Detection of Streptococcus Pneumoniae and elimination of carryover contamination by multiple cross displacement amplification coupled with antarctic thermal sensitive uracil-DNA-glycosylase

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Abstract

Background *Streptococcus pneumoniae* (*S. pneumoniae*), as an important human pathogenic bacterium, causes high mortality and morbidity, especially among children younger than 5 years. Accurate and rapid detection of *S. pneumoniae* is critical important. **Methods** A ply gene-based multiple cross displacement amplification (MCDA), which amplifies DNA under isothermal conditions (65 °C) for 40 min, was established for accurate and rapid detection of *S. pneumoniae*. The amplicons were indicated using lateral flow biosensor (LFB). **Results** The MCDA assay could detect as little as 10 fg purified *S. pneumoniae* genomic DNA, and 447 CFU/mL spiked sputum samples with a sensitivity 100 times that of PCR. The specificity of MCDA primer targeting ply gene was validated using 15 *S. pneumoniae* strains and 25 non-*S. pneumoniae* strains, indicating that ply gene-based MCDA assay was highly selective for *S. pneumoniae*. Moreover, the MCDA assay coupled with antarctic thermal sensitive uracil-DNA-glycosylase (AUDG) can effectively eliminate the carryover contamination, and then preventing false-positive results. **Conclusion** MCDA assay in combination with AUDG was a simple, rapid, sensitive, specific and reliable method for the diagnosis of *S. pneumoniae* infection, which appears to be a valuable tool for clinical screening.

Background

Streptococcus pneumoniae (*S. pneumoniae*) is a gram-positive bacterium, regularly colonize in the upper respiratory tract, which is the most common pathogen of human meningitis, septicemia, community-acquired pneumonia, otitis media and sinusitis [1, 2]. According to the 2005 data of World Health Organization (WHO), *S. pneumoniae* infection causes 1.6 million deaths worldwide each year, of which 700,000 to 1 million are children under the age of 5, and mostly from developing countries [3]. Thus, accurate and rapid detection of *S. pneumoniae* is the primary task of preventing spread of the pathogen and reducing the harm caused by disease.

The traditional detection technique for *S. pneumoniae* is bacterial culture-colonial morphology biochemical-biochemical examination, which is time-consuming, usually requiring three or more days. It is of vital importance for clinical treatment to accurately and rapidly detecting *S. pneumoniae*. Molecular biology techniques, such as polymerase chain reaction (PCR)-based assays have been used for detection of *S. pneumoniae* with primers specific to pneumolysin [4-6]. Pneumolysin, which is encoded by the *ply* gene, is a virulence factor from *S. pneumoniae* and is involved in pathogenesis [7, 8], representing the potential diagnostic target. However, the PCR reaction system is complex, complicated to perform, and requiring expensive thermal cycle apparatus and professional technicians [9]. Thus, PCR-based assays are not available in some backward clinical laboratories and “on-site” detection.

In recent years, Wang et al. [10] devised a novel nucleic acid isothermal amplification method, multiple cross displacement amplification (MCDA). Compared with PCR, MCDA has superior performance, which amplifies DNA under isothermal temperature (60°C-69°C) with higher efficiency, specificity and only a simple thermostatic equipment is required. This method has been used for detecting *Klebsiella*

pneumoniae [11], *Vibrio parahaemolyticus* [12], *Listeria monocytogenes* [13] and so on. MCDA reaction system requires ten specially designed primers that recognize ten distinct regions of target gene and the reaction can be completed within 40 min. Therefore, MCDA is a potential valuable method for rapid detection of *S. pneumoniae*, especially when resource settings are limited. In addition, four detection methods for MCDA products including colorimetric indicator, real-time turbidity, lateral flow biosensor (LFB) and gel electrophoresis have been performed [14].

In this study, we established a highly sensitive and specific MCDA assay for accurate and rapid detection of *S. pneumoniae*. *Ply* gene, a *S. pneumoniae*-specific gene that encodes pneumolysin, is used as the target gene. We determined the optimal temperature, sensitivity, specificity and the clinical sensitivity of *S. pneumoniae* MCDA assay. Furthermore, MCDA was coupled with antarctic thermal sensitive uracil-DNA glycosylase (AUDG) to eliminate carryover contamination and prevent the false-positive results.

Methods

Primer design

Pneumolysin, a virulence factor, encoded by the *ply* gene, is a specific target for the detection of *S. pneumoniae*. Five pairs of MCDA primers were designed on the *ply* gene from the *S. pneumoniae* standard strain of ATCC49619 according the principle of MCDA developed by Wang et al [13, 14]. Primer Primer 5.0 and PrimerExploer V4 were used. The pneumolysin (*ply*)-encoding primer sets are listed in **Table 3** and **Figure 5**. The 5'- ends of the primers C1 and D1 were labeled with fluorescein isothiocyanate (FITC) and biotin, respectively. Primers were synthesized in Aoke Dingsheng Biotechnology Co., Ltd. (Beijing, China).

Bacteria strains

The bacteria strains used in this study included 15 *S. pneumoniae* strains and 25 non-*S. pneumoniae* strains (**Table 1**). The QIAamp DNA minikits (Hilden, Germany) was used for the extraction of genomic DNA from bacteria strains. Protocols were carried out in accordance with the manufacturer's instructions. The *S. pneumoniae* standard strain ATCC49619 was used for confirmation of the performance, determination of the optimal temperature and analysis of sensitivity. DNA of *S. pneumoniae* ATCC49619 was serially diluted (10ng, 10pg, 1pg, 100fg, 10fg, 1fg and 0.1fg/μL) for sensitivity analysis.

S. pneumoniae MCDA assay

The *S. pneumoniae* MCDA assay was conducted based on previously published methods of Wang et al. [13, 14]. The isothermal amplification kits (Haitaizhengyuan, Beijing, China) was used in this study. *S. pneumoniae* MCDA assay was carried out in a 25μl reaction mixture including 1 μl of DNA template, 12.5μl of 2×Reaction buffer, 0.4μM of F1 and F2, 1.6μM of CP1 and CP2, 0.8μM of C1 (C1*), C2, D1 (D1*), D2, R1 and R2, and 1μl of *Bst* DNA polymerase (10U). DNA of *Staphylococcus aureus* and *Salmonella typhi* were used as negative controls. Distilled water was used as blank control. The reaction systems were isothermally amplified at 63°C for 40 min, followed by 85 °C for 5 min to stop the amplification.

In this study, four methods were used to determine the amplification results, including colorimetric indicator (Malachite Green, MG), turbidimeter (Loopamp Realtime Turbidimeter LA-320C), lateral flow biosensor (LFB) and 2% agarose gel electrophoresis. The above four methods were performed as previously described [14].

Optimal temperature of *S. pneumoniae* MCDA assay

The optimal amplification temperature of *S. pneumoniae* MCDA assay was determined at isothermal temperature ranging from 62 to 67°C with 1 °C intervals. DNA templates of *Staphylococcus aureus* and *Salmonella typhi* were used as negative control, and distilled water was used as blank control. The temperature that contributed to produce higher amplification products and occur turbidity earlier was considered for the optimal temperature. The experiment was repeated three times.

Sensitivity of *S. pneumoniae* MCDA assay

Sensitivity of MCDA assay was determined using serial dilutions of *S. pneumoniae* ATCC49619 genomic DNA. A total of 7 different concentrations of DNA templates (10ng, 10pg, 1pg, 100fg, 10fg, 1fg, 0.1fg/μL) were acquired. 1 μl of each dilution were added into the amplification mixture and then to be amplified at optimal temperature for 40min. Color indicator, real time turbidity, LFB detection and gel electrophoresis were conducted to analyze the MCDA products and then determine the limit of detection of *S. pneumoniae* MCDA assay. Distilled water was used as blank control. The sensitivity experiment was repeated in triplicate.

Eliminating carryover contamination by using AUDG enzyme

The *S. pneumoniae* MCDA products in the absence of AUDG enzyme were quantified using ultraviolet spectrophotometer (NanoDrop ND-1000, Calibre, China), and then to be serially diluted from 1×10⁻¹⁰ to 1×10⁻²⁰ g/μL. These dilutions were served as the source of simulated carryover contamination and were used as templates in *S. pneumoniae* MCDA assay. To demonstrate that simulated carryover contamination (UTP-incorporated products) can contaminate the MCDA reaction and to confirm that AUDG enzyme can eliminate the false-positive results caused by carryover contaminants, the sensitivity of MCDA-AUDG and MCDA were compared by using the above serial diluted MCDA products (1 μl).

Specificity of *S. pneumoniae* MCDA assay

To determine the specificity of *S. pneumoniae* MCDA assay, DNA templates from 40 bacteria strains including 15 *S. pneumoniae* strains and 25 non-*S. pneumoniae* strains were applied to the MCDA assay (**Table 1**). The products were analyzed using color indicator and LFB detection. The specificity experiment was repeated three times.

Evaluation of clinical sensitivity of *S. pneumoniae* MCDA assay

The sensitivity of *S. pneumoniae* MCDA assay in clinical application was evaluated by artificially adding serial dilutions of colony forming units (CFUs) of *S. pneumoniae* ATCC49619 into the sputum samples. The single colony of ATCC49619 was enriched and cultured. Then, the number of ATCC49619 CFUs was counted, and cultured suspensions were added into the *S. pneumoniae* negative sputum samples. The concentration of ATCC49619 in 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. Then, 100 μ L of artificially contaminated sputum samples were subjected to extract the genomic DNA, which were eluted in 10 μ L of Qiagen elution buffer (Qiagen, Germany). Next, 1 μ L of DNA templates were used for MCDA assay and the PCR method was also adopted. The experiment was performed three times independently.

Results

Confirmation of *S. pneumoniae* MCDA products.

To confirm the feasibility of the MCDA primers in detecting *S. pneumoniae*, MCDA amplification was performed at 63 °C for 40 min with and without target DNA. The MCDA product of *S. pneumoniae* ATCC49619 was detected visually as lake green color in the tube (**Fig.1A**), two red lines (control line and test line) on the LFB (**Fig.1B**) and ladder-like bands on the gel (**Fig.1C**), which indicated the amplification with target sequence. While the MCDA products of negative control and blank control were present as colorless (**Fig.1A**), only one red line (control line) (**Fig.1B**) and no ladder-like bands (**Fig.1C**). Our results indicated that the *S. pneumoniae*-specific primers targeting *ply* gene were suitable for MCDA assay.

Optimal temperature of *S. pneumoniae* MCDA assay.

To identify the optimal amplification temperature for *S. pneumoniae* MCDA assay, *S. pneumoniae* ATCC49619 DNA was used (1 pg/reaction) to be amplified at different temperatures ranging from 62°C to 67°C at 1 °C intervals. The real time turbidity of the reactions was monitored. From the Kinetic curves as shown in **Figure 2**, we found that 65°C was the optimal amplification temperature, because the turbidity of the reaction occurred earlier and the amount of MCDA products were higher than other temperatures (**Fig. 2D**). 65°C was used in the subsequent studies.

Sensitivity of *S. pneumoniae* MCDA assay.

To determine the limit of detection (LoD), serial dilutions of *S. pneumoniae* ATCC49619 DNA (10ng, 10pg, 1pg, 100fg, 10fg, 1fg, 0.1fg/ μ L) were subjected to be amplified at 65°C. The results showed that the LoD of *S. pneumoniae* MCDA assay was 10fg (**Fig.3**). The positive amplicons were represented in the color indicator (**Fig.3A**), real-time turbidity (**Fig.3B**), LFB (**Fig.3C**) and gel electrophoresis (**Fig.3D**) as lake green, turbidity curve, two red lines and ladder-like bands, respectively. It is worth noting that the results of the above four methods were completely consistent.

MCDA assay combined with AUDG enzyme eliminates false-positive results caused by carryover contamination.

To determine whether carryover contamination from UTP-incorporated MCDA products would contaminate the MCDA reactions, we evaluated the sensitivity of MCDA-AUDG and MCDA using serial diluted MCDA products with the concentrations ranging from 1×10^{-10} to 1×10^{-20} g/ μ L. Our results showed that MCDA-AUDG can only detect 1×10^{-14} g/ μ L or more of simulated carryover contamination (Fig.4). Without AUDG enzyme, MCDA can detect as little as 1×10^{-19} g/ μ L of simulated carryover contamination (Fig.4). These results indicated that carryover contamination with low concentrations (1×10^{-19} g/ μ L) can cause false-positive results. In addition, our results also demonstrated that AUDG enzyme is able to eliminate the carryover contamination to a certain extent. Therefore, MCDA assay coupled with AUDG enzyme can significantly reduce the possibility of false-positive amplifications of *S. pneumoniae*.

Specificity of *S. pneumoniae* MCDA assay.

To determine the specificity of *S. pneumoniae* MCDA assay with AUDG digestion in detecting *S. pneumoniae*, DNA from 15 *S. pneumoniae* strains and 25 non-*S. pneumoniae* strains were tested. All of the DNA from *S. pneumoniae* strains showed positive results, while the DNA from other *Streptococcus* species and non-*Streptococcus* strains showed negative results (Table 1). These results indicated that the MCDA assay with AUDG enzyme was 100% specific for *S. pneumoniae* detection.

Clinical sensitivity of *S. pneumoniae* MCDA assay in sputum samples.

To explore the sensitivity of MCDA assay with AUDG digestion in clinical application, the artificially contaminated sputum samples with different concentrations of *S. pneumoniae* ATCC49619 were analyzed by using MCDA-AUDG assay. The MCDA-AUDG assay occurred positive amplification when the amount of *S. pneumoniae* in the spiked sputum samples were more than 447 CFU/ml (equivalent to 2.9 CFU per reaction) (Table 2). The sensitivity of MCDA-AUDG assay was 100 times that of PCR, the LoD of PCR was 44700 CFU/ml (equivalent to 290 CFU per reaction) (Table 2).

Discussion

S. pneumoniae, an important human pathogenic bacterium, causes high mortality and morbidity. Statistics show that in China, the cases of pneumococcal infection account for 12% of the global cases, which is also one of the countries with the highest deaths caused by pneumococcal infection in children younger than 5 years [15]. Because of the severity of *S. pneumoniae* infection, simple and accurate methods are needed to detect *S. pneumoniae* and timely guide clinical treatment. Mitsuko et al. has reported a rapid method based on loop-mediated isothermal amplification (LAMP) for the diagnosis of *S. pneumoniae* infection, which is highly selective because it uses four primers to recognize six different regions of the target gene [16, 17]. Studies have reported that increasing the primers of isothermal amplification could improve the sensitivity [18]. This study developed a novel method, MCDA in combination with AUDG, to specifically detect *S. pneumoniae* and prevent false positive results.

Within the present study, we designed five pairs primers targeting ten distinct regions of the *ply* gene. *Ply* gene, which encodes pneumolysin, a well-characterized virulence factor of *S. pneumoniae* and

participating in the pathogenesis, has been used as the specific target gene of *S. pneumoniae* [7, 8]. Our results demonstrated that the primers are highly specific for the detection of *S. pneumoniae*. Only the DNA from *S. pneumoniae* strains were positively amplified, while the DNA from other *Streptococcus* species and non-*Streptococcus* strains were not amplified (**Table 1**). Therefore, the selectivity of the MCDA assay is extremely high in detecting *S. pneumoniae*.

In addition, we evaluated the sensitivity of MCDA assay by testing serial dilutions of *S. pneumoniae* ATCC49619 DNA. The detection limit of MCDA assay was determined to be 10 fg (**Fig.3**). It has been reported that LAMP can detect *S. pneumoniae* as few as 25 fg [19] or 20 fg [20]. We also assessed the clinical sensitivity of this assay by using spiked sputum samples. The MCDA can detect *S. pneumoniae* as low as 447 CFU/ml (equivalent to 2.9 CFU per reaction), which is similar with that of LAMP [19]. Thus, MCDA appears to be a sensitive method of detecting *S. pneumoniae* infection.

However, owing to the high sensitivity of MCDA assay, false-positive results caused by carryover contamination became a barrier to accurate and rapid diagnosis of *S. pneumoniae*. Moreover, because of the opening indicating operation of MCDA products (such as LFB), the aerosol droplets containing high concentration of products cannot be avoided. In this study, our results showed that trace amounts of carryover contamination (1×10^{-19} g/ μ L) can lead to false-positive amplification. Thus, we prevent the false-positive results arising from carryover contamination by performing AUDG enzyme digestion, which specifically cleave uracil bases and permit the nature templates to be amplified normally [11, 19], and can eliminate as much as 1×10^{-15} g/ μ L of simulated contaminants. These results suggested that MCDA-AUDG assay can effectively eliminate carryover contamination and then reduce false-positive amplifications.

About products analysis, four methods including colorimetric indicator, real-time turbidity, LFB and gel electrophoresis were used. The results of these four methods were highly consistent. However, comparing with conventional analysis methods, LFB was probably the optimal monitoring method. In the MCDA system, one primer was labeled with FITC and one primer was labeled with biotin. Subsequently, the positive amplification products were labeled with FITC and biotin. Within the LFB, the FITC and biotin labeled products combined the anti-FITC antibody immobilized on the test line and dye streptavidin coated on the control line, respectively, and the results are displayed as two red lines within 2 minutes. The advantages of LFB included simple to use, rapid, cost-effective, objective and no equipment requirement. Thus, LFB is an effective method for the products analysis of MCDA assay.

In conclusion, we established a simple, rapid, sensitive, specific and reliable MCDA-LFB method in combination with AUDG for the diagnosis of *S. pneumoniae* and prevention of false-positive results caused by carryover contamination. LFB detection provided a potential tool for accurate and rapid detection of *S. pneumoniae*. The sensitivity, specificity and clinical sensitivity of MCDA assay in the diagnosis of *S. pneumoniae* were evaluated by using pure cultures and spiked sputum samples. Therefore, the MCDA-LFB coupled with AUDG is potentially a valuable tool for the detection of *S. pneumoniae*.

Abbreviations

AUDG: Antarctic thermal sensitive uracil-DNA glycosylase

CFUs: colony forming units

FITC: fluorescein isothiocyanate

LAMP: loop-mediated isothermal amplification

LFB: lateral flow biosensor

LoD: limit of detection

MCDA: multiple cross displacement amplification

MG: Malachite Green

PCR: polymerase chain reaction

Ply: pneumolysin

WHO: World Health Organization

Declarations

Acknowledgments

Not applicable

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LY, FZ, LN, SH designed the research and wrote the manuscript; LY, FZ and LN performed the experiments and analyzed the data; YC, LW, XZ, YW and JN contributed the reagents and materials; SH supervised the study. All authors reviewed the manuscript.

Ethics approval and consent to participate

This study was carried out in accordance with the recommendations of Declaration of Helsinki, Ethics Committee of Peking University Shougang Hospital with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Peking University Shougang Hospital.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Bacteria strains used in the *S. pneumoniae* MCDA assay.

ID	Strains	Sources	MCDA result
1	<i>Streptococcus pneumoniae</i>	ATCC49619	Positive
2-15	<i>Streptococcus pneumoniae</i>	Isolated strains from sputum	Positive
16	<i>Shigatoxin-producing E. coli (STEC)</i>	<i>E. Coli</i> 933	Negative
17	<i>Enteroaggregative E. coli (EAEC)</i>	<i>E. Coli</i> 042	Negative
18	<i>Enteroinvasive Escherichia coli (EIEC)</i>	<i>E. Coli</i> 44825	Negative
19	<i>Enteropathogen Escherichia coli (EPEC)</i>	<i>E. Coli</i> 2348/69	Negative
20	<i>Enterotoxigenic Escherichia coli (ETEC)</i>	<i>E. Coli</i> 10407	Negative
21	<i>Salmonella typhi</i>	Isolated strains from feces	Negative
22	<i>Citrobacter freundii</i>	Isolated strains from sputum	Negative
23	<i>Listeria ivanovii</i>	BAA678	Negative
24	<i>Listeria monocytogenes</i>	Isolated strains from blood	Negative
25	<i>Klebsiella pneumoniae</i>	ATCC2146	Negative
26	<i>Streptococcus suis</i>	Isolated strains from feces	Negative
27	<i>Proteus mirabilis</i>	Isolated strains from urine	Negative
28	<i>Streptococcus salivarius</i>	Isolated strains from dialysate	Negative
29	<i>Neisseria perflava</i>	Isolated strains (unknown)	Negative
30	<i>Staphylococcus cohnii</i>	Isolated strains from environment	Negative
31	<i>Pseudomonas aeruginosa</i>	Isolated strains from sputum	Negative
32	<i>Enterobacter cloacae</i>	ATCC700323	Negative
33	<i>Serratia marcescens</i>	Isolated strains from sputum	Negative
34	<i>Candida krusei</i>	Isolated strains from urine	Negative
35	<i>Bacillus cereus</i>	Isolated strains from blood	Negative
36	<i>Escherichia coli</i>	ATCC25922	Negative
37	<i>Acinetobacter baumannii</i>	Isolated strains from sputum	Negative
38	<i>Pseudomonas aeruginosa</i>	ATCC27853	Negative
39	<i>Escherichia coli</i>	ATCC35218	Negative
40	<i>Streptococcus agalactiae</i>	Isolated strains from sputum	Negative

Table 2. Clinical sensitivity of *S. pneumoniae* MCDA assay in sputum samples.

Method	LoD	Sputum
MCDA	2.9 CFU/reaction	447 CFU/ml
PCR	290 CFU/reaction	44700 CFU/ml

Table 3. Primers used in this study.

Primera	Sequence and modification (5'-3')	Lengthb
F1	AGGATTTAAACAGAGAGGAATT	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 for the MCDA-LFB assay; D1*, 5'-labeled with Biotin for the MCDA-LFB assay.

b nt, nucleotide; mer, monomeric.

Figures

Figure 1

Confirmation of *S. pneumoniae* MCDA products.

Figure 2

Optimal temperature of *S. pneumoniae* MCDA assay.

Figure 3

Sensitivity of *S. pneumoniae* MCDA assay.

Figure 4

MCDA assay coupled with AUDG enzyme eliminates false-positive results caused by carryover contamination.

Figure 5

Location of the primers on the ply gene used for MCDA assay.