ORIGINAL ARTICLE

High Resolution Melting Curve Analysis Method for Detecting of Carbapenemases Producing *Pseudomonas aeruginosa*

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Abstract:

Background: The, carbapenems have the broadest spectrum of activity among all β-lactam antibiotics, and also, carbapenem resistant *P. aeruginosa* may be susceptible to other β-lactam antibiotics. There are several methods for detecting to Carbapenems resistance of *Pseudomonas aeruginosa* (*P. aeruginosa)* strains. *Aim and Objectives:* In the current study, High Resolution Melt curve analysis (HRM) is evaluated to detect carbapenem resistant *P. aeruginosa. Material and Methods:* This experimental study was done on standard isolates of *P. aeruginosa* and carbapenemase producing strains in Hamadan. *16srRNA* of *P. aeruginosa* and *KPC* gene of carbapenemase producing strains were applied to detect by HRM. Also, sensitivity and specificity of primers have been evaluated and carbapenemase producing *P. aeruginosa* were determined based on melt curve temperature range. Finally, melt curve profiles were assessed and analyzed using StepOne Software v2.3 and HRM Software v3.0.1 software. *Results:* According to dilutions ratios of 10^{8} to 10^{-4} it was found that the designed primers have the ability to identify 10^{-3} CFU/ml of bacteria for *P. aeruginosa* and 10^{-1} CFU/ml for carbapenemase producing strains. The melt curve was also shown at 80.9±0/5 °C for all DNA dilutions of *P. aeruginosa* and 82.4±0/5 °C for all DNA dilutions of Carbapenemases strains. *Conclusion:* The sensitivity and specificity of HRM method indicated that it is highly reliable, rapid and cost-effective to detect carbapenemase producing *P. aeruginosa.*

Keywords: *Pseudomonas aeruginosa,* High-Resolution Melting Curve Analysis, Carbapenemases, Antibiotic Resistance

Introduction:

Antibiotic resistance is the ability of micro-organism to resist and survive when exposed to an antimicrobial agent. The extensive use of antimicrobial drugs is considered to be the main reason for the emergence of drug-resistant organisms [1]. Choosing an appropriate anti-microbial therapy for critically ill patients is a major problem for healthcare providers due to increased prevalence of drug-resistant micro-organisms in the environment of hospitals. Treatment and management of patients infected by resistant strains has become more difficult than those infected by susceptible organisms [2]. This is mainly because the infection caused by resistant organisms will increase hospitalization time, mortality rate, and overall healthcare costs [3].

Pseudomonas aeruginosa (*P.aeruginosa*) is an opportunistic pathogen and will lead to infection in immunocompromised patients [4]. In hospitalized patients, Pseudomonas is a leading cause of nosocomial infections via colonization of catheters, skin wounds, ventilator-associated pneumonia and it is also a cause of respiratory infections in individuals with Cystic Fibrosis (CF)' [5]. The carbapenems are β-lactam antibiotics with

the widest spectrum of antibacterial activity of the currently available antibiotics. *P. aeruginosa* is considered as a poor target for ertapenem, but the reasons have so far not been elucidated yet [6]. Structurally two features distinguish the carbapenems from other β-lactams, namely the introduction of a hydroxyethyl side chain in trans configuration at position 6, and the lack of a sulfur or oxygen atom in the thiazolidinic ring [7]. It is also apparent that imipenem lacks a heterocyclic side chain, which explains why this antibiotic is not a substrate for the multi-drug efflux pumps of *P. aeruginosa*. Carbapenem resistant *P. aeruginosa* may retain susceptibility to other β- lactams such as piperacillin/tazobactam, ceftazidime and cefepime [8]. Still, MDR phenotypes resistant to all β-lactams are increasingly observed. Some isolates may also be resistant to aminoglycosides, and fluoroquinolone in the all over the world [9].

There are several methods for detecting *P.aeruginosa* strains with carbapenemases enzyme. The time consuming and high cost of these tests have made it necessary to use molecular techniques [10, 11]. Molecular techniques have been developed in order to improve the sensitivity and to detect clinical specimen infection earlier [12]. one of these molecular methods based on Real Time PCR is HRM [11].

High Resolution Melting Analysis (HRM or HRMA) is a fast, cost-effective, and closed-tube method that screens sequence variations within amplicon by analyzing the melting behavior of DNA duplexes [13]. Advances in instrumentation and DNA intercalating dyes enable HRM to be applied for various genetic testing, including SNP genotyping, mutation scanning, and the assessment of promoter methylation [14]. DNA intercalating dyes (e.g. EvaGreen, SYTO 9 and LC Green) which replace conventional fluorescent probes can be used at fully saturating concentrations without interference with PCR amplification [15]. In addition, those DNA intercalating dyes fluorescence when they are bound to double-stranded DNA whereas they lose fluorescence when they are dissociated from double-stranded DNA.

Objective:

In this study, HRM technique was used to identify the carbapenemase producing strains of *P.aeruginosa*.

Material and Methods:

Selection of the bacterial strain:

In this experimental study, which was done on clinical and standard isolates of *P. aeruginosa* stored in the microbiology laboratory in Hamadan University of Medical Sciences in 2018. *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 700603 and *Escherichia coli ATCC 25922* were used as the reference strains in each assay.

Preparation of DNA extraction:

In order to extract genomic DNA, the gram-negative kit (Cinna Gen, Iran) was used based on the manufacturer's instructions. In summary, a single colony was transferred in 5 ml BHI broth and incubated for 24 hours at 37° C. then, 1.5 ml of the suspension was transferred in a sterile 1.5 ml Eppendorf tube and then centrifuged for 10 minutes (4500rpm). DNA was extracted accompanying with Lysozyme and the sediment from the centrifuged bacteria in BHI. DNA concentration and light absorption were measured by a spectrophotometer called Nanodrop (A& E lab, U.K).

PCR and sequencing:

PCR was carried out in a final volume of a 25µl reaction mixture containing 12.5µl master mix (Ampliqon, Germany), 1µl forward primers and 1µl reverse primers (10pmol), 1µl of DNA and sterile DW. DNA amplification was carried out with thermal cycling (Eppendorf thermocycler, Germany) conditions consisting of an initial denaturation step at 94°C for 5 min, followed by 30 cycles included: denaturation at 95°C for1 min, annealing at 55°C for55 sec, and extension at 72°C for 5 min. *P. aeruginosa ATCC27853* and *Escherichia coli ATCC 25922* were used as a positive and negative controls in all PCR experiments. PCR products were purified and sequenced for identifying *P. aeruginosa,* by Korea Bioneer Company, (Representative Pishgam Company/Iran)

Evaluation of sensitivity and specificity and HRM assay:

The sensitivity of the designed primers was tested by using quantitated DNA of appropriate *P. aeruginosa* diluted in 10-fold serial dilutions. Each were tested in triplicate to determine the lower limit of detection in three consecutive days. *P. aeruginosa ATCC 27853(16srRNA* gene*)* and *K. pneumoniae ATCC 700603* (*KPC* gene) were used as positive control and *Escherichia coli ATCC 25922* was used as a negative control in order to test the specificity of the primers. The melting temperature of each product was determined in singlet PCR with Eva® Green. To ensure that the difference in the Tm values between the primers was at least 2°C in order to avoid overlapping of peaks, different combinations of primers for each of three genes were chosen. HRM assay and amplification were performed by using a Real time PCR (ABI step one plus, USA). Reactions were carried out in a total volume of 20µl including: Master Mix HRM (HOT FIREPol® EvaGreen HRM Mix) 4µl, 1µl of each primer (20pmol), 1µl of bacterial DNA, and DEPS water. The cycling conditions were as follows: denaturing at 95°C for 15 min, followed by 40 cycles of 15 sec at 95°C, 54°C for 55sec. In order to find a melting curve for the separation of the genes, the temperature cycle was as follows: 95°C for 15s,. The range temperature was 60 to 95°C in order to determine the melting temperature and readings consecutive, Ramp temperature was 0/3° C in terms of province. All steps were completed by using StepOne Software v2.3 and HRM Software v3.0.1 software.

Results:

Species Identification by PCR and Sequencing:

The *16srRNA* gene with the length of 155bp and *KPC* gene primers with the length of 254bp were successfully amplified for identifying of all the *P. aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 700603 in this study. Gel electrophoresis confirmed that the amplicons.

Analytical Sensitivity and Specificity of Primers:

In all dilutions, the observed melting temperatures shown in the melting curves of gene amplification were equal to 80.9±0/5˚C for *16sRNA* gene, 82.4 ± 0/5˚C for *KPC* gene; all of these melting temperatures were completely consistent with the results of primer blasts in NCBI database. The analysis of the threshold melting curves of the intended genes indicated a successful onset of gene replication in all prepared dilutions in different cycles. Obtaining the standard concentrations in the dilutions with ratios of 10^{8} to 10^{-3}, it was found

that the designed primer has the ability to identify 10^{-1} CFU bacteria for *P. aeruginosa* and 10^{-3} CFU bacteria for Carbapenemase producing strain. The temperatures observed at the end of the reaction according to the Melting curves showed that the primers used in the present study were not able to identify other bacteria such as, *Escherichia coli ATCC 25922* and *Pseudomonas aeruginosa ATCC27853* (Fig. 1) (Fig. 2).

Detecting Carbapenemase producing strain by HRM Assay:

The results of the HRM test for Carbapenemase producers were identified from a dilution of 10^{8} to 10^{-3} genomic DNA dilutions. The melting curve was also shown at 82.4 °C for all dilutions (Fig. 3).

Detection of *Pseudomonas aeruginosa* by HRM Assay:

The results of the HRM test for *Pseudomonas aeruginosa* were identified from a dilution of 10^{8} to 10^{-1} genomic DNA dilutions. The melting curve was also shown at 80.9 °C for all dilutions (Fig. 4).

Table 1: Oligonucleotide Sequences Used in This Study

Gene	Sequence of Primers	Tm.p	Product size (bp)	References
16 SrRNA	F: TGGAGCATGTGGTTTAATTCGA R: TGCGGGACTTAACCCAACA	79/57	155	[16]
KPC	F: GATACCACGTTCCGTCTGG R: GCAGGTTCCGGTTTTGTCTC	83/5	254	[17]

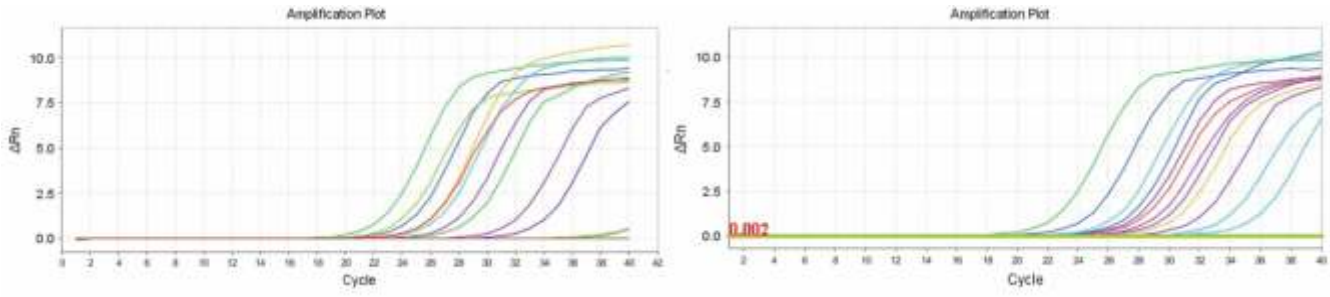

Fig. 1: Amplification and CT value of serial dilution of *Pseudomonas aeruginosa* (Right) and Carbapenemasese strain (Left) of genomic DNA

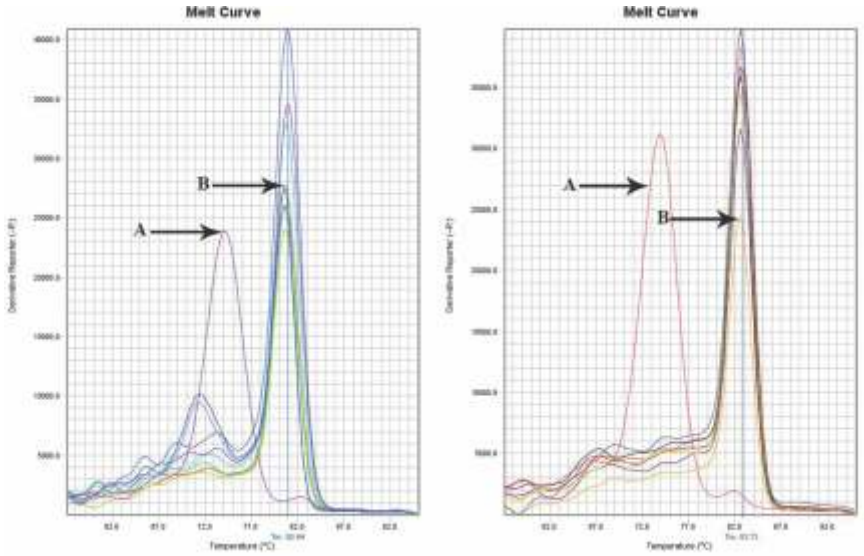

Fig. 2: Sensitivity and specificity test by Real Time-PCR MCA for primers used to detect *Pseudomonas aeruginosa Carbapenemaseses strain* with a melting point of 82.4±0/5 °C (left) and *Pseudomonas aeruginosa* with a melting point of 80.9 ± 0/5°C (right). Left: A: Positiv control *K. pneumoniae* ATCC 700603. B: negative control *Escherichia coli ATCC*. Right: A: Positiv control *P. aeruginosa ATCC 27853*. B: negative control *Escherichia coli*

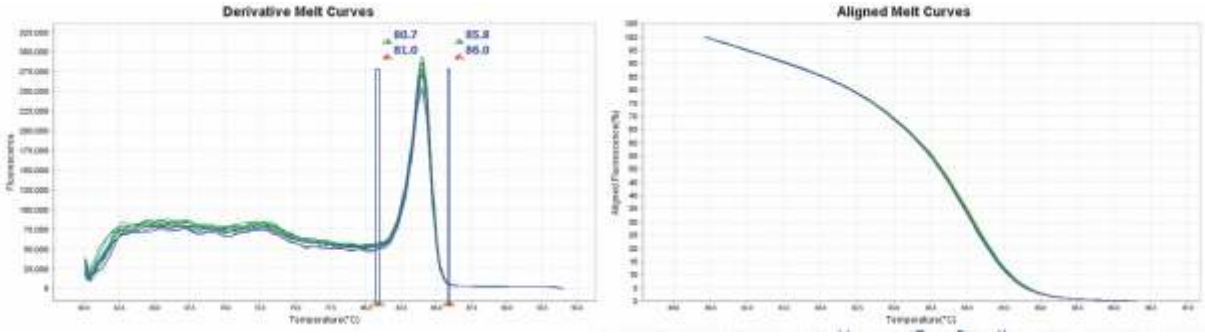

Fig. 3: Melting curve and HRM analysis for the *Pseudomonas aeruginosa Carbapenemaseses strain*

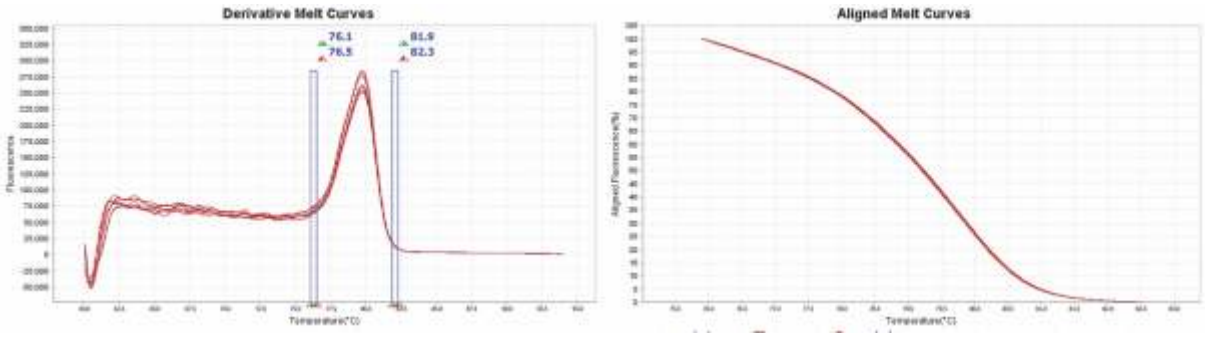

Fig. 4: Melting curve and HRM analysis for the *Pseudomonas aeruginosa*

Discussion:

Our study indicated that HRM analysis can give rapid and accurate identification of *P. aeruginosa* and carbapenemase producers. The development of molecular methods, which use HRM assay, has the advantage of being sensitive, specific and can detect *P. aeruginosa* and carbapenemase producers. Khosravi *et al.* demonstrated that the use of HRM method can be very effective in identifying antibiotics resistant genes, and it has high sensitivity and specificity compared to phenotypic methods [18]. In our study, it is aimed to develop HRM assays, which can be used as a screening tool. Phenotypic methods do not have high power to detect *P. aeruginosa* and Carbapenemas strains. Hence, it is possible to identify the genus, species and strains of bacteria by using quantitative methods such as HRM.

In this experimental study, using HRM method, different dilutions of DNA were performed to detect *P. aeruginosa* and carbapenemase producers. DNA dilutions showed that *P. aeruginosa* was diluted to 10^{-3} and carbapenemase producing strains up to dilution of 10^{-1}. Also, different dilutions of genomic DNA revealed the same results for melting curves. In Geyer *et al.* and Roth *et al.* studies that identified antibiotic-resistant strains, it was shown that HRM should demonstrate the same function at different dilutions of genomic DNA. In this way, at the lowest dilution and maximum dilution levels, the melting temperature must be the same [8, 19]. Hence, various factors contribute to enhance the quality of HRM, such as: the DNA purity, the single-step or two-step reaction and the length of primers [18]. In this study, a high purity DNA extraction kit was used and specific primers with appropriate amplicon length were used to detect *P. aeruginosa* and carbapnemase producing strain too. Monteiro *et al.*, Martini *et al.*, and de Lima *et al.* showed that the HRM method is more effective in detecting different bacterial species compared to the culture method. In these studies, it was determined that a culture method with low specification, poor diagnostic range, inability to differentiate bacterial species, and the involvement of human error with false positives and false negatives can affect the results [11, 20, 21]. The similar results has been observed in the current study which, some *P. aeruginosa* strains were not easily detected by culture method and were excluded from the study. Hence, in HRM assay, the use of sensitive primers is one of the most important points to consider, because if the appropriate target is not selected, additional techniques should be used to eliminate these errors [22]. In studies by Krawczyk *et al.* [23] design of melt profiles was used in conjunction with the PFGE method to increase the sensitivity of the melt-based method. In this study, the specific temperature for *P. aeruginosa* primer was 83.4 ± 0/5°C and 79.4 ± 0/5°C for KPC primer. Li *et al.*, Tahmasebi *et al.*, and Heydari *et al.* showed that specific temperature in different dilutions of genomic DNA in identifying bacteria is one of the most important issues to be considered in the melting curve method. Our results indicated that all dilutions had the same temperature for *P. aeruginosa* and carbapenemase producers. The similarity of our study with the above studies suggests that with the upgrade of the HRM method, bacteria can be detected even in small quantities [13, 24, 25].

The results from our study showed that, there is some practical limitations, for example, the HRM method should be used in various clinical specimens such as blood infection, in order to determine the sensitivity and specificity of identifying the number of bacteria, using limited dilutions to determine the sensitivity and also using a two-step reaction instead of a one-step. Of course, it is advisable to use duplex and multiplex methods instead of a single method. Because of the high frequency of these bacteria in clinical and food samples, most of them need to be diagnosed. This would impose a heavy financial burden on the laboratory in the case of using costly tests. In studies conducted by Tan TY *et al.*, the proposed Real Time Tetraplex PCR and the simultaneous use of all primers in a tube, not only confirm the cost-effectiveness of this method, but also they approve final result with phenotypic methods for species evaluation resistant, which ultimately underscored the value of using this method [26].

Conclusion:

The HRM assay described in this study could be practical and useful tool for the identifying carbapenemases producing *Pseudomonas aeruginosa*, and it has highly sensitivity and specific. Is easy to perform and could provide results in less than 4 h. In addition, the risks of PCR products contamination are minimized.

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