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ORIGINAL RESEARCH

Rapid simultaneous detection of *bla*$_{oxa-23}$, *Ade-B*, *int-1*, and *ISCR-1* in multidrug-resistant *Acinetobacter baumannii* using single-tube multiplex PCR and high resolution melting assay

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Objective: The aim of this study was to develop a multiplex PCR system for the rapid and simultaneous detection of *bla*$_{oxa-23}$, *Ade-B, int-1,* and *ISCR-1* genes in multidrug-resistant *Acinetobacter baumannii* (MDRAB) using high resolution melting (HRM) assay.

Methods: Four pairs of primers were designed, and PCR amplification products were sequenced and compared with NCBI GeneBank sequences to ensure primer specificity. Multiplex PCR was performed using a dedicated HRM reagent, and melting curves and temperatures were able to distinguish the four genes. This method was subsequently used to detect these genes in 79 MDRAB isolates from the Third Affiliated Hospital of Southern Medical University in southern China.

Results: Using the HRM assay, 73 out of 79 isolates were found to carry both *bla*$_{oxa-23}$ and *Ade-B*, one isolate carried *int-1*, two isolates carried both *int-1* and *ISCR-1*, and three isolates carried *Ade-B, int-1*, and *ISCR-1*. No isolates carried all four genes.

Conclusion: Compared with traditional resistance gene detection methods–PCR and agarose gel electrophoresis-based resistance gene detection methods–the multiplex PCR and HRM assay method was simple, rapid, highly efficient, and cost-effective. Our results showed that *bla*$_{oxa-23}$ and *Ade-B* were the main resistance genotypes in MDRAB.

Keywords: *bla*$_{oxa-23}$, *Ade-B*, *int-1*, *ISCR-1*, multiplex PCR, high resolution melting

Introduction

Acinetobacter baumannii is an aerobic, non-fermenting, Gram-negative opportunistic pathogen that causes pneumonia, secondary meningitis, and a variety of other infections following incisions or wounds to the skin and soft tissues.[1–3] The ability of *A. baumannii* to survive under a wide range of environmental conditions for a long time ensures this species is a prevalent nosocomial pathogen worldwide, especially in the intensive care, neurosurgery, and burns departments of hospitals.[4–6] Owing to the ability of *A. baumannii* to develop and/or acquire a variety of resistance mechanisms, currently susceptible bacteria may soon evolve into multidrug-resistant *A. baumannii* (MDRAB) that are resistant to a wide variety of antibacterial agents including β-lactams, cephems, carbapenems, aminoglycosides, tetracyclines, fluoroquinolones, and folate pathway inhibitors.[7] MDRAB infections can ultimately lead to increased morbidity and mortality.

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Genomics analysis revealed that MDRAB contain several large resistance islands that contain 24 genes associated with antibiotic resistance and 16 genes associated with resistance to heavy metal salts or quaternary ammonium compounds commonly used in disinfectants. These resistance genes were reportedly acquired from *Pseudomonas aeruginosa, Salmonella*spp., *Escherichia coli*, and other Gram-negative bacteria.[8] Multiple mechanisms are involved in resistance in *A. baumannii*, including the production of inactivating enzymes, deletion, or mutation of outer membrane proteins, expression of multidrug efflux systems, changing or protecting the target sites of antibacterial agents, and transferring resistance genes through mobile genetic elements.[9] The $bla_{\text{oxa-23}}$ gene is the most common carbapenem resistance gene in MDRAB clinical isolates from most regions of the world. This gene encodes a carbapenem-hydrolyzing class D oxacillinase, and production of this enzyme is not inhibited by clavulanic acid.[10–12] Multidrug efflux systems such as *Ade-ABC* have a tripartite structure comprising an inner membrane component (*AdeA*), an antibacterial agent transport component (*AdeB*), and an outer membrane component (*AdeC*). Resistance to β-lactams, erythromycin, aminoglycosides, tetracyclines, and chloramphenicol is dependent on overexpression of *Ade-ABC*.[13–15] The class 1 integron *int-1* is a three-part bacterial mobile genetic element found in *A. baumannii*. The 5′ conserved region of *int-1* encodes a site-specific integrase, the central variable region encodes several gene cassettes, and there is also a 3′ conserved region.[16] The class 1 integron can capture resistance genes and integrate the associated gene cassettes into the *A. baumannii* genome to generate MDRAB.[17] Insertion sequence common region 1(*ISCR-1*) is a special mobile genetic element that is similar to IS91-like and shares a sustaining transpose rolling-circle mechanism.[18] Like the class 1 integron, *ISCR-1* has the ability to capture resistance genes from other bacteria.[19] The ability to detect *OXA-23, Ade-ABC*, class 1 integron, and *ISCR-1* resistance genes is crucial for identifying and treating MDRAB.

Recent improvements in the molecular methods used to detect resistance genes have decreased turnaround time and increased the specificity and sensitivity of testing procedures.[20,21] There are existing molecular assays that can detect $bla_{\text{oxa-23}}$, *Ade-B, int-1*, and *ISCR-1*, but these are time-consuming and unable to detect all four resistance genes in a single assay.[19,22] Although detection of these genes may not revolutionize clinical treatment, their identification will assist epidemiological investigations and help to control hospital-acquired infections. Our objective was to develop a single-tube multiplex PCR assay that can detect these four genes simultaneously using a dedicated high resolution melting (HRM) assay reagent. The established method was used to characterize the four resistance genes in MDRAB isolates collected in 2014 from the Third Affiliated Hospital of Southern Medical University in southern China.

Materials and methods

Definition of MDRAB

MDRAB is defined as non-susceptible to at least one agent in ≥3 antimicrobial categories of aminoglycosides, antipseudomonal carbapenems, antipseudomonal fluoroquinolones, antipseudomonal penicillins + β-lactamase inhibitors, extended-spectrum cephalosporins, folate pathway inhibitors, penicillins + β-lactamase inhibitors, and tetracyclines.[23]

Bacterial strains and antibiotic susceptibility testing

After exclusion of strains isolated repeatedly from one patient, a total of 87 MDRAB strains were isolated from various clinical specimens at the Third Affiliated Hospital of Southern Medical University, a comprehensive teaching hospital in southern China. All isolates were identified and tested for drug sensitivity using Microscan Walkaway 40 Plus (Beckman Coulter, West Sacramento, USA), and the results were interpreted according to the Clinical and Laboratory Standards Institute 2017 guidelines.

DNA isolation

Total DNA was extracted from well-established colonies using the Rapid Ezup Column Genomic DNA Extraction Kit (Sangon Biotech, Shanghai, China) according to the recommendations of the manufacturer. DNA was eluted with 100 μL CE buffer and stored at –80°C.

Primer design

With reference to sequences from the NCBI database (http://www.ncbi.nlm.nih.gov/gene/), we designed two pairs of primers for each of the four genes, one of which was used for preliminary screening of the target gene, and another that was used to establish the multiplex PCR assay. The four sets of primers used for multiplex PCR were designed to have a similar annealing temperature, and to amplify predicted products of different sizes and melting temperatures (Table 1).

Table 1 Primers used in this study

Primer name	Sequence(5′→3′)[a]	Primer T_m (°C)[b]	Product size[b]	Product predicted T_m (°C)[b]	Product actual T_m (°C)[c]
Preliminary primers					
OXA-23 F	AGGTCATTTACCGCTTGG	54.1	396 bp	ND	ND
OXA-23 R	TCCATCTGGCTGCTCAAC	53.1			
Ade-B F	AGATTTCAAAGAGCGGACTA	52.2	263 bp	ND	ND
Ade-B R	CTTGTGGCAACCCTTCAT	53.3			
int-1 F	GCAAGGTTTCGGTCTCCAC	57.5	477 bp	ND	ND
int-1 R	AGAACCACGGCCAGGAAT	57.2			
ISCR1 F	AATCGCCCACTCAAACAA	54.1	576 bp	ND	ND
ISCR1 R	CATCTTCGGCATAGACACC	53.2			
Primers for establishing the multiplex PCR with HRM assay					
OXA-23 F	AAACGTATTGGTTTCGGTAAT	54.0	139 bp	82.0	79.5
OXA-23 R	TTTCACTAAATGGAAGCTGTG	53.3			
Ade-B F	TATTGGCTACGAGTGGACAG	53.5	390 bp	85.1	82.4
Ade-B R	CCACAGGTAAATGCAAGTGA	54.1			
int-1 F	CTTACGAACCGAACAGGC	53.6	234 bp	91.5	90.3
int-1 R	CGAGGTCTTCCGATCTCC	54.5			
ISCR1 F	CGCTAAATCTCAATGTCCAC	53.1	187 bp	88.6	87.1
ISCR1 R	GCATCACGCTCCAAAATC	54.0			

Notes: [a] The sequence of primer was designed by using primer premier 5.0 software.[b] The primer T_m, product size and product predicted T_m were provided by primer premier 5.0 software.[c] The product actual T_m were observed in this assay.
Abbreviation: ND, not determined.

Preliminary screening of bla$_{oxa-23}$, *Ade-B*, int-1, and ISCR-1

Four sets of preliminary primers were used to characterize the four genes in the 8 of 87 MDRAB isolates. Each 20 μL PCR contained 10 μL of 2× PCR master mix (Generay Biotech, Shanghai, China), 0.6 μL of 10 μM forward and reverse primers, 1.4 μL of template DNA, and 7.4 μL of double-distilled water. Amplification was performed using the following conditions: initial denaturation at 95°C for 3 mins, followed by 40 cycles of denaturation (95°C, 30 s), annealing (56°C for *bla*$_{oxa-23}$, 55°C for *Ade-B*, 59°C for *int-1*, 56°C for *bla*$_{oxa-23}$, 45 s), extension (72°C, 1 min), and a final extension at 72°C for 5 mins. To validate the PCR products they were separated by agarose gel electrophoresis, stained with GoldView ™(nontoxic) and photographed on a UV transilluminator.

DNA sequencing and actual melting temperature (t_m) determination

To ensure the specificity of the primers used for the multiplex PCR with HRM assay, the four sets of the primers were used to detect the four genes that were amplified using the preliminary primers. Reactions were similar to the preliminary screening conditions but contained 10 μL of 2× HRM FAST PCR master mix (Kapa Biosystems, Cape Town, South Africa), 0.6 μL of 10 μM forward and reverse primers, 1.4 μL of template DNA, 2 μL of 25 mM $Mgcl_2$, and 5.4 μL of double-distilled water. Amplifications were performed as described above but with an annealing temperature of 56°C for all four sets of primers. PCR products were separated by agarose gel electrophoresis and sequenced, and data were analyzed using the Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

In addition to determining the actual melting temperature (T_m) using HRM analysis with the LightScanner System (Idaho Technology Inc., Cape Town, South Africa), melting curves were performed by measuring the decrease in fluorescent signal with increasing temperature by ramping at 0.02°C/s with 25 acquisitions/°C. T_m is defined as the temperature at which 50% of the amplified product dissociated into single-stranded DNA, and the actual T_m of the four genes was used as a reference to determine which gene was present in amplification reactions from the isolates.

Development of the multiplex PCR and HRM assay

Template DNA used in multiplex PCR was a mixture of isolates, and these were tested for the presence of *bla*$_{oxa-23}$, *Ade-B, int-1*, and *ISCR-1*. Multiplex PCR was performed in single-tube 20 μL reactions containing 10 μL of 2× HRM FAST PCR master mix, 0.4 μL of 10 μM forward and reverse primers (total of 3.2 μL), 1.4 μL of compound template DNA, 2 μL of 25 mM $Mgcl_2$, and 3.4 μL of double-distilled water. To ensure the sensitivity and specificity of multiplex PCR, annealing was tested at 54°C, 56°C, 58°C, and 60°C. All other reaction conditions were as described above. Melting curves from all multiplex PCR amplification products were generated using a LightScanner System, and products were separated by agarose gel electrophoresis following HRM analysis.

Screening of clinical isolates

Seventy-nine of the remaining 87 MDRAB strains were screened for the presence of the four genes using the multiplex PCR and HRM assay described above. We also tested for the presence of the four genes using traditional PCR and agarose gel electrophoresis-based methods (one PCR reaction detecting one gene) to verify the accuracy of the multiplex PCR and HRM assay.

Results

Preliminary screening for bla$_{oxa-23}$, *Ade-B*, int-1, and ISCR-1

Preliminary screening of eight MDRAB isolates for the four genes was performed to provide positive controls for developing multiplex PCR of unknown samples. Agarose gel electrophoresis demonstrated the presence of *Ade-B, int-1,* and *ISCR-1* in strain 1, and *bla*$_{oxa-23}$ and *Ade-B* in all of the other seven strains (Figure 1).

DNA sequencing and differentiation of the four genes using HRM curve analysis

Strains 1 and 2 were used as positive controls for establishing the multiplex PCR with HRM assay. Four sets of the primers were used to detect the four genes in these strains, and agarose gel electrophoresis resulted in bands of 139, 390, 234, and 187 bp as expected (Figure 2). The BLAST results confirmed that the sequences of the PCR amplification products were the same as those previously published.

The PCR amplification products were differentiated by HRM curve analysis. The presence of *bla*$_{oxa-23}$ was revealed by a single dominant peak with a melting temperature of 79.5°C in the melting curve analysis (Figure 3A), while *Ade-B int-1* and *ISCR-1* corresponded to single dominant peaks at 82.4°C, 90.3°C, and 87.1°C, respectively (Figure 3B–D). All melting

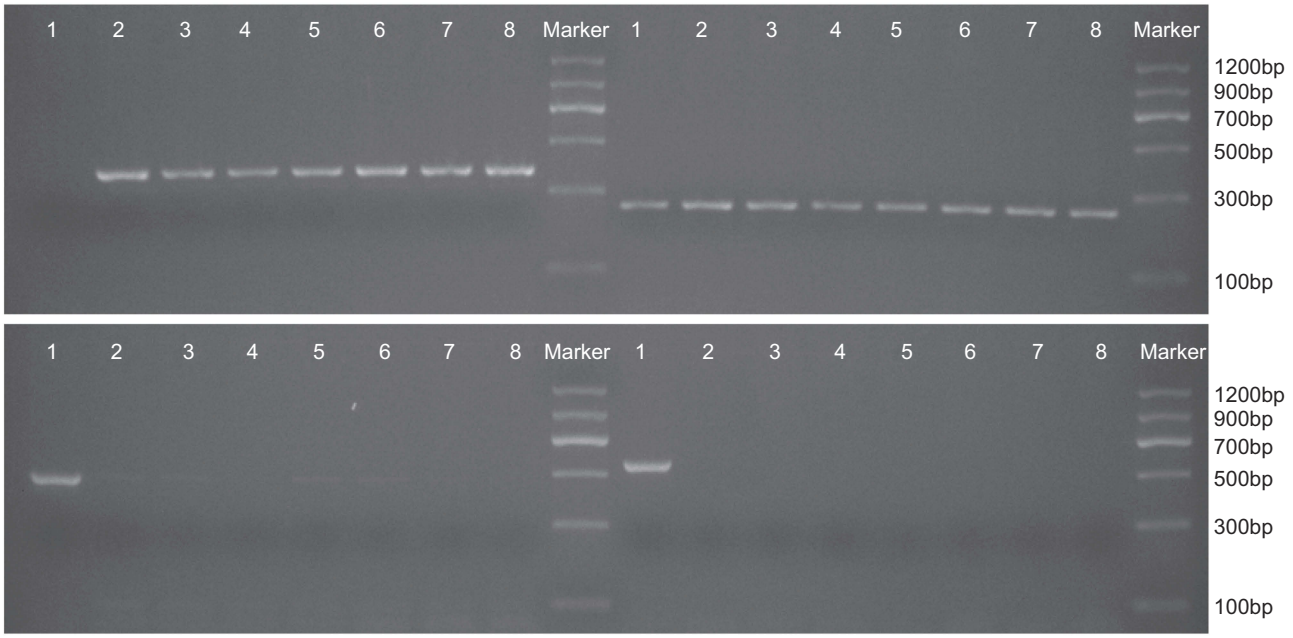

Figure 1 Agarose gel electrophoresis of the PCR amplification products of the four genes from eight MDRAB isolates using preliminary primers.

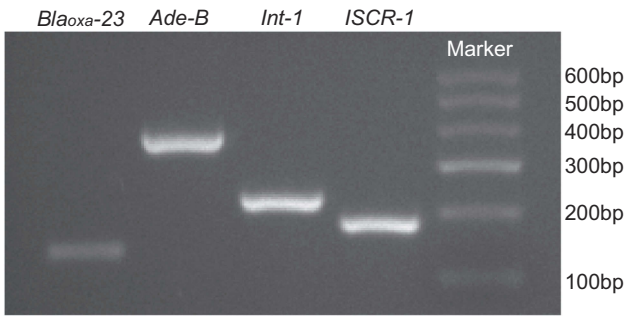

Figure 2 Agarose gel electrophoresis of PCR amplification products of the four genes using multiplex PCR primers.

temperatures were lower than predicted, and differences in the actual melting temperatures allowed the four genes to be easily distinguished by HRM curve analysis (Figure 4).

Development of the multiplex PCR and HRM assay

Multiplex PCR amplification products with different annealing temperatures were separated by agarose gel electrophoresis and photographed, and melting curve analysis was performed after electrophoresis. Multiplex PCR amplification generated four products with sizes corresponding to the four genes (Figure 5A). Additionally, HRM analysis showed that the melting curves of multiplex PCR amplification products produced four peaks with the same melting temperature as the four genes (Figure 5B–C). Agarose gel electrophoresis showed that the $bla_{\text{oxa-23}}$ amplification product decreased with increasing annealing temperature (Figure 5A), and the melting curve of $bla_{\text{oxa-23}}$ similarly decreased with increasing annealing temperature (Figure 5D). Since the $bla_{\text{oxa-23}}$ peak was the lowest of all four peaks, we selected 54°C as the most appropriate annealing temperature to improve the sensitivity of $bla_{\text{oxa-23}}$ detection in the multiplex PCR with HRM assay.

Screening clinical isolates for the presence of the four genes

Seventy-nine MDRAB clinical isolates were screened for the presence of the four genes using the multiplex PCR with HRM assay. From the melting curves, the HRM assay identified $bla_{\text{oxa-23}}$ and *Ade-B* in 73/79 isolates, while *int-1* was present one isolate, both *int-1* and *ISCR-1* were present in two isolates, and *Ade-B, int-1* and *ISCR-1* were present in three isolates (Figure 6). No isolates were found to be carried all four genes (Figure 6). The presence of the four genes detected using the multiplex PCR with HRM

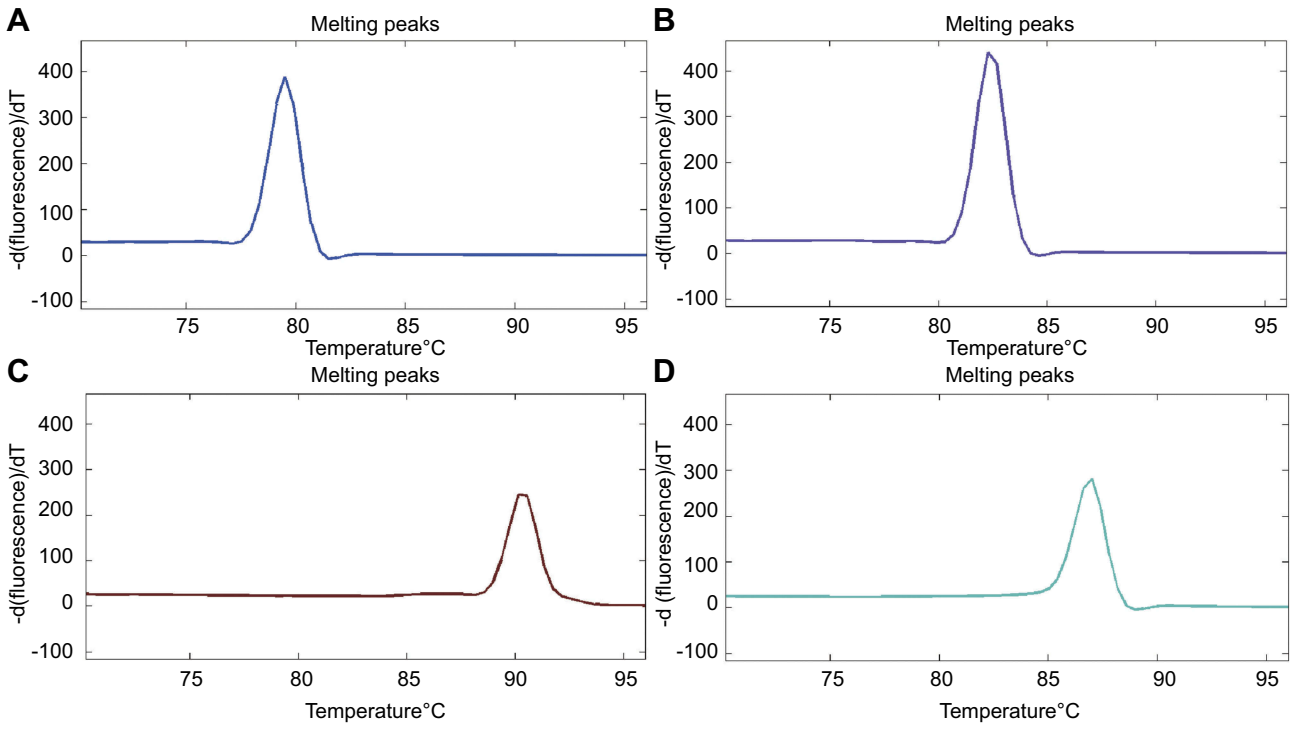

Figure 3 Melting curves of PCR amplification products of the four genes used in HRM analysis. –d[Fluorescence]/dT (*y*-axis) is plotted against temperature (C) on the *x*-axis. The melting curves of $bla_{\text{oxa-23}}$, *Ade-B, int-1*, and *ISCR-1* are shown in **A**, **B**, **C**, and **D**, respectively.
Abbreviation: HRM, high resolution melting.

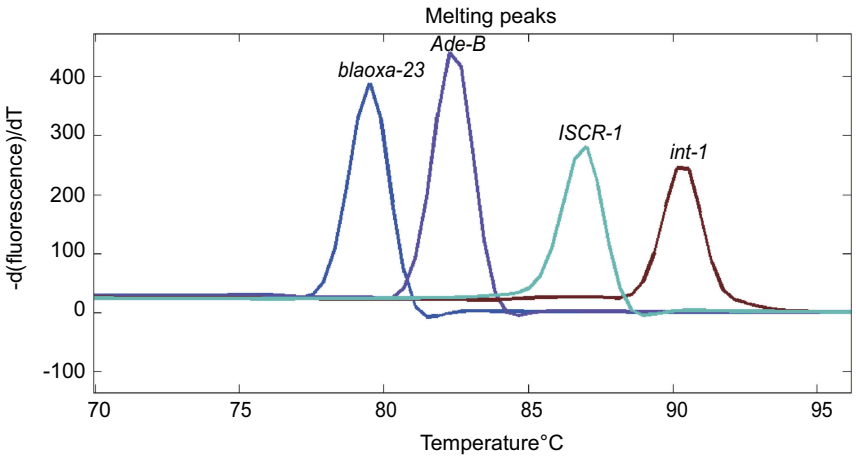

Figure 4 Melting curves of the four genes form HRM analysis plotted on the same graph.
Abbreviation: HRM, high resolution melting.

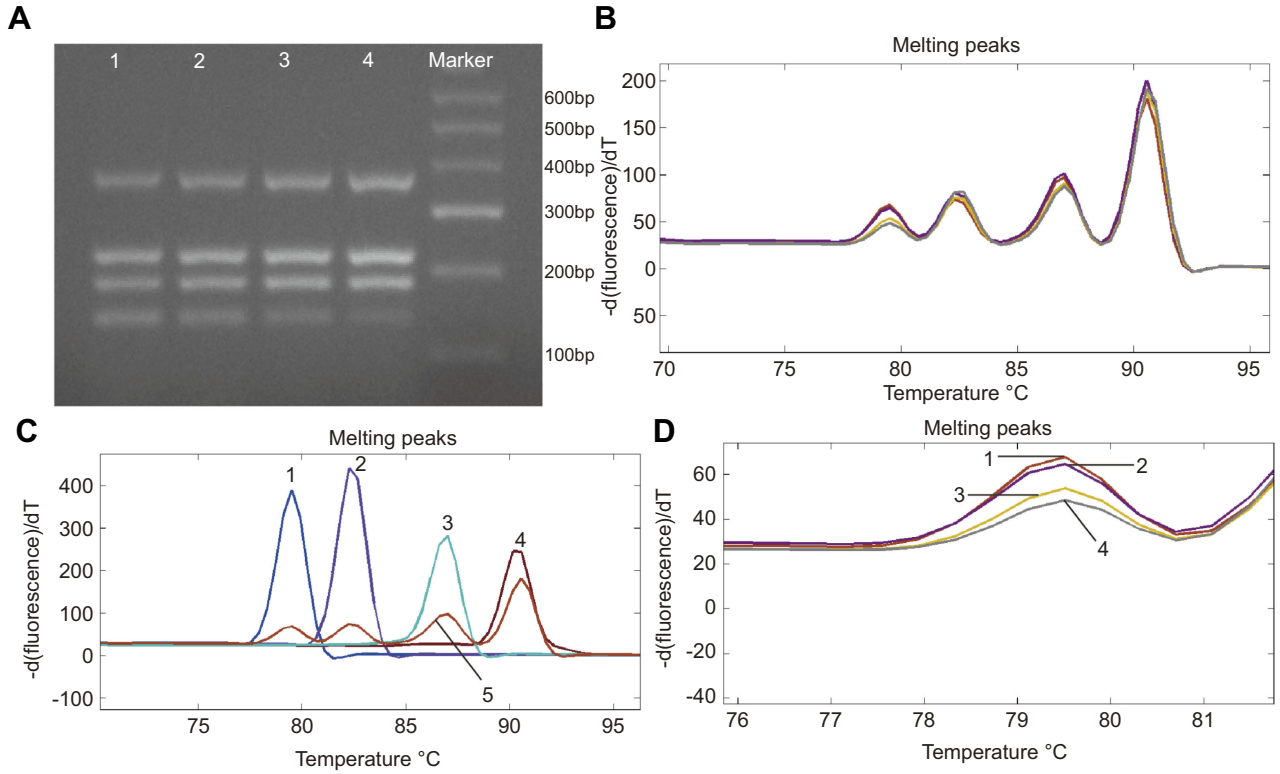

Figure 5 (**A**) Representative agarose gel electrophoresis of multiplex PCR products of the four genes with different annealing temperatures amplified in a single tube. 1–4= annealing temperatures of 54°C, 56°C, 58°C, and 60°C, respectively. (**B**) Representative melting curves of the single-tube multiplex PCR with HRM assay performed with different annealing temperatures. (**C**) Melting curves of the four genes (1–4) and that of the multiplex PCR with HRM assay (5) plotted on a single graph. (**D**) Representative enlargement of bla_{oxa-23} peaks from melting curves derived from a single-tube multiplex PCR with HRM assay with different annealing temperatures. 1–4= annealing temperatures of 54°C, 56°C, 58°C, and 60°C, respectively.

assay was consistent with data from traditional methods (Figure7, Table 2).

Discussion

A. baumannii causes nosocomial infections in hospitals and health care departments and is difficult to treat in multidrug-resistant organisms that often carry *OXA-23, Ade-ABC*, Class 1 integron, and *ISCR-1*.[9,24] Accurate and rapid detection of bla_{oxa-23}, *Ade-B, int-1*, and *ISCR-1* in clinical microbiology laboratories is important for prevention and treatment of MDRAB.[25] Conventional assays for screening these *genes* are expensive and time-consuming.

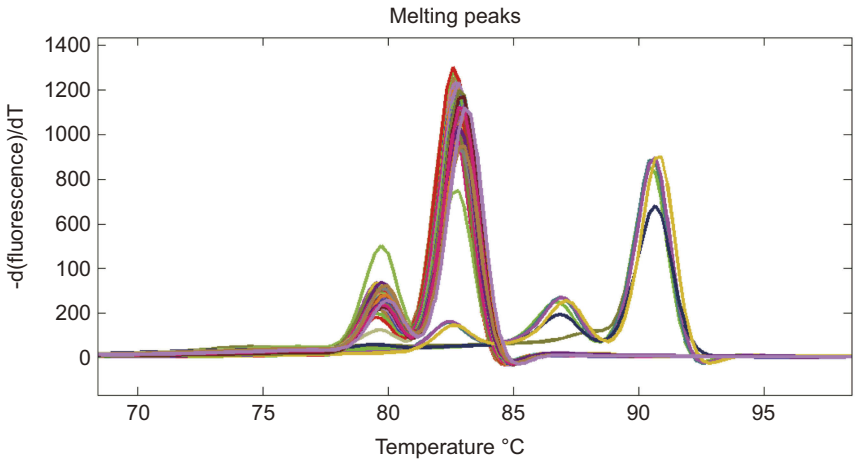

Figure 6 Melting curves of the four genes detected in 79 clinical isolates using the single-tube multiplex PCR with HRM assay.
Abbreviation: HRM, high resolution melting.

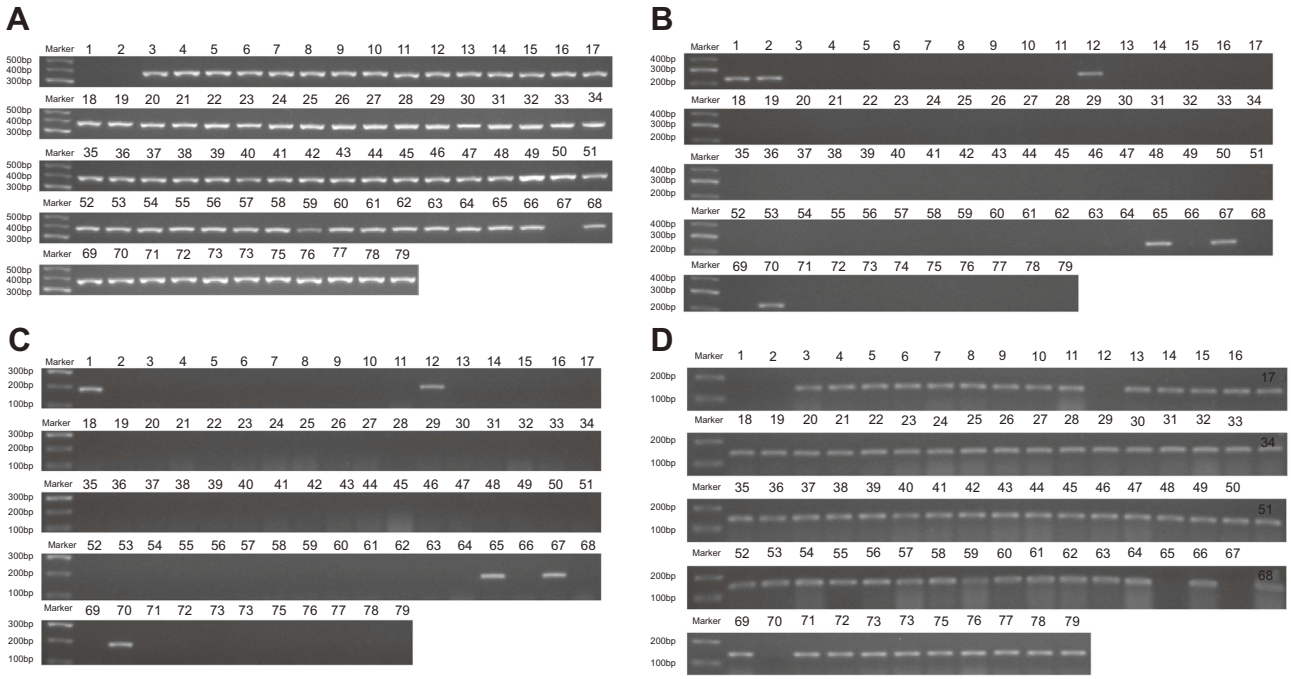

Figure 7 (**A**) Agarose gel electrophoresis of the resistance gene $bla_{oxa\text{-}23}$ in 79 clinical isolates. (**B**) Agarose gel electrophoresis of the resistance gene *int-1* in 79 clinical isolates. (**C**) Agarose gel electrophoresis of the resistance gene *ISCR-1* in 79 clinical isolates. (**D**) Agarose gel electrophoresis of the resistance gene *Ade-B* in 79 clinical isolates.

In contrast, HRM analysis is a rapid, cost-effective, and high-throughput method. We, therefore, sought to develop a rapid assay for detecting these genes without the need to perform agarose gel electrophoresis in this study.

We designed two sets of primers for each gene and PCR amplification products were sequenced to ensure the specificity of the multiplex PCR with HRM assay. The melting temperatures of the amplified products were different enough to allow us to distinguish between them.

Compared with traditional phenotypic assays, our multiplex PCR with HRM assay has a number of advantages; (1) It is significantly faster and more high-throughput. All four genes are detected simultaneously in the multiplex PCR with HRM assay in a single tube, minimizing contamination and improving efficiency since an agarose gel electrophoresis step is not required. The multiplex PCR with HRM assay could test 96 clinical isolates in 2 hrs 30 mins. (2) The novel assay is cost-effective. The approximate cost of this assay from DNA template extraction to

Table 2 Presence of the four genes in clinical isolates detected by multiplex PCR with HRM assay and traditional method

Gene	Isolates (Detection by multiplex PCR with HRM assay)	Isolates (Detection by traditional method)
bla_{oxa-23} and *Ade-B*	73	73
int-1	1	1
int-1 and *ISCR-1*	2	2
Ade-B, int-1 and *ISCR-1*	3	3
Total	79	79

Abbreviation: HRM, high resolution melting.

multiplex PCR with HRM analysis is $2 per sample for the detection of all four genes. (3) The novel assay is non-destructive and environmentally friendly since no harmful ethidium bromide is used because an agarose gel electrophoresis step is not performed.

The four genes are known to play an important role in the evolution if resistance in *A. baumannii*, and in MDRAB in particular. In the 79 clinical isolates tested, bla_{oxa-23} and *Ade-B* were the most common, in accordance with published literature.[26,27] Our results also demonstrate the co-existence of *ISCR-1* and *int-1* in bacteria. This may be related to the fact that ISCR1 can insert DNA into the 3′ conserved regions of class 1 integrons to generate complex class 1 integrons carrying the sul1, resX, and trIb resistance genes.[24,28]

In conclusion, we have developed a single-tube multiplex PCR with HRM assay which can reliably detect the presence of bla_{oxa-23}, *Ade-B, int-1*, and *ISCR-1* separately or in combination. This assay is rapid, cost-effective, non-destructive, easy to set-up, and has high-throughput in nature.

Disclosure

The authors report no conflicts of interest in this work.

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