Multiple Cross Displacement Amplification—a more applicable technique in detecting Pseudomonas Aeruginosa of Ventilator-associated Pneumonia (VAP)

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Abstract

**Background** Early and rapid identification of *Pseudomonas aeruginosa* (*P. aeruginosa*) in patients with suspected ventilator-associated pneumonia (VAP) provides theoretical clinical advantages in therapeutic optimization strategies.

**Methods** The *PA-MCDA* assay was conducted at an isothermal temperature during the amplification stage, and products were visually detected by color changes. The entire process was completed within 1 h. A total of 77 strains, including *P. aeruginosa* species and various other species of non-*P. aeruginosa* were used to evaluate *PA-MCDA* assays. Bronchoalveolar lavage fluid (BALF) of suspected VAP patients were examined by the MCDA assay.

**Results** The MCDA assay exhibited a 100 percent analytical specificity in detecting *PA* from all 77 strains, and the limit of detection were as low as 100 fg DNA per reaction. A temperature of 65ºC was recommended as standard during the amplification stage. The agreement between *PA-MCDA* and bacteria culture was 91.18% (κ = 0.787; p = 0.000) in identification of *P. aeruginosa* in BALF from suspected VAP. The *PA-MCDA* assay showed values of 92.31%, 90.78%, 77.41% and 97.18% for sensitivity, specificity, positive predictive value and negative predictive value, respectively. *PA-MCDA* had higher detective rate of *P. aeruginosa* than bacteria culture in patients with antipseudomonal therapy.

**Conclusions** The instrument-free platform of the MCDA assay makes it a simple, rapid and applicable procedure for “on-site” diagnosis and point-of-care testing for the presence of *P. aeruginosa* without the need for specific bacterial culture.

**Background** Ventilator-associated pneumonia (VAP) develops in intensive care unit (ICU) patients that have been mechanically ventilated for at least 48 h[1]. The infection rate was related to disease severity and the degree of organ failure[2]. Further, the EU-VAP study[3] identified that the overall incidence of VAP was 18.3 episodes per 1000 ventilator-days. Moreover, *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Staphylococcus aureus* were the most frequently isolated pathogens in patients with VAP. In a multicenter study[4], *P. aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumonia* were found
the most frequent bacteria (97/212, 45.8%) in VAP patients. In addition, VAP induced a more prolonged need for mechanical ventilation, number of ICU stays and hospitalization, far worse outcomes, and increased associated healthcare costs[5, 6]. Rapid completion of antibiotic administration might decrease the incidence of subsequent organ dysfunction and could be associated with a lower risk-adjusted in-hospital mortality rate [7]. The conventional detection of *P. aeruginosa* in the clinical setting was generally achieved by growing the target pathogen on agar plate surfaces and cultures[8]. Although the culture-based technique is reliable, the time required for conducting it is at least for a period of 48 h [9]. Furthermore, published guidelines recommended an initial empiric combinatorial coverage with antibiotics targeted to Gram-negative and Gram-positive bacteria Methicillin-resisitant Staphylococcus aureus (MRSA) in the setting of high-risk VAP patients prior to obtaining culture results[1]. Inappropriate initial antimicrobial therapy and antibiotic exposure attributed to antibiotic-resistance[10, 11]was associated with increased in-hospital mortality rates [12]. Rapid and accurate identification of the suspected pathogens was thus warranted to provide the potential to maximize administration of appropriate specific antibiotic and possibly avoid a need for empiric broad-spectrum antibiotic therapy. Rapid excluding some specific and common pathogens would be possible to avoid unnecessary antibiotic exposure and minimize some undesirable consequences[13].

More recently, multiple cross displacement amplification (MCDA, Chinese IP Office Patent Application CN201510280765.X), auto-cycling, and strand displacement DNA synthesis was devised and validated as a possible replacement for PCR-based assays and applicability in the detection of specific nucleic-acid sequences[14, 15]. The assay employed an isothermal temperature during amplification, and the products were visually detected by color changes. The entire process is completed in 1 h and benefits from being an instrument-free, simple and practical procedure for ‘on-site’ diagnosis and point-of-care testing. The current study is the first to report application of the novel MCDA assay to rapidly detect the target pathogen, *P. aeruginosa*.

Methods

**PA-MCDA assay primer design**
Based on the mechanism of MCDA, a set of MCDA primers used for *P. aeruginosa* detection was designed that targeted the *oprL* gene, which encodes L-lipoprotein. The details of MCDA primers used in the report are shown in Figure 1 and Table 2. The primers were commercially synthesized and purified by Tsingke (Beijing, China).

**PA-MCDA reactions**

MCDA reactions were performed in a one-step reaction in a 25 μl mixture containing 12.5 μl 2× the supplied buffer (Beijing- Hai Tai Zheng Yuan Technology Co., Ltd.), 0.1 μl each of the displacement primers F1 and F2, 0.2 μl each of the amplification primers C1, C2, R1, R2, D1 and D2, 0.4μl each of the cross primers CP1 and CP2, 1μl (8U) of *Bst* 2.0 DNA polymerase, 1μl of the DNA template and 0.8 μl of the colorimetric indicator. Moreover, negative control mixtures contained 10 ng of the *Staphylococcus aureus* and *Klebsiella pneumoniae* genomic templates, and blank control mixtures contained 1μl of double distilled water (DW). To evaluate the feasibility of the MCDA primer set that was designed to detect *P. aeruginosa*, we initially conducted the MCDA reactions at 63°C for 45 min and terminated the MCDA reaction by heating at 85°C for 5 min. Then, the optimal amplification temperature of the MCDA primer set was examined at fixed temperatures from 59°C-68°C at steps of 1°C intervals. In particular, MCDA products were detected using a colorimetric indicator and agarose gel electrophoresis.

**Bacterial strains and genomic template preparation**

A total of 124 bacterial strains and 14 fungi of positive culture were isolated in the clinical microorganism laboratory of the Third Hospital of Xiamen from 26th June to 26th July, 2017. The bacterial strains list of standard culture is detailed in Additional file 1. The positive bacterial strains including 6/124(4.84%) polymicrobial growth and 32/124(22.81%) Multidrug-Resistant (MDR) strains were isolated from 118 clinical samples in which the tracheal aspirate and BALF in 38 cases, the secretion and drainage in 33 cases, the blood in 16 cases, urine, faeces, catheter and other samples in 31 cases. We chose top 13 bacteria strains, 77 samples to design the PA-MCDA reactions (Table 3). The bacteria strains identified by conventional cultivation method automatic bacterial identification system (VITEK 2 Bio-Merieux, France) were stored in a 15% (w/v) glycerol broth at -70°C. After
refreshing the culture three times on a nutrient agar plate at 37°C, the genomic templates were then extracted from all cultured strains using DNA extraction kits Qiagen Co., Ltd. Beijing, China), and subsequently tested with an ultraviolet spectrophotometer and stored under -20°C before use.

**Specificity of the PA-MCDA assay**

To evaluate the analytical specificity of the PA-MCDA assay, MCDA reactions were conducted under conditions that were described above with the 77 P. aeruginosa and non-P. aeruginosa pure genomic templates that were derived from all pure bacterial strains.

**Sensitivity of the PA-MCDA assay**

The genomic templates of P. aeruginosa were serially diluted (10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg per microliter) with the intent of verifying the limit of detection (LoD), and 1μl of each serial dilution was then added to the MCDA reaction mixtures. The LoD of the MCDA assay was confirmed by the genomic DNA concentration of the template. MCDA results were detected using a colorimetric indicator, malachite green, MG (Beijing Hai Tai Zheng Yuan Technology Co., Ltd.) and 2.5% agarose gel electrophoresis.

**Verification of the PA-MCDA assay**

This study was conducted in the 30-bed ICU of The Third Hospital of Xiamen in Fujian province, which is a 1200-bed hospital in China. A total of 102 patients enrolled in this study who were suspected VAP from 1st January 2018 to 14th March 2020. Patients satisfied two or more of the following criteria: fever > 38.5°C, leukocytosis > 10⁹/L or leukopenia < 4×10⁸/L, purulent tracheobronchial secretions, and a new or persistent infiltrate on chest radiography. The following data were recorded: demographic characteristics, indication(s) for ICU admission, prior antimicrobial therapy within 2 months before VAP, duration of mechanical ventilation before VAP, Clinical pulmonary infection score (CPIS[16]) including temperature, blood leukocytes, tracheal secretions, oxygenation and pulmonary radiography, usual biochemical and hematological tests.

The BALF were abstracted in one bottle, following which, one half (5ml) processed for standard culture by the clinical microorganism laboratory of the Third Hospital of Xiamen and the other 5ml stored at -70°C until the time of DNA extraction. BALF was plated on chocolate, sheep blood, and
MacConkey agar plates and incubated for 48-72 h according to routine clinical protocol. The DNA extraction from BALF method was described before. In this procedure, 1μl of the extracted DNA template of the BALF specimen was added to the PA-MCDA assay and the reactions were performed at an optimal amplification temperature for 45 min. The products were detected by a color change and compared to the results of a standard clinical culture, which was blinded to the research investigators. This study was approved by the local ethics committee of the Third Hospital of Xiamen, and performed according to the ethical standards of the latest revision of the Declaration of Helsinki. Written and informed consent was obtained from family members or the appropriate responsible parties.

Statistical analysis
Continuous variables of patients’ characteristics were reported as the means±standard deviations (SD) or the medians (interquartile ranges (IQR)), and categorical variables were reported as numbers (%). The accuracy of the PA-MCDA assay was compared with the microbiological culture in a cross-sectional analysis. P value < 0.05 was considered significant. Statistical analysis was performed using SPSS version 20.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results
Successful establishment of the PA-MCDA assay
To verify the feasibility of PA-MCDA primers, the MCDA reactions were initially carried out in the presence or absence of genomic DNA templates within 45 min at a constant temperature of 63ºC[14]. A color shift of positive amplification in PA-MCDA tubes was directly observed to change from one of colorless to one of green, while the negative control tube remained colorless by the naked eye (Fig. 2A). The positive MCDA products were seen as ladder-like patterned bands on ethidium bromide-stained 2.5% agarose gels that were resolved by electrophoresis; however, these were not seen in the Staphylococcus aureus, Klebsiella pneumonia or blank control (Fig.2B). Hence, the designed MCDA primer was a good candidate to establish the MCDA methodology for detecting P. aeruginosa.

Optimizing the temperature for the PA-MCDA assay
To confirm the optimal reaction temperature for the PA-MCDA assay, the P. aeruginosa strain was
used as a positive control at a concentration of 1ng per tube and the MCDA amplifications were monitored by a real-time turbidity technique. Performing the PA-MCDA assay at temperatures that ranged from 59°C to 67°C at 1°C increments, verified that 65°C was an optimal temperature for amplification – with a faster amplification procedure obtained from assay temperatures of 65°C (Fig. 3).

**Specificity and sensitivity for P. aeruginosa detection by MCDA assays**

When genomic templates were used in MCDA assays, only the genomic DNAs that were isolated from the *P. aeruginosa* strains (tubes 1 to 17) generated positive results. Genomic templates from all non-*P. aeruginosa* strains (tubes 8 to 34) did not provide production of detectable amplification products (Fig. 4). The color change was observed in positive MCDA tubes (tubes 1 to 17), and a ladder-like pattern was seen on an ethidium bromide-stained 2.5% agarose gel via electrophoresis resolution. These were not seen in negative tubes (Fig. 4).

Serial dilution of the *P. aeruginosa* genomic DNA (10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg per microliter) were used in MCDA assays. Fig. 5A and 5B). It indicated that the LoD, and the sensitivity of the PA-MCDA assay was 100 fg genomic templates per reaction.

**Application of PA-MCDA to clinical samples**

The MCDA assay was used to examine 102 BALF from patients who were suspected of presenting with VAP. Demographic and clinical characteristics of our patients are shown in Table 1. The median duration of mechanical ventilation was 7 days (IQR 4.00-32.50) before suspected VAP onset and 97 patients had received antibiotics within two months before suspected VAP. The clinical diagnosis of VAP was given by physician judgment with respect to particular patients or special clinical situations combination with culture results. A total of 94 positive bacteria results were detected by conventional culture in 82/102 (80.39%) BALFs in which Polymicrobial growth was 12/102 (11.76%) and Polymicrobial included *P. aeruginosa* were 4/102 (3.92%). Bacterial strains list of BALF by standard culture is detailed in Additional file 2. After extracting DNA from these BALF specimens and adding 1μl of the DNA template to the PA-MCDA assay, the reactions were carried out at 65°C for 45 min and the results were compared to those of the standard culture. The *P. aeruginosa* was detected in 26
samples by microbiological culture and in 31 samples by PA-MCDA. Two methods unanimously detected 24 \( P. \) \( aeruginosa \) strains while the MCDA detected 7 \( P. \) \( aeruginosa \) strains in culture negative patients who received antipseudomonal therapy. The positive results of PA-MCDA and microbiological culture were 24/76(31.58%) and 17/76(22.37%) respectively in 76 patients who had received antipseudomonal therapy. In the cross-sectional analysis, the agreement between the tests was 91.18% (\( \kappa = 0.787; \ p = 0.000 \)), likelihood ratio positive was 10.02 and likelihood ratio negative was 0.08. The PA-MCDA assay showed values of 92.31%, 90.78%, 77.41% and 97.18% for sensitivity, specificity, positive predictive value and negative predictive value, respectively.

Discussion

PA-MCDA reaction was performed with a set of 10 oligonucleotide primers, which specifically recognized 10 distinct sites on the target sequence, wherein the optimal temperature for amplification was 65°C. In particular, a colorimetric indicator (malachite green, MG) had been applied and the color changed from colorless to a light green color when the reaction was positive. The specificity of the PA-MCDA assay was 100 percent, and the sensitivity achieved a level as low as 100fg of the template. The entire procedure, including that of specimen processing (15 min), the isothermal reaction, and result reporting (45 min), could be completed in approximately 1h. The agreement between PA-MCDA and bacteria culture was 91.18% (\( \kappa = 0.787; \ p = 0.000 \)) in identification of \( P. \) \( aeruginosa \) in BALF from suspected VAP. PA-MCDA had higher detective rate of \( P. \) \( aeruginosa \) than bacteria culture in patients who had received antipseudomonal therapy.

A rapid, simple and accurate detection method of pathogenic microorganisms was necessary for the timely administration of appropriate therapy and arriving at a time to discontinue unnecessary antibiotic(s). Delayed receipt of an appropriate antibiotic(s) was independently associated with poorer clinical and economic outcomes in patients with serious Gram-negative bacterial infections, regardless of any resistance status[9]. Molecular diagnostic assays, such as PCR-based methods (e.g., conventional PCR, real-time PCR [17], and PCR-Electro Spray Ionization MS (PCR/ESI-MS)[18], permit more rapid detection of targeted bacterium by nucleic acid amplification, and have been established and applied in the clinic. However, these PCR-based techniques have some shortcomings, which
include the following: (i) the instrument used is extremely expensive; (ii) the diagnostic specificity is highly affected by the amplification conditions and the primer design; (iii) use of these techniques indicate that PCR results require gel electrophoretic analysis or real-time analytical apparatus. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF)[19] consistently decreased the time for successful identification of the pathogenic organism, shorten the period of time for administering an effective and optimal antibiotic and thus arrive at improved patient outcomes. However, it also needs a bacterial culture and expensive instruments.

For diagnosis of VAP, not the standard culture but the MCDA assay are intended to supplant physician judgment respect to particular patients or special clinical situations. The PA-MCDA assay just represents a rapid, sensitive and nearly instrument-free method of \textit{P. aeruginosa} detection. Only a water bath or heat block was needed during the reaction stage and the cost is about $8 per sample compared to standard culture is $10 per sample. Due to MCDA being able to provide results within only one hour, the clinician can save time to provide targeted therapy for patients, especially in the context of severe sepsis or septic shock patients. The negative test indicated that there were no \textit{P. aeruginosa} growth or the number of \textit{P. aeruginosa} was very below the $10^4$ cfu/ml in the BALFs as the high sensitivity of PA-MCDA assay[20]. The value was that empiric combination coverage for \textit{P. aeruginosa} might not be necessary and the discontinuation of antibiotic therapy with antipseudomonal activity needed to be considered by clinicians[13]. Although, the bacterium specific-MCDA assay cannot differentiate the infection from colonization for it cannot quantitative the bacteria strains of BALF that is the methodology to diagnose VAP, the different time of color change of positive MCDA assay may relate to the amount of DNA templates of bacteria because its reaction products could be detected by real-time fluorescence and less time of positive reactions were produced in more specific-DNA templates[20]. Whether a threshold time of color change for MCDA reflects the quantitative or semiquantitative bacterium for clinical application needs a precise design and analysis. The PA-MCDA assay also detected 7 positive \textit{P. aeruginosa}, while standard culture was negative in 76 patients who had received antipseudomonal therapy. The interpretation might suggest the MCDA approach had a higher sensitivity than standard culture[15, 21], or that the culture
negativity might reflect the presence of active culture inhibitors in the samples[22], or that the *P. aeruginosa* had become non-viable before or even between the standard culture periods since the growth conditions had changed. Clinical factors should also be taken into account because they might alter the decision of whether to withhold or continue antibiotics. The ultimate clinical determination of VAP, pathogens and regarding antibiotics application was made by the physician in the light of each patient’s individual circumstances[13].

In order to use MCDA method to clinical application, this study also has limitations. The first is the bacterium specific-MCDA assay cannot differentiate the infection from colonization because it cannot provide the results of quantitative bacterium of BALF. A more precise study will be designed recently to explore the relationship between a cutoff time for MCDA color change and the quantitative or semiquantitative bacterium in clinical samples. In addition, whether or not the assay could detect the target pathogen in other specimens such as blood, urine or serous effusion needs further study. Consequently, both the negative and positive results of PA-MCDA cannot rule out the presence of other pathogens, Gram-positive or Gram-negative bacteria or fungi, for polymicrobial growth always existed. MCDA cannot provide precise information to prescribe or withdrew pathogen-specific therapy[23] until more pathogen specific-MCDA and resistance-associated genes be designed[20]. We propose to assign some microorganism-specific MCDA assays and resistance-associated genes (e.g., the nfxB gene and the blaPER-1 gene)[18, 19, 24, 25] that are aligned to common pathogens found in ICU, which would include *Staphylococcus. aureus* (MRSA)[26] , *Acinetobacter baumannii*, *Escherichia coli*, fungal species, and others in one template.

**Conclusions**

The PA-MCDA assay for rapid detection of *P. aeruginosa*, which was based on the oprL gene was successfully developed. This approach enabled its reaction products to be identified by the naked eye, and the assay established a high degree of both specificity and sensitivity for target template analysis. The PA-MCDA assay does not only have the benefit of a rapid, reliable and nearly instrument-free procedure, but it can differentiate *P. aeruginosa* from pure strains of bacteria and clinical specimens without the need for time-consuming bacterial culture and its clinical significance
needs further establishment.

**Declarations**

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**Availability of data and materials**

The dataset analysed during the current study is available from the corresponding author on reasonable request.

**Authors’ contributions**

The corresponding author (BC) was in charge of study design. The first author (JXW) was responsible for manuscript writing and cooperated with the rest three authors (HMC, XML and CYJ) in clinical research work. JXW was in charge of data collection and experiment, BC was responsible for data analysis. HMC, XML and CYJ were responsible for sample collection, experiment technical and material support during the study. All authors have read and approved the publication of this manuscript.

**Ethics approval and consent to participate**

All procedures in the study were performed in accordance with the ethical standards of the institutional research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Written and informed consent was obtained from family members or the appropriate responsible parties.

**Consent for publication**

Not applicable

**Competing interests**

All authors declared that they have no competing interests.
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**Tables**

Table 1. Demographic, clinical and biological characteristics of patients

| Characteristics at ICU admission | Total [n=102] |
|----------------------------------|--------------|
| Male sex, n(%)                  | 75(73.5%)    |
| Age(years), median(IQR)         | 54.62±19.59, 57(39.75-70.75) |
| Medical patients, n (%)         | 39(38.2%)    |
| Surgery patients, n (%)         | 63(61.8%)    |
| Prior antibiotics in 90 days, n (%) | 61(59.8%) |

| Characteristics upon VAP onset |               |
|-------------------------------|---------------|
| ICU stay (days), median(IQR)   | 37.87±80.45, 8(4.00-37.75) |
| Duration of MV (days), median(IQR) | 35.42±80.05, 7(4.00-32.50) |
| Septic shock, n (%)            | 57(55.9%)     |
| CPIS                           | 6.59±1.70,7(5.00-8.00) |
| CRP                            | 84.59±57.98, 69.45(46.00-104.80) |
| PCT                            | 17.34±24.29, 10(2.34-21.30) |

| Antibiotics onset   |               |
|---------------------|---------------|
| None                | 5(4.9%)       |
| Monotherapy         | 41(40.2%)     |
| Combination antibiotic therapy | 56 (54.9) |
| covering PA         | 76(74.5%)     |
| Change of antibiotics, n (%) | 45(44.1%) |

| Clinical diagnosis of VAP |          |
|--------------------------|----------|
|                          | 68(66.7%) |

*: duration of mechanical ventilation before VAP, Characteristics upon suspected VAP onset, CPIS, Clinical pulmonary infection score.
Table 2. Primers used for multiple cross displacement amplification in this study.

| Primers | Sequences (5’-3’)                  | Length |
|---------|------------------------------------|--------|
| CP1     | GCCGAATTCAGCATTTCCATCATG-CCTGAACTGACGGTGCC | 43mer  |
| CP2     | CGATGCTTCCGGTGGAAGGTGC-AACGGCACGGCTGGTG | 37mer  |
| F1      | GCCTTCCCTGGTCCCTTA                 | 18nt   |
| F2      | CGGCTTCGTCGCTCAG                  | 16nt   |
| C1      | GCCGAATTTTCAGCATTTCCATCATG        | 25nt   |
| C2      | CGATGCTTCCGGTGGAAGGTGC            | 21nt   |
| D1      | ACTCCTAATGAACCCCATGT              | 19nt   |
| D2      | ACCCGAACGCGAGCTATG                | 18nt   |
| R1      | CAGAGCCACGCAGCA                   | 16nt   |
| R2      | GGCTGTTGGCTGTTGGGT               | 16nt   |
| P1      | CCTGAACTGACGGTGCGCC              | 18nt   |
| P2      | AACGGACCCGGCTGGTG                | 16nt   |

Table 3. List of bacterial strains

A total of 77 bacterial strains, which included 17 PA and 60 non-PA strains were identified by MALDI-TOF (microflex LT/SH, Bruker corporation, Karlsruhe, Germany) by the clinical microorganism laboratory of the Third Hospital of Xiamen.

| Bacteria                        | Strains | Bacteria                        | Strains |
|---------------------------------|---------|---------------------------------|---------|
| *Pseudomonas. aeruginosa*       | 17      | *Streptococcus agalactiae*      | 5       |
| *Escherichia coli*              | 5       | *Enterococcus faecalis*         | 5       |
| *Staphylococcus. aureus*        | 5       | *Salmonella typhimurium*        | 5       |
| *Acinetobacter baumannii*       | 5       | *Klebsiella. pneumoniae*        | 5       |
| *Staphylococcus epidermidis*    | 5       | *Enterobacter cloacae*          | 5       |
| *Staphylococcus capitis*        | 5       | *Stenotrophomonas maltophilia*  | 5       |
| *Streptococcus pyogenes*        | 5       |                                 |         |

Figures
Figure 1

Schematic depiction of the primer sequences and positions for MCDA. The location and nucleotide sequence of the PA oprL gene that assisted in designing MCDA primers. The primer site sequences are underlined. Right and left arrows indicate sense and complementary sequences that were used in the assay.
Confirmation and detection of products. (A) The color change seen in MCDA tubes: tube 1 is the positive amplification of PA, the green color was observed directly; tubes 2 and 3 are the negative amplifications of S. aureus and K. pneumoniae respectively; tube 4 is the negative amplification of the control (no DNA); tubes 2, 3 and 4 remained colorless. (B) 2.5% agarose gel electrophoresis applied to MCDA; lane 0:DL 100-bp DNA marker; lane 1: positive MCDA products of PA; lane 2 and 3: negative products of S. aureus and K. pneumoniae respectively; and lane 4, negative control (no DNA).
Figure 3

Optimal reaction temperature for the PA-MCDA primer assay. MCDA reactions when detecting the PA gene were monitored by real-time measurement of turbidity. A turbidity of >0.1 was considered positive. Nine kinetic graphs were obtained at various temperatures (59–67 °C, at 1 °C intervals) with PA DNA at a concentration of 1 ng per tube. The graphs showed that 65 °C was an optimal temperature for amplification.
Figure 4

Specificity for PA detection by MCDA assays. Of all 77 pure genomic templates, only the genomic DNAs from the PA strains generated positive results. The color shift in the PA-MCDA tubes (tubes 1-17) was directly observed as a green color. A grey color was seen in tubes 18-34, in which 18-21 were E.coli(ETEC, EAEC, EIEC and EHEC). Tubes 22-23 were S. aureus, of which, tubes 24-25 were A. baumannii, and tubes 26-34 were respectively S. epidermidis, S. capitis, Streptococcus pyogenes, S. agalactiae, E. faecalis, S. typhimurium, K. pneumoniae, E. cloacae, and S. maltophilia. A 2% agarose gel electrophoresis assay was
applied to detect PA MCDA; lane 0, DL 100-bp DNA marker; lanes 1-17 were positive MCDA products that corresponded to PA tubes 1-17; and lanes 18-34 were negative and corresponded to tube 18-34 respectively.
Figure 5

Sensitivity of the MCDA assays using serially diluted PA genomic DNA. (A) PA genomic DNA was serially diluted to 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg per microliter. When the dilution was more than 100 fg/μL, the green color by MG and the laddering pattern by agarose gel electrophoresis were directly observed. The LoD of the PA MCDA assay was as low as 100 fg per microliter (white arrow). (B) Real-time turbidity was applied to analyze the amplification products. Genomic DNA levels >100 fg per reaction produced positive reactions.
Supplementary Files

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