Rapid and Sensitive Detection of *Pseudomonas aeruginosa* Using Multiple Cross Displacement Amplification and Gold Nanoparticle-based Lateral Flow Biosensor Visualization

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Abstract

Pseudomonas aeruginosa causes nosocomial infections of burn patients and other immunocompromised individuals, but the conventional diagnosis of *P. aeruginosa* infection depends on time-consuming culture-based methods. Hence, a simple, fast, sensitive technique for detection of *P. aeruginosa* using multiple cross displacement amplification (MCDA) and gold nanoparticle-based lateral flow biosensors (LFB) was developed. By using this technique, the reaction could be completed at an optimized constant temperature (67°C) within only 40 min. The reaction product could be detected visually using a LFB, eliminating the need for special equipment. The *P. aeruginosa*-MCDA-LFB method was highly specific, and accurately distinguished *P. aeruginosa* from other pathogens. Just 10 fg of genomic DNA template (from pure culture) could be detected. The assay could also detect *P. aeruginosa* in clinical sputum samples and showed the same sensitivity and specificity as the reference (culture-biochemical) method. In the future, this rapid, simple and accurate *P. aeruginosa*-MCDA-LFB technique might be applied in clinical practice.

Keywords: *Pseudomonas aeruginosa*; Multiple cross displacement amplification; Gold nanoparticles; Lateral flow biosensor; clinical detection

Introduction

Pseudomonas aeruginosa, a Gram-negative obligate aerobe, is an opportunistic pathogen and that often causes nosocomial infections (Sekiguchi et al. 2007). *P. aeruginosa* is a particular risk to burn patients (Church et al. 2006) and immunocompromised individuals, such as cystic fibrosis (Lau et al. 2005), transplant (Johnson et al. 2009), cancer (Elting et al. 1997), and AIDS patients (Meynard et al. 1999). *P. aeruginosa* shows wide-scale multidrug resistance. Accurate identification of *P. aeruginosa* is critical to patient management.

It can take >2 days to identify *P. aeruginosa* from clinical samples using traditional culture-biochemical methods. Therefore, it is desirable to develop a fast, sensitive, and accurate assay to detect this pathogen. Antibody-based detection cannot be used because of a lack of high avidity antibodies, so nucleic acid-based assays are generally used for detection of *P. aeruginosa*. Many researchers have reported the use of PCR for *P. aeruginosa* detection (Anuj et al. 2009; da Silva Filho et al. 1999; De Vos et al. 1997; Jaffe et al. 2001; Khan and Cerniglia 1994; Lavenir et al. 2007; McIntosh et al. 1992; Spilker et al. 2004; Xu et al. 2004). L lipoprotein is a *P. aeruginosa* outer membrane protein that is involved in resistance of the bacterium to antimicrobials. This protein is found only in this organism, so the protein or its gene could be suitable target for rapid identification of *P. aeruginosa* by molecular methods (Douraghi et al. 2014; Jami Al-Ahmadi and Zahmatkesh Roodsari 2016). However, such assays (e.g. PCR) often require equipment (Anuj et al. 2009; Douraghi et al. 2014; Jami Al-Ahmadi and Zahmatkesh Roodsari 2016) that is not available in many settings.

Multiple cross displacement amplification (MCDA), a nucleic acid amplification technique (Wang et al. 2015), has been applied in the detection of bacteria including *Vibrio parahaemolyticus*, *Shigella* spp. and *Listeria monocytogenes* (Wang et al. 2016a; Wang et al. 2016b; Wang et al. 2017). MCDA assays are conducted isothermally (at 60–69°C), so only require a simple heater. MCDA methods are easy, fast, specific, and very sensitive (requiring as few as three bacterial cells). Amplicon detection can be performed using gold nanoparticle-based lateral flow biosensors (LFB) (Wang et al. 2016a; Wang et al. 2016b; Wang et al. 2017), a simple and equipment-free method.

Here, we aimed to establish a MCDA-LFB assay to detect *P. aeruginosa* via the *oprL* gene (a *P. aeruginosa*-specific gene that encodes L lipoprotein) in pure cultures, then to apply the assay to clinical sputum samples. We also compared the performance of this method to routine methods used in the clinical laboratory, with a view to the application of our method in clinical practice.

Materials and Methods

Reagents

QIAamp DNA Mini Kits (Qiagen, Beijing, China) were used for DNA extraction from cultured pure bacterial strains. QIAamp DNA Microbiome Kits were used for DNA extraction from clinical sputum samples. Loopamp™ kits and Loopamp Fluorescent Detection Reagent were from Eiken Chemical (Beijing). Malachite Green (MG) was obtained from Bei Jing-Hai Tai Zheng Yuan Technology Co., Ltd. (Beijing). Biotinylated bovine serum albumin (biotin-BSA) and rabbit anti-fluorescein antibody (anti-FITC Ab) were obtained from Abcam Co., Ltd. (Shanghai, China). Streptavidin-immobilized 30-nm gold nanoparticles (SA-G) were from Resenbio Co., Ltd. (Xi'an, China). Other LFB detection materials including membrane backing materials, sample and conjugate pads, nitrocellulose (NC) membrane, and absorbent pads were from JieYi Biotechnology Co., Ltd. (Shanghai).

Primers and gold nanoparticle-based dipstick biosensor preparation

The primers used in this study are listed in Table 1. Gold nanoparticle-based dipstick biosensors were prepared as described (Wang et al. 2016a). HPLC-purified primers were synthesized by TsingKe Biotech Co., Ltd. (Beijing). Assembled biosensors (4-mm wide) were stored at room temperature in a desiccant gel.

Bacteria

The bacteria used in this study are listed in Table 2. All strains were identified by reference methods (culture and biochemical methods) in the Department of Clinical Laboratory, Peking University Shougang Hospital; they were maintained in pure culture, and stored in 15% (w/v) glycerol broth at −70°C. Before use, strains were subcultured on nutrient agar plates at 37°C. Genomic DNA was extracted from all strains using a DNA Mini Kit and stored at −20°C. *P. aeruginosa* strain SGH-PA001 DNA was used for analysis of assay performance and sensitivity, and for determination of the optimal reaction temperature, while DNA from *Klebsiella pneumoniae* SGH-KP001 and *Staphylococcus epidermidis* SGH-SE001 strains were used as negative controls. For sensitivity analysis, DNA of *P. aeruginosa* SGH-PA001 was serially diluted as indicated.

MCDA assay

MCDA reactions (25 μl) were performed as described previously (Wang et al. 2015; Wang et al. 2016a; Wang et al. 2016b) with some modification. Briefly, the reaction mixture

included: 12.5 μl 2× reaction mix (Loopamp DNA Amplification Kit); 0.4 μM displacement primers (F1 and F2); 0.8 μM amplification primers C1 and C2; 1.2 μM amplification primers R1, R2, D1 and D2; 1.2 μM cross primer CP1; 2.4 μM cross primer CP2; 1.25 μl *Bst* DNA polymerase (10 U); and DNA template (1 μl). Negative control mixtures contained 10 ng genomic DNA of *K. pneumoniae* SGH-KP001 or *S. epidermidis* SGH-SE001, and blank controls used 1 μl pure water.

To verify the primers we designed for the *P. aeruginosa*-MCDA-LFB assay, the reactions were initially set at 62°C for 40 min and then terminated the amplification by heating at 85°C for 5 min. The optimal reaction temperature for *P. aeruginosa*-MCDA assay was then determined in reactions at fixed temperatures from 60 to 69°C (in 1°C intervals).

Amplicons were analyzed by four different methods: a colorimetric indicator (MG), 2% agarose gel electrophoresis, by using a turbidimeter (Loopamp@Realtime Turbidimeter LA-320C), and by LFB detection. When using MG, the amplified products cause a change in the solution from colorless to sky blue (negative/blank controls remain colorless). In electrophoresis, reaction products were separated by applying one hundred volts across the gel for at least 30 min. DNA amplifications were visualized by exposing the gel to UV light, a specific ladder of bands is observed for positive amplifications, but not negative controls. On the LFB, two red lines (the test line and the control line) are observed for positive reactions, but the control line only is visualized for negative and blank control reactions.

Assessment of MCDA-LFB assay

Specificity of the MCDA-LFB assay for *P. aeruginosa* was analyzed using DNA from 40 bacterial strains (Table 2) including two other *Pseudomonas* species, and 28 non-*Pseudomonas* strains. The detection limit was defined in terms of the amount of the genomic DNA template. Assays were repeated at least twice. Detection of *P. aeruginosa* by MCDA-LFB was compared to that using the other three methods (see above) for three replicates of each template diluted to 10 ng/μl –10 fg/μl.

Application of *P. aeruginosa*-MCDA-LFB assay to human clinical sputum samples

To explore the application of the *P. aeruginosa*-MCDA-LFB assay to human clinical samples, 120 human sputum samples were collected from the Department of Clinical Laboratory, Peking University Shougang Hospital, which were previously identified by culture and biochemical methods (reference methods) and stored at −70°C until use. By the reference method, 60 (50%) of the 120 samples were identified as *P. aeruginosa*-positive, while the remainder were identified as containing other bacteria or negative. The consistency of results from *P. aeruginosa*-MCDA-LFB assays were compared with those from the reference method

and also with the conventional PCR method. All patients who provided samples gave written informed consent. This study was approved by the Ethics Committee of Shougang Hospital, and conducted according to the regulations of the Ministry of Health, China.

Results

Detection of *P. aeruginosa*-MCDA products

To confirm the effectiveness of the primers targeting the *oprL* gene of *P. aeruginosa* (Table 1), MCDA assays were performed at 62°C for 40 min using DNA from pure culture as the template. Amplification product was observed when the template was DNA from *P. aeruginosa* SGH-PA001, but not with DNA from *K. pneumoniae* SGH-KP001 or *S. epidermidis* SGH-SE001 (negative controls), or for the blank control (Fig. 1). Therefore, the *P. aeruginosa*-MCDA primer set in current study was suitable for development of the MCDA-LFB assay.

Optimal assay temperature

P. aeruginosa SGH-PA001 DNA was used (1 pg/reaction); reactions were monitored by real-time turbidity. The MCDA assay was conducted from 60 to 69°C. The kinetic data in Fig. 2 show that 67°C was the optimum reaction temperature.

Assay specificity and sensitivity

When DNA from 40 bacterial strains was tested by MCDA-LFB assay, only *P. aeruginosa* DNA produced positive results. Amplification products were not detected when the template was DNA from other *Pseudomonas* species or from non-*Pseudomonas* strains (Table 2).

Just 10 fg of *P. aeruginosa* template DNA gave amplicons that could be detected colorimetrically, by turbidity, by electrophoresis, or by LFB (Fig. 3). The results obtained using the LFB agreed completely with those by the other methods. Only 40 min was required to complete the reaction.

Detection of *P. aeruginosa* in clinical sputum samples by MCDA-LFB

A total of 120 clinical sputum samples were detected using *P. aeruginosa*-MCDA-LFB and conventional PCR (Table 3). By the reference method, 60 of the 120 clinical samples were positive for *P. aeruginosa*. The MCDA-LFB assay results were in complete agreement. However, the PCR assay only identified 52 positive samples.

Discussion

This study shows that multiple cross displacement amplification targeting the *oprL* gene with detection by a gold nanoparticle-based LFB can be used to detect *P. aeruginosa* with excellent specificity and sensitivity. *oprL* is a *P. aeruginosa*-specific gene encoding L lipoprotein; this gene/protein has been used previously in detection methods for *P. aeruginosa* (De Vos et al. 1997; Douraghi et al. 2014; Goto et al. 2010; Jami Al-Ahmadi and Zahmatkesh Roodsari 2016). The assay produced positive results for all *P. aeruginosa* strains tested, both from pure cultures and clinical samples, and negative results for all other species of bacteria and blank controls. Thus, the *P. aeruginosa*-MCDA-LFB method is highly selective for *P. aeruginosa*.

When the MCDA products were directly analyzed using the LFB (Wang et al. 2016a; Wang et al. 2016b; Wang et al. 2017), detection took <5 min, which is faster than gel electrophoresis (at least 30 min), less subjective and error-prone than colorimetric detection, and does not require instrumentation (as does the turbidity method). Thus, LFB detection is preferable and advantageous for determining the MCDA assay results.

The *P. aeruginosa*-MCDA-LFB assay required only simple incubation at 67°C for 40 min. Easy-to-use, suitable equipment is available, such as battery-powered hot blocks that can hold 96 reaction tubes at a time. The MCDA amplification can be conducted using commercial isothermal amplification kits; the cost of each reaction is about $US3.5, and the LFB costs around $US 2 per test. The assay does not require highly-trained personnel or a certified laboratory, thus labor costs can be low. Overall, therefore, the assay is very cost effective.

Importantly, we successfully applied the *P. aeruginosa*-MCDA-LFB assay to clinical sputum samples, and the sensitivity and specificity were the same as those of the reference method (Table 3). The whole *P. aeruginosa*-MCDA-LFB procedure except for sample processing could be completed within 45 min. Our study suggests that the *P. aeruginosa*-MCDA-LFB assay has the potential for fast detection of *P. aeruginosa* in clinical settings. Soon, we will focus on other clinical sample types, such as blood, urine and throat swabs, for clinical application of the *P. aeruginosa*-MCDA-LFB assay.

In conclusion, a reliable, fast, inexpensive, technically simple, sensitive and specific *oprL*-MCDA-LFB assay was successfully developed to detect *P. aeruginosa*, a common cause of nosocomial infections in burn patients and other immunocompromised individuals. The use of a lateral flow biosensor provided an objective and easily interpretable output. This newly developed method has the same sensitivity and specificity as the established reference method and could be applied in clinical settings.

Acknowledgments

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Conflicts of Interest

The authors declared no conflict of interest.

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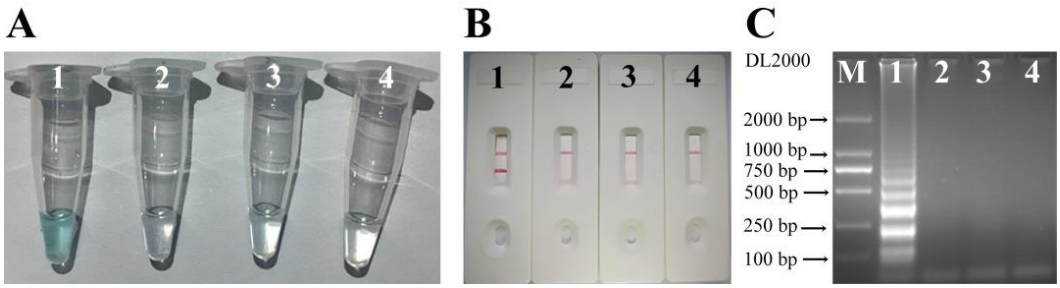

Fig. 1. Detection of *P. aeruginosa*-MCDA products. (a) Using Malachite Green (MG) – amplification products in the *P. aeruginosa*-MCDA assay were detected visually by a color change (sky blue for positive samples, colorless for negative samples); (b) Lateral flow biosensor (LFB) applied for visual detection of *P. aeruginosa*-MCDA products; (c) 2% agarose gel electrophoresis of *P. aeruginosa*-MCDA products. Lane M, DNA maker DL2000. Tube 1/Biosensor 1/Lane 1: amplification of *P. aeruginosa* strain SGH-PA001; Tube 2/Biosensor 2/Lane 2: negative control (*Klebsiella pneumoniae* strain SGH-KP001); Tube 3/Biosensor 3/Lane 3: negative control (*Staphylococcus epidermidis* strain SGH-SE001); Tube 4/Biosensor 4/Lane 4: blank control (double distilled water).

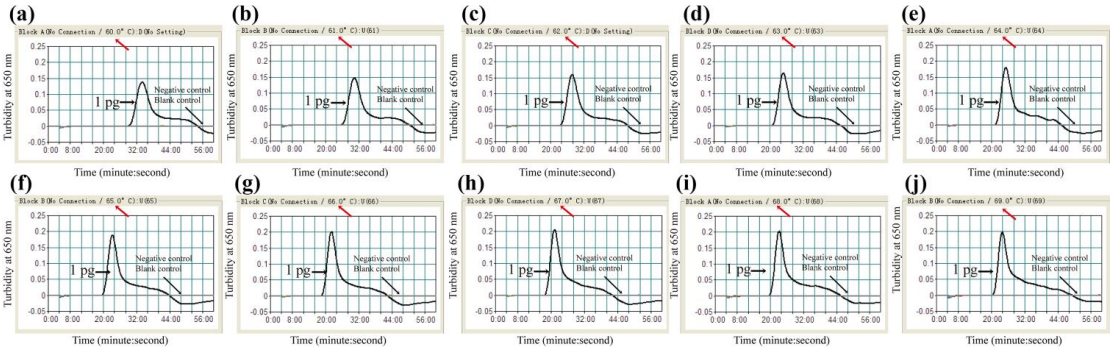

Fig. 2. Optimization of amplification temperature for *P. aeruginosa* MCDA. MCDA reactions for detection of *P. aeruginosa* were monitored by real-time measurement of turbidity. The panels show the corresponding concentrations of DNA. *K. pneumoniae* was used as the as negative control, and sterile double distilled water as the blank control. Turbidity of >0.1 was considered positive. Kinetic curves (a-j) were generated at 60–69°C (1°C intervals) respectively with 1 pg DNA per reaction.

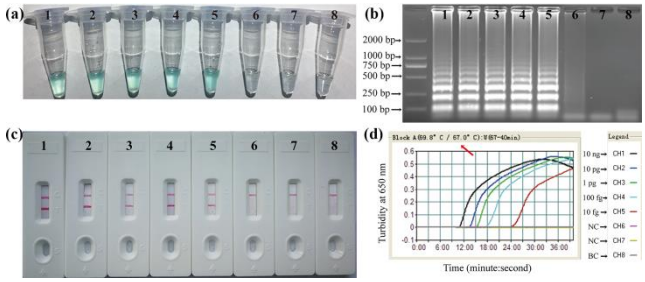

Fig. 3. Sensitivity of MCDA-LFB assay using serially diluted *P. aeruginosa* strain SGH-PA001 genomic DNA. (a) A colorimetric indicator (MG), (b) gel electrophoresis, (c) lateral flow biosensor, (d) and real-time turbidity, were applied for analysis of the amplification products. Serial dilutions of the template (10 ng, 10 pg, 1 pg, 100 fg and 10 fg) were subjected to standard MCDA reactions. Tubes (panel a)/Lanes (panel b)/Biosensors (panel c)/Turbidity signals (panel d) 1–8 respectively represent *P. aeruginosa* DNA levels of 10 ng, 10 pg, 1 pg, 100 fg, and 10 fg per reaction, a negative control (10 pg of *K. pneumoniae* genomic DNA), a negative control (10 pg of *S. epidermidis* genomic DNA), and a blank control (double distilled water).

Table 1. Primers used in this study

Primer	Length	Sequence and modifications (5'-3')
F1	16 nt[1]	CGTGCGATCACCACCT
CP1	40 mer[2]	GTACGTCCAGAGCGCGCATGTCTACTTCGAGT ACGACAGC
C1	20 nt	GTACGTCCAGAGCGCGCATG
C1*[3]	20 nt	Biotin-GTACGTCCAGAGCGCGCATG
D1	16 nt	CTTCCGGCTTCAGGTC
D1*[4]	16 nt	FITC-CTTCCGGCTTCAGGTC
R1	18 nt	TGCCTTTCAGGTCTTTCG
R2	17 nt	AGGCCACACCGACGAAC
D2	17 nt	GTGCCAAGGCCGTTCAG
C2	20 nt	GCACCCGCGAGTACAACATG
CP2	37 mer	GCACCCGCGAGTACAACATGCCTGCAGCACCA GGTAG
F2	17 nt	AAACCAGTTCCAGCGTG

[1] nt, nucleotide; [2] mer, monomeric; [3] C1*, 5′-labeled with biotin when used in the MCDA-LFB assay; [4] D1*, 5′-labeled with fluorescein isothiocyanate (FITC) when used in the MCDA-LFB assay.

Table 2. Bacterial strains used in this study

Bacteria	Strain no./source	No. of strains	MCDA result
Pseudomonas aeruginosa	SGH-PA001[1]	1	Positive
	Isolated strains from Shougang Hospital	9	Positive
Other Pseudomonas strains	Isolated strains from Shougang Hospital	2	Negative
Klebsiella pneumoniae	SGH-KP001[2]	1	Negative
Staphylococcus epidermidis	SGH-SE001[3]	1	Negative
staphylococcus hominis	Isolated strains from Shougang Hospital	2	Negative
Staphylococcus aureus	Isolated strains from Shougang Hospital	4	Negative
Streptococcus agalactiae	Isolated strains from Shougang Hospital	2	Negative
Acinetobacter baumannii	Isolated strains from Shougang Hospital	2	Negative
Acinetobacter caviae	Isolated strains from Shougang Hospital	2	Negative
Aeromonas veronii	Isolated strains from Shougang Hospital	2	Negative
Shigella dysenteriae	Isolated strains from Shougang Hospital	1	Negative
Shigella boydii	Isolated strains from Shougang Hospital	1	Negative
Enterohemorrhagic *E. coli*	Isolated strains from Shougang Hospital	1	Negative

Enteropathogenic *E. coli*	Isolated strains from Shougang Hospital	2	Negative
Enterotoxigenic *E. coli*	Isolated strains from Shougang Hospital	2	Negative
Enteroaggregative *E. coli*	Isolated strains from Shougang Hospital	1	Negative
Enterococcus faecalis	Isolated strains from Shougang Hospital	2	Negative
Enterococcus faecium	Isolated strains from Shougang Hospital	2	Negative

[1–3] SGH, Shougang Hospital; PA, *Pseudomonas aeruginosa*; KP, *Klebsiella pneumoniae*; SE, *Staphylococcus epidermidis*

Table 3. Comparison of methods for detection of *P. aeruginosa* in clinical samples

Detection method	**Clinical sputum samples (n = 120)**	
	Positive	**Negative**
Culture	60	60
MCDA-LFB	60	60
PCR	52	68