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A novel isothermal amplification-based method for detection of *Corynebacterium striatum*

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ABSTRACT

Corynebacterium striatum is an emerging multidrug-resistant pathogen causing increasing numbers of infections and nosocomial outbreaks worldwide. Thus, a simple, rapid and accurate method for *C. striatum* is urgently required for improving diagnosis efficiency. In this study, a *C. striatum*-multiple cross displacement amplification (MCDA) with visual detection reagent (VR) assay (*C. striatum*-MCDA-VR), which was a novel isothermal amplification-based method, was established to detect the species-specific *ftr1* gene of *C. striatum*. Amplification was performed at a constant temperature (68 °C) for only 40 min, and the reaction results could be easily elucidated by observation of reaction mixture color when employing the VR. The limit of detection of this method was 10 fg of pure *C. striatum* DNA. No cross-reaction was observed with non-*C. striatum* strains. In testing of clinical sputum samples, the *C. striatum*-MCDA-VR assay showed excellent sensitivity and specificity when compared with sputum smear tests and PCR. The *C. striatum*-MCDA-VR assay is a simple, rapid and cost-effective approach for identifying *C. striatum* in microbiological laboratories, especially in resource-limited settings.

1. Introduction

Corynebacterium striatum, a Gram-positive, non-sporulating, non-motile short rod bacterium, normally colonizes human skin and mucous membranes (Funke et al., 1997) and is considered as an opportunistic pathogen. In recent years, outbreaks of this pathogen and nosocomial cases have been increasingly reported (Baio et al., 2013; Nudel et al., 2018; Renom et al., 2014; Verroken et al., 2014). The organism is responsible for infections including pneumonia (Diez-Aguilar et al., 2013), sepsis (Ishiwada et al., 2016) and endocarditis (Tran et al., 2012), especially in chronically ill and immunocompromised patients (Otsuka et al., 2006). Many studies have shown that *C. striatum* infections are linked to prolonged duration of hospitalization, prolonged use of invasive medical procedures, and repeated antibiotic exposure (Nudel et al., 2018; Otsuka et al., 2006; Wang et al., 2016b). It is noteworthy that this emerging pathogen usually shows multidrug resistance (Alibi et al., 2017; Otsuka et al., 2006; Wang et al., 2016b) and thus clinical treatment is difficult.

Accurate identification is essential for correct diagnosis and treatment of infection. In clinical microbiology laboratories, microorganisms are routinely identified by biochemical and morphological investigation, for example, sputum smear tests (SSTs). However, it is difficult to identify *Corynebacterium* spp. to the species level by these methods. Thus, additional tests such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and 16S rRNA gene sequencing are required (Bao et al., 2017; Diez-Aguilar et al., 2013; Gomila et al., 2012; Nudel et al., 2018; Otsuka et al., 2006; Verroken et al., 2014). Nevertheless, these methods are culture-based and time-consuming, and particular equipment is required for them.

The *ftr1* gene has been identified as a species-specific gene of *C. striatum* and can be used to differentiate *C. striatum*, *C. amycolatum* and *C. xerosis* by PCR (Santos et al., 2017). However, this method still requires sophisticated instruments to execute tedious thermal cycling amplification, and the validity and effectiveness of the PCR assay has not been confirmed with clinical samples. Multiple cross displacement amplification (MCDA), a simple, rapid, highly sensitive and specific assay method, has been applied to detection of many species (Li et al.,

Abbreviations: DDW, double distilled water; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MCDA, multiple cross displacement amplification; PCR, polymerase chain reaction; SST, sputum smear test; VR, visual detection reagent

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2019; Niu et al., 2018; Wang et al., 2015; Wang et al., 2016a; Wang et al., 2018). The MCDA method is based on isothermal strand-displacement polymerization reaction. A set of ten primers, including one pair of cross primers, one pair of displacement primers, and three pairs of amplification primers, are designed to amplify ten distinct regions of the target sequence. This approach amplifies nucleic acid fragments in isothermal conditions without the thermal denaturation of templates, and thus a simple heater is sufficient for this assay.

In the present study, we established an MCDA assay targeting the *ftr1* gene to identify *C. striatum* simply, rapidly, sensitively and accurately. The assay was verified using pure cultures and clinical sputum samples, and was compared with the PCR assay (Santos et al., 2017) and SSTs.

2. Materials and methods

2.1. Reagents and instruments

A Wizard® Genomic DNA Purification Kit was purchased from Promega Corporation (Madison, WI, USA). 2× *EasyTaq*® PCR SuperMix was purchased from Transgen Biotech Co., Ltd. (Beijing, China). Isothermal® Amplification Kits were purchased from HaiTaiZhengYuan Technology Co., Ltd. (Beijing, China). Visual detection reagent (VR) was from ZhenMin Biotech Co., Ltd. (Beijing, China).

2.2. Bacterial strains and chromosomal DNA preparation

C. striatum reference strain ATCC 6940 was chosen to establish the *C. striatum*-MCDA assay. One hundred *C. striatum* clinical isolates from different sources, including ninety-four isolates from sputum, three isolates from urine, two isolates from blood, and one isolate from bronchoalveolar lavage fluid (Table S1), and 31 non−*C. striatum* organisms (Table 1) were used to test the *C. striatum*-MCDA assay. All the isolates used in this study were stored in 20% (*w/v*) glycerol broth at −80 °C. The species of all isolates was confirmed by 16S rRNA gene sequencing. *Corynebacterium* and non-*Corynebacterium* isolates were cultured three times on Columbia blood plates (OXOID, USA) and suitable nutrient agar plates, respectively, at 37 °C. Genomic DNA was extracted from cultured strains using the Wizard® Genomic DNA Purification Kit according to the technical manual and quantified using a NanoDrop ND-1000 instrument (Calibre, Beijing, China). *C. striatum* ATCC 6940 genomic DNA was serially diluted from 1 ng to 1 fg at 10-fold intervals for sensitivity analysis of the MCDA method.

2.3. PCR assay for C. striatum

According to previous research (Santos et al., 2017), primers Cst_1-F and Cst_1-R were used to amplify the *ftr1* gene from *C. striatum*. Twenty-microliter PCR reactions contained: 10 μL 2× *EasyTaq*® PCR SuperMix, 0.4 μM each primer, and 1 μL template DNA. Cycling was performed as described by Santos et al. (2017).

2.4. Design of C. striatum-MCDA assay primers

Based on the *ftr1* gene (GenBank accession no. EEI79352.1), five pairs primers (F1, F2, CP1, CP2, C1, C2, D1, D2, R1 and R2) were designed using PrimerExplorer V4 (Eiken Chemical, Japan) and Primer Premier 5.0 software. BLAST analysis was used to check the specificity of MCDA primers. Integrated DNA Technologies design tools (http://www.idtdna.com/pages/scitools) were used to ensure that hairpin structures would not be formed. Information on *C. striatum*-MCDA primers is shown in Table 2 and Fig. 1. All primers were synthesized by TsingKe Biotech Co., Ltd. (Beijing, China) at HPLC purification grade.

Table 1
Bacterial strains used in this study.

Bacteria	Strain no.	No. of strains
Corynebacterium striatum	ATCC 6940	1
	Isolated strains	100
Corynebacterium simulans	Isolated strains	1
Corynebacterium propinquum	Isolated strains	2
Acinetobacter baumannii	Isolated strains	1
Burkholderia cepacia	Isolated strains	1
Citrobacter freundii	ATCC 43864	1
	ATCC 8090	1
Citrobacter braakii	Isolated strains	1
Citrobacter youngae	Isolated strains	1
Clostridium difficile	ATCC BAA-1803	1
Enterococcus faecalis	Isolated strains	1
Escherichia coli	ATCC 25922	1
Klebsiella pneumoniae	Isolated strains	1
Klebsiella rhinoscleromatis	Isolated strains	1
Listeria monocytogenes	EDG-e	1
Morganella morganii	Isolated strains	1
Plesiomonas shigelloides	Isolated strains	1
Pseudomonas aeruginosa	ATCC 15442	1
Salmonella enteritidis	Isolated strains	1
Serratia marcescens	Isolated strains	1
Shigella dysenteriae	Isolated strains	1
Shigella flexneri	Isolated strains	1
Staphylococcus aureus	Isolated strains	1
Staphylococcus epidermidis	Isolated strains	1
Staphylococcus haemolyticus	Isolated strains	1
Streptococcus bovis	Isolated strains	1
Streptococcus pneumoniae	ATCC 49619	1
Streptococcus pyogenes	Isolated strains	1
Streptococcus sanguis	Isolated strains	1
Streptococcus suis	GZ1	1
Yersinia enterocolitica	Isolated strains	1

Table 2
Primers used in this study.

Primer	Sequence (5′-3′)	Length[a]
F1	GGCTCCACCATGACGC	16 nt
F2	CGCAATGACGTTAGAGATGT	20 nt
CP1	TTGATGCGCACTGCGCCGTAGCTTTCCCTAGGCATCCTCA	40 mer
CP2	GTCGTGGCTGCGGGCACGAAGGCGAGTGTGTG	32 mer
C1	TTGATGCGCACTGCGCCGTAG	21 nt
C2	GTCGTGGCTGCGGGCA	16 nt
D1	TACATCGCTACGGCTAC	17 nt
D2	ACGGACTTGCAGGAAGCT	18 nt
R1	GTGACTTTAAAGAAGGTGC	19 nt
R2	CGGCATCTTGCTGGTC	16 nt

[a] nt, nucleotide; mer, monomeric.

2.5. Standard C. striatum–MCDA-VR assay

The MCDA reaction was performed in a final volume of 25 μL based on a protocol described in previous studies (Wang et al., 2015). In brief, each reaction contained 0.6 μM each of displacement primers F1 and F2, 2.4 μM each of cross primers CP1 and CP2, 1.2 μM each of amplification primers C1, C2, D1, D2, R1 and R2, 12.5 μL 2× reaction mix (Isothermal® Amplification Kit), 1.25 μL of *Bst 2.0* DNA polymerase (10 U; New England Biolabs, USA), 1 μL colorimetric indictor (VR), and 1 μL template DNA.

The isothermal amplification reaction products were detected by real-time turbidimeter (Loopamp, LA-320c), colorimetric indicator, and 2% gel electrophoresis. When employing the VR, the positive reaction mixture containing the amplicons changed to bright blue, while the negative control and blank control changed to colorless.

The MCDA reaction mixtures were incubated isothermally for 1 h and then heated to 85 °C for 5 min to stop the reaction. Mixtures with 1 μL genomic DNA of *C. simulans* and *C. propinquum* were used as

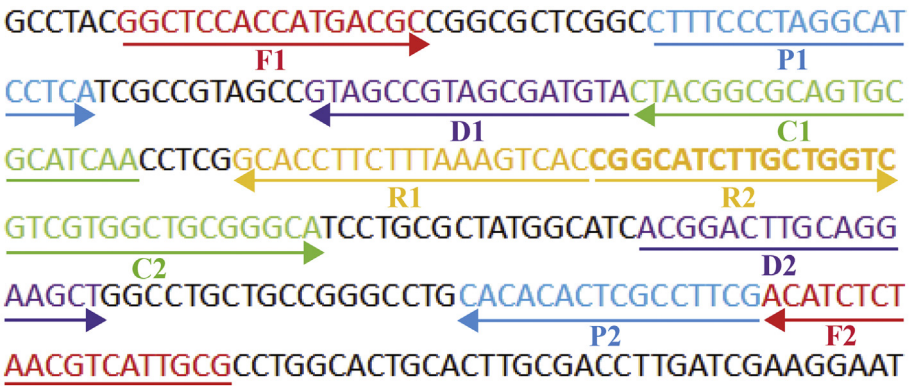

Fig. 1. Sequence of *ftr1* gene used to design multiple cross displacement amplification (MCDA) primers. The nucleotide sequence of the sense strand of the *ftr1* gene is shown. Right-pointing arrows and left-pointing arrows indicate sense and complementary sequences that were used, respectively.

negative controls, and mixture with 1 μL double distilled water (DDW) was chosen as a blank control.

2.6. Sensitivity and specificity of C. striatum-MCDA assay

To determine the limit of detection (LoD), serial dilutions of *C. striatum* ATCC 6940 genomic DNA (1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg per microliter) were tested as templates. The sensitivity of the *C. striatum*–MCDA assay was examined by the three methods mentioned above. The specificity of the MCDA assay was analyzed with DNA templates from 131 bacterial strains (Table 1). All the experiments mentioned above were repeated at least twice.

2.7. Application of C. striatum-MCDA-VR assay to clinical sputum samples

After the *C. striatum*-MCDA-VR assay was established, 84 clinical sputum samples were used to evaluate its applicability, and the results were compared with both SSTs and PCR. The clinical sputum samples were collected from 84 elderly hospitalized patients with an average age of 83.3 years, who had clinical symptoms of respiratory infection, such as fever and cough. Each sputum sample tested was divided into two parts: one part was used to perform a SST directly, and the other was used for DNA extraction according to the technical manual of the DNA purification kit after digestion by 4% sodium hydroxide solution. The resultant DNA solutions were stored at −20 °C and used for *C. striatum*-MCDA-VR assay and PCR.

2.8. Ethics statement

This study was approved by the Research Ethics Committee of the Chinese Center for Disease Control and Prevention. All experiments were conducted according to relevant regulations.

3. Results

3.1. Confirmation and detection of C. striatum-MCDA-VR products

To confirm the effectiveness of the *C. striatum*-MCDA primers (Table 2), *C. striatum* MCDA assays with DNA from pure cultures were performed at 68 °C for 1 h. Three monitoring techniques—real-time turbidimetry, a colorimetric indicator and 2% agarose gel electrophoresis—were employed to verify the *C. striatum*-MCDA-VR assay. As shown in Fig. 2, positive amplification only occurred in the tube with DNA from *C. striatum* ATCC 6940, and not in the negative controls (with *C. simulans* or *C. propinquum* DNA) or the blank control (DDW).

3.2. Optimal reaction temperature for C. striatum-MCDA-VR assay

To determine the optimum amplification temperature, *C. striatum* ATCC 6940 was used (10 pg genomic DNA per reaction). Reactions were conducted at 60–69 °C with 1 °C increments and were monitored by real-time turbidity. According to data generated, 68 °C was determined to be the most suitable temperature for the *C. striatum*-MCDA-VR assay, because it showed fast amplification and a high level of products (Fig. 3).

3.3. Sensitivity of C. striatum-MCDA-VR assay

Serial dilutions of genomic DNA from *C. striatum* ATCC 6940 were used to determine the LoD of the *C. striatum*-MCDA-VR assay. Ultimately, 10 fg of template DNA was confirmed as the LoD by the three monitoring methods employed in this study (turbidity, VR and electrophoresis) (Fig. 4).

All the experiments mentioned in this section were repeated at least twice.

3.4. Optimal reaction time for C. striatum-MCDA-VR assay

With 10 fg genomic DNA template, 30 min was sufficient for the amplification (Fig. 5). However, the presence of adverse factors in sputum samples (such as residual sodium hydroxide from specimen processing) may delay the amplification (Liu et al., 2018). Therefore, we recommend 40 min as the reaction time for the *C. striatum*-MCDA-VR assay to avoid false negative results.

3.5. Specificity of C. striatum-MCDA-VR assay

In total, pure cultures of 131 strains, including 100*C. striatum* strains and 31 other bacteria (Table 1), were tested by the *C. striatum*-MCDA-VR assay developed in this study. Only *C. striatum* strains gave positive results; non−*C. striatum* strains and the blank control exhibited negative results (Fig. 6).

3.6. Application of C. striatum-MCDA-VR assay to clinical sputum samples

To demonstrate the feasibility of the *C. striatum*-MCDA-VR assay as a reliable detection method for *C. striatum* in clinical specimens, we analyzed 84 sputum samples by SST, PCR and *C. striatum*-MCDA-VR assay, in parallel. Of the 84 sputum samples, 71 gave consistent results by the three methods, including SST^+ PCR^+ $MCDA^+$ ($n = 48$) and SST^- PCR^- $MCDA^-$ ($n = 23$). However, 13 sputum samples did not give consistent results: SST^+ PCR^- $MCDA^+$ ($n = 6$), SST^+ PCR^- $MCDA^-$ ($n = 4$) and SST^- PCR^+ $MCDA^+$ ($n = 3$).

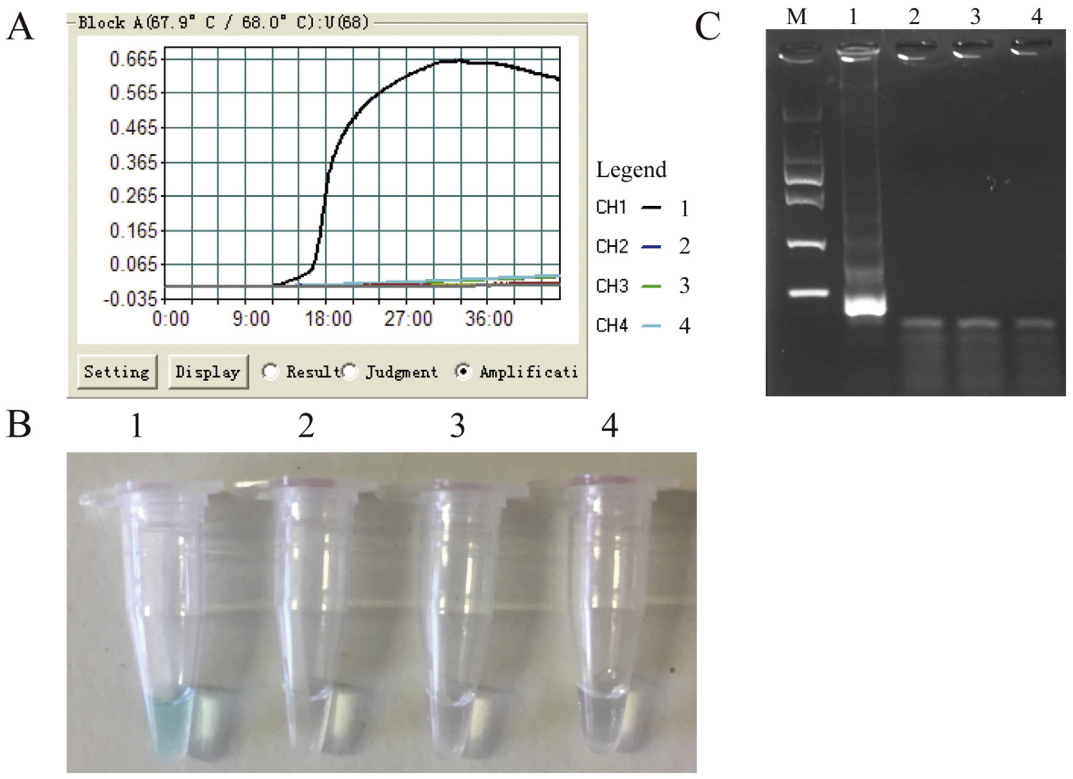

Fig. 2. Confirmation and detection of *Corynebacterium striatum*-MCDA-VR products. (A) The amplified products of the *C. striatum*-MCDA-VR assay were monitored by real-time measurement of turbidity. Turbidity > 0.1 was considered to be positive. (B) Using visual detection reagent (VR), positive amplification resulted in bright blue samples (tube 1), and negative samples were colorless (tubes 2–4). (C) Positive amplification products showed characteristic ladder bands by agarose gel electrophoresis. 1, positive amplification (*C. striatum* ATCC 6940); 2, negative control (*C. simulans*); 3, negative control (*C. propinquum*); 4, blank control (double distilled water; DDW). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

C. striatum is one of the commonly isolated coryneform bacteria in clinical microbiology laboratories (Martinez-Martinez et al., 1995), and is capable of causing serious infections in humans (Ishiwada et al., 2016). Because of the increasing number of reports of outbreaks of multidrug-resistant *C. striatum* infection and the growing incidence of *C. striatum* cases, a rapid and accurate diagnostic method for *C. striatum* is urgently required. In the present study, we established an isothermal amplification-based method, the *C. striatum*-MCDA-VR assay, for detection of *C. striatum*. It was able to detect *C. striatum* from pure cultures and sputum specimens simply, rapidly and cost-effectively. The *C. striatum*-MCDA-VR primers were designed based on *ftr1*, a *C. striatum*-specific gene (Santos et al., 2017).

The specificity of the *C. striatum*-MCDA-VR assay was demonstrated. *C. simulans* and *C. striatum* are biochemically and morphologically very similar and share 98% 16S rRNA gene sequence homology (McMullen et al., 2017; Wattiau et al., 2000), which causes difficulty in distinguishing between them. The *C. striatum*-MCDA-VR assay could differentiate these two organisms efficiently, with high selectivity.

In addition to excellent specificity, the newly developed *C. striatum*-MCDA-VR assay was able to detect as little as 10 fg of genomic DNA

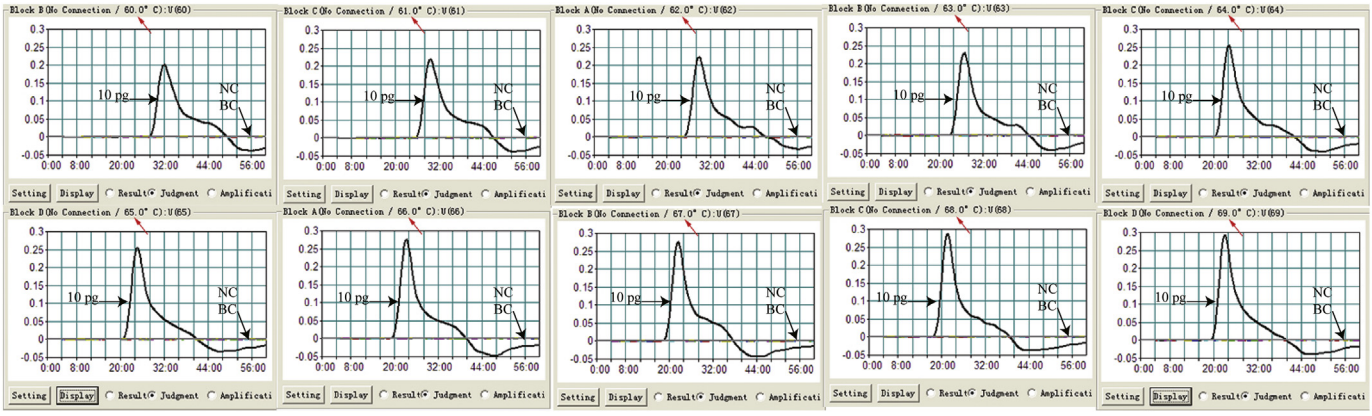

Fig. 3. Optimal reaction temperature for *C. striatum*-MCDA-VR assay. MCDA reactions for detection of *C. striatum* were monitored by real-time measurement of turbidity and the corresponding curves for concentrations of DNA are shown in the figure. Turbidity > 0.1 was considered positive. Ten kinetic graphs were generated at various temperatures (60 to 69 °C, 1 °C intervals) with target pathogen DNA at 10 pg per reaction. DNAs of *C. simulans* and *C. propinquum* were used as negative controls, and DDW was used as the blank control. NC, negative control; BC, blank control.

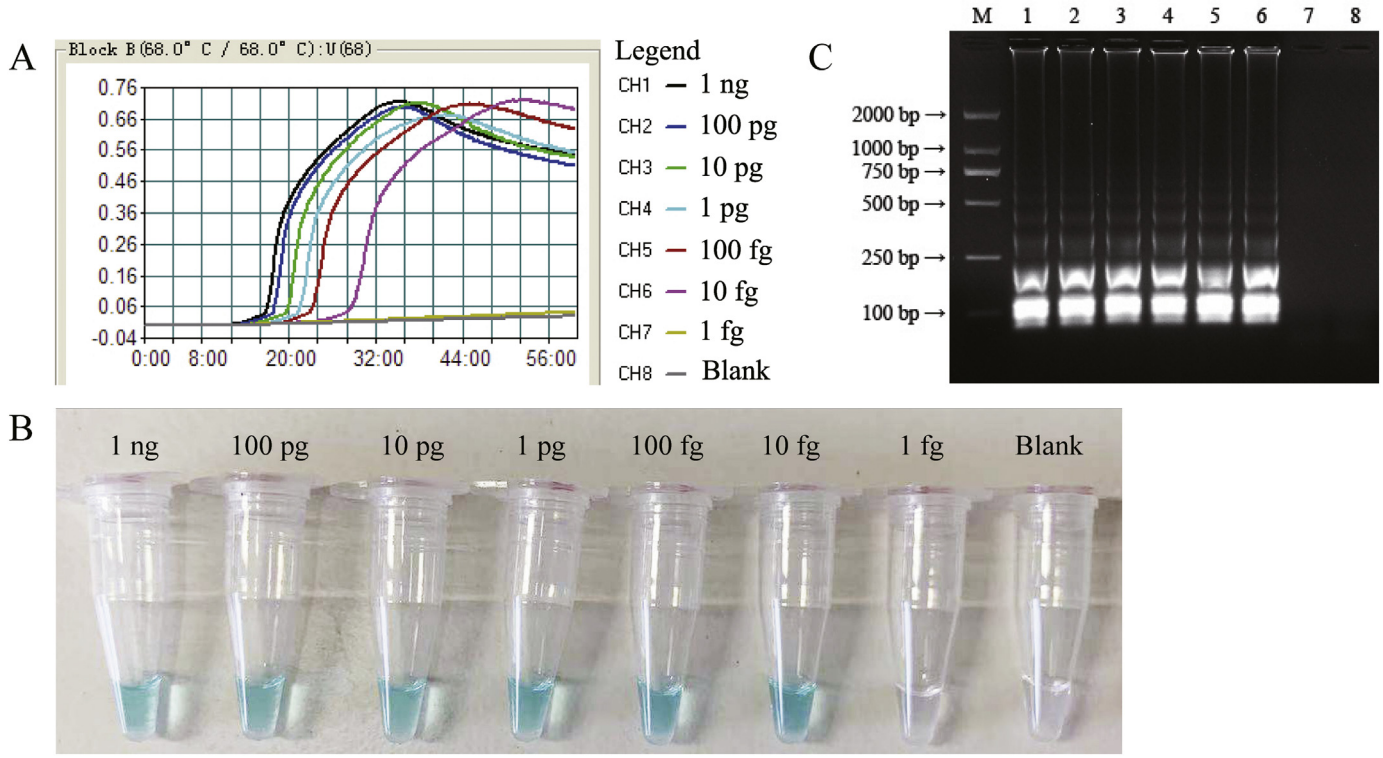

Fig. 4. Analytical sensitivity of *C. striatum*-MCDA-VR assay using serially diluted genomic DNA of *C. striatum* ATCC 6940. Three monitoring methods, real-time turbidity (A), VR (B) and gel electrophoresis (C), were applied to analyze the reaction products. Serial dilutions (1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg) of DNA template were subjected to standard MCDA reactions. (C) Lanes 1–8 represent 1 ng to 1 fg of DNA per reaction and a blank control (DDW), respectively. Genomic DNA at ≥10 fg per reaction showed positive results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

extracted from a pure culture (Fig. 4). The *C. striatum*-MCDA-VR assay was thus 10^6-fold more sensitive than the PCR method, which needed at least 15 ng of template DNA per reaction (Santos et al., 2017). Furthermore, the amplification products could be assessed using a colorimetric detection reagent (bright blue for positive samples, colorless for negative); therefore, a heat block or water bath maintaining a constant temperature of 68 °C, which is available in most laboratories, was sufficient for the assay. The MCDA test can be performed using commercial kits (such as Isothermal® Amplification Kits and Eiken Loopamp Kits), and each MCDA reaction costs < 5 USD. The entire procedure including specimen processing and DNA extraction (45 min), as well as isothermal amplification and result assessment (40 min), could be completed within 85 min.

Identification of *Corynebacterium* spp. to the species level can be difficult, but is necessary in clinical specimens (Funke et al., 1997). Additionally, it is worthwhile identifying coryneform bacteria to the species level to detect and describe unknown species, or to attribute potential pathogenicity to those organisms which were hitherto thought nonpathogenic (von Graevenitz et al., 1994). In clinical microbiology laboratories, the identification of *Corynebacterium* spp. in sputum samples usually relies on SSTs and culture-based methods, such as MALDI-TOF MS and RapID CB Plus. However, according to previous

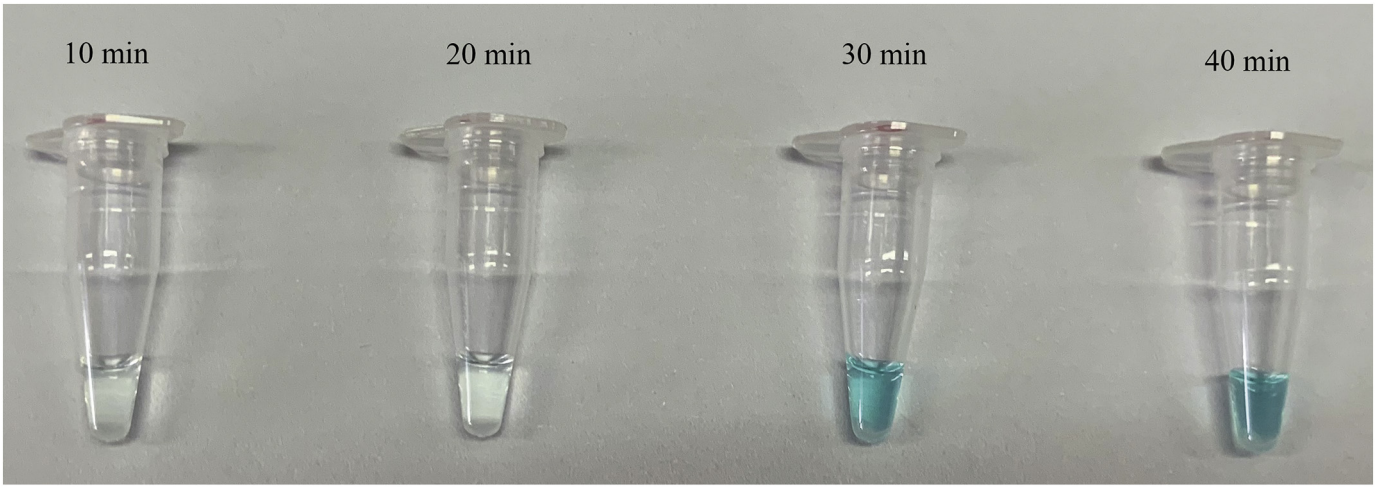

Fig. 5. Optimal reaction time for *C. striatum*-MCDA-VR assay. In this assay, 10 fg genomic DNA of *C. striatum* ATCC 6940 (the LoD of the *C. striatum*-MCDA-VR assay) were used. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

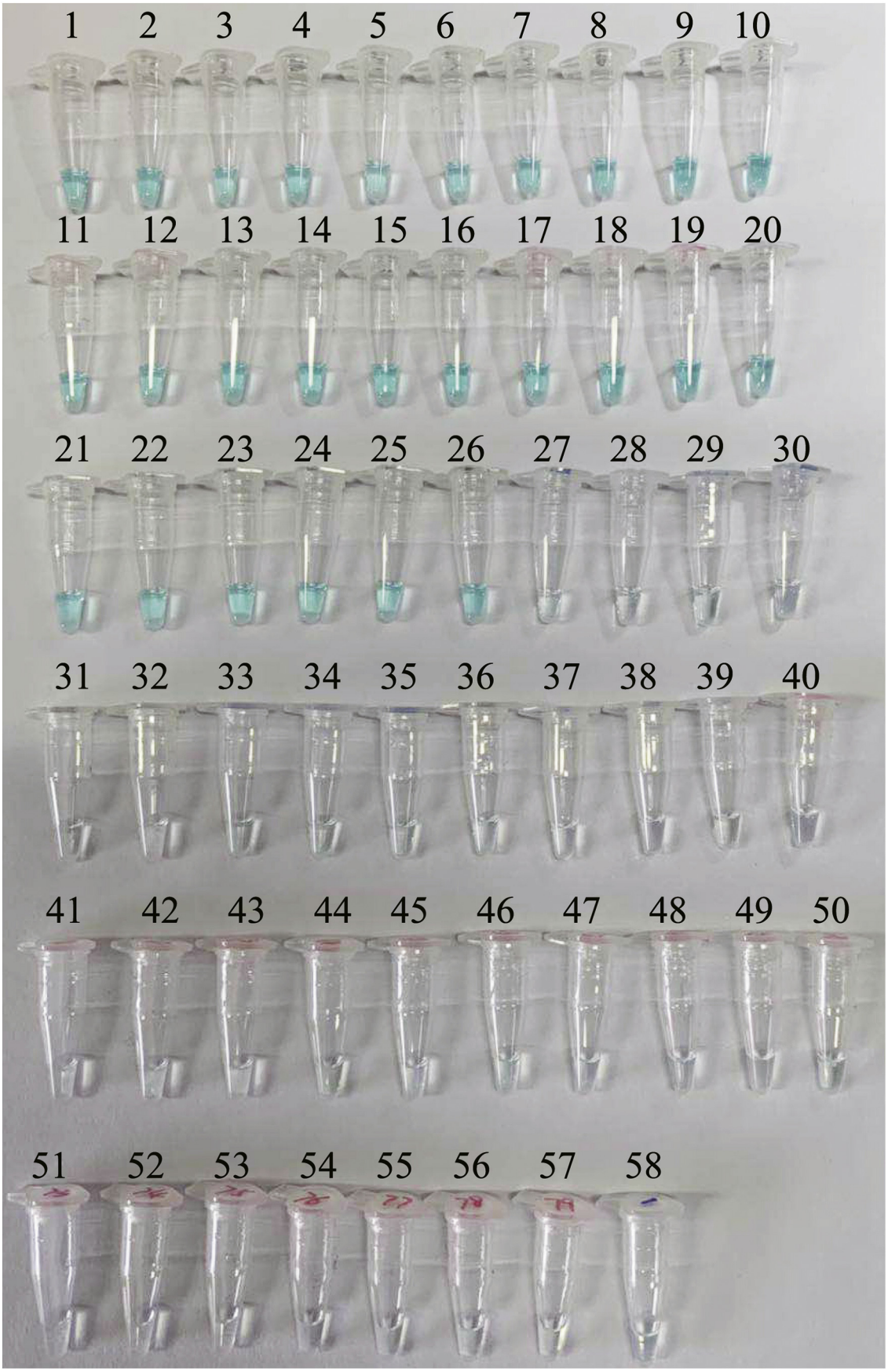

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Fig. 6. Analytical specificity of *C. striatum*-MCDA-VR assay. MCDA reactions were carried out using different DNA templates and analyzed by means of VR. Tube 1, *C. striatum* reference strain ATCC 6940; Tubes 2–26, *C. striatum* clinical isolates; Tube 27, *C. simulans*; Tubes 28–29, *C. propinquum*; Tubes 30–57, *Acinetobacter baumannii*, *Burkholderia cepacia*, *Citrobacter freundii* (ATCC 43864), *Citrobacter freundii* (ATCC 8090), *Citrobacter braakii*, *Citrobacter youngae*, *Clostridium difficile*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella rhinoscleromatis*, *Listeria monocytogenes*, *Morganella morganii*, *Plesiomonas shigelloides*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Serratia marcescens*, *Shigella dysenteriae*, *Shigella flexneri*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Streptococcus bovis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus sanguis*, *Streptococcus suis*, *Yersinia enterocolitica*; Tube 58, blank control (DDW). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

study (McMullen et al., 2017), *C. tuberculostearicum* and *C. simulans* could be misclassified as *C. striatum* by these methods. Additionally, because a SST requires rich experience and is subjective, it may also result in misidentification or missed detection. Moreover, some organisms probably cannot be observed because of the death of bacteria after application of antibiotics (Hu et al., 2019) or during long term storage (Liu et al., 2018). Molecular methods provide rapid and accurate alternative options without subjective factors and culture-limited conditions. In the present study, the three techniques used (SST, MCDA and PCR) did not give concurrent results for 15.5% (13/84) of the sputum samples. So, we attempted to isolate and culture *C. striatum* strains on Columbia blood plates at 37 °C. As a result, *C. striatum* strains were isolated successfully from nine samples (SST^+ PCR^- $MCDA^+$ and SST^- PCR^+ $MCDA^+$), and not from the other four samples (SST^+ PCR^- $MCDA^-$). Our results thus indicated that the *C. striatum*-MCDA-VR assay was more sensitive than the PCR method, as well as more effective and more accurate than SSTs. Additionally, colorimetric detection of MCDA amplicons did not require expensive instruments or well-trained personnel. Hence, the *C. striatum*-MCDA-VR assay developed in this study is an excellent option to identify *C. striatum* strains and diagnose *C. striatum* infections.

5. Conclusions

In the present study, we established an isothermal amplification-based method, the *C. striatum*-MCDA-VR assay, to detect *C. striatum* in pure cultures and clinical sputum samples. *C. striatum* could be identified visually through simple color changes in a short time without sophisticated instruments or well-trained personnel. As a fast, sensitive and specific method, the *C. striatum*-MCDA-VR assay can be considered a high-efficiency alternative tool for screening of *C. striatum* isolates in clinical and environmental specimens in clinical and microbiological laboratories, especially in resource-limited settings.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105675.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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