

# Prevalence and Molecular Typing of Colistin-Resistant *Pseudomonas aeruginosa* (CRPA) Among $\beta$ -Lactamase-Producing Isolates: A Study Based on High-Resolution Melting Curve Analysis Method

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**Background:** The frequency and production of  $\beta$ -lactamase enzymes may be different in colistin-resistant *Pseudomonas aeruginosa* (CRPA) strains compared to susceptible strains. The purpose of this study was to investigate the relationship between colistin resistance and  $\beta$ -lactamase enzymes in different Sequence Types (ST) of *P. aeruginosa*.

**Methods:** A total of 101 *P. aeruginosa* isolates were collected from different samples. The antimicrobial susceptibilities of the bacterial isolates were examined by disk diffusion and MIC E-test methods. Also, real-time PCR and high-resolution melting curve analysis (HRMA) assay were performed to detect the resistance genes.

**Results:** Out of the 101 *P. aeruginosa* isolates, four isolates (3.96%) were resistant to colistin. Also, 39 isolates (38.61%) were considered as MDR, and eight isolates (7.92%) were considered as XDR. Further, 25 (24.75%) and 26 isolates (25.74%) were produced ESBL and carbapenemase enzymes, respectively. According to HRMA results, four isolates (3.96%) were positive for *pmrA*, three isolates (2.97%) were positive for *mcr-1*, 25 isolates (24.75%) were positive for *bla*TEM, 24 isolates (23.76%) were positive for *bla*SHV, 26 isolates (25.75%) were positive for *bla*KPC, and 23 isolates (22.77%) were positive for *bla*IMP genes. Furthermore, ST108 and ST250 showed the highest distribution in *P. aeruginosa* isolates. Also, ST217, ST1078, and ST3340 were reported as novel types in CRPA strains.

**Conclusion:** Concerns about the prevalence of CRPA strains should be taken seriously. Also, our results showed that the *mcr-1* gene plays a vital role in the distribution of ESBL and KPC-producing *P. aeruginosa* strains.

**Keywords:** *Pseudomonas aeruginosa*, high-resolution melting curve analysis assay, HRMA assay,  $\beta$ -lactamases, colistin resistance, sequence types

## Introduction

*Pseudomonas aeruginosa* has been shown to produce the following  $\beta$ -lactamases: Class A extended-spectrum  $\beta$ -lactamases (ESBL) and Class B Metallo- $\beta$ -lactamases that degrade all  $\beta$ -lactams, including the carbapenems.<sup>1,2</sup> Carbapenemases, mostly classified as Ambler class B Metallo- $\beta$ -lactamases (MBLs), are zinc-dependent and have an extensive substrate profile, such as carbapenems and expanded-spectrum cephalosporins.<sup>3,4</sup> However, resistance to colistin in  $\beta$ -lactamase producing strains is higher than strains lacking this enzyme.<sup>5,6</sup>

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The emergence of multi-drug resistance (MDR) strain of *P. aeruginosa* has resulted in the use of older drugs such as colistin.<sup>7</sup> Colistin is bactericidal and displays excellent activity against pathogens such as *P. aeruginosa*, which is highly resistant to currently available antibiotics such as carbapenems.<sup>8</sup> These modifications occur as a result of the activation of the PmrA-PmrB system—this system regulated by the PhoP-PhoQ system.<sup>9</sup> In addition to the known chromosomal-mediated resistance mechanisms, a plasmid-mediated colistin resistance gene named *mcr-1* was discovered in China and subsequently elsewhere around the world.<sup>10</sup> Carbapenemase producers, mainly nosocomial *P. aeruginosa* isolates, are usually MDR, and therapeutic options for treating infections with carbapenemase producers remain limited.<sup>11</sup>

However, the sensitivity and specificity of phenotypic methods are less than molecular methods.<sup>12</sup> The speed and accuracy of these methods are also low compared to new molecular assay.<sup>13</sup> HRMA is the characteristic of a targeted PCR product melting profile resulting from gradually increasing melting temperature ( $T_m$ ) by tiny increments in the presence of high-saturating DNA binding dye.<sup>14</sup> Moreover, analysis based on HRMA has been used in different clinical laboratory applications, such as the identification of many different bacterial isolates.<sup>15</sup> Understandably, HRMA assay has endless applications and just as many advantages. The technology that runs and analyzes samples undergoing HRMA is a cost-effective one. HRMA also is a straightforward technology that can perform both the PCR and HRM Analysis in one instrument.<sup>12</sup>

CRPA, have all been reported in clinical isolates; therefore, rapid and accurate identification of these strains become particularly important.<sup>9</sup> Hence, we used the HRMA technique to identify the different isolates of *P. aeruginosa* in the clinical specimens. Hence, the present study involving the epidemiological surveillance of colistin-resistant *P. aeruginosa* from Hamadan, Iran. We further describe the molecular epidemiology of ESBL and KPC-producing strains leading to increase colistin resistance in *P. aeruginosa*.

## Materials and Methods

### Study Design and *P. Aeruginosa* Isolates

Out of the 402 isolates collected from different clinical specimens which were part of the routine hospital laboratory procedure, a total of 101 *P. aeruginosa* isolates were

collected from different samples from November 2017 to December 2018, from different wards of the Hamedan Hospitals and University Centers, Iran. Biochemical tests were used to differentiate between closely related bacterial species or genera. These tests were conducted according to the standard specifications described and applied in the Microbiology Laboratory at Hamedan University of medical sciences. Colonies morphologically resembled *P. aeruginosa* and had a positive oxidase test selected for further identification. Then colonies were examined by various biochemical tests such as oxidase, catalase, urease, arginine dehydrogenase, and oxidation-fermentation. Bacterial strains were stored at  $-20^{\circ}\text{C}$  in Brain-Heart Infusion (BHI) (Merck, Germany) broth supplemented with 25% v/v glycerol. This study was approved by the ethics committee of Hamadan University of Medical Sciences (No: IR.UMSHA.REC. 1396.637).

### Determination of Antibiotic Profiles

The disk diffusion (Kirby-Bauer) method was used for the detection of antimicrobial resistance patterns. Nine separate antimicrobial susceptibility discs, including imipenem, doripenem, amikacin, ceftazidime, aztreonam, gentamicin, ciprofloxacin, piperacillin, and cefepime (MAST, UK) were used. Plates were incubated for 18–24 hours in ambient air at  $35^{\circ}\text{C}\pm 2$  and the diameters of zones of inhibition around the antimicrobial discs recorded to the nearest whole millimeter using a graduated ruler. Results were reported as “sensitive,” “intermediate,” or “resistant” based on the cut-off values recommended by CLSI 2018.<sup>16</sup>

MIC test was performed for all the *P. aeruginosa* isolates based on the standard CLSI guidelines 2018 using E. test (Liofilchem, Italy) and broth microdilution method for the following antimicrobials: amikacin, ciprofloxacin, ceftazidime, aztreonam, imipenem, meropenem, ceftazidime, and colistin. For all phenotypic tests, *P. aeruginosa* ATCC 15,442 and *P. aeruginosa* ATCC 27,853 were used as a positive control, and *P. aeruginosa* PAO-1 was used as a negative control.

### Phenotypic Detection of ESBL and KPC-Producing

Combined disc method (CDT) (Mast, UK) and modified Hodge test (MHT) was used for the detection of ESBL and KPC-producing strains, respectively. Discs containing cefotaxime and ceftazidime with and without clavulanic acid were used. An ESBL phenotype was defined as an increase

of  $\geq 5$  mm in the zone around the disc containing clavulanic acid compared to the zones of corresponding discs without clavulanic acid.<sup>17</sup> In each batch of the experiment, *K. pneumoniae* ATCC BAA-1705 was included as the positive control, and a carbapenemase-negative *K. pneumoniae* strain ATCC BAA-1706 was included as a negative control.

## Extraction of DNA

Genomic DNA was isolated using the Qiagen miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Also, a plasmid was isolated from 5mL of overnight culture using Qiagen miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA and plasmid concentrations were determined using a spectrophotometer Nanodrop-200 (Hangzhou Allsheng Instruments Co., Ltd, China), and the samples were stored at  $-20^{\circ}\text{C}$  for further DNA analysis.

## Evaluation of Sensitivity and Specificity and HRMA Assay

Real-time PCR was carried out in 20 $\mu\text{L}$  reactions on the ABI step one plus (USA) instrument (Table 1). For each reaction, 4 $\mu\text{L}$  of the reaction mix (HOT FIREPol EvaGreen HRMA Mix), 1 $\mu\text{L}$  of each primer (5pmol).<sup>9,18-21</sup> Furthermore, PCR grade water to a final volume of 12 $\mu\text{L}$

was added to 2 $\mu\text{L}$  of the target DNA. PCR amplification was carried out according to the following parameters; a denaturation cycle at  $95^{\circ}\text{C}$  for 10min following by 40 amplification cycles which include, denaturation at  $95^{\circ}\text{C}$  for 10 sec, annealing at  $58^{\circ}\text{C}$  for 30 sec with single fluorescence measurement and extension at  $72^{\circ}\text{C}$  for 20 sec. This was followed by a melting curve ( $T_m$ ) analysis program for one cycle ( $95^{\circ}\text{C}$  for 1 sec,  $70^{\circ}\text{C}$  continuous using a temperature transition rate of  $0.3^{\circ}\text{C}/\text{sec}$  and a cooling period of  $40^{\circ}\text{C}$  for 30 sec. Then, the detection of pathogens was performed by melting curve analysis. *P. aeruginosa* ATCC 15,442 and *P. aeruginosa* ATCC 27,853 were used as a positive control, and *P. aeruginosa* PAO-1 was used as a negative control.

## MLST Sequences Typing

All strains of colistin and  $\beta$ -lactamase producers were selected for sequencing. The seven housekeeping genes: *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE* were selected for amplification and sequencing, according to the MLST scheme of *P. aeruginosa* website (<http://pubmlst.org/paeruginosa/>). PCR amplification reactions (volumes, nucleotides, and primer concentrations) were performed in an Eppendorf thermocycler as previously described. The amplified products were purified by Pishgam biotech co (Tehran, Iran).

**Table 1** Oligonucleotide Sequences Used in This Study

Target	Primer Name	Sequence of Primers	Melting $T_m$	Product Size (bp)	GenBank Accession numbers	References
KPC	<i>blaKPC</i>	F: GATACCACGTTCCGTCTGG R: GCAGGTTCCGGTTTTGTCTC	$83.2 \pm 0.5^{\circ}\text{C}$	254	JX682705.1	[12]
	<i>blaIMP</i>	F: GGCTTAATTCTCGATCTATCCC R: CTAGCCAATAGTTAACTCCGC	$80.6 \pm 0.5^{\circ}\text{C}$	114	AB983593.1	[5]
ESBL	<i>blaTEM</i>	F: GCATCTTACGGATGGCATGA R: GTCCTCCGATCGTTGTCAGAA	$82.5 \pm 0.5^{\circ}\text{C}$	101	KX688041.1	[1]
	<i>blaSHV</i>	F: TCCCATGATGAGCACCTTTAAA R: TCCTGCTGGCGATAGTGGAT	$83 \pm 0.5^{\circ}\text{C}$	105	MGI88748.1	[1]
Colistin	<i>mcr-1</i>	F: CGGTCAGTCCGTTTGTTTC R: CTTGGTCCGTCTGTAGGG	$90.05 \pm 0.5^{\circ}\text{C}$	309	KX016796.1	[8]
	<i>mprA</i>	F: CACCAGGTGACCCTGTCC R: CGTAGAGGCTCTGCTCCAGT	$91.7 \pm 0.5^{\circ}\text{C}$	124	CP044006.1	[14]

## Data Analysis

The WHO.net 2018 software was used for entry and preliminary analysis of microbiology data. SPSS software version 16 (SPSS, Chicago, IL) and chi-square and *t*-test were used to examine the relationship between different variables. The instrument measured the fluorescence, and ABI Thermo Fisher software plotted the fluorescence versus temperature in a graph. Reagent blanks were used to ensure there was no contamination in the reagents.

## Results

Out of the 402 isolates collected from different clinical specimens, 101 *P. aeruginosa* isolates were identified as follows: 22 isolates (21.78%) from blood, 30 isolates (29.70%) from wounds, 18 isolates (17.82%) from the burn, 14 isolates (13.86%) from urine, seven isolates (6.93%) from the catheter, and ten isolates (9.9%) from discharge. Eighty-eight isolates (87.12%) were isolated from female patients, and 13 isolates were collected (12.87%) from male patients.

## Prevalence of Antibiotic Resistance Profile

The resistance profile of *P. aeruginosa* isolates was detected as follows: 28 strains (27.72%) resistant to imipenem, 19 strains (18.81%) resistant to doripenem, 13 strains (12.87%) resistant to amikacin, 19 strains (18.81%) resistant to ceftazidime, 11 strains (10.89%) resistant to aztreonam, 96 strains (95.04%) resistant to gentamicin, 97 strains (96.03%) resistant to ciprofloxacin, 23 strains (22.77%) resistant to piperacillin, and 29 strains (28.71%) resistant to cefepime (Figure 1A). Besides, 39 isolates (38.61%) were resistant to six antibiotics and were considered as MDR strains, and eight isolates (7.92%) were resistant to all antibiotics and were considered as XDR strains (Figure 1).

Out of the 101 isolates, 28 isolates (27.72%) showed MIC >8 µg/mL for imipenem, 20 isolates (19.18%) demonstrated MIC >32 µg/mL for ceftazidime, 13 isolates (12.87%) were detected with MIC >4 µg/mL for amikacin, 10 isolates (9.9%) indicated MIC >4 µg/mL for aztreonam, 98 isolates (96.03%) showed MIC >4 µg/mL for ciprofloxacin, and 4 isolates (3.96%) were observed with MIC >4 µg/mL for colistin (Figure 1).

## Prevalence of ESBL and KPC-Producing Strains

Out of the 101 isolates of *P. aeruginosa*, 25 isolates (24.75%) were considered as ESBL producing, and 26

isolates (25.74%) were considered as KPC-producing (carbapenemase-producing), and 13 isolates were considered as ESBLs/carbapenemase-producing.

## Analytical Sensitivity and Specificity of Primers

The melting profile was characterized by a peak of 83.2 ±0.5°C for *blaKPC* gene, 80.6±0.5°C for *blaIMP* gene, 83 ±0.5°C for *blaSHV* gene, 82.5±0.5°C for *blaTEM* gene, 91.7±0.5°C for *pmrA* gene, and 90.0± 0.5°C for *mcr-1* gene. The results of the standard curves for the various dilutions of DNA are shown in Figures 2 and 3. Moreover, the primers used were not able to detect *Staphylococcus aureus* at specific T<sub>m</sub> (Figure 4).

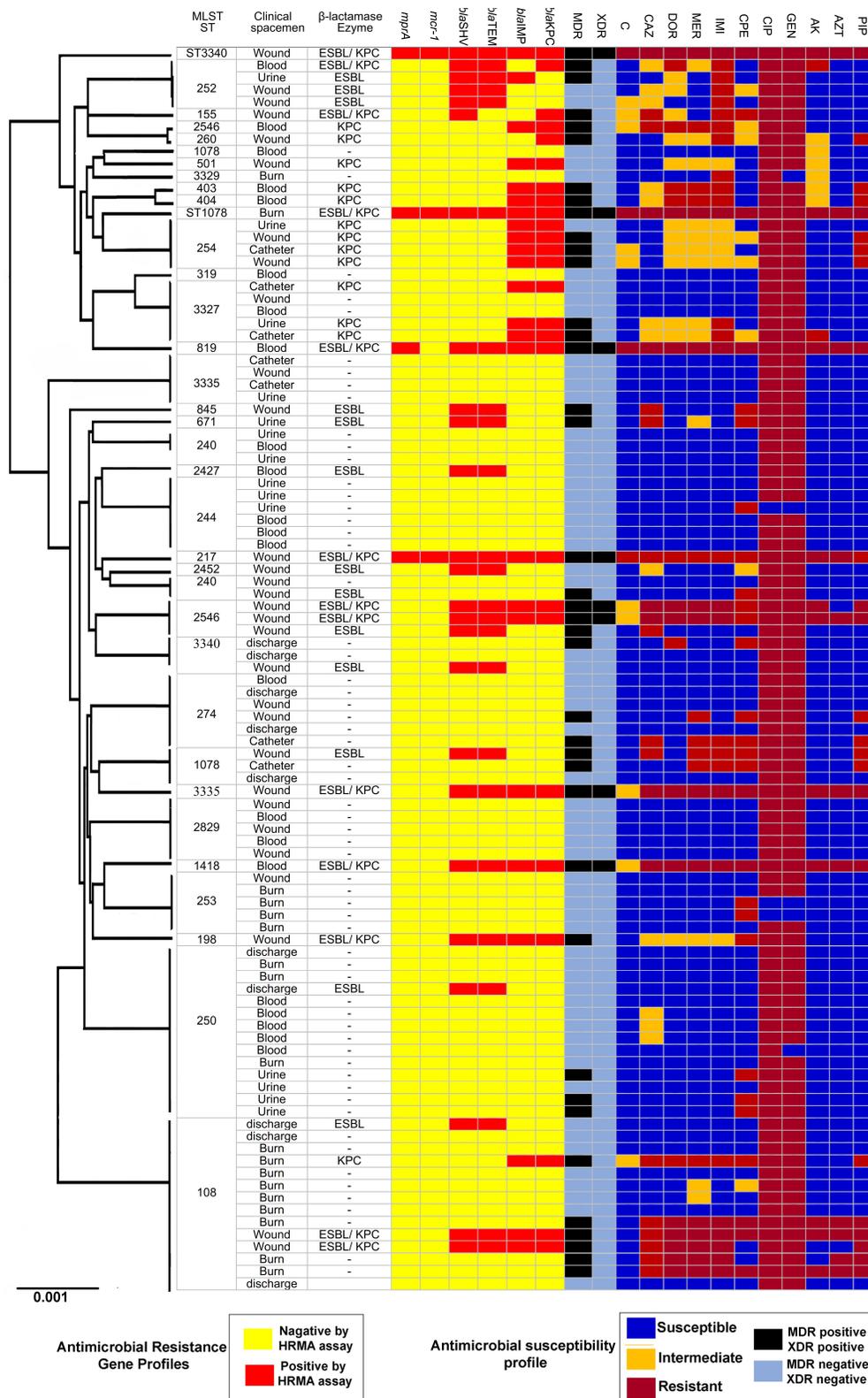
## Detection ESBL and KPC Producer, and Colistin-Resistant Strains by HRMA Assay

The frequency of beta-lactamase and colistin genes by the HRMA method was as follows: 25 isolates (24.75%) carrying *blaTEM* gene, 26 isolates (25.74%) carrying *blaKPC* gene, 24 isolates (23.76%) carrying *blaSHV* gene, 23 isolates (22.77%) carrying *blaIMP* gene, four isolates (3.96%) carrying *pmrA* gene, and three isolates (2.97%) carrying *mcr-1* gene (Figures 4 and 5).

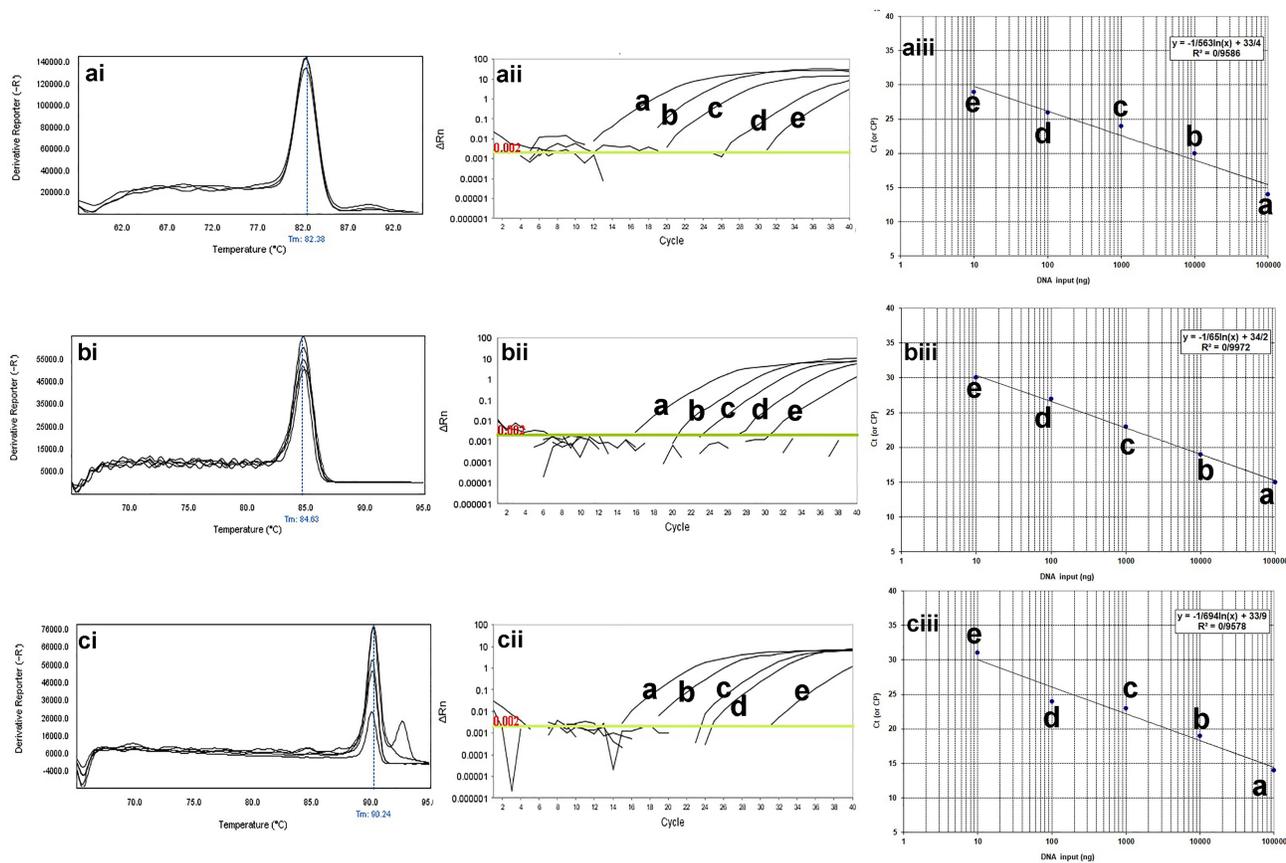
## Phylogenetic Analysis of the MLST Sequences

Among the 101 isolates that were genotyped, 35 different STs were identified. ST108 and ST250 were found in many strains. Therefore, a lower level of genetic diversity was observed among the isolates were obtained from male patients comparing to the isolates were collected from female patients. In total, 40% of all colonized female patients harbored *P. aeruginosa* STs that were also found in other male patients, and 30% were colonized with isolates with unique STs. Moreover, ST217, ST1078, and ST3340 were characterized as novel types among the CRPA, ESBL, and carbapenemase-producing *P. aeruginosa*. Typing by MLST further illustrated the enormous diversity within the strains, as isolates with similar β-lactamase genes had different STs (Figure 1).

Allelic profiles were investigated to find a representative isolate circulating among the different samples, including wound, blood, and urine culture. The MLST revealed a new ST, namely ST3340, belonging to a clonal complex previously has not been described. Further, 20 ST types were identified, of which two were shared between multiple clinical samples. ST108 and



**Figure 1** Analysis of 101 sequenced *P. aeruginosa* genomes. A maximum-likelihood tree and MLST analysis, presence of beta-lactamases, and enzymes. **Abbreviations:** MDR, multidrug-resistant; C, colistin; CAZ, ceftazidime; DOR, doripenem; MER, meropenem; IMI, imipenem; CPE, cefepime; GEN, gentamycin; CIP, ciprofloxacin; AK, amikacin; AZT, aztreonam; PIP, piperacillin.



**Figure 2** The qPCR dilution series for analytical sensitivity of real-time PCR for *mcr-1*, *bla<sub>TEM</sub>*, and *bla<sub>SHV</sub>* genes in clinical isolates of *P. aeruginosa*. The melting profiles of *mcr-1*, *bla<sub>TEM</sub>*, and *bla<sub>SHV</sub>* genes showed (i) Figures. The Ct value of different dilutions was shown in (ii) Figures. The primer efficiency slope and standard curves of *mcr-1*, *bla<sub>TEM</sub>*, and *bla<sub>SHV</sub>* genes are shown in (iii) Figures. The melting temperature profile was as follows:  $83.2 \pm 0.5^\circ\text{C}$  for *bla<sub>KPC</sub>* gene,  $80.6 \pm 0.5^\circ\text{C}$  for *bla<sub>IMP</sub>* gene,  $83.0 \pm 0.5^\circ\text{C}$  for *bla<sub>SHV</sub>* gene,  $82.5 \pm 0.5^\circ\text{C}$  for *bla<sub>TEM</sub>* gene,  $91.7 \pm 0.5^\circ\text{C}$  for *pmrA* gene, and  $90.0 \pm 0.5^\circ\text{C}$  for *mcr-1* gene. Also, the efficiency of the primer was 98.19% for the *bla<sub>SHV</sub>* gene, 99.97% for the *bla<sub>TEM</sub>* gene, and 97.01% for the *mcr-1* gene. For evaluation of the sensitivity of the real-time PCR assays, a series of five-fold diluted DNA of the positive controls were initially tested on the ABI Step-One Plus system. The mean of a: 100,000 ng; b: 10,000 ng; c: 1000 ng; d: 100 ng; e: 10 ng of DNA dilutions. Horizontal lines represent the cycle threshold of real-time PCR. The melting and slope temperature profile were as follows:  $83.2 \pm 0.5^\circ\text{C}$  for *bla<sub>KPC</sub>* gene,  $80.6 \pm 0.5^\circ\text{C}$  for *bla<sub>IMP</sub>* gene,  $83.0 \pm 0.5^\circ\text{C}$  for *bla<sub>SHV</sub>* gene,  $82.5 \pm 0.5^\circ\text{C}$  for *bla<sub>TEM</sub>* gene,  $91.7 \pm 0.5^\circ\text{C}$  for *pmrA* gene, and  $90.0 \pm 0.5^\circ\text{C}$  for *mcr-1* gene.

ST250 were common among wound, urine, burn, and discharge specimens, and ST254 and ST274 were shared by the wound, catheter, burn, and discharge specimens (Figure 1).

## Analytical Analysis

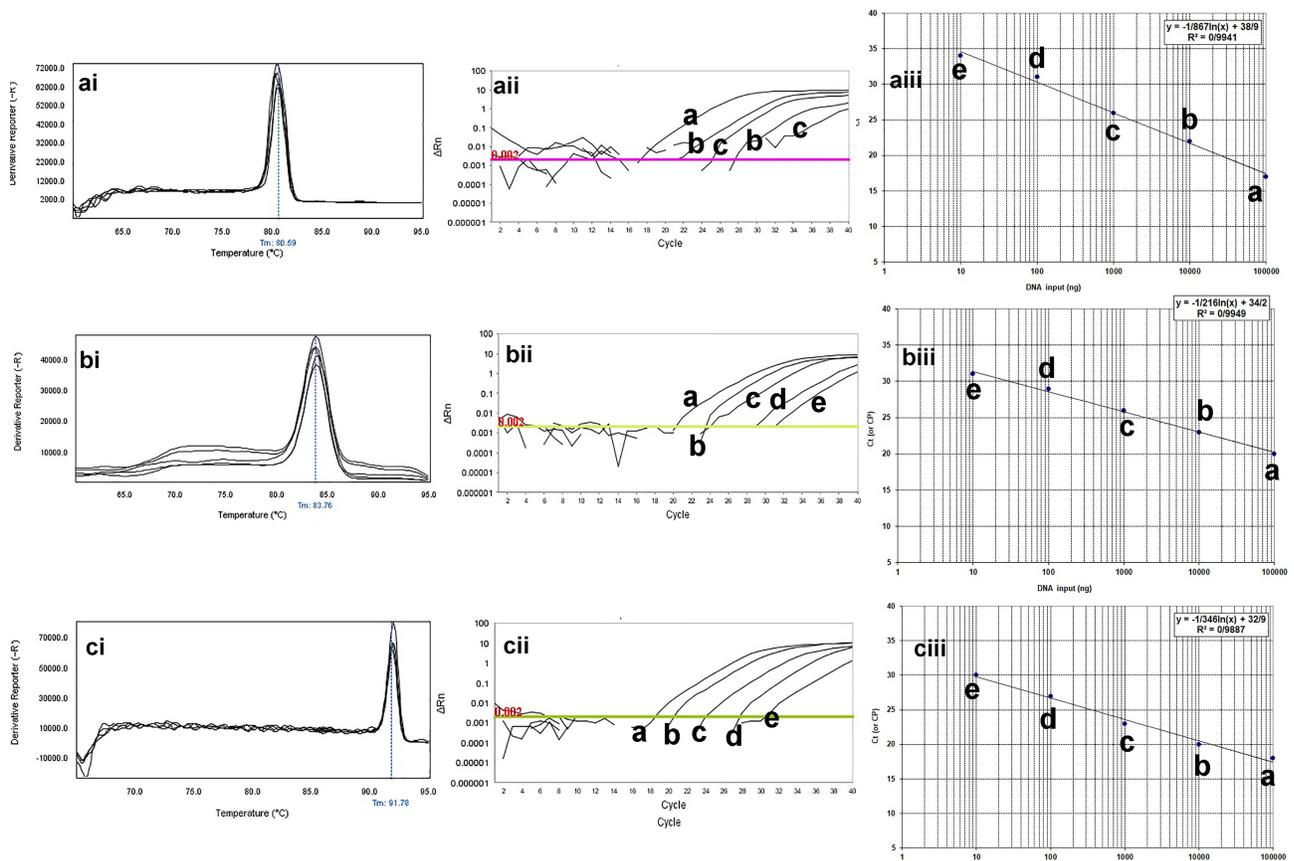
The most prevalent STs, ST1078 and ST3340, had a higher prevalence in the blood and wound infections. The type of the samples and antibiotics residence were correlated with the carriage of STs ( $P = 0.001$ ). In general, ST3340 strains were more sequence type in MDR and XDR strains. Further, Both ST1078 and ST3340 strains were more often resistant to colistin ( $P = 0.001$ ) (Table 2).

## Discussion

*P. aeruginosa* is an important pathogen associated with severe infections in healthcare settings and a significant

cause of morbidity and mortality. However, colistin is rapidly emerging as the last resort antibiotic for the treatment of MDR/XDR *P. aeruginosa*.<sup>15</sup>

In this study, according to phenotypic methods, gentamicin and ciprofloxacin-resistant strains had the highest frequency with 95.04% and 96.03%, respectively, and amikacin (6.66%) and colistin (3.96%) showed the lowest abundance. Moreover, MDR and XDR strains were counted as 38.61% and 7.92% of isolates, respectively. These results are in agreement with different studies of Venezuela,<sup>22</sup> China,<sup>23</sup> and Germany,<sup>24</sup> that demonstrated high rates of antibacterial resistance of *P. aeruginosa*, where they showed similar resistance to the antibiotics examined in this study. In contrast to the current study, some reports showed a low rate of resistance in the last three years, except for colistin, in which the same findings were described.<sup>25,26</sup> Also, the



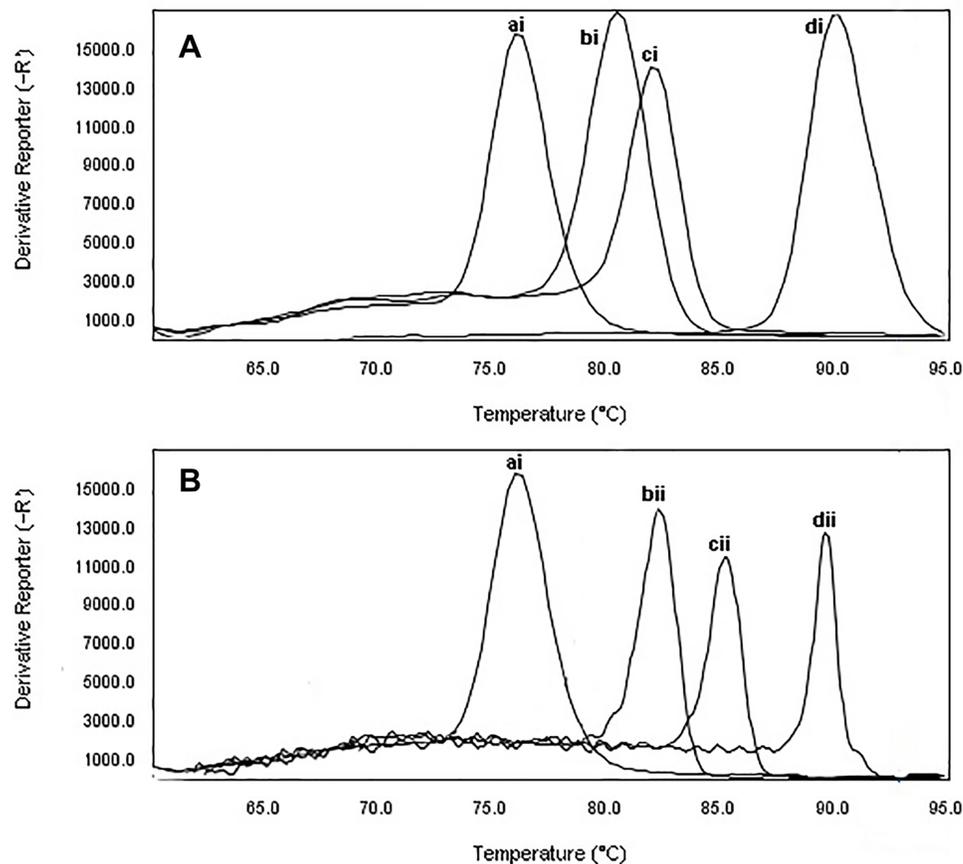
**Figure 3** The qPCR dilution series for analytical sensitivity of real-time PCR for *blaIMP*, *blaKPC*, and *pmrA* genes in clinical isolates of *P. aeruginosa*. The melting profiles of *blaIMP*, *blaKPC*, and *pmrA* genes showed (i) Figures. The Ct value of different dilutions was shown in (ii) Figures. The primer efficiency slope and standard curves of *blaIMP*, *blaKPC*, and *pmrA* genes are shown in (iii) Figures. The melting temperature profile was as follows:  $83.2 \pm 0.5^\circ\text{C}$  for *blaKPC* gene,  $80.6 \pm 0.5^\circ\text{C}$  for *blaIMP* gene, and  $91.7 \pm 0.5^\circ\text{C}$  for *pmrA* gene. Also, the efficiency of the primer was 99.31% for the *blaKPC* gene, 97.99% for the *blaIMP* gene, and 98.12% for the *pmrA* gene. For evaluation of the sensitivity of the real-time PCR assays, a series of five-fold diluted DNA of the positive controls were initially tested on the ABI Step-One Plus system. The mean of a: 100,000 ng; b: 10,000 ng; c: 1,000 ng; d: 100 ng; e: 10 ng of DNA dilutions. Horizontal lines represent the cycle threshold of real-time PCR.

prevalence of KPC, ESBL-producing, and CRPA strains (as illustrated in Figure 1) was 25.74%, 24.75%, and 3.96%, respectively, which was in line with other studies.<sup>27,28</sup> However, other studies described the different abundance of *P. aeruginosa* isolates of KPC, ESBL-producer, and resistant to colistin.<sup>1,2</sup>

In the current study, the distribution of ESBL and KPC-producing strains were reported 58.92%, 48.71% from MDR strains, and 100% from XDR strains of *P. aeruginosa*, respectively. Moreover, in MDR strains, 33.33% of isolates were KPC-ESBL producing strain, and in XDR, 100% of *P. aeruginosa* isolates were KPC-ESBL producers. Those findings correlate with other reports indicated that the prevalence of MDR and XDR strains in KPC-ESBL producing strains of *P. aeruginosa* higher than that of non-KPC-ESBL producing strains.<sup>1,29</sup> Therefore, based on Table 2, there was a significant relationship between the prevalence

of MDR-XDR strains and the production of ESBL and carbapenemases producing *P. aeruginosa* ( $p=0.001$ ). Some researchers reported that the same result and stated that the frequency of  $\beta$ -lactamases in XDR strains is higher than MDR ones.<sup>1,3,30,31</sup> However, to increase the predictive value of this association, precise and sensitive molecular methods should be used in epidemiological studies.<sup>32</sup>

In this study, we developed an HRMA assay for the detection of ESBL producing, KPC-producing, and CRPA strains by melting curve analysis technology. Based on Melting temperatures, as showed in Figures 2 and 3, melt curves of gene amplification were equal to  $91.7 \pm 0.5^\circ\text{C}$  for *pmrA* gene and  $90.0 \pm 0.5^\circ\text{C}$  for *mcr-1* gene. Besides, DNA melting temperatures for the *blaTEM*, *blaSHV*, *blaKPC*, and *blaIMP* genes were  $82.5 \pm 0.5^\circ\text{C}$ ,  $83 \pm 0.5^\circ\text{C}$ ,  $83.2 \pm 0.5^\circ\text{C}$ , and  $80.6 \pm 0.5^\circ\text{C}$ , respectively. According to our study in Figure 5,



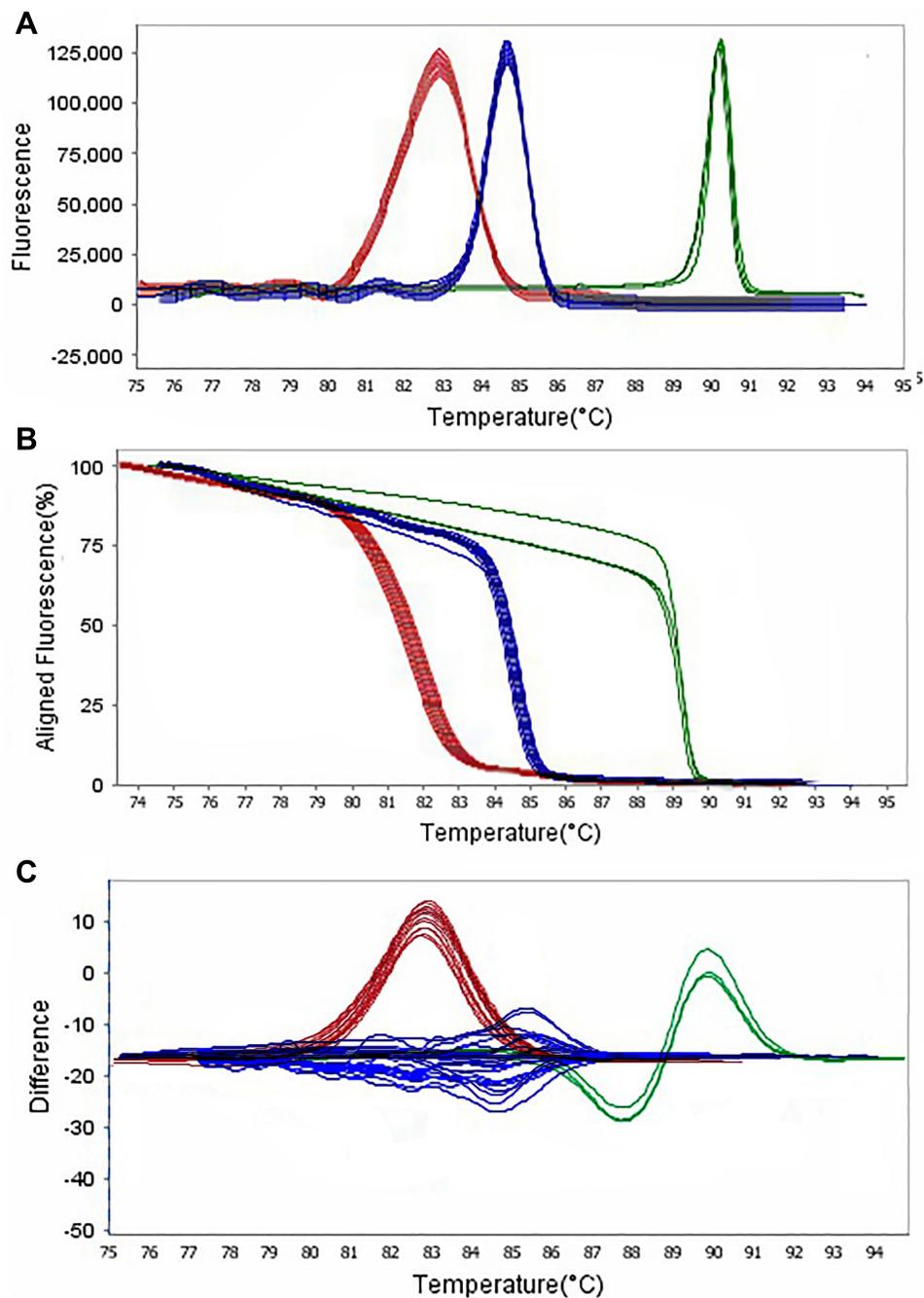
**Figure 4** The specificity of real-time PCR for primers used to detect of CRPA and  $\beta$ -lactamase producing *P. aeruginosa*. Melting curve analysis showing the melting temperature peaks ( $T_m$ ) of *bla*IMP (bi), *bla*KPC(ci), *mprA*(di), *bla*TEM(bii), *bla*SHV(cvi), and *mcr-1*(dii). ai: *Staphylococcus aureus* ATCC25923.

CRPA strains had a prevalence of 2.44% and detected by the HRMA method in agreement with a similar study,<sup>11,12</sup> as well as Sekyere in a study showed that different methods for identifying colistin resistance strains are not of the same sensitivity and specificity.<sup>18</sup> Also, the mechanism of colistin resistance in *P. aeruginosa* has not been fully understood, and some functions may be performed by genes not cover in this study.

According to Figures 5 and 6, HRMA results revealed that 88.23%, 82.35%, and 94.11% of *P. aeruginosa* were positive for *bla*TEM and *bla*SHV genes. Likewise, the frequency of MDR-ESBL producing, MDR-KPC producing, XDR-KPC producing, and XDR-KPC producing strains were reported in 83.52%, 76.47%, 87.5%, and 37.5% of *P. aeruginosa*, respectively. A strong and significant relationship was observed between the MDR-XDR strains and the production KPC and ESBL producer in *P. aeruginosa* strains, whereas the frequency of KPC-ESBL producing strains in MDR and XDR strains were

83.52% and 62.5%, respectively. Besides, our phenotypic and HRMA findings confirmed a significant association between colistin resistance and the prevalence of beta-lactamases. Also, the high prevalence of CRPA was reported in MDR and XDR strains. Thus, carbapenem resistance has led to the emergence of the colistin. On the other hand, the emergence of colistin resistance in XDR-KPC producing strains is higher than in MDR-KPC producing strains and is mediated by the *mcr-1* gene. The *mcr-1* gene can be transmitted through the plasmid to colistin-sensitive strains.<sup>33–36</sup> Another concern is the emergence of colistin resistance during therapy among KPC-producing strains of *P. aeruginosa* in Switzerland,<sup>37</sup> USA,<sup>38</sup> and Australia.<sup>8</sup>

As the main benefit of this study was to develop an HRMA assay to detect low levels KPC-producing, ESBL producing simultaneously, and CRPA strains, we next selected the KPC/IMP/*mprA* primer set1 and TEM/SHV/*mcr-1* primers set2 combination for a multiplex assay assessment. Although the results of the HRMA



**Figure 5** Representative high resolution melting graphs corresponding to one high resolution melting analysis of MDR and XDR strains of *P. aeruginosa* (n = 39). Curves of tested samples identified as *bla*<sub>IMP</sub> carrier are shown in red, *bla*<sub>KPC</sub> carrier are shown in blue, and *mprA* carrier are shown in green.  $\beta$ -lactamases genes were amplified successfully using the EvaGreen Dye in the ABI Step-OnePlus machine by one-sept protocol. (A) Melting curves; (B) Normalized plot; and (C) Difference plot. Positive control: *P. aeruginosa* NCTC 13,359.

assay and phenotypic methods for identifying  $\beta$ -lactamase producing strains in *P. aeruginosa* showed close results; however, the HRMA assay has several advantages. The significant advantage of the HRMA assay in this study was the identification of co-existence of CRPA and  $\beta$ -lactamase producing strains

in a short time. Bentaleb et al<sup>39</sup> and Otaguiri et al<sup>40</sup> demonstrated that HRMA based methods have an improved level of sensitivity when compared to culture and, thus, can detect lower levels of bacteria.

In Korea, it has previously been argued that some STs are highly abundant in CRPA strains.<sup>6</sup> In the present

**Table 2** Analysis of Variables Significantly Associated STs, Antimicrobial Resistance and Clinical Samples in Clinical Isolates of *P. aeruginosa*

Antibiotics	β-Lactam Resistant Genes				β-Lactamase Producing Strains		Colistin-Resistant Strains		STs
	<i>blaIMP</i>	<i>blaKPC</i>	<i>blaSHV</i>	<i>blaTEM</i>	ESBL	Carbapenemase	By MIC	By HRM	
Imipenem	p*=0.046	p=0.597	p=0.016	p=0.036	p=0.036	p=0.019	p=0.019	p=0.019	p=0.025
Doripenem	p=0.018	p=0.824	p=0.025	p=0.038	p=0.038	p=0.05	p=0.035	p=0.05	p=0.045
Amikacin	p=0.093	p=0.080	p=0.019	p=0.035	p=0.035	p=0.017	p=0.044	p=0.117	p=0.050
Ceftazidime	p=0.009	p=0.071	p=0.007	p=0.032	p=0.032	p=0.062	p=0.097	p=0.062	p=0.097
Aztreonam	p=0.046	p=0.097	p=0.002	p=0.065	p=0.065	p=0.012	p=0.009	p=0.212	p=0.012
Gentamicin	p=0.017	p=0.305	p=0.045	p=0.005	p=0.005	p=0.009	p=0.004	p=0.009	p=0.024
Ciprofloxacin	p=0.046	p=0.597	p=0.055	p=0.005	p=0.005	p=0.045	p=0.019	p=0.045	p=0.060
Cefepime	p=0.218	p=0.824	p=0.022	p=0.03	p=0.03	p=0.020	p=0.052	p=0.020	p=0.055
Piperacillin	p=0.047	p=0.052	p=0.030	p=0.029	p=0.029	p=0.071	p=0.055	p=0.071	p=0.146
Clinical samples									
Wound	p=0.071	p=0.040	p=0.097	p=0.097	p=0.097	p=0.020	p=0.001	p=0.020	p=0.042
Urine	p=0.097	p=0.020	p=0.009	p=0.009	p=0.009	p=0.080	p=0.009	p=0.080	p=0.074
Blood	p=0.193	p=0.080	p=0.043	p=0.043	p=0.043	p=0.025	p=0.003	p=0.025	p=0.060
Catheter	p=0.146	p=0.597	p=0.365	p=0.365	p=0.365	p=0.035	p=0.035	p=0.035	p=0.085
Burn	p=0.218	p=0.824	p=0.003	p=0.003	p=0.003	p=0.015	p=0.283	p=0.015	p=0.305
Discharge	p=0.007	p=0.045	p=0.035	p=0.023	p=0.127	p=0.079	p=0.007	p=0.052	p=0.065

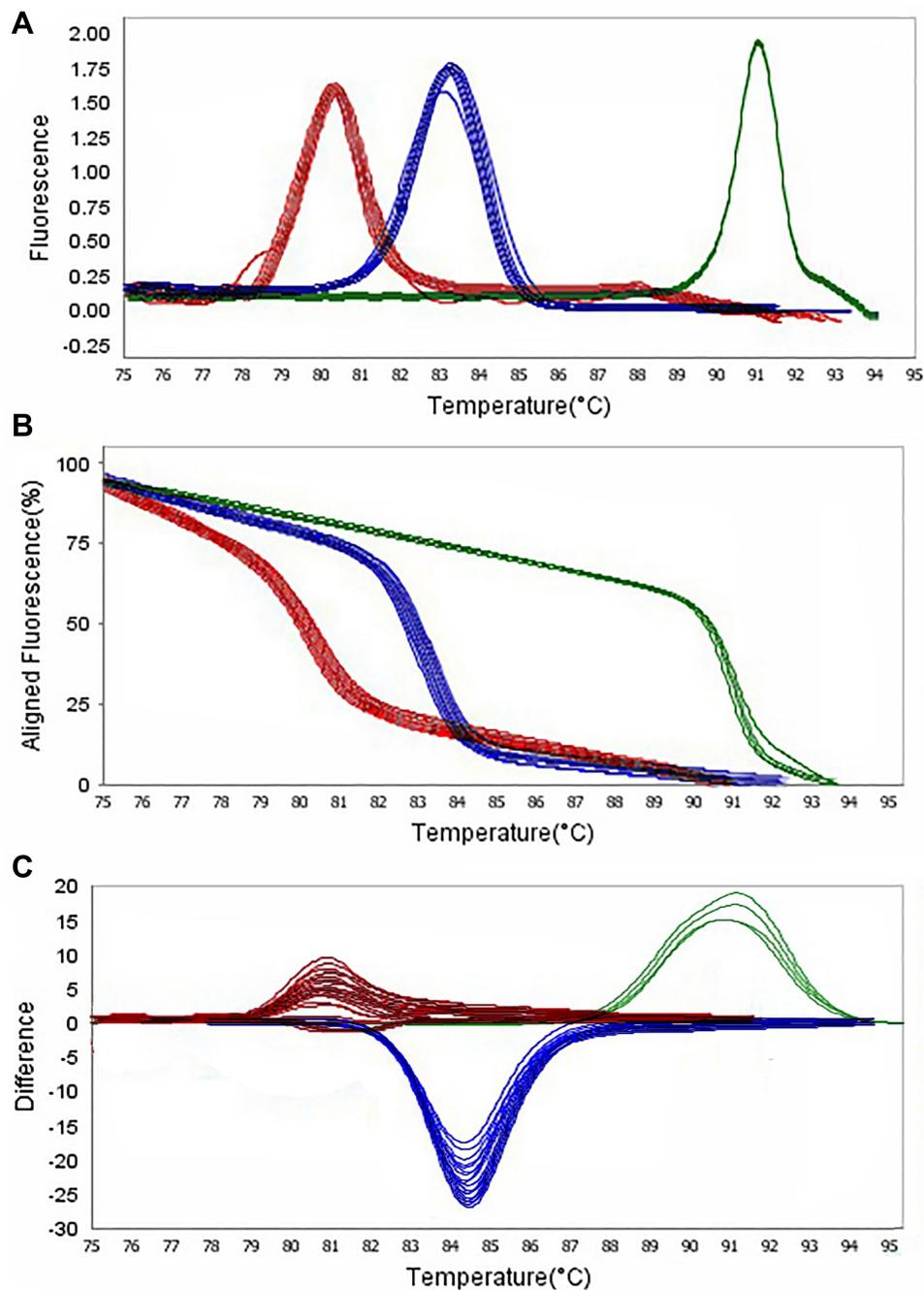
**Notes:** \*Fisher and the chi-square tests were performed for the comparison of data. P values of less than 0.05 were considered statistically significant for all the statistical tests performed.

study, Figure 1 reported that the most prevalent STs, ST108, and ST250, had a higher prevalence in the blood and burn-wound infections. The gender of the patients was correlated with the carriage of STs ( $P = 0.001$ ). Compared to strains of all other STs, strains of ST1078 and ST3340 could be characterized by a specific antibiotic resistance profile. In general, ST3340 strains were more resistant to other antibiotics and susceptible to amikacin. Both ST1078 and ST3340 strains were more often resistant to colistin. In contrast to this study, some researchers reported ST235 and ST108 as the essential MDR strains in colistin-sensitive *P. aeruginosa*.<sup>5,6</sup> This difference may be due to resistance to colistin in *P. aeruginosa*.

In this study, we would like to point out these limitations; the role of colistin resistance in the prevalence of *P. aeruginosa* β-lactamase producing strains needs further studies. Besides, the HRMA method is limited due to the use of dedicated primers and sensitive fluorescent dyes. It should be noted that software analysis of the melting curve of HRMA has a significant impact on the quality of the results.

## Conclusions

Colistin resistance in clinical isolates of *Pseudomonas aeruginosa* is one of the most critical recent threats. The results of the present study showed that colistin resistance is directly related to ESBL and KPC enzymes. Analysis of new sequence types (ST3340 and ST1078) from *P. aeruginosa* isolates revealed that both STs were resistant to colistin and producer of ESBL and KPC enzymes. Those observations suggest that colistin resistance may provide resistance to beta-lactam and pneumonia antibiotics. Moreover, colistin resistance was observed only in MDR-ESBL producing, MDR-KPC producing, XDR-KPC producing, XDR-KPC producing. These indicate that the optimization of an accurate and sensitive method for the identification of CRPA strains is essential. Compared with phenotypic methods, HRMA assay can identify colistin and β-lactam resistant strains simultaneously with high speed and accuracy. Moreover, we reported that the acquisition of a novel ST of colistin-resistant from Iran, further indicating that the emergence of CRPA strains among important human pathogens is becoming broadened.



**Figure 6** Representative high resolution melting graphs corresponding to one high resolution melting analysis of MDR and XDR strains of *P. aeruginosa* (n = 39). Curves of tested samples identified as *bla*<sub>TEM</sub> carrier are shown in red, *bla*<sub>SHV</sub> carrier are shown in blue, and *mcr-1* carrier are shown in green.  $\beta$ -lactamases genes were amplified successfully using the EvaGreen Dye in the ABI Step-OnePlus machine by one-sept protocol. (A) Melting curves; (B) Normalized plot; and (C) Difference plot. Positive control: *P. aeruginosa* NCTC 13,359.

## Abbreviations

MDR, multiple drug resistance; XDR, extensively drug-resistant; KPC, *Klebsiella pneumoniae* carbapenemase; ESBL, extended-spectrum  $\beta$ -lactamases; SHV, sulfhydryl variable; TEM, temoneira; *mcr-1*, mobilized colistin

resistance-1; MIC, minimum inhibitory concentration; HRMA, high-resolution melting analysis; STs, sequence type; MLST, multilocus sequence typing; CRPA, colistin-resistant *P. aeruginosa*; CDT, combined disk method; TSB, tryptic soy broth; WHO, World Health Organization.

## 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). Written informed consent was taken from all participants.

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## Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed on the journal to which the article will be submitted; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors report no conflicts of interest for this work.

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