Evaluation of High-Resolution Melting Curve Analysis (HRM) assay for Detection of Pseudomonas aeruginosa PASGNDM699: A dangerous New Delhi metallo-β-lactamase (NDM) strain

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Abstract

Background: New Delhi metallo-β-lactamase (NDM-1) is a broad spectrum β-lactamase that is able to inactivate all β-lactams except aztreonam, as is typical of metallo-β-lactamases. NDM-1 producers in Pseudomonas aeruginosa, especially PASGNDM699 strain, cause a range of infections such as urinary tract, diarrhoea and soft tissue infections. The aim of this study was to Standardization of High-Resolution Melting Curve Analysis (HRM) assay for detection of P. aeruginosa, especially PASGNDM699 strain. Methods: The HRM method was done on standard strains of P. aeruginosa strains. 9-fold Serial dilutions of known DNA concentrations, extracted from standard isolates were prepared and tested by Real Time Melting curve and HRM assay. Data analysis was performed using the StepOne Software v2.3 and HRM Software v3.0.1 (Applied Biosystems, Ltd). Results: Based on the results of the Real Time PCR assay and melt curve analysis, melting point temperatures of the N-1, N-2 and N-3 amplicon for isolates identified as NDM strains were 87.57°C, 76.92°C and 82.97°C, respectively. Furthermore, melting point temperatures of the blaVIM, blaSPM and blaSIM amplicon for isolates identified as MBL strains were 84.56°C, 85.35°C and 86.62°C, respectively. Due to the analytical specificity of the primers, all dilutions with a similar Tm and melt peaks were obtained in the melting curves. Moreover, the analytical sensitivity of NDM primer were able to detected 100CFU/mL, 103CFU/mL and 104CFU/mL of standard DAN by N-1, N-2 and N-3 primers, respectively. Also, according to analytical sensitivity of MBL primers, blaVIM was able detected of 100CFU/mL, blaSPM primer 105CFU/mL and blaSIM primer 102CFU/mL of PASGNDM699 strain. HRM results showed that N-1 primers with 55 bp and blaVIM primers with 124 bp had the highest sensitivity and specificity for P. aeruginosa PASGNDM699 strain identification. Conclusion: The data from our study indicated that the sensitivity and specificity of the HRM method linked to the primer length and the fluorescent dye. Further, we can identify antibiotic resistance in substrates such as P. aeruginosa PASGNDM699 by software analysis and melting curve analysis.

Background

Pseudomonas aeruginosa is one of the major microorganisms involved in urinary, bloodstream, pulmonary, soft tissue, and surgical site infections in compromised individuals as those in intensive care units[1]. β-lactam antibiotics make up more than 50% of all commercially prescribed antibiotics for treatment of bacterial infections. The general mechanism of action of β-lactam antibiotics is inhibition of peptidoglycan synthesis which constitutes a major portion of bacterial cell wall synthesis[2]. The attachment of β-lactam antibiotics to penicillin-binding protein leads to the inhibition of transpeptidase that eventually leads to the death of bacteria. Several mechanisms to attain resistance against β-lactam antibiotics[3, 4]. These include: mutations to the active site of penicillin-binding-protein(PBP) to prevent drug binding, modification of the cell wall to prevent drug entry and assist active removal of antibiotic compounds, and producing the class of enzyme known as β-lactamase, which includes serine β-lactamases and metallo-β-lactamase (MBLs) [5, 6]. These strains hydrolyze the β-lactam ring of drug compound, thereby inactivating them. In contrast to serine β-lactamases, MBLs use at least one but more commonly two Zn2+ ions in their active site to catalyze the hydrolysis of β-lactam rings[2, 3]. There are

various methods for identifying MBL and NDM strains, which fall into two phenotypic and genotypic groups. Usually, phenotypic methods have low specificity, low speed, and error in results. Therefore, it is necessary to use molecular methods along with phenotypic methods. High-resolution melt (HRM) analysis is one of the most sensitive and precise molecular methods based on Real Time PCR[7].

HRM is used to characterize samples according to their dissociation behavior as they transition from dsDNA to ssDNA with increasing temperature and fluorescence detection. HRM melt curves is a function of the amplicon DNA sequence, which allows discrimination of amplicons with different nucleotide sequence based on melt curve shape, regardless of the amplicon Tm[8, 9]. The melt curve analysis is generally used in conjunction with HRM. It is generated after PCR amplification and indicates a change in fluorescence as temperature is raised by a fraction of a degree, from 60^{o}C and slowly increasing to 95^{o}C[10, 11]. Quenched amplicons produce a significant change in fluorescent signal, as they are denatured. The difference in fluorescence is used to determine the melting temperature (Tm), the temperature at which amplicon dissociation occurs. HRM and that the melting temperature (Tm) of PCR products can vary based on the length and DNA sequence[12, 13]. This method has been shown to provide a successful platform for the identification of microbial pathogens, and for discriminating Single Nucleotide Polymorphisms (SNPs) that confer a decrease in susceptibility to antimicrobials. The bases “G” and “C” have 3 hydrogen bonds connecting them whereas the bases “A” and “T” have only 2[10, 13]. This small difference will affect the melting temperature in which the bond will break by requiring a higher melting temperature for DNA with larger amounts of GC content[14]. HRM has the potential to be a powerful tool in the clinical microbiology laboratory, providing rapid detection of genetic determinants conferring antibiotic resistance to complement current phenotypic antimicrobial susceptibility testing methods[15].

The overarching aim of the current study was to define the genetic events that take place when resistance to third generation β-lactamase is selected in PASGNDM699 strain of *P. aeruginosa* by HRM technique.

Methods

Study design and *P. aeruginosa* strains

Standard cultures of *P. aeruginosa* PASGNDM699 (detection in clinical isolates), *Enterococcus faecalis* NCTC13779 and *Staphylococcus aureus* ATCC25923 were purchased from Pasteur Institute (Tehran, Iran). Cultures were grown at 37°C in Tryptic Soy broth (Merck, Germany). Biochemical tests were used to differentiate between closely related bacterial species or genus. These tests were conducted according to the standard specifications described and applied in the Microbiology Laboratory at Hamedan medical science. *P. aeruginosa* colonies were identified based on morphology, Gram staining, pyocyanin production and a biochemical test (such as oxidase, catalase, urease, arginine dehydrogenase and oxidation-fermentation). Bacterial strains were stored at -20°C in Brain-Heart Infusion (BHI) broth supplemented with 25% v/v glycerol. This study was approved by the Ethics Committee of Hamadan University of Medical Sciences (Code No: IR.UMSHA.REC.1396.637).

DNA Extraction, PCR assay and Sequencing

P. aeruginosa DNA extraction was performed using the DNA extraction kit (Qiagen, Germany), the steps were followed according to the kit protocol. DNA concentration was determined using a spectrophotometer Nanodrop-200 (Hangzhou Allsheng Instruments Co., Ltd, China). Primers sequence were initially set up as outlined in Ly et al. [16], Bordin et al. [6], Monteiro et al. [17], Kosykowska et al[18], and Alkasaby et al[19]. PCR amplification was done in 50µl reaction volumes containing 0.5µM of each primer, 25µM 2X mastermixe (Ampliqon, Denmark) 1µl DNA extract, and 5µl of supplied deionized water. Amplification was done using PCR thermocycler 1001C (BioRad, Germany) using the following profile: initial denaturation at 95°C for 5min, 30 cycles of denaturation at 95°C for 1 min, annealing at 61°C for 1min, and extension at 72°C for 1 min, and a final extension at 72°C for 5min. Amplified products were detected by agarose gel electrophoresis in 1% Tris-Acetate-EDTA (TAE) agarose (Sigma-Aldrich, USA). In this study, we performed the Sangar chain termination method for sequencing for all genes. All PCR products were sent to Pishgam Company (Tehran, Iran) for sequencing.

Real-time PCR and primers sensitivity and specificity

Primers sequence were initially set up as outlined in Sekyere (Osei Sekyere 2019) and Lee and Ko (Lee and Ko; 2014). For sensitivity and specificity of primers, nine-fold serial dilutions of 0.5 McFarland DNA ($1.5X10^8$ CFU/mL) were made ($1:1^{-1}$, $1:1^{-2}$, $1:1^{-3}$, $1:1^{-4}$, $1:1^{-5}$, $1:1^{-6}$, $1:1^{-7}$, $1:1^{-8}$). Primers efficiencies of both target genes and reference genes were tested by serial dilution Real-time PCR. Standard curves were constructed by the Ct (y-axis) versus log DNA dilution (x-axis). The primer efficiency (E) of one cycle in the exponential phase was calculated according to the equation: $E=10^{-(1/slope)-1}X100$ [20]. Briefly, 2µL 0.5µM of each primer, 2µL DNA template, 4µL EvaGreen, and made up to a final volume of 20µL using ddH2O. Real-time PCR reactions were performed on the ABI Real-time machine (ABI step one plus, USA). The thermal cycles were set for reverse transcription steps: 55°C for 5 min, 95°C for 10 min, 95°C for 20 sec followed by PCR steps: 95°C for 15 sec, 59°C for 30 sec repeated for 40 cycles. An arbitrary threshold value of 0.002 was set for all real-time PCR reactions. This value conferred a threshold that crossed the real-time PCR amplification curves during the exponential phase of amplification, enabling the determination of a cycle time (CT) value for each reaction. Strains included *Enterococcus faecalis* NCTC13779 and *Staphylococcus aureus* ATCC25923 were utilized as controls to assess analytical specificity. To calculate the efficiency of reaction, the slope value was calculated from serial dilutions for each gene, which was then used to determine the efficiency of reaction. In order to obtain accurate and reproducible results, reactions should have efficiency close to 2 (100%), which means that the template doubles with each cycle during exponential amplification. A slope of - 3.32 indicates optimal PCR efficiency.

Evaluation of sensitivity and specificity of HRM assay

The efficiency and the analytical sensitivity of the HRM-PCR were evaluated by triplicate testing of a 10-fold serial dilution series of each of the three reference strains. The standard curve was constructed by

using the serially diluted DNA of 0.5 McFarland ($1/5X10^8$ CFU/mL) preparation as template in the optimized HRM assay. A negative control was included in very run and HRM curve assay was performed after amplification to confirm the Tm of the amplification product. The Applied Biosystems step one plus, Real-Time PCR System was used to amplify and detect products. The reaction mix was prepared using the following components for each of the samples: 4µl of Master Mix HRM (HOT FIREPol EvaGreen HRM Mix), 1µM of each respective primer and 12µL of DMSO (Sigma-Aldrich, USA). Each reaction contained 2 µl of DNA diluted 1:10 in nuclease-free water. The following cycle parameters were used: 2 min at 50°C, 10min at 95°C, followed by 40 cycles with denaturing for 15sec at 95°C and by annealing/elongation for 1 min at 60°C. Melting curves were generated after each run to confirm a single PCR product (from 60°C to 95°C, increasing 1°C/3 sec).

Data analysis

Sequencing results were analyzed by BioEdit 7.4 software (Caredata, Inc, USA) to identify the sequences that are reliable and conclusive for mutational analysis and to eliminate sequences with noises that give inconclusive and unreliable data. Peak Tm Calling Analysis was performed after each real-time PCR reaction run within the ABI Thermo Fisher software (release 2018, Version 3.0.2.), based on which clear melting peaks and shoulders were revealed for the amplicons. The temperatures at the melting peaks are recorded as Tm values. Reliable and conclusive sequences obtained after analysis were used to locate the mutation using nucleotide BLAST tool of the National Center for Biotechnology Information (NCBI). Reagent *bla*nks were used to ensure there was no contamination in the reagents.

Results

Species Identification by PCR and Sequencing:

Sequencing of standard strains was determined for *P. aeruginosa* PASGNDM699 with CP020704 accession number.

Analytical Sensitivity and Specificity of Primers:

Due to the use of 9-fold dilutions, a high CT was observed in the 10^0 CFU/mL and low CT in the 10^8 CFU/mL. The CT values for these cell densities were within the 9 to 40 cycle range in the amplification process, while higher DNA concentrations appeared within 9 to 31 cycles. As seen in Figure 1 and Figure 2, relative to linear range of each standard curve, melting peaks can be seen for many of the lower DNA concentrations, however the concentrations could not be reliably quantified. The actual quantitative, linear portion of the calibration curves therefore did not extend as low as the limit of detection. Melting curves displayed a single melting Tm, 87.57°C for N-1 gene, 76.92°C for N-2 gene, 82.97°C for N-3 gene, 84.56°C for *bla*VIM gene, 86.62°C for *bla*SIM gene and 85.35°C for *bla*SPM gene (Figs 1 and 2). Samples containing DNA exhibit positive real time PCR amplification and negative controls failed to show amplification, also, Serial dilutions of positive control DNA amplification curves showed Ct values that were inversely related to the concentration of template DNA (Fig 3).

Reaction efficiencies were found to be within the range of 3 to 3.5 when calculated from the standard curves using the ABI Thermo Fisher analysis software (Version 2.3.2) with a formula of $E = 10^{(-1/slope)}-1$. For the N-1, N-2 and *bla*VIM primer set, the reaction efficiency reached a value slightly greater than 3, at 3.2, which would suggest an efficiency of 101%. Efficiencies greater than 100% can be obtained. All the investigated dilutions showed low efficiencies: N-3, E=98.8%; *bla*SMP, E=95.588% and *bla*SIM, E=96.493(Figs 1 and 2).

For the N-1 and *bla*VIM primer set, the linear range was determined to extend as low as 10^0 CFU/mL, N-2 was 10^3 CFU/mL, N-3 was 10^4 CFU/mL, *bla*SMP was 10^2 CFU/mL and *bla*SIM was 10^5 CFU/mL as indicated by the lowest DNA concentration value on each of the standard curves. Points which caused the curves to deviate from linearity (mostly those with lower concentrations) were excluded (Figs 1 and2).

Sensitivity and specificity of HRM Assay:

Fluorescence data were analysed using the tools for HRM analysis incorporated in the ABI Thermo Fisher analysis software. HRM PCR amplification curves of samples analyzed for the presence of *P. aeruginosa* PASGNDM699 are shown in Figure 4, 5 and 6. Difference plots of normalized data show the difference in fluorescence between each sample of DNA. Derivative plots display the rate of fluorescence change; the peak indicates the melting temperature of a sample. All plot displayed a single melting domain, typically between 87.07°C – 87.57°C for N-1 gene, 76.42°C – 76.92°C for N-2 gene, 82.47°C – 82.97°C for N-3 gene, 84.06°C – 84.56°C for *bla*VIM gene, 86.12°C – 86.62°C for *bla*SIM gene and 85.30°C – 85.35°C for *bla*SPM gene in accordance with different product sizes.

The results of this representative experiment show that all samples containing *P. aeruginosa* DNA had measurable amplification as detected by exponential fluorescence and all samples containing negative control DNA did not (Fig 3) and all the DNA dilutions of *P. aeruginosa* PASGNDM699 were identified (dilution 10^8 to 10^0 CFU/mL). In addition, the N-1 and *bla*VIM genes in *P. aeruginosa* PASGNDM699 was detected in all dilution of DNA. Moreover, N-2, N-3, *bla*SPM and *bla*SIM primers can able to detect bacterial DNA in dilutions of 10^3CFU/mL, 10^4CFU/mL, 10^2CFU/mL, and 10^5CFU/mL, respectively (Figs 4 and 5).

The software automatically analyzed the raw melting curve data and set the starting (pre-melt) and ending (post-melt) fluorescence signals of all data to uniform values to aid interpretation and analysis (Figs 4 and 5). The cursors for these two points are defaulted to the ends of the curve but these regions were manually adjusted to encompass representative baseline for the pre-melt and post-melt phases. Widening the normalization regions into the melt phase was avoided to ensure that curves normalize effectively. Moreover, we performed a melt curve analysis of HRM PCR samples to assess the specificity of the amplicon. The results of the HRM showed a very similar melt peak for all Serial dilutions of *P. aeruginosa*.

Discussion

present study revealed that the sensitivity and specificity of the method for identifying resistant strains have a significant impact on the speed and accuracy of detection. However, to obtain specificity of the Real Time PCR and primers, DNA melting curve analysis (MCA) and standard curve in different dilutions were used. The efficiency of the dilution for all genes was at least 99.99%, the r2 was >0.99.99, and melt curves yielded single peaks. These features are exemplified in Figure 1 and 2 showing the difference between a nonoptimized and optimized standard curve. Interestingly, the slope of the Ct vs DNA relationship varied little across the 9 fold- dilution tested, ranging from -3.589 to -3.955. Also, No amplification, i.e. CT > 40, was obtained for *P. aeruginosa* PASGNDM699 strain. According to Lalonde et al[21] and Heydari el al[22] studies, this can be justified because short fragment binds less fluorescent and compensated by its higher primer concentration. However, sometimes the peak height of short amplicon increases in different replicate. This problem gets extremely worse in the MCA that sometimes we lost the long amplicon even at the primer ratio of 1:1. Furthermore, this results agree with Mentasti et al[2]. They proved that the MCA method for the detection of NDM strains has high efficiency and precision, which is best achieved by adjusting the primer dilutions.

Morever, three NDM-1 primers with different amplicon length and MBL primers were used to detect *P. aeruginosa* PASGNDM699 strain. Based on Melting temperatures of different dilutions of standard DAN, melt curves of gene amplification were equal to 87.7±0.5˚C, 76.6±0.5˚C, 82.6±0.5˚C for NDM-1 primers, and 90.0± 0.5˚C for *bla*VIM, 90.0± 0.5˚C for *bla*SIM, 90.0± 0.5˚C for *bla*SIM primers for MBL genes, respectively. This indicated that the specificity of primers with 5°C error range can detect *P. aeruginosa* PASGNDM699 strains. Andini et al[23] showed that accurate analysis of the melting curve could play a very important role in the diagnosis. Ashrafi et al [10]found that to obtain the best performance in sophisticated methods such as HRM, the melting temperature of DNA must be monitored in various dilutions to obtain accurate sensitivity and specificity. Tahmasebi et al[7] also conformed that efficiency is probably due to the shorter length of products of primers, which enabled better amplification in PCR.

In this study, in Figure 1 showed that for the *NDM-1* primer set *with 55bp*, the linear range was determined to extend as low as 10^0 CFU/mL, *NDM-1 with 85 bp* was 10^3 CFU/mL and *NDM-1 with 155bp* was 10^1 CFU/mL as indicated by the lowest DNA concentration value on each of the standard curves. Further, Figure 2 indicates that for the *blaSHV* primer set, the linear range was determined to extend as low as 10^0 CFU/mL, *blaSPM* was 10^3 CFU/mL and *blaVIM* was 10^1 CFU/mL. This is consistent with observation of Smiljanic et al[24]. They illustrated that identification of NDM and MBL strains in Gram-negative non-fermentative is difficult, because the resistance to carbapenems in these bacteria is encoded by similar sequences. Thus, the use of a sensitive and precise method such as HRM along with specific primers could enable the identification of strains such as MBL and NDM.

In the present study, HRM and different primers were used to identify *P. aeruginosa* PASGNDM699 strain. In a study,by Ding et al.[25] Proposed the resistance of PASGNDM699 strain to a wide range of antibiotics. They also confirmed the clinical importance of PASGNDM strains in causing resistant infections. Based on Figure 4 and 5, all the DNA dilutions of *P. aeruginosa* PASGNDM699 strain were identified (dilution 10^8 to 10^0 CFU/mL). The results were different from the obtained by Naas et al[26] and

Smiljanic et al[27] studies. They confirmed the identification of NDM strains at dilution 103 and stated that the HRM method had limitations in the detection of different dilutions. Although identification of MBL and NDM strains has been performed in various studies in Sweden[28], USA[29], Australia[30], and Italy[31] in gram-negative Bacteria by HRM method, the novelty of the present study was the use of HRM method to identify *P. aeruginosa* PASGNDM699 strain. It was also found that the HRM method is highly potent in detecting PASGNDM699 strains that are resistant to colistin and carbapenem.

According to our results, the short primers (N-1 with 55bp and *bla*VIm with 111bp) had the best sensitivity and specificity in the HRM assay, in addition, they identified the NDM-1 and MBL genes in all dilutions. On the other hand, the purity of the extracted DNA was also a factor. This makes us to think that the amplification of long fragment heavily depends on the DNA quality. Słomka et al[13] demonstrated that when the DNA quality is low, saying DNA degradation or long DNA breaks during extraction makes the long template harder to be amplified. This problem gets worse in digital PCR because the reagent range in the reaction gets more stringent and template is much more diluted. Meanwhile in the digital PCR, as there is only one molecule in the well, the high ratio of primer for short amplicon might not be necessary and even cause the long template can't be amplified because of the primer competition.

However, HRM assay is used to amplify and concurrently quantify a targeted DNA molecule and enables both detection and quantification of DNA. HRM PCR needs a fluorescent reporter that binds to the formed product and reports its presence by fluorescence. The EvaGreen® Dye was used in this study, because it is an saturating dye which do not interfere with PCR reactions, even if they used at the largest level of saturation which gives the maximum fluorescence; That the Eischeid[32] study confirms these results.

It is necessary to point out limitations of the HRM approach in this study. The length of the selected primers should be considered to identify the bacterial sub-strains. If the ratios of the different agents are higher than 1:10, the system does not detect the infectious agent which is in lower quantities. However, the determination of Tm is very sensitive to the composition of the PCR reaction mixture,especiallyto the ionic strength. To avoid Tm bias due to thepipetting errors between PCR runs, the application of mastermixes is recommended. Limitation of the method can be thatvarious mastermixes offered by differentsuppliers vary in reagent composition. This may influence the Tm values. Besides, in case of different mastermixes from different suppliers, calibration is necessary to establish the newTmdata on the fungal strains.

In conclusion, we demonstrated that the HRM assay is a rapid and sensitive pre-sequence screening tool which allows the detection of point low concetration of a DNA. It eliminates much of the labour and cost involved in performing DNA sequencing of an entire gene and direct DNA sequencing is therefore only required as confirmation of a mutation or polymorphism. Compared with existing methods it is not only more cost-effective, but is also capable of detecting new functional mutations that will have importance in cascade screening of affected subjects. Finally, the analysis of the melting curves is an important step in the identification of heterozygous base changes. The selection of the melting temperature range is

important to the analysis as there needs to be sufficient data both prior to and following the melting transition, to allow reliable normalisation of the melting curves. HRM has also the capacity to greatly increase the scope and sensitivity of haplotype analysis.

Abbreviations

HRM: High Resolution Melting Analysis; SIM: Seoul IMipenemase; VIM: Verona integron-borne metallo-β-lactamase; NDM: Extended-spectrum β-lactamases; MBL: Metallo-β-Lactamase; SMP: Sao Paulo metallo-β-lactamase; HRM: High Resolution Melting Analysis; Tm: Temperature; CT: Cycle Threshold.

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of Hamadan University of Medical Sciences ((No: IR.UMSHA.REC.1396.637).Consent for publication Not applicable

Availability of data and materials

The data can be accessible to the interested researchers by the corresponding authors on reasonable request.

Consent for publication

Not Applicable.

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Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

MRA and HT proposed, designed, carried out the study, HT and SD analyzed the generated data and drafted the manuscript and performed the data analysis. MRA provided with some of the strains and HT

participated in proofreading of the manuscript and in critical revision. All authors read and approved the fnal manuscript.

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Table

Table 1. Oligonucleotide sequences used in this study

Toreget	Primer Name	Sequence of Primers	Melting Tm	Product size(bp)	References
NDM-1	*N-1*	F: GACCGCCCAGATCCTCAA R: CGCGACCGGCAGGTT	87.57	55	[6]
	N-2	F: TTGGCCTTGCTGTCCTTG R: ACACCAGTGACAATATCACCG	76.92	85	[17]
	N-3	F: GCGCAACACAGCCTGACTTT R: CAGCCACCAAAAGCGATGTC	82.97	155	[16]
MBL	*blaSIM*	TACAAGGGATTCGGCATCG TAATGGCCTGTTCCCATGTG	85.35	577	[19]
	blaVIM	F: TCTCCACGCACTTTCATGAC R: GTGGGAATCTCGTTCCCCTC	84.56	124	[18]
	blaSPM	F: AAAATCTGGGTACGCAAACG R: ACATTATCCGCTGGAACAGG	86.62	271	[19]

Figures

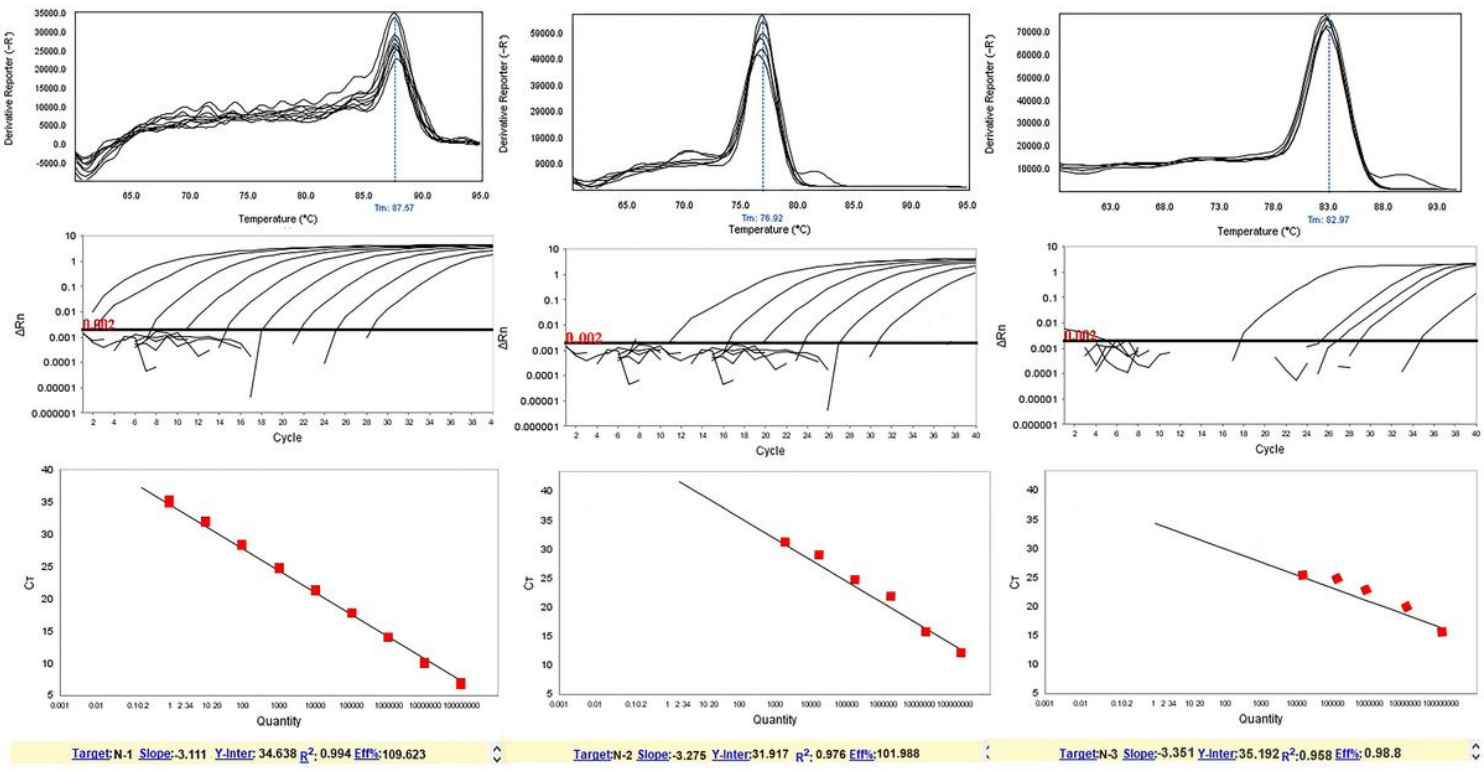

Figure 1

Analytical sensitivity of Real-Time PCR and examples of optimization of primer pairs based on melting curve analysis for NDM primers used to detect P. aeruginosa PASGNDM699. The melting curves for each primer pair were investigated. Right: N-1 gene with a melting point of 87.53±0/5oC, Middle: N-2 gene with a melting point of 76.92±0/5oC; Left: N-3 gene with a melting point of 82.97±0/5oC. The mean of a: 108;

b: 107; c: 106; d: 105; e: 104; f: 103; g: 102; h: 101 and i: 100 CFU/mL of DNA dilutions. Bold black Horizontal lines represent cycle threshold of Real Time PCR. One peak with a shoulder corresponds to genomic DNA amplification; no peak corresponds to no amplification. Eva Green color and single tube reaction were used in this test. Also, Real Time PCR was performed as single-step.

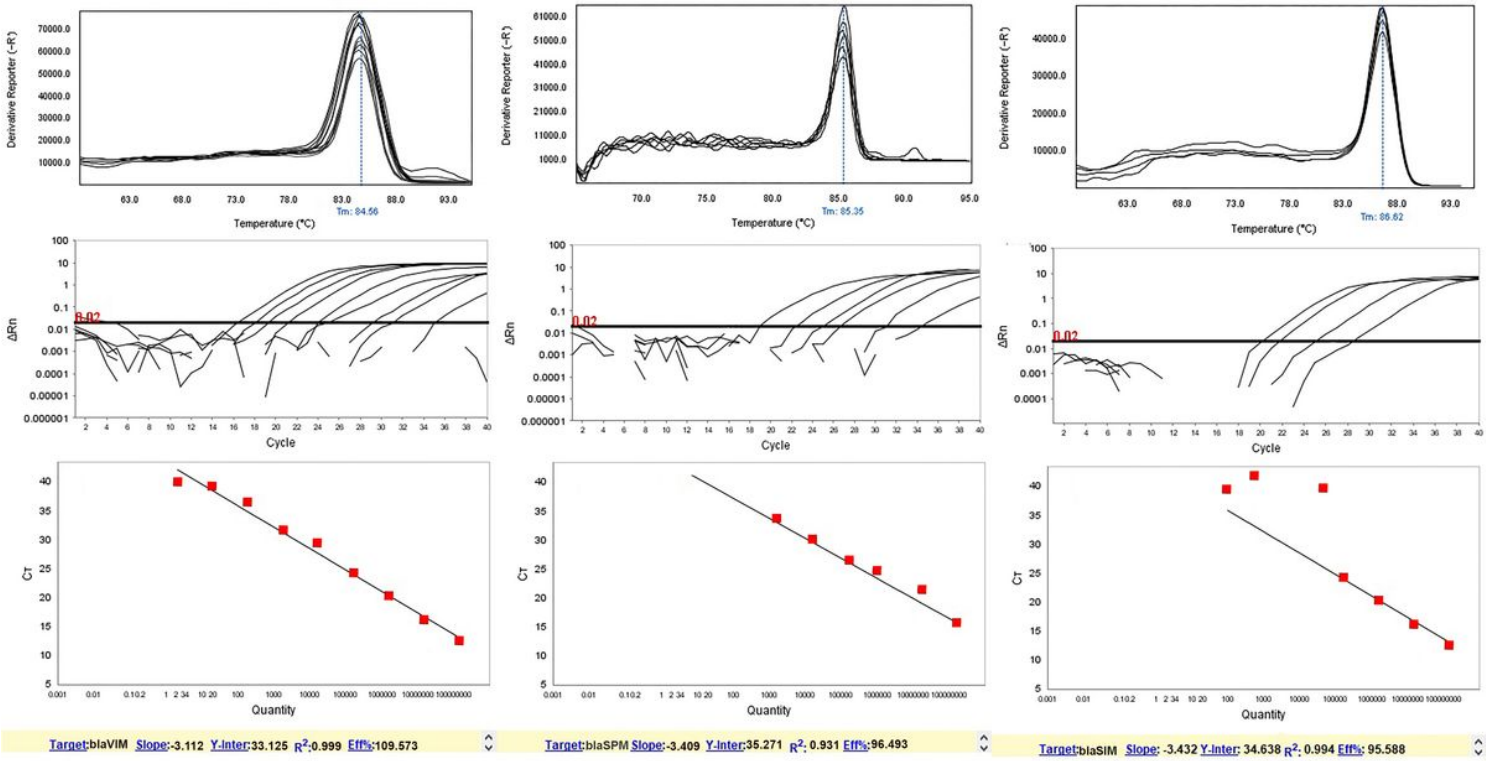

Figure 2

Analytical sensitivity of Real-Time PCR and examples of optimization of primer pairs based on melting curve analysis for MBL primers used to detect P. aeruginosa PASGNDM699. The melting curves for each primer pair were investigated. Left: blaVIM gene with a melting point of 84.56±0/5oC, Middle: blaSPM gene with a melting point of 85.35±0/5oC; Right: blaSIM gene with a melting point of 86.62±0/5oC. The mean of a: 108; b: 107; c: 106; d: 105; e: 104; f: 103; g: 102; h: 101 and i: 100 CFU/mL of DNA dilutions. Bold black Horizontal lines represent cycle threshold of Real Time PCR. One peak with a shoulder corresponds to genomic DNA amplification; no peak corresponds to no amplification. Eva Green color and single tube reaction were used in this test. Also, Real Time PCR was performed as single-step.

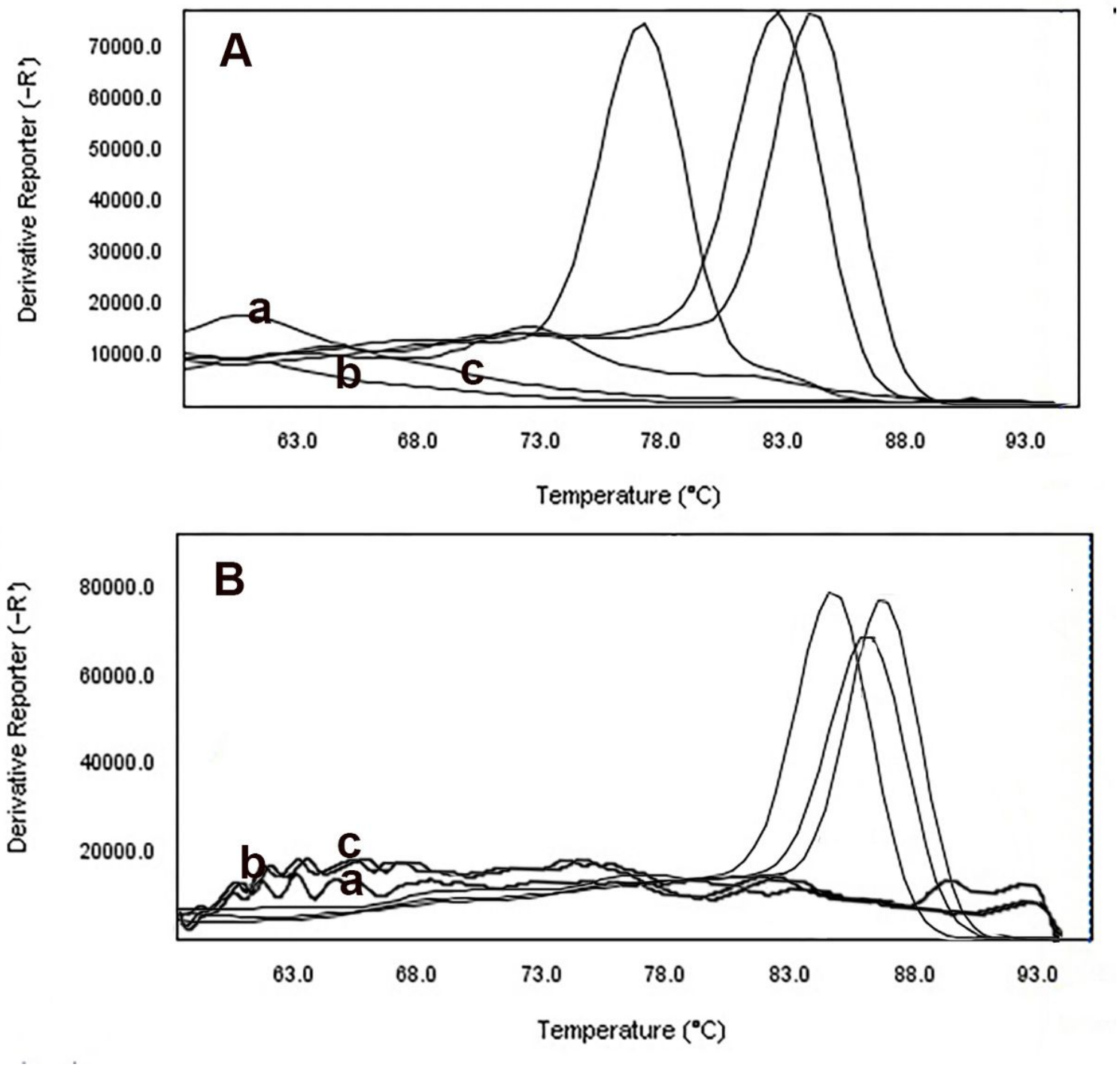

Figure 3

Melting curve analysis and analytical specificity of Real-Time PCR for NDM primers (A) and MBL primers (B) used to detect P. aeruginosa PASGNDM699. a: Blank tube; b: Enterococcus faecalis NCTC13779; and c: Staphylococcus aureus ATCC25923. One peak with a shoulder corresponds to genomic DNA amplification; no peak corresponds to no amplification. Eva Green color and single tube reaction were used in this test. Also, Real Time PCR was performed as single-step. 0.5-McFarland concentration (1.5x 108 CFU/mL of DNA) was used to determine primer specificity.

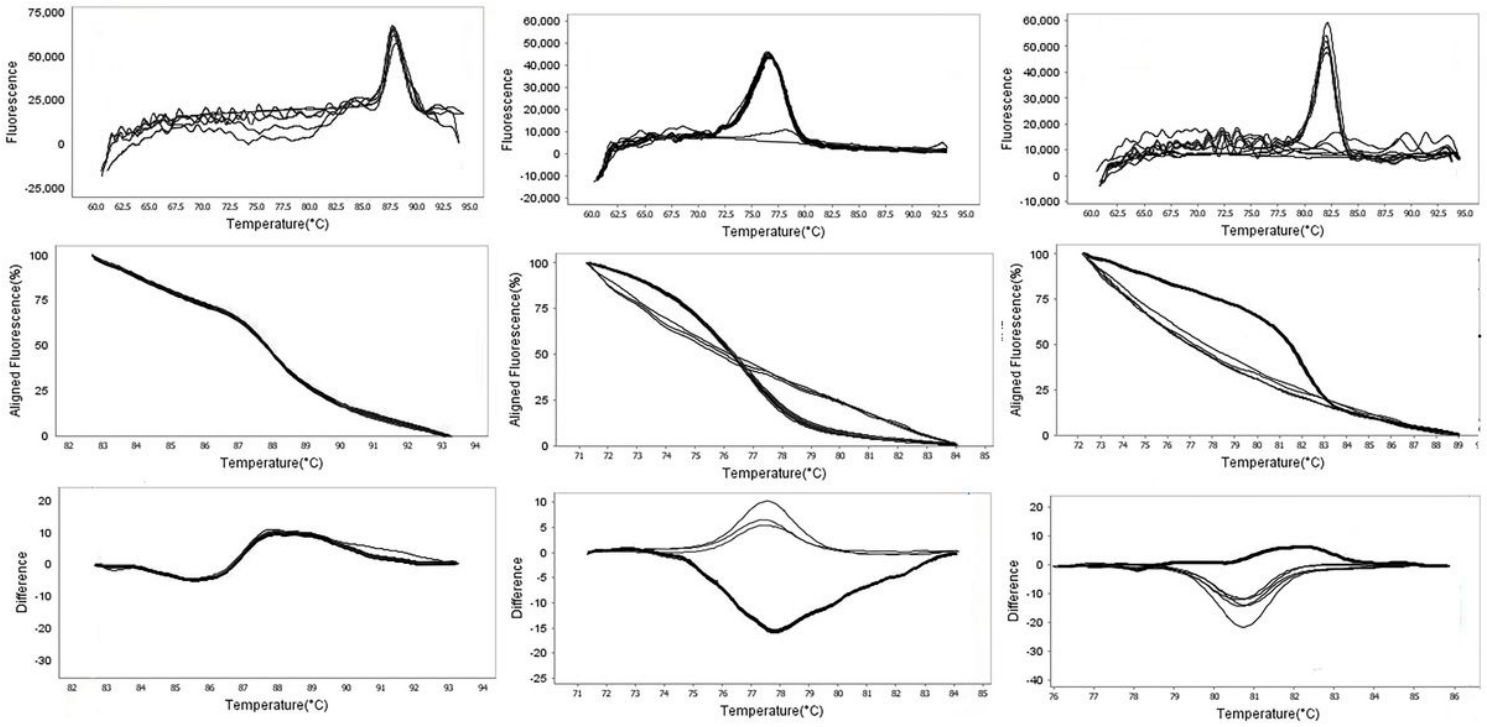

Figure 4

HRM graphs corresponding to one high resolution melting analysis of a subset of P. aeruginosa PASGNDM699 by N-1 (Left), N-2 (Middle) and N-3 (right) genes. DNA samples from all the dilutions involved in this study were prepared and amplified successfully using the EvaGreen dye-based method in the ABI instrument. Primers specific melting peaks (Tm) were obtained via HRM analysis, allowing the differentiation of all investigated β-lactamase enzymes. Due to the highly saturating EvaGreen dye and the HRM analysis, the accuracy of the resolution was ±0. 1–0.5 ˚C.

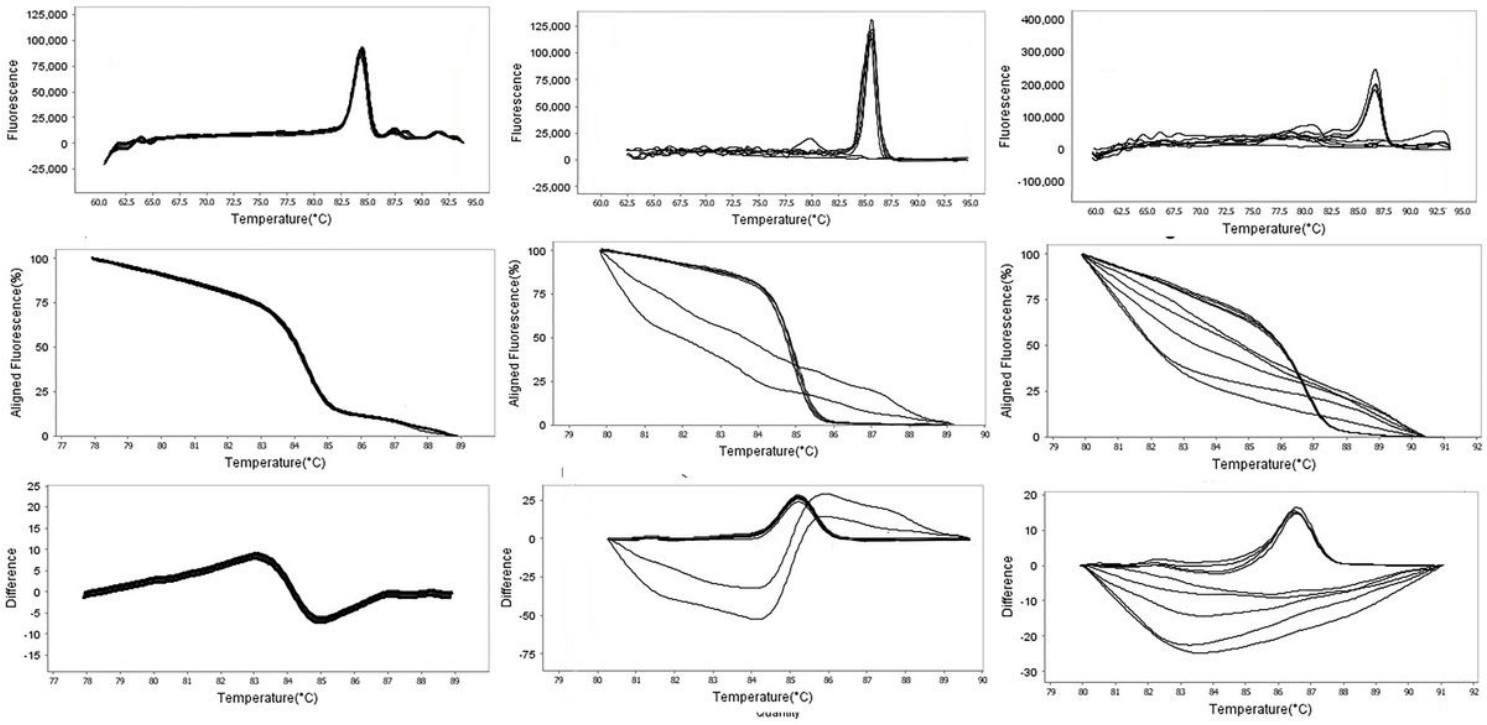

Figure 5

HRM graphs corresponding to one high resolution melting analysis of a subset of P. aeruginosa PASGNDM699 by blaVIM (Left), blaSIM (Middle) and blaSPM (right) genes. DNA samples from all the dilutions involved in this study were prepared and amplified successfully using the EvaGreen dye-based method in the ABI instrument. Primers specific melting peaks (Tm) were obtained via HRM analysis, allowing the differentiation of all investigated β-lactamase enzymes. Due to the highly saturating EvaGreen dye and the HRM analysis, the accuracy of the resolution was ±0.1˚C – 0.5˚C.