Infection and Drug Resistance

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ORIGINAL RESEARCH

Distribution of Class B and Class A β-Lactamases in Clinical Strains of *Pseudomonas aeruginosa*: Comparison of Phenotypic Methods and High-Resolution Melting Analysis (HRMA) Assay

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Background: There are various phenotypic methods for identifying class B and class A β-lactamase enzymes in *Pseudomonas aeruginosa*. The purpose of this study was to compare the sensitivity and specificity of different phenotypic methods with HRMA assay to detect β-lactamase-producing *P. aeruginosa* strains.

Methods: Eighty-eight of *P. aeruginosa* isolates were collected from different specimens. Conventional double-disk test (DDT) and EDTA-imipenem microbiological (EIM) were performed to detect ESBL and MBL-producing strains, respectively. Meanwhile, the Modified Hodge test and Carba-NP test were performed on all carbapenem-resistant strains. HRMA method and sensitivity and specificity of primers were determined based on the melt curve temperature range. In all comparisons, PCR was considered as the gold standard.

Results: Of the 402 isolates collected from different clinical specimens, 88 isolates of *P. aeruginosa* were identified. However, 43 strains were (48.88%) ESBL-producing, and 7 strains (7.95%) were MBL-producing. Also, using the Modified Hodge test and Carba-NP method, 11 (12.5%) and 19 (21.59%) strains were carbapenemase-producing, respectively. The results of the HRMA test revealed that genes coding for bla_{SHV}, bla_{TEM}, bla_{KPC}, bla_{IMP}, bla_{VIM}, and bla_{GES} were detected in 44.31%, 22.72%, 13.63%, 14.7%, 5.6%, and 2.27% of *P. aeruginosa* isolates. Nonetheless, for bla_{KPC} and bla_{GES} genes, sensitivity and specificity of the Carba-NP test were 90.47%, 94.87%, and 83.36%, 94.80%, respectively. However, sensitivity and specificity of MHT was 91.66%, 98.70%, and 77.77%, 96.42%, respectively. For bla_{SHV} and bla_{TEM} genes, sensitivity and specificity of DDT were 95.55%, 95.55%, and 86%, 83.50%, respectively. However, sensitivity and specificity of EMI were 77.77%, 97.59%, and 91.66%, 97.43% for bla_{VIM} and bla_{IMP}, respectively.

Conclusion: The HRMA is a powerful, accurate, closed-tube, rapid method for detecting β-lactamase genes in *P. aeruginosa*. The high sensitivity and specificity of this method, along with phenotypic tests, play a useful role in increasing the predictive value of clinical reports.

Keywords: *Pseudomonas aeruginosa*, high-resolution melting curve analysis, HRMA, β-lactamases, drug resistance

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Background

Pseudomonas aeruginosa is one of the leading nosocomial pathogens worldwide.[1] Nosocomial infections caused by this organism are often hard to treat because of both the intrinsic resistance of the species and its remarkable ability to acquire further mechanisms of β-lactamases enzymes to multiple groups of antimicrobial

agents.[2,3] However, in 1980 the β-lactamases were classified into four classes (A, B, C, and D).[4,5] Class A and D include the classic and extended-spectrum β-lactamases (ESBLs) and are mostly composed of the bla_{TEM}, bla_{SHV}, CTX-M, and OXA enzymes, class B comprises the Metallo-β-lactamases (MBL), and finally, class C contains the AmpC β-lactamases (ACBL).[6,7] Except for class B metalloenzymes, β-lactamases belong to the family of serine-reactive hydrolases.[4,8,9] Carbapenemases, enzymes possessing the ability to inhibit almost all β-lactam antibiotics, including carbapenems, have been mainly detected in mentioned species.[10] Until the early 1990s, carbapenemases were considered as species-specific, chromosomally encoded β-lactamases. Identification of genes encoding for carbapenemases on mobile genetic elements emerged the possible horizontal spread of these enzymes.[4,11,12]

There are various phenotypic methods for identifying β-lactamases enzyme-producing of *P. aeruginosa*, each with disadvantages and benefits.[3] Double-disk synergy test for detection of strains belongs to class A β-lactamases, Modified Hodge test to detect class A β-lactamases and meropenem E-test strip to identify class B β-lactamases is common tests.[13,14] One of the most critical advantages of phenotypic methods is their cost-effectiveness. Of course, the fatality of these methods and the low speed of diagnosis should be mentioned from its main disadvantages, and the need to use sensitive and rapid molecular methods along with these phenotypic methods is essential.[10,15]

High-Resolution Melting Analysis (HRMA) is an emerging technique used to discriminate DNA sequence variants.[8] HRMA is based on accurate determination of the relationship between temperature and the extent of dissociation of a PCR amplicon. HRMA is almost performed universally in real-time PCR thermocyclers post-PCR.[16] In HRMA, the fluorescence produced by a DNA intercalating dye (eg, SYBERGreen) is monitored during strand dissociation events during the melt phase. While phenotype-based detection methods have many advantages, molecular real-time amplification techniques have gained significant acceptance because they may provide more rapid detection, increased sensitivity, and specificity, and lack the risk of carry-over contamination associated with earlier methods.[17,19] However, normalized and difference graphs were generated to assess the ability of the HRMA method to differentiate between bacterial strains.[18]

This study aimed to investigate different methods in diagnosing different strains of *P. aeruginosa*. Besides, by optimizing the HRMA assay, the errors and shortcomings of this method were investigated based on local and experimental conditions. Also, by comparing the sensitivity and specificity of phenotypic methods and the HRMA assay, the advantages, and disadvantages of different methods were discussed.

Methods

Study Design and Sampling

During 9 months (Dec 2017 to Sep 2018), 88 of *P. aeruginosa* strains were collected from various clinical isolates, including blood, sputum, urine, ulcers, and secretions. All isolates were transferred to the Microbiology Laboratory of Hamedan University of Medical Sciences for differential tests.

P. aeruginosa Isolates

Isolates suspected to be *P. aeruginosa* were identified by using Pseudomonas Cetrimide agar (Merck, Germany), and plates were incubated for 48 hours at 42ºC under aerobic conditions. Then, colonies grown on the Cetrimide agar were examined by various biochemical tests. Biochemical tests were performed to confirm *P. aeruginosa* using methyl-red and Voges – Proskauer (MRVP) tests, Oxidase, Catalase, hydrolysis of citrate utilization, Indole production, and fermentation of various sugars (All from Sigma-Aldrich, USA). Bacterial strains were stored at −20°C in Brain-Heart Infusion (BHI) broth (Merck, Germany) supplemented with 25% v/v glycerol.

Antibiotic Disc Susceptibility Testing

Antibiotic susceptibility testing was done as per the disc diffusion method (Bauer et al, 1966) following the guidelines of the Clinical Laboratory Standard Institute (CLSI) 2018 against 13 antibiotics of different classes. However, disc diffusion based on using antibiotic discs (MAST, UK); as ceftazidime (30 µg), doripenem (10 µg), meropenem (10 µg), imipenem (10 µg), cefepime (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), amikacin (30 µg), norfloxacin (10 µg), aztreonam (30 µg), piperacillin/tazobactam (100/10µg), Tobramycin (10 µg), and piperacillin (100 µg).

Detection of ESBL Strains by Double-Disk Confirmatory Test (DDCT)

Conventional DDCT was performed to detect ESBLs in a representative *P. aeruginosa* strain using cefotaxime + clavulanate (30/10 µg) with cefotaxime (30 µg) alone and

ceftazidime + clavulanate (30/10 µg) with ceftazidime (30 µg) alone extended-spectrum cephalosporins (Mast, UK). Modifications of DDCT were applied according to Sahni et al,-[20] following the guidelines of Clinical Laboratory Standard Institute (CLSI) 2018, ESBL positive isolates show an increase of 5 mm zone of inhibition with clavulanic acid as compared to the zone size for CTX and CZD alone. *K. pneumonia* ATCC700603 and *P. aeruginosa* PAO-1 were applied as a positive and negative control, respectively.

Detection of MBL Strains by EIM Assay

EDTA-imipenem microbiological (EIM) assay was performed, as described by Marchiaro et al[21] following the guidelines of the Clinical Laboratory Standard Institute (CLSI) 2018. MBL combined disc test was considered positive if the zone diameter difference between imipenem + EDTA and imipenem discs (MAST, UK) was larger than 7 mm. *P. aeruginosa* PAO-1 and *P. aeruginosa* NCTC 13359 were used as a positive and negative control, respectively.

Detection of Carbapenem-Resistant Strains by Modified Hodge Test and Carba-NP Test

The Modified Hodge test (MHT) was performed on all carbapenem-resistant isolates as described by Lee et al using *E. coli* ATCC 25922 as an organism sensitive to carbapenems, *P. aeruginosa* 18524928.2 (negative control) and *Enterobacter cloacae* 91419421 strain as a positive control. Two imipenem and ceftazidime sensitive strains were also chosen as additional negative controls. The surface of a Mueller Hinton agar plate was inoculated using a cotton swab with an overnight culture suspension of the *E. coli* 0.5 McFarland Standard. After several minutes of drying, 10 µg meropenem (Mast, UK) disk was placed at the center of the Muller Hinton (MH) plate, and suspected isolates from the overnight cultures were streaked heavily from the drug disk to the edge of the agar plate. Results were recorded after the overnight incubation at 36°C±1°C in an ambient-air incubator. The true positive results were regarded as the appearance of the "cloverleaf" inhibition zone due to enhanced growth of indicator strain toward the meropenem disk alongside the test strain.[22]

Preparation of DNA Extraction

Plasmid and DNA were extracted with a plasmid DNA purification kit (Qiagen, Germany). Extraction was performed based on the kit protocol.

Evaluation of Sensitivity and Specificity of Primers and HRMA Assay

Standard strains, including *P. aeruginosa* NCTC 13359 and *P. aeruginosa* ATCC 27853, were used to optimize HRMA assay. To the examination of the sensitivity of primers, a standard bacteria with a concentration of 0.5 McFarland (1.5×10^8 CFU/mL) was provided into serial dilutions of 10^7 to 10° CFU/mL. A real-time PCR test was performed for all the dilutions. The melting temperature of each product was determined in Singleplex PCR with SYBRGreen. 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 the three genes were chosen.

Optimize of HRMA Assay

Singleplex HRMA assay and amplification were performed using a real-time PCR (ABI step one plus, USA). Reactions were carried out in a total volume of 20 µL included: HRMA Master Mix (HOT FIREPol® EvaGreen HRMA Mix) 4 µL, 1 µL of each primer (20pmol), 1 µL 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, 59°C for one 30sec. Also, the melting curve was obtained following the melting steps: DNA double strands opening step – 95°C, 15 s, gradually heating step – 55°C, 15 s to 95°C with a temperature increase of 2% of the maximum setting of the machine. A ramping rate of 1% was also tested to see whether slower speed helps show more information about the melting curve.

Statically Analysis of Data

All statistical analyses were performed using the SPSS program, version 16.0 (SPSS Inc., Chicago, USA). The chi-squared test and Fisher's exact test (two-sided) were used in order to analyze the qualitative data. The normal distribution of the quantitative data was tested for using the Shapiro–Wilk test. Also, melt curve profiles were assessed and analyzed using ABI Step one software version 2.3 (ABI Thermo Fisher Scientific, Inc., USA). HRMA data analyzed by ABI Step one High-Resolution Melt (HRMA) software v3.01 (ABI Thermo Fisher Scientific, Inc., USA). Data analysis was performed using a bivariate random-effects model to estimate pooled sensitivity, specificity, positive (PPV), and negative predictive values (NPV). PCR method was used as a gold-standard test.

Sanger Sequencing PCR and Phylogenic Tree

Sequencing was performed on standard PCR products on both the forward and reversed strands. PCR amplification was performed in a 25 ul mixture containing 12.5 µL master mix (Amplicon, Denmark), 1 µL forward primers and 1 µL reverse primers (according to Table 1), 1 µL of DNA and sterile DW (Sigma-Aldrich, St Louis, MO). Amplification of the target regions was performed in 35 cycles consisting of initial heat activation at 95°C for 10 min, denaturation at 95°C for 45 s, and elongation at 71°C for 1 min, with a final elongation at 72°C for 5 min. Annealing Tm and DNA amplification were carried out (Eppendorf thermocycler, Germany) with thermal cycling conditions consisting of Table 1. Electropherograms of generated sequences were inspected with MEGA6 software and Chromas software version 2.8 (Technelysium Pty. Ltd., Helensvale, Australia). Obtained DNA sequences were analyzed with the BLAST program, available from URL: http://www.ncbi.nlm.nih.gov. Primers used for sequencing are listed in Table 1.

Results

In total, 88 *P. aeruginosa* isolates were collected. Of these isolates, 19 (21.59%) of the isolates were collected from blood, 26 (29.54%) from the wound, 10 (11.36%) from urine, and 11 (12.5%) from catheters. Tony-tow (25%) isolates were collected from other samples. However, 53 isolates (60.22%) were isolated from female patients, and 35 isolates (39.77%) from male patients.

Antibiotic Sensitivity Profile

Antibiotic susceptibility profiles were obtained after testing by the Kirby Bauer disk diffusion method according to CLSI guidelines 2018. The overall antibiotic resistance of clinical isolates is summarized in Table 2, which also illustrates the increased resistance level for ciprofloxacin (76.13%), followed by gentamicin (67.04%), ceftazidime (44.31%), imipenem (35.22%), piperacillin (32.94%), and doripenem and cefepime (30.68%). Additionally, the levels of non-susceptible to at least one agent in three or more antimicrobial categories hence considered as MDR, were unexpectedly high, affecting 42.04% of *P. aeruginosa* clinical isolates included in this study.

Prevalence of Class B and Class A β-Lactamases in *P. aeruginosa* Isolates by Phenotypic Methods

Results of phenotypic tests for the detection of different classes of β-lactamase enzymes for *P. aeruginosa* are shown in Table 2. In this case, 43 strains (48.88%) of *P. aeruginosa* isolates were ESBL-producing (Figure 1ai). Also, in class B β-lactamases, seven isolates (7.94%) of *P. aeruginosa* were MBL-producing (Figure 1aii). Carbapenemase-producing *P. aeruginosa* was identified in 11 (12.5%), and 19 (21.5%) isolates, by Modified Hodge test (Figure 2ai) and Carba-NP test (Figure 2aii) methods, respectively.

Table 1 Oligonucleotide Sequences Used in This Study

Gene	Primer Name	Sequence of Primers	Melting Tm	Annealing Tm/ Time	Product Size (bp)	References
bla_{SHV}	SHV	F: TCCCATGATGAGCACCTTTAAA R: TCCTGCTGGCGATAGTGGAT	88.57±0.5°C	59	105	[5]
bla_{TEM}	TEM	F: GCATCTTACGGATGGCATGA R: GTCCTCCGATCGTTGTCAGAA	82.4± 0.5°C	58	101	[5]
bla_{KPC}	KPC	F: GATACCACGTTCCGTCTGG R: GCAGGTTCCGGTTTTGTCTC	83.55±0.5°C	60	254	[8]
bla_{IMP}	IMP	F: GGCTTAATTCTCGATCTATCCC R: CTAGCCAATAGTTAACTCCGC	80.16±0.5°C	61	114	[3]
bla_{VIM}	VIM	F: TCTCCACGCACTTTCATGAC R: GTGGGAATCTCGTTCCCCTC	88.57±0.5°C	60	124	[3]
bla_{GIM}	GES	F: GTTTTGCAATGTGCTCAACG R: TGCCATAGCAATAGGCGTAG	86.86±0.5°C	61	387	12

Table 2 Antibiotic Susceptibility Profiles in Clinical Isolates of *P. aeruginosa*

Antibiotics Resistance	β-lactamase genes														
	Total(%)			*bla*$_{SHV}$(%)		*bla*$_{TEM}$(%)		*bla*$_{KPC}$(%)		*bla*$_{IMP}$(%)		*bla*$_{VIM}$(%)		*bla*$_{GES}$(%)	
	S	I	R	S	R	S	R	S	R	S	R	S	R	S	R
Ceftazidime	33(37.5%)	16(18.1%)	39(44.3%)	4(12.1%)	33(84.6%)	0(0%)	19(48.7%)	0(0%)	11(28.2%)	0(0%)	13(33.3%)	0(0%)	5(12.8%)	0(0%)	2(5.1%)
Doripenem	46(52.2%)	15(17.0%)	27(30.6%)	0(0%)	25(92.5%)	0(0%)	20(74.0%)	0(0%)	12(44.4%)	0(0%)	12(44.4%)	0(0%)	5(18.5%)	0(0%)	2(7.4%)
Meropenem	54(61.3%)	13(14.7%)	21(23.8%)	1(1.8%)	17(80.9%)	0(0%)	20(95.2%)	0(0%)	12(57.1%)	0(0%)	13(61.9%)	0(0%)	5(23.8%)	0(0%)	2(9.5%)
Imipenem	40(45.4%)	17(19.3%)	31(35.2%)	3(7.5%)	25(80.6%)	0(0%)	20(64.5%)	0(0%)	12(38.7%)	0(0%)	13(41.9%)	0(0%)	5(16.1%)	0(0%)	2(6.4%)
Cefepime	50(56.8%)	11(12.5%)	27(27.2%)	1(2.0%)	26(96.2%)	0(0%)	19(70.3%)	0(0%)	12(44.4%)	0(0%)	10(37.0%)	0(0%)	5(18.5%)	0(0%)	2(7.4%)
Gentamycin	18(20.4%)	11(12.5%)	59(67.0%)	0(0%)	39(66.1%)	1(5.5%)	18(31.0%)	0(0%)	12(20.3%)	0(0%)	13(22.0%)	0(0%)	5(8.4%)	0(0%)	2(3.3%)
Ciprofloxacin	12(13.6%)	9(10.2%)	67(76.1%)	0(0%)	39(58.2%)	0(0%)	20(29.8%)	0(0%)	12(17.9%)	0(0%)	13(19.4%)	0(0%)	5(7.4%)	0(0%)	2(2.9%)
Amikacin	52(59.1%)	17(19.3%)	19(21.5%)	0(0%)	15(78.9%)	1(1.9%)	15(78.9%)	0(0%)	12(63.1%)	0(0%)	13(68.4%)	0(0%)	5(26.3%)	0(0%)	2(10.5%)
Norfloxacin	50(56.8%)	18(20.4%)	20(22.7%)	7(14.0%)	11(55.0%)	2(4.0%)	13(65.0%)	2(4.0%)	9(45.0%)	5(1.0%)	7(35.0%)	0(0%)	2(10.0%)	0(0%)	2(1.0%)
Aztreonam	67(76.1%)	9(10.2%)	12(13.6%)	0(0%)	11(91.6%)	0(0%)	11(91.6%)	5(7.1%)	5(41.6%)	0(0%)	11(91.6%)	0(0%)	5(41.6%)	0(0%)	2(16.6%)
Tobramycin	68(77.2%)	9(10.2%)	11(12.5%)	0(0%)	11(100%)	0(0%)	11(100%)	0(0%)	11(100%)	0(0%)	11(100%)	0(0%)	3(27.2%)	0(0%)	2(18.1%)
Piperacillin	48(54.5%)	11(12.5%)	29(28.4%)	3(5.1%)	25(86.2%)	3(5.1%)	14(48.2%)	2(3.4%)	6(20.6%)	2(3.4%)	8(27.5%)	0(0%)	2(6.8%)	0(0%)	2(6.8%)
Piperacillin/tazobactam	48(54.4%)	16(18.1%)	24(27.2%)	2(4.1%)	19(79.1%)	3(6.2%)	11(45.8%)	3(6.2%)	7(29.1%)	3(6.2%)	6(25.0%)	0(0%)	2(8.3%)	0(0%)	2(8.3%)
MDR	37(22.4%)			35(94.5%)		17(45.9%)		12(32.4%)		12(32.4%)		5(13.5%)		2(5.4%)	
β-lactamase-producer strain															
class A β-lactamases (ESBL)	43(22.4%)			39(90.6%)		20(46.5%)		10(23.2%)		8(18.6%)		5(11.6%)		2(4.6%)	
class B β-lactamases (MBL)	7(22.4%)			5(71.4%)		5(71.4%)		7(100%)		7(100%)		5(71.4%)		2(28.5%)	
class D β-lactamases (KPC)	11 (12.5%) (Hodge test)19 (21.5%) (Carba-NP)			11 (100%)17 (89.7%)		10 (90.9%)14 (73.6%)		11 (100%)12 (63.1%)		11 (100%)9 (47.3%)		5 (45.4%)4 (21.0%)		2 (18.1%)2 (10.5%)	
Clinical isolates															
Wound	26(29.5%)			17(65.3%)		14(53.8%)		8(30.6%)		11(42.3%)		11(42.3%)		2(7.6%)	
Blood	19(21.5%)			13(68.4%)		5(26.3%)		2(10.5%)		2(10.5%)		2(10.5%)		0(0%)	
Urine	10(11.3%)			5(50.0%)		1(1.0%)		1(1.0%)		1(0%)		1(0%)		0(0%)	
Catheter	11(12.5%)			3(27.2%)		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)	
Other case	22(25%)			0(0%)		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)	

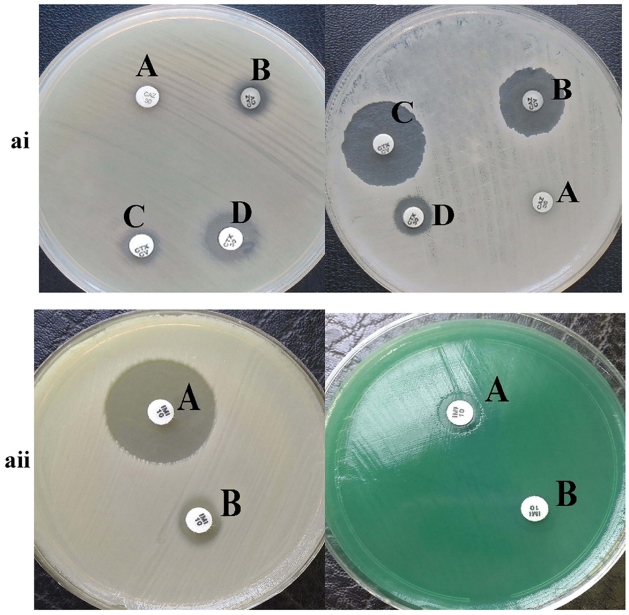

Figure 1 ai: The result of phenotypic detection of ESBL-producing *Pseudomonas aeruginosa* by Double-Disk Confirmatory Test (DDCT) in *Pseudomonas aeruginosa*. A: Ceftazidime (30 µg); B: ceftazidime + clavulanate (30/10µg); C: cefotaxime + clavulanate (30/10µg); D: cefotaxime (30 µg). Top: ESBL negative strain; Bottom: ESBL positive strain; ESBL positive isolates show an increase of 5 mm zone of inhibition with clavulanic acid as compared to the zone size for CTX and CZD alone. aii: The result of phenotypic detection of MBL-producing *Pseudomonas aeruginosa* by EDTA-imipenem microbiological (EIM) test in *Pseudomonas aeruginosa*. A: EDTA + imipenem; B: imipenem. Top: MBL positive strain; Bottom: MBL negative strain; MBL considered positive when zone diameter difference between imipenem + EDTA and imipenem discs was larger than 7 mm.

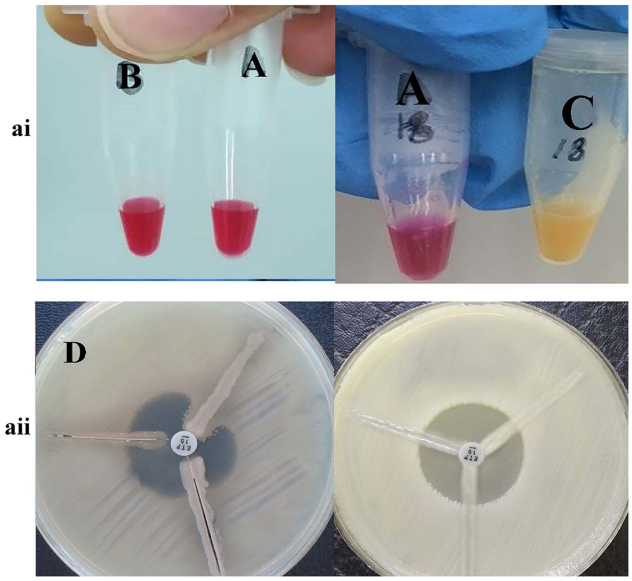

Figure 2 The result of phenotypic detection of carbapenemase-producing *Pseudomonas aeruginosa* by Modified Hodge test (right) and Carba-NP test test (left) in *Pseudomonas aeruginosa*. ai A: control negative strain; B: KPC negative strain by Carba-NP test test; C: KPC positive strain by Carba-NP test test. aii D: KPC positive strain by Modified Hodge test; (E) KPC negative strain by Modified Hodge test.

Prevalence of Class B and Class A β-Lactamases in *P. aeruginosa* Isolates by PCR Assay

Thirty-nine isolates (44.31%) with bla_{SHV} gene, 20 isolates (22.7%) with bla_{TEM} gene, 12 isolates (13.63%) with bla_{KPC} gene, 13 isolates (14.77%) with bla_{IMP} gene, 5 isolates (5.6%) with bla_{VIM} gene, and 2 isolates (2.2%) with bla_{GES} gene were detected. Details of the frequency of β-lactamase genes on different strains and clinical samples are shown in Table 2.

Phylogenetic Relationships of β-Lactamase Genes

According to Figure 3, phylogenetic trees were constructed using the Neighbor-Joining technique, where evolutionary history and taxa were taken from 500 replicates to prepare the bootstrap consensus tree confidence limits. The phylogenetic tree was designed by determining the nucleotides sequence of bla_{SHV}, bla_{TEM}, bla_{KPC}, bla_{IMP}, bla_{VIM}, and bla_{GES} genes by sanger method. Indistinct and ambiguous sites were removed for each sequence pair. However, phylogenetic classification showed a high percentage of class A β-lactamases in *P. aeruginosa* strains (44.31%). At lower frequencies, percentages of 5.68% for the class B β-lactamases. Details of the different strains topping based on β-lactamase genes are illustrated in Figure 3.

Analytical Sensitivity and Specificity of Primers by Real-Time PCR

The observed Tm shown in the melting curves of gene amplification was equal to 83.5±0.5°C for bla_{KPC} gene, 80.0±0.5°C for bla_{IMP} gene, 84.8±0.5°C for bla_{VIM} gene, 86.9±0.5°C for bla_{GES} gene, 88.5±0.5°C for bla_{SHV} gene and 82.2±0.5°C for bla_{TEM} gene. 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. The sensitivity of the real-time PCR calculated from eight dilutions of the positive control monitored by the use of ABI Software V 3.2 and shown in Figures 4 and 5.

Detection of Class B and Class A β-Lactamases in *P. aeruginosa* Isolates by HRMA Assay

The results of the HRMA test revealed that out of 88 clinical isolates of *P. aeruginosa*, 39 isolates (44.31%)

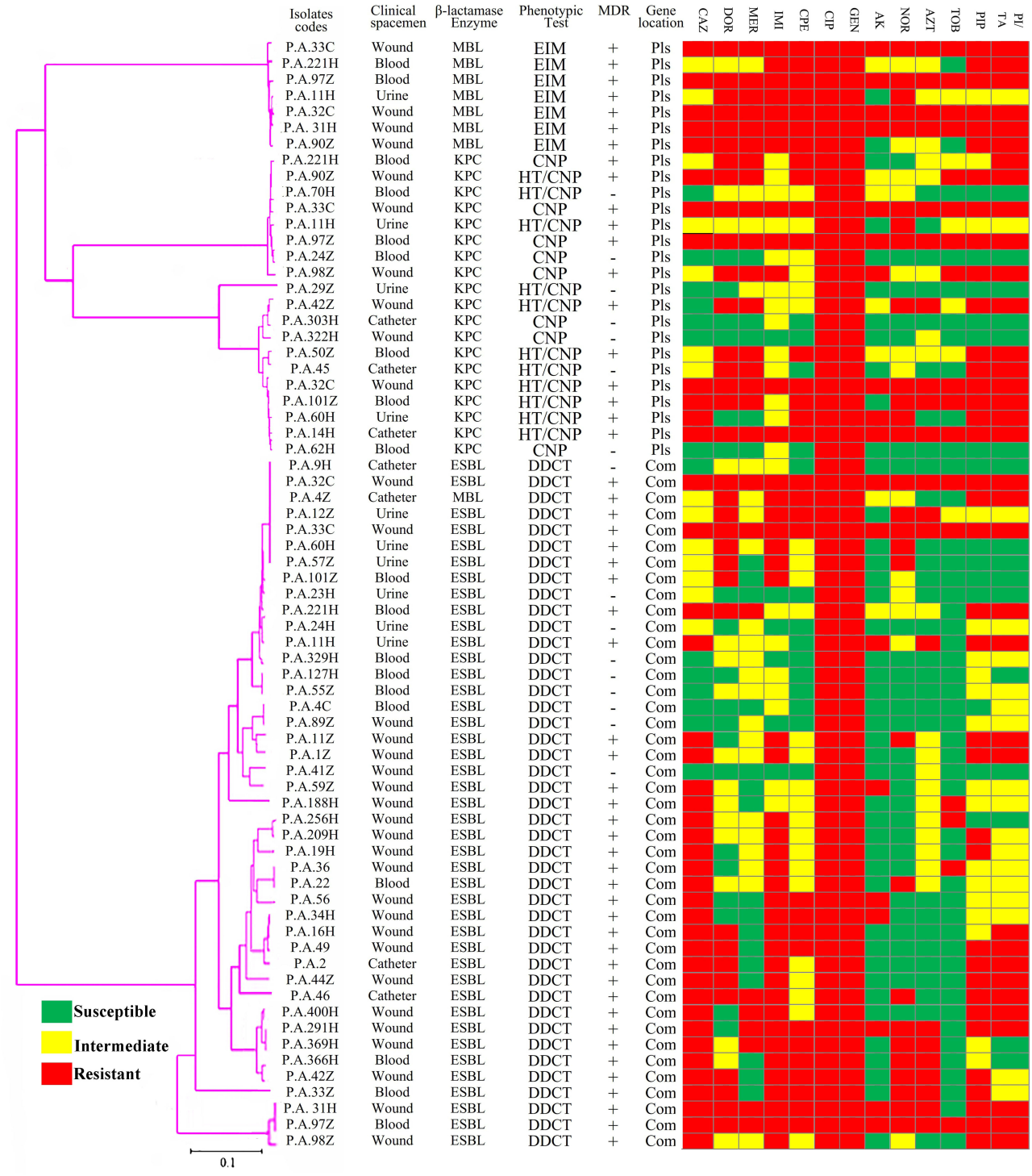

Figure 3 Phylogenetic tree of β-lactamases genes based on the gene sequencing of the class A and class B β-lactamases in *P. aeruginosa* isolates.
Abbreviations: MDR, multidrug-resistant; Com, chromosomal genes; Pls, plasmid genes; CAZ, ceftazidime; DOR, doripenem; MER, meropenem; IMI, imipenem; CPE, cefepime; GEN, gentamycin; CIP, ciprofloxacin; AK, amikacin; NOR, norfloxacin; AZT, aztreonam; TOB, tobramycin; PIP, piperacillin; PI/TA, piperacillin/tazobactam.

were bla_{SHV} gene, 20 isolates (22.7%) were bla_{TEM} gene. Also, 12 isolates were bla_{KPC} gene (13.6%) and 14 isolates (15.9%) were bla_{IMP} gene. Also, five isolates (5.6%) with bla_{VIM} gene and two isolates (2.2%) with bla_{GES} gene. The result of HRMA from DNA amplification of the positive strains monitored by the use of ABI Step one High-Resolution Melt (HRMA) software v3.01 and shown in Figures 6 and 7.

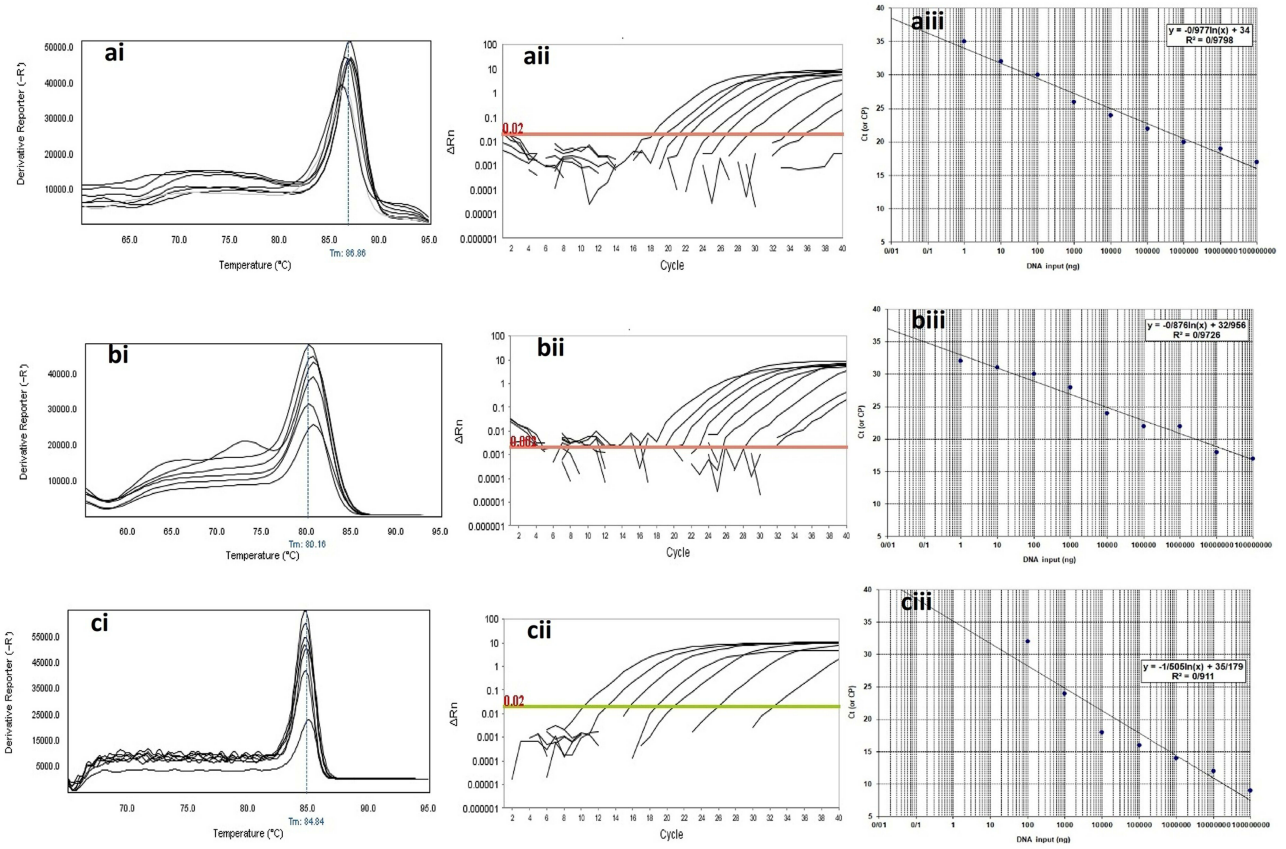

Figure 4 Analytical sensitivity of Real-time PCR for primers used to detect class B β-lactamases genes in clinical isolates of *P. aeruginosa*. ai to aiii: bla_{GES}; ai: Melting curve in 86.86°C; aii: Amplification curve; and aiii: Standard curve with efficiency=1.808. bi to biii: bla_{IMP} gene; bi: Melting curve in 80.16°C; bii: Amplification curve; and biii: Standard curve with efficiency=1.742. ci to ciii: bla_{VIM} gene; ci: Melting curve in 84.84°C; cii: Amplification curve; and ciii: Standard curve with efficiency=1.575. The mean of a: 10^8; b: 10^7; c: 10^6; d: 10^5; e: 10^4; f: 10^3; g: 10^2; h: 10^1 and i: 10° CFU/mL of DNA dilutions. 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. SYBR Green I Dye and single-tube reaction were used in this test. Also, Real-Time PCR was performed as single-step.

Sensitivity and Specificity of Methods

Based on Table 3 and statistical analysis of the results of the HRMA test for detection of class A and class B β-lactamases in *P. aeruginosa* strains, sensitivity and specificity of the HRMA method was 100%. Nonetheless, for the detection of carbapenemase-producing strains, sensitivity and specificity of Carba-NP test was 90.47% and 94.87%, respectively. However, sensitivity and specificity of MHT were 91.66% and 98.70%, respectively (All reported for bla_{KPC} gene). For the detection of ESBL-producing strains, the sensitivity and specificity of the DDT were 95.55% and 95.55% (for bla_{SHV} gene). Besides, for MBL-producing strains, the sensitivity and specificity of the EIM were 77.77% and 97.59%, respectively (for bla_{VIM} gene).

As shown in Table 4, the sensitivity and specificity of the HRMA method was 100%. Also, the sensitivity and specificity of the Carba-NP test were 83.36% and 94.80%, respectively. However, sensitivity and specificity of MHT were 77.77% and 96.42%, respectively (All reported for bla_{GES} gene). For the detection of ESBL-producing strains, the sensitivity and specificity of the DDT were 86% and 83.50%, respectively (for the bla_{TEM} gene). In addition, for MBL strains, the sensitivity and specificity of the EIM were 91.66% and 97.43%, respectively (All reported for bla_{IMP} gene).

According to Table 5, a significant relationship was observed between antibiotic resistance patterns, different classes of β-lactamase, and sensitivity/specificity of phenotypic methods and HRMA ($p \leq 0.05$). Thus, strains that had multiple antibiotic resistance played an essential role in enhancing the sensitivity and specificity of phenotypic and HRMA methods.

Discussion

One of the many concerning characteristics of *P. aeruginosa* is its low antibiotic susceptibility. It has a propensity to develop resistance during therapy, even evolving an

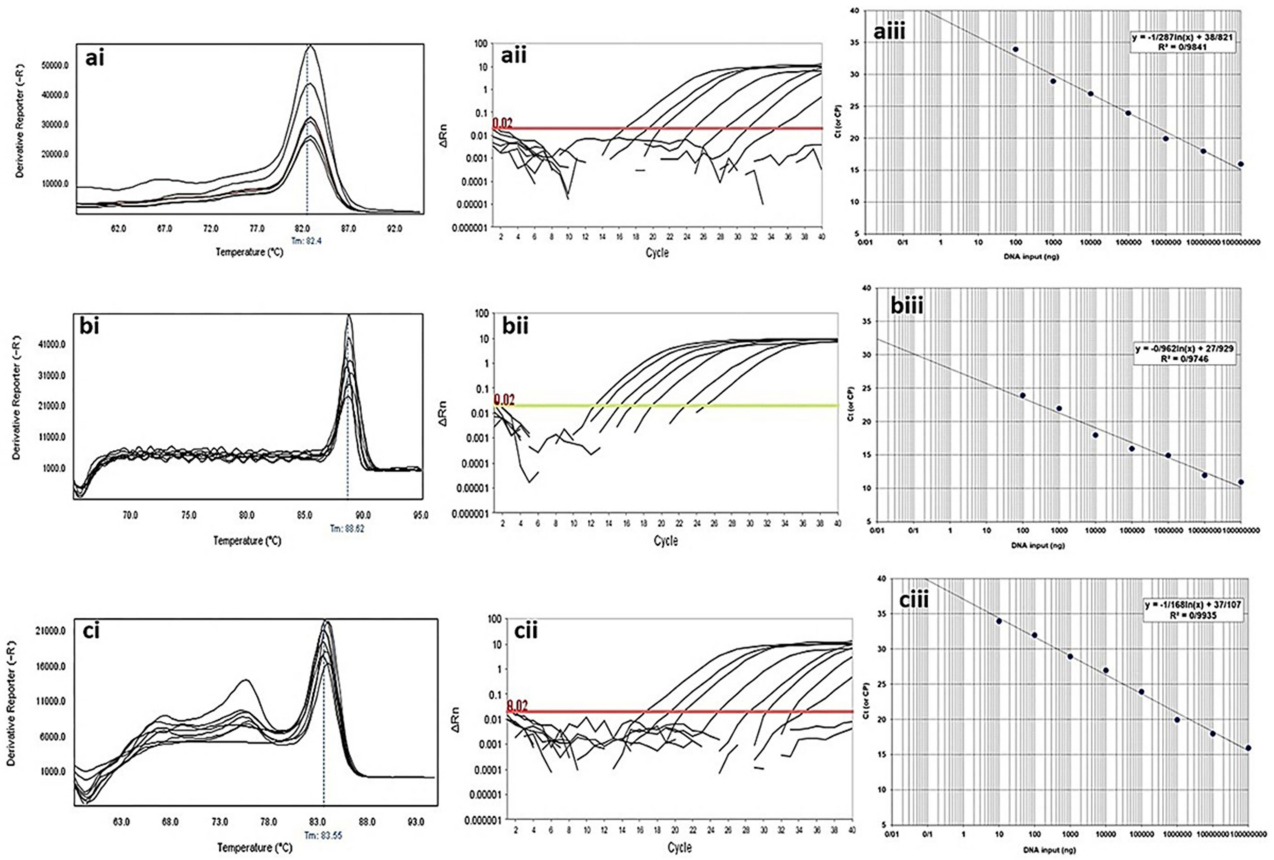

Figure 5 Analytical sensitivity of real-time PCR for primers used to detect class A β-lactamases genes in clinical isolates of *P. aeruginosa*. ai to aiii: bla_{TEM} gene; ai: Melting curve in 82.4°C; aii: Amplification curve; and aiii: Standard curve with efficiency=1.429. bi to biii: bla_{SHV} gene; bi: Melting curve in 88.52°C; bii: Amplification curve; and biii: Standard curve with efficiency=1.742. ci to ciii: bla_{KPC} gene; ci: Melting curve in 83.55°C; cii: Amplification curve; and ciii: Standard curve with efficiency=1.575. The mean of a: 10^8; b: 10^7; c: 10^6; d: 10^5; e: 10^4; f: 10^3; g: 10^2; h: 10^1 and i: 10° CFU/mL of DNA dilutions. 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. SYBR Green I Dye and single-tube reaction were used in this test. Also, Real-Time PCR was performed as single-step.

MDR phenotype. Based on Table 2, a very high percent of *P. aeruginosa* isolates were resistant to ciprofloxacin (76.13%) and gentamycin, which (67.04%) is in agreement with the report of Kotwal et al,[23] who found 82% and 77% resistance, respectively. This finding differs from an earlier report of Choudhary et al,[24] who recorded 40% ciprofloxacin resistance among *P. aeruginosa* isolates; however, resistance to gentamycin found was 37%. Besides, the prevalence of carbapenemase-producing, ESBL-producing (both belong to class A β-lactamase), and MBL-producing (belong to class b β-lactamase) strains were 48.86%, 12.5%, and 7.95%, respectively. Under the present results, previous studies have demonstrated that the prevalence of MBL, ESBL, and carbapenemase-producing in *P. aeruginosa* isolates were more than 10%.[23] The levels observed in this investigation are far below those observed by Farhan et al,[25] who report 54% of ESBL-producing, 21% of carbapenemase-producing, and 52% of MBL-producing in *P. aeruginosa* isolates.

In the current study, based on Figure 3, phylogenetic diagrams of different *P. aeruginosa* strains were described. In this classification based on β-lactamase genes, the class A β-lactamase was the most abundant, and the bla_{TEM} and bla_{SHV} genes showed the most variation. These results reflect those of Karami et al,[26] who also found that class A β-lactamase was the most prevalent.

In determining the specificity and sensitivity of the diagnostic method, the serial dilution plays a significant role. In some studies, Svec et al[27] showed that the serial dilution of the DNA for the identification of different bacteria based on real-time PCR was of particular importance in increasing the sensitivity and specificity of the method. In Figures 4 and 5, the specificity/sensitivity of the method for class A and class B β-lactamase by real-time melting curve was described. The straight line was built through nine-fold serial dilutions, with a range from10^8 to 10°. Further, the temperature and length of the primer also play an essential role in the sensitivity of

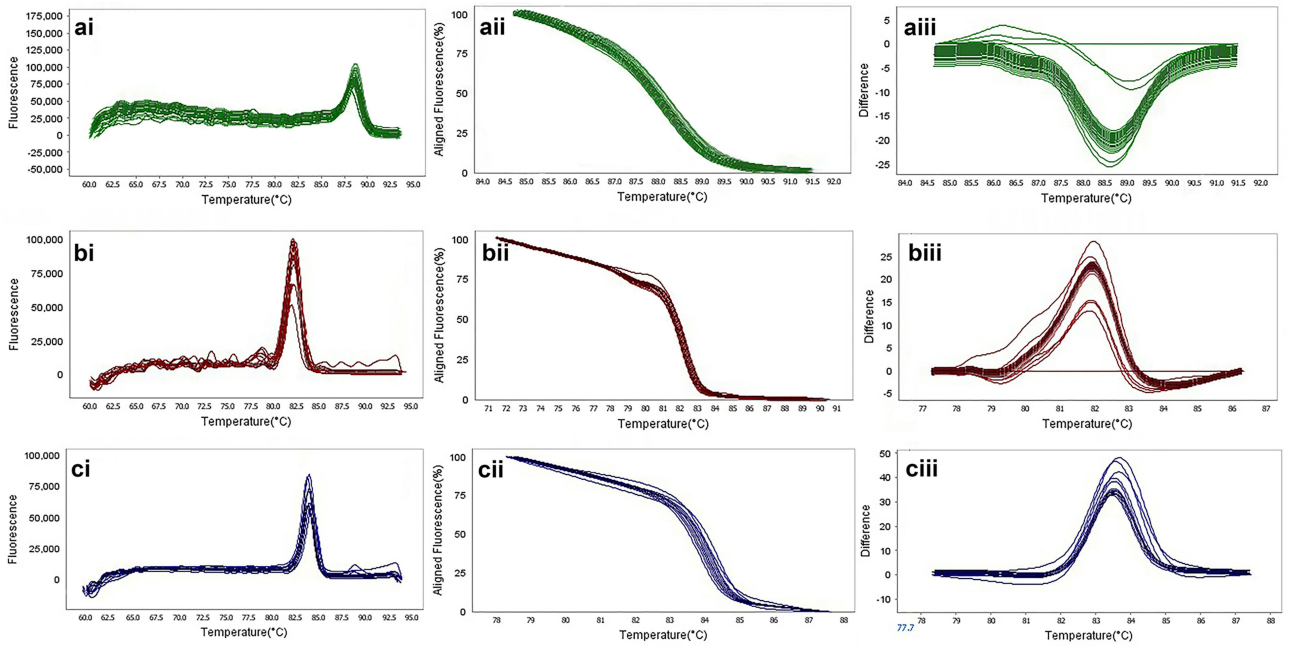

Figure 6 The result of molecular detection of class A β-lactamases genes by HRMA assay. a: bla_{SHV} with 245 bp length and a melting temperature of 88.57±0/5°C; b: bla_{TEM} with 101 bp length and a melting temperature of 82.4±0/5°C; c: bla_{KPC} with 245 bp length and a melting temperature of 83.55±0/5°C. β-lactamases producing genes were amplified successfully using the EvaGreen Dye in the ABI Step-OnePlus machine by one-sept protocol. Primers specific melting peaks were obtained via HRMA analysis, allowing the differentiation of all investigated β-lactamase enzymes. Due to the highly saturating EvaGreen dye and the HRMA analysis, the accuracy of the resolution was ±0. 1–0.5 °C. (ai, bi, and ci) Melting curves; (aii, bii, and cii) normalized plot; and (aiii, biii, and ciii) difference plot.

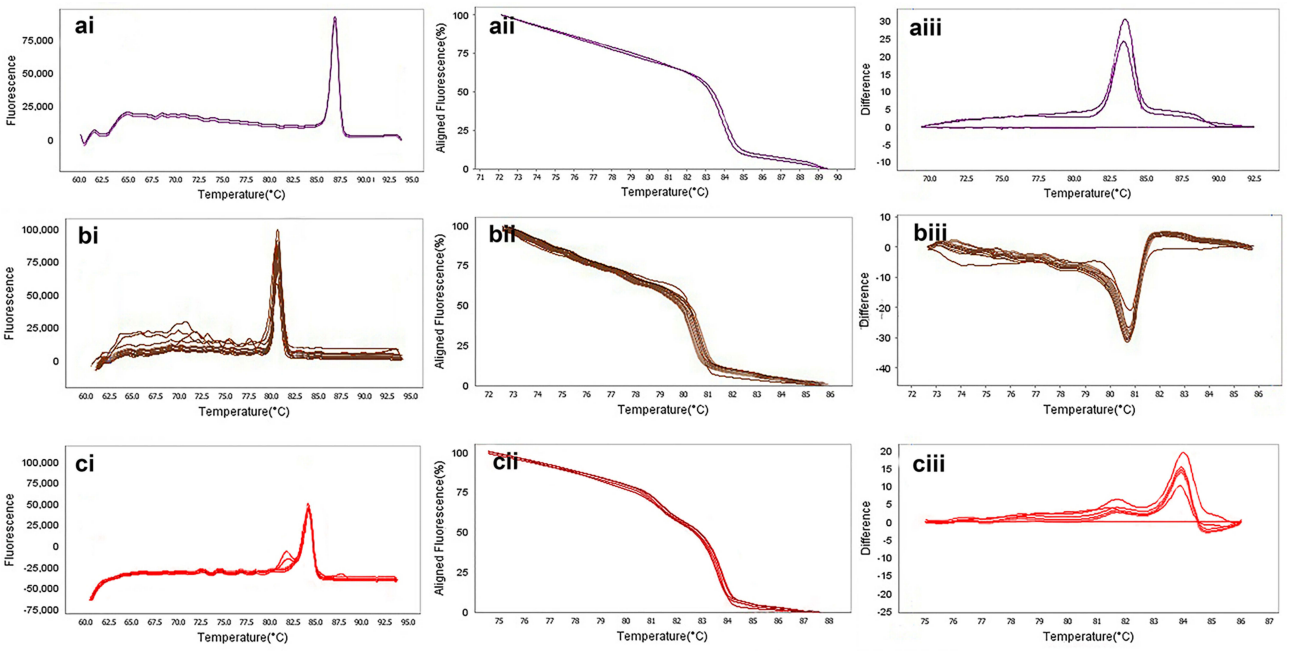

Figure 7 The result of molecular detection of class B β-lactamases genes by HRMA assay. a: bla_{GES} with 72 bp length and a melting temperature of 86.86±0/5°C; b: bla_{IMP} with 114 bp length and a melting temperature of 80.16±0/5°C; c: bla_{VIM} with 105 bp length and a melting temperature of 84.80±0/5°C. β-lactamases producing genes were amplified successfully using the EvaGreen Dye in the ABI Step-OnePlus machine. Primers specific melting peaks were obtained via HRMA analysis, allowing the differentiation of all investigated β-lactamase enzymes. Due to the highly saturating EvaGreen dye and the HRMA analysis, the accuracy of the resolution was ±0. 1–0.5 °C. (ai, bi, and ci) Melting curves; (aii, bii, and cii) normalized plot; and (aiii, biii, and ciii) difference plot.

Table 3 Sensitivities, Specificities, Positive Predictive and Negative Predictive Values for Phenotypic and HRMA (*bla*$_{SHV}$ Gene for ESBL-Producing, *bla*$_{KPC}$ Gene for Carbapenemase-Producing and *bla*$_{VIM}$ Gene for MBL-Producing) Methods in β-Lactamase-Producing Strain of *P. aeruginosa*

Methods	No. of *P. aeruginosa* Strains (Total n = 88)															
	KPC Strains (Total n = 12)				MBL Strains (Total n = 5)				ESBL Strains (Total n = 39)				Sen (%)	Sp (%)	PPV (%)	NPV (%)
	True Positive	False Positive	False Negative	True Negative	True Positive	False Positive	False Negative	True Negative	True Positive	False Positive	False Negative	True Negative				
DDT	0	0	0	0	0	0	0	0	43	4	2	86	95.55	95.55	91.48	97.72
EIM assay	0	0	0	0	7	2	2	81	0	0	0	0	77.77	97.59	97.59	77.77
Hodge test	11	1	1	76	0	0	0	0	0	0	0	0	91.66	98.70	91.66	98.70
Carba-NP	19	4	2	74	0	0	0	0	0	0	0	0	90.47	94.87	97.36	82.60
HRM	12	0	0	76	5	0	0	83	39	0	0	69	100	100	100	100
Gold Standard	12	0	0	76	5	0	0	83	39	0	0	69	100	100	100	100

Abbreviations: Sen, sensitivity; Sp, speciation; PPV, positive predictive values; NPV, negative predictive values.

Table 4 Sensitivities, Specificities, Positive Predictive and Negative Predictive Values for Phenotypic and HRMA (*bla*$_{TEM}$ Gene for ESBL-Producing, *bla*$_{IMP}$ Gene for Carbapenemase-Producing and *bla*$_{GES}$ Gene for MBL-Producing) Methods in β-Lactamase-Producing Strain of *P. aeruginosa*

Methods	No. of *P. aeruginosa* Strains (Total n = 88)															
	MBL Strains (Total n = 13)				KPC Strains (Total n = 2)				ESBL Strains (Total n = 20)				Sen (%)	Sp (%)	PPV (%)	NPV (%)
	True Positive	False Positive	False Negative	True Negative	True Positive	False Positive	False Negative	True Negative	True Positive	False Positive	False Negative	True Negative				
DDT	0	0	0	0	0	0	0	0	43	16	7	81	86	83.50	72.88	92.04
EIM assay	7	3	2	81	0	0	0	0	0	0	0	0	77.77	96.42	70	91.01
Hodge test	0	0	0	0	11	2	1	76	0	0	0	0	91.66	97.43	84.61	98.70
Carba-NP	0	0	0	0	19	4	3	73	0	0	0	0	83.36	94.80	82.60	96.05
HRM	2	0	0	85	13	0	0	76	20	0	0	68	100	100	100	100
Gold Standard	2	0	0	85	13	0	0	76	20	0	0	68	100	100	100	100

Abbreviations: Sen, sensitivity; Sp, speciation; PPV, positive predictive values; NPV, negative predictive values.

Table 5 Relationship of Antibiotic Susceptibility Profiles, β-Lactamase Class and Sensitivity/Specificity of Phenotypic and HRMA in Clinical Isolates of *P. aeruginosa*

Antibiotics Resistance	Methods					
	DDT	EIM	Hodge test	Carba-NP	HRMA	Gold Standard
Ceftazidime	0.071	0.040	0.832	0.097	0.033	0.077
Doripenem	0.097	0.020	0.065	0.009	0.832	0.089
Meropenem	0.193	0.080	0.073	0.043	0.193	0.193
Imipenem	0.169	0.061	0.340	0.032	0.169	0.169
Cefepime	0.071	0.040	0.832	0.097	0.033	0.056
Gentamycin	0.097	0.020	0.065	0.009	0.832	0.117
Ciprofloxacin	0.193	0.080	0.073	0.043	0.193	0.146
Amikacin	0.169	0.061	0.340	0.032	0.169	0.088
Norfloxacin	0.071	0.040	0.832	0.097	0.033	0.077
Aztreonam	0.097	0.020	0.065	0.009	0.832	0.089
Tobramycin	0.193	0.080	0.073	0.043	0.193	0.193
Piperacillin	0.169	0.061	0.340	0.032	0.169	0.169
Piperacillin/tazobactam	0.071	0.040	0.832	0.097	0.033	0.056
MDR	0.218	0.824	0.343	0.383	0.647	0.052
β-lactamase-producer strain						
class A β-lactamases (ESBL)	0.077	0.252	0.005	0.050	0.027	0.079
class B β-lactamases (MBL)	0.089	0.085	0.063	0.006	0.045	0.035
class A β-lactamases (KPC)	0.193	0.080	0.073	0.043	0.025	0.044

the real-time PCR reaction and efficiency of primers in the current study above 140%. The resulting plot showed an excellent correlation between the temperature and length of the primer and the method efficiency. This finding corroborates the ideas of Author links open overlay panel Wu et al,[28] who suggested that the temperature and length of the primer for the identification of different bacteria based on real-time PCR was of particular importance in increasing the sensitivity and specificity of the method.

Based on Table 4 and a statistical analysis of the results of the HRMA test for the detection of carbapenemase-producing strains in *P. aeruginosa*, sensitivity, and specificity of the HRMA method was 100%. Also, the sensitivity and specificity of Carba-NP test was 90.47% and 94.87%, respectively. Also, sensitivity and specificity of MHT was 91.66% and 98.70%, respectively (All reported for bla_{KPC} gene). For the detection of ESBL-producing strains, the sensitivity and specificity of the DDT were 95.55% and 95.55%, respectively (for bla_{SHV} gene). In addition, for the detection of MBL-producing strains, the sensitivity and specificity of the EIM test were 77.77% and 97.59%, respectively. Given these results, we determined that the molecular identification of antibiotic-resistant bacteria is directly related to the resistance gene. Some genes have higher sensitivity and specificity to detect β-lactamase enzymes in *P. aeruginosa*. Peter et al,[29] and Dortet et al,[30] showed that phenotypic detection methods for class A and class B β-lactamase had been developed and used globally. While combined disk tests for MBL-producing (class B β-lactamase) *P. aeruginosa*, MHT for carbapenemase-producing, and double-disk test for ESBL-producing strains (class A β-lactamase) are frequently used due to cost-effectiveness and user-friendly manner. The specificity and sensitivity of the abovementioned methods are reported variously, 70–84% sensitivity, and 86–90% specificity.[29,31,32]

In our study, sensitivity and specificity of the Carba-NP test and MHT tests for the identification of carbapenemase-producing strains (class A β-lactamase) were 91.66%, 98.70% and 91.66%, 98.70%, respectively, Dortet et al,[32] reported 100% sensitivity and specificity for the Carba-NP method. However, we showed that the sensitivity and specificity of the Carba-NP test were higher than the HTM test, which was reported by Bayramoğlu et al.[22] They showed that phenotypic tests have high susceptibility and specificity to detect strains produced by *P. aeruginosa*. Also, for ESBL-producing strains, the sensitivity and specificity of the DD method were 95.55% and 95.55%, respectively, while for MBL-producing strains by EDTA-imipenem disk method, the sensitivity was 77.77% and specificity was 97.59% while Marchiaro

et al[21] and Bogiel et al[33] reported that the sensitivity and specificity of the diagnostic method for MBL-producing strains is less than 85%, which is not consistent with our observations.

Our observations showed that the sensitivity and specificity of the HRMA method for the detection of ESBL-producing *P. aeruginosa* was 97.9% and 99.56%, respectively. For the identification of MBL-producing strains, the susceptibility and specificity of the HRMA method were 99.2% and 99.7%, respectively, and were 100% reported for the detection of carbapenemase-producing. Studies in UK,[19] Denmark,[34] and USA[35] have shown that the sensitivity and specificity of HRMA to detect class A and class B β-lactamase are more than all phenotypic methods. However, in these studies, some phenotypic methods were used to detect strains producing ESBL and bla_{KPC} with false positives and false negatives. It should be noted that phenotypic methods, although easy to access, but have many limitations. One of the most critical limitations of phenotypic methods is the inability to detect strains producing several enzymes. For example, routine phenotypic methods can not detect class A and class B β-lactamase enzymes simultaneously because phenotypic methods have limitations on implementation.

Table 5 shows that a relationship between antibiotic resistance and sensitivity and specificity of phenotypic tests. Elhariri et al[36] reported that the frequency of the bla_{SHV} gene is higher than that of the bla_{TEM} gene. Rezai et al[37] reported a high frequency of bla_{SHV} genes in the study of the frequency of ESBL strains in *P. aeruginosa*. In the studies of Malkoçoğlu et al,[38] and Giani et al,[39] frequency of bla_{VIM} gene was higher in comparison to the bla_{GES} gene in *P. aeruginosa* strains.

The present study showed that the high sensitivity and specificity of the HRMA method in the detection of antibiotic-resistant strains in *Pseudomonas aeruginosa* clinical isolates compared to phenotypic methods explains its cost-effectiveness. Of course, optimizing real-time PCR in many cases can reduce the cost of response. One significant advantage of real-time chemistry platforms is that they do not require post-amplification processing to verify the specificity of the amplification products. Tong and Giffard[40] demonstrated that real-time chemistries with exponential measurements clearly provide quantitative advantages of fast, precise, and accurate results. Besides, the number of reactions required to perform HRMA also plays an essential role in reducing costs. Murai et al[41] found that the cost of real-time PCR reaction for a large number of bacterial isolates was more economical than for a small number.

However, based on our results, other advantages of an HRMA assay and real-time PCR technology are its ability to detect non-viable, fastidious, and unculturable organisms that would otherwise be missed by culture. Sirous et al,[42] in other bacterias, showed that HRMA assay is a rapid, accurate, and cost-effective method possessing high sensitivity and specificity for the determination of antibiotic resistance. However, A PCR-positive, culture-negative specimen may reflect a real pathogen, yet detecting them would lead to a biased lower sensitivity and specificity value of the HRMA test. It should be noted that false positives could also be due to cell-free pathogen DNA circulating in the blood, from an old or controlled infection or contamination. Moreover, the quality and accuracy of these methods can be significantly enhanced by examining the distribution of genes responsible for resistance to β-lactamases and comparing these genes with the results of phenotypic methods. Because in some strains, it is possible that the resistance appears in a phenotypic form but does not have a resistance gene.

Limitations

There are several limitations to our study. First, the present study was conducted on a small number of clinical isolates of *P. aeruginosa*, while in order to reach a definitive and complete result, the population of the population should be increased and the number of strains with other resistance was also examined. Secondly, the focus on MDR and XDR strains is especially felt so that strains with broad resistance to different antibiotic groups can be detected in the shortest possible time. Hence, the detection of other strains, such as New Delhi Metallo-β-lactamase (NDM) strains, AmpC, and resistance to colistin, can also increase the strength of the study. Thirdly, the use of other molecular methods along with the HRMA method and the determination of their sensitivity and specificity can be helpful in choosing different molecular methods by researchers.

Conclusions

Our knowledge from this study suggests that some phenotypic methods are not suitable for identifying ESBL and MBL-producing *P. aeruginosa* strains. Therefore, cost-effectiveness (for a high number of samples), high speed and accuracy, and excellent sensitivity of the HRMA method can play an essential role in increasing the accuracy of clinical reporting. Moreover, methods such as

HRMA can be used to reduce the diagnostic error in phenotypic methods with high speed and precision in identifying antibiotic-resistant bacteria. Hence, it can be concluded that the HRMA approach in identifying clinical isolates of *P. aeruginosa* carrying carbapenemase, MBL, and ESBL enzymes high efficiency.

Abbreviations

PPV, positive predictive values; TEM, temoneira; VEB, Verona integron-encoded metallo-β-lactamase; OXA, oxacillin hydrolyzing capabilities; MBL, metallo-β-lactamase; bla_{GES}, Guiana extended-spectrum; bla_{IMP}, imipenem; bla_{KPC}, *Klebsiella pneumoniae* carbapenemase; PER, *Pseudomonas* extended resistant; ESBL, extended-spectrum beta-lactamase; PSE, *Pseudomonas*-specific enzymes; HRMA, high-Resolution Melt.

Data Sharing Statement

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Hamadan University of Medical Sciences (CodeNo: IR.UMSHA.REC.1398.573).

Consent for Publication

All the authors agree to publish the manuscript in Infection and Drug Resistance journal.

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Author Contributions

HT and SD performed microbiological and molecular tests and wrote the manuscript. MYA and FK play a role in Project Administration. MA supervised all of the stages of designing the study, conducting the research, and writing the manuscript. All authors contributed to data analysis, drafting or revising the article, 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 declare that they have no competing interests.

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