the lower detection limit, serial dilutions of *S. pneumoniae* ATCC49619 genomic DNA (10 ng, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg and 0.1 fg/μL) were subjected to amplification at 65°C. The results suggested that the lower detection limit of the *ply*-MCDA assay was 10 fg (Fig. 3). The positive amplicons were identified using the color indicator (Fig. 3A), real-time turbidity (Fig. 3B), the LFB (Fig. 3C) and gel electrophoresis (Fig. 3D) as lake green color, a turbidity curve, two red lines and ladder-like bands, respectively. Moreover, the results of the above four methods were completely consistent.

The AUDG enzyme eliminates carryover contamination

To determine whether the carryover contamination from the dUTP-incorporated *ply*-MCDA products could contaminate the *ply*-MCDA reaction, the sensitivity of *ply*-AUDG-MCDA and *ply*-MCDA were evaluated using serial dilutions of *S. pneumoniae ply*-MCDA products (ranging from 1×10^{-10} g/μL to 1×10^{-20} g/μL).

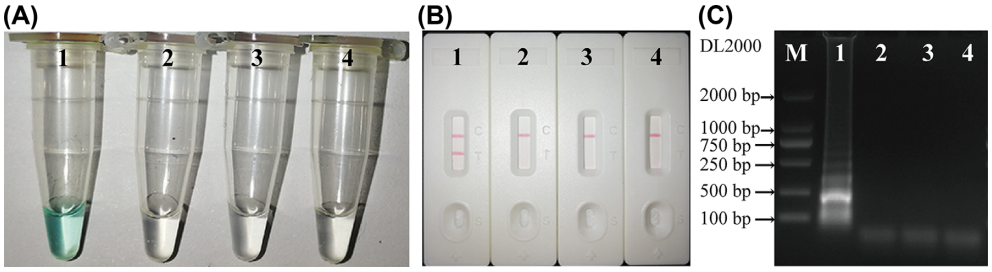

Figure 1. Confirmation of *ply*-MCDA products. **(A)** Colorimetric indicator of *S. pneumoniae ply*-MCDA products (Malachite Green, lake green indicates positive and colorless indicates negative); **(B)** LFB to visually detect *S. pneumoniae ply*-MCDA products (positive with two red lines, one control line and one test line); **(C)** Gel electrophoresis of *S. pneumoniae ply*-MCDA products (positive with ladder-like bands). Reaction 1, positive control, *S. pneumoniae* ATCC49619; Reaction 2, negative control, *Staphylococcus aureus*; Reaction 3, negative control, *Salmonella typhi*; Reaction 4, blank control.

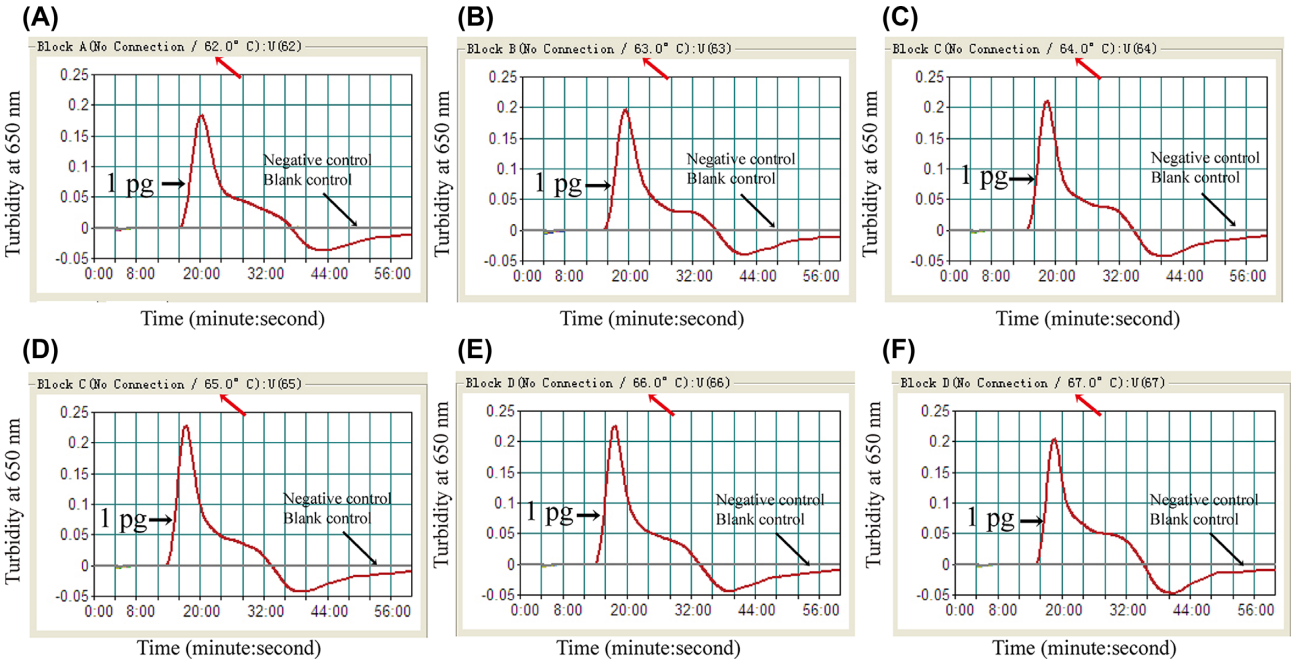

Figure 2. Optimal temperature of the *ply*-MCDA assay. The DNA templates of *S. pneumoniae* ATCC49619 (1 pg per reaction) were amplified at 62–67°C (1°C intervals), respectively. The real-time turbidities were monitored at 650 nm **(A–F)**. *Staphylococcus aureus* DNA and *Salmonella typhi* DNA were negative controls, and distilled water were blank control. Turbidity > 0.1 was defined as positive.

Our results showed that *ply*-AUDG-MCDA could detect simulated carryover contamination of 1×10^{-14} g/μL or more (Fig. 4). However, *ply*-MCDA without AUDG enzyme could detect simulated carryover contamination of 1×10^{-19} g/μL (Fig. 4). The results indicated that false-positive results would occur when the concentration of carryover contamination reached 1×10^{-19} g/μL. Our results also demonstrated that the AUDG enzyme could eliminate the carryover contamination. Therefore, false-positive results could be effectively avoided by adding the AUDG enzyme into the *ply*-MCDA reaction system.

Specificity of the *ply*-AUDG-MCDA assay

To identify the specificity of *ply*-AUDG-MCDA assay to detect *S. pneumoniae*, DNA templates from 15 *S. pneumoniae* strains and 25 non-*S. pneumoniae* strains were tested. The 15 strains of *S. pneumoniae* showed positive results, while other *Streptococcus* species and non-*Streptococcus* strains showed negative results (Table 2). The results suggested that the *ply*-AUDG-MCDA assay was highly specific for *S. pneumoniae*.

Analytical sensitivity of *ply*-AUDG-MCDA assay in clinical specimen

To determine the analytical sensitivity of the *ply*-AUDG-MCDA assay in clinical specimens, sputum samples artificially contaminated with serial dilutions of *S. pneumoniae* ATCC49619 were tested. Positive amplification occurred when the number of *S. pneumoniae* ATCC49619 in the sputum samples reached 447 CFU/mL. Moreover, the sensitivity of *ply*-AUDG-MCDA was 100 times greater than that of conventional PCR; the lower detection limit of conventional PCR was 44 700 CFU/mL.

DISCUSSION

S. pneumoniae is an important human pathogenic bacterium with high morbidity and mortality. Statistics show that in China, cases of pneumococcal infection account for 12% of the global cases, which is also one of the countries with the highest rate of deaths caused by pneumococcal infection in children under 5-years-old (O'Brien *et al.* 2009). The severity of *S. pneumoniae* infection means that simple and accurate methods are needed

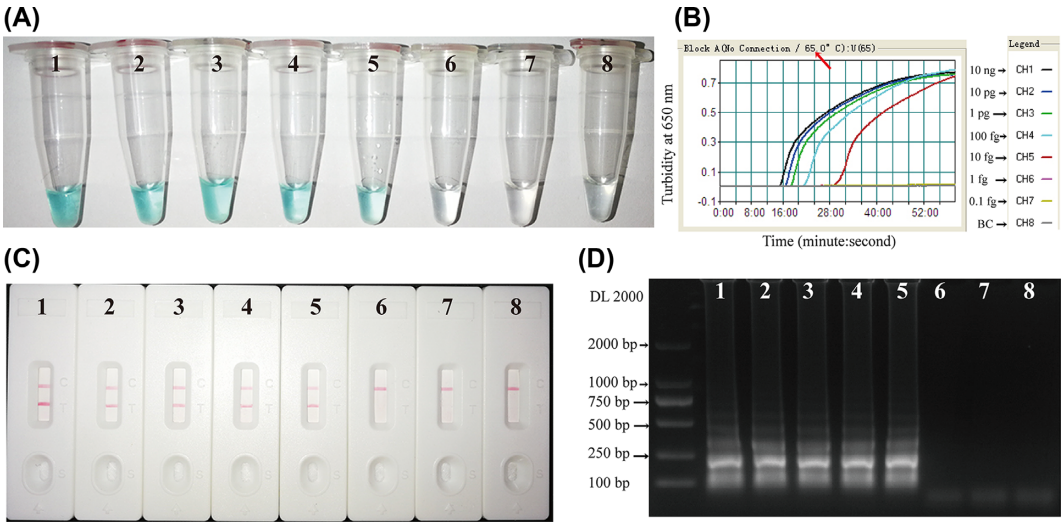

Figure 3. Sensitivity of the *ply*-MCDA assay. Serial dilutions of *S. pneumoniae* ATCC49619 genomic DNA (10 ng, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg and 0.1 fg/μL) were subjected to be amplified at 65°C for 40 min. Distilled water was the blank control (BC). **(A)** Colorimetric indicator (Malachite Green, lake green indicates positive and colorless indicates negative). **(B)** Real-time turbidity curves of eight reactions were recorded at 650 nm. Turbidity > 0.1 was defined as positive. **(C)** LFB detection of *ply*-MCDA products (two red lines represents positive and one red line represents negative). **(D)** Gel electrophoresis of *ply*-MCDA products (ladder-like bands indicate positive). 1–8: 10 ng, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 0.1 fg and BC.

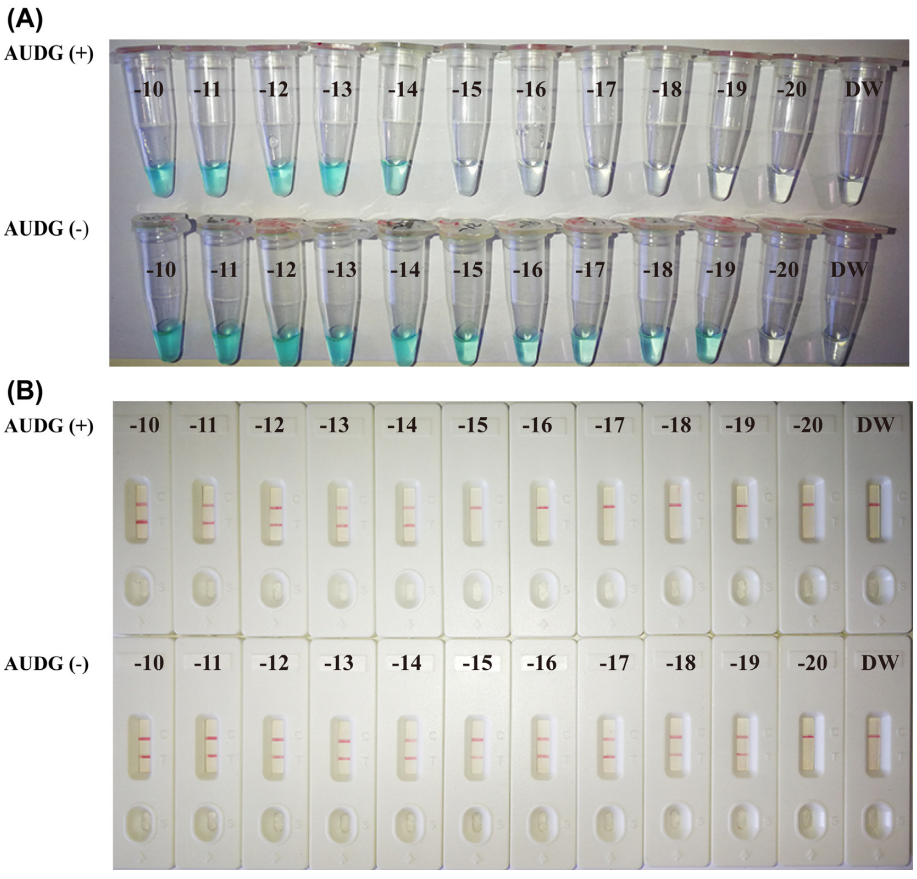

Figure 4. The AUDG enzyme eliminates carryover contamination. The sensitivity of the *ply*-MCDA assay with AUDG (AUDG+) and without AUDG (AUDG-) were evaluated using 10-fold serial diluted simulated carryover contamination (dUTP-incorporated *ply*-MCDA products, concentrations between 1×10^{-10} and 1×10^{-20} g/μL). The products were analyzed using a colorimetric indicator **(A)** and LFB **(B)**.

to detect *S. pneumoniae* to guide timely clinical treatment. Mitsuko *et al.* reported LAMP could be applied to detect *S. pneumoniae* infection, which was highly selective because it can identify six different regions of the target gene using four primers (Notomi *et al.* 2000; Seki *et al.* 2005). Studies have shown that increasing the number of isothermal amplification primers can improve the sensitivity of the method (Soo *et al.* 2013). In this study, we developed a novel method, named AUDG-MCDA-LFB, to simultaneously detect *S. pneumoniae* and prevent false-positive results.

In the present study, we designed five pairs of primers targeting ten distinct regions of the *ply* gene, which encodes pneumolysin (a well-characterized virulence factor of *S. pneumoniae* that is involved in pathogenesis), which has been used as a specific diagnostic target of *S. pneumoniae* (Berry *et al.* 1989; Rossjohn *et al.* 1998). Our results demonstrated that the *ply* gene-targeted primer sets were highly specific for *S. pneumoniae*. All the strains of *S. pneumoniae* were amplified successfully, while other *Streptococcus* species and non-*Streptococcus* strains were not amplified (Table 2). Therefore, the specificity of *ply*-AUDG-MCDA assay to detect *S. pneumoniae* was extremely high, especially in combination with clinical manifestations.

In addition, the sensitivity of the *ply*-MCDA assay was evaluated by testing serial dilutions of *S. pneumoniae* ATCC49619 genomic DNA. The lower detection limit of the *ply*-MCDA assay was 10 fg (Fig. 3). It has been reported that LAMP can detect as little as 25 fg (Wang *et al.* 2018a) or 20 fg (Wang *et al.* 2019) of *S. pneumoniae*. We also assessed the analytical sensitivity of this assay in clinical specimens using spiked sputum samples. The *ply*-AUDG-MCDA assay could detect as low as 447 CFU/mL of spiked sputum samples, which was similar to that of LAMP (Wang *et al.* 2018a). Thus, the *ply*-AUDG-MCDA assay was a sensitive method of *S. pneumoniae* detection.

However, because of the high sensitivity of the MCDA assay, false-positive results caused by carryover contamination have become an extremely important interference factor for *S. pneumoniae* detection. For example, the confirmation procedure of MCDA products by agarose gel electrophoresis or LFB require opening the reaction tube, thus the aerosol droplets containing high concentrations of products may be produced. Our results demonstrated that trace amounts of carryover contamination (1×10^{-19} g/μL) could generate false-positive results. Thus, prevention of carryover contamination is critical for reliable and accurate detection. To avoid the false-positive results arising from carryover contamination, we merged the MCDA assay with AUDG cleavage, which can simultaneously detect target sequences and prevent carryover contamination. In this study, the carryover contamination was successfully eliminated using the AUDG enzyme and dUTP. First, dUTP was substituted for dTTP and incorporated into all amplicons. Next, AUDG cleavage and MCDA amplification were performed in a one-pot, closed-tube. Before MCDA amplification, the amplification mixture added with the AUDG enzyme was reacted at room temperature for 5 min. The AUDG enzyme specifically cleave uracil bases in contaminants, while the uracil-free natural templates remain unaffected. In the MCDA amplification stage, the cleaved contaminants failed to be replicated by *Bst* 2.0 polymerase and were degraded by hydrolysis, thus eliminating the re-amplification of contaminants. The AUDG enzyme, a heat-labile enzyme, is rapidly deactivated at elevated temperatures. The genuine MCDA amplification products generated from the target sequences are not cleaved, permitting natural templates to be amplified normally (Wang *et al.* 2018a,b). In the present study, AUDG could eliminate up to 1×10^{-15} g/μL of simulated contaminants, suggesting that the *ply*-AUDG-MCDA assay could effectively reduce the likelihood of false-positive results.

To analyze the results, a colorimetric indicator, real-time turbidity, LFB and gel electrophoresis were used. The results using these four methods were highly consistent. However, compared with the conventional analysis methods, LFB was probably the optimal monitoring method. In the MCDA system, one primer is labeled with FITC and another primer is labeled with biotin. Subsequently, positive amplicons are simultaneously labeled with FITC and biotin. In the LFB, FITC- and biotin-labeled products are combined with anti-FITC antibodies immobilized on the test line and streptavidin dye coated on the control line, respectively. The results appeared as two red lines within 2 min. The LFB method is simple, rapid, cost-effective, objective and has no equipment requirement. Thus, LFB is an effective method for indicating MCDA results. Notably, the entire analysis procedure of *S. pneumoniae* AUDG-MCDA-LFB can be completed in approximate 47 min, including AUDG cleavage (5 min), the MCDA reaction (40 min) and results indication by the LFB (2 min), which is technically simple, requiring minimal training and one worker is sufficient to perform the assay. However, given the diversity of pathogens that cause similar clinical symptoms, a panel that can simultaneously detect a comprehensive set of pathogens would be promising. Therefore, a multiplex-MCDA-LFB system to detect multiple pathogens should be developed in the future study.

In conclusion, we established a simple, rapid and accurate *ply*-AUDG-MCDA-LFB method for the simultaneous detection of *S. pneumoniae* and prevention of false-positive results. AUDG can effectively eliminate carryover contamination. The LFB provided a potential tool for the rapid and objective indication of the MCDA results. The sensitivity, specificity and analytical sensitivity in clinical specimens of the *ply*-AUDG-MCDA assay were evaluated successfully using cultured strains and spiked sputum samples. Therefore, the developed *ply*-AUDG-MCDA-LFB system is a valuable method to detect *S. pneumoniae*, which provides a practical solution for resource-limited clinical laboratories and 'on-site' testing.

DISCLOSURES

Linlin Yan, Shoukui Hu, Fan Zhao, Lina Niu, Yu Cai, Lei Wu, Xiaoxue Zhu, Naishu Gao, Jinqing Nong and Zhe Xing have filed for a patent from the State Intellectual Property Office of the People's Republic of China, which includes the sequences and the MCDA-LFB method used for detecting *S. pneumoniae* in this manuscript [application number CN 201910806244.1].

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