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ORIGINAL ARTICLE

Characterization of Plasmid-Mediated AmpC and Carbapenemases among Iranain Nosocomial Isolates of *Klebsiella pneumoniae* Using Phenotyping and Genotyping Methods

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Abstract
Objectives: Plasmid-mediated AmpC β-lactamases (PMABLs) and carbapenemases are emerging groups of antimicrobial-resistance determinants. The aims of the study were to evaluate the occurrence of PMABLs and carbapenemases in clinical isolates of *Klebsiella pneumoniae* and compare the test performance of various phenotypic methods for detection of these enzymes in Iran.
Methods: A total of 100 *K. pneumoniae* isolates were collected from clinical specimens obtained in Valiasr Hospital. AmpC production in all isolates was determined using the AmpC disk test, the cephamycin Hodge test, the AmpC Etest, and the boronic acid combined-disk test. In addition, carbapenemase production was determined using the modified Hodge test, the EDTA disk synergy test, and the boronic acid combined-disk test. The performances of various phenotypic methods were evaluated by the comparison of their results with polymerase chain reaction (PCR) method as the gold standard.
Results: Of the 100 isolates, 19 (19%) were demonstrated to harbor the PMABL-resistance gene by the multiplex PCR method. The PCR result indicated the presence of carbapenemase genes in 12 isolates. The performance of various phenotypic tests carried out for detection of carbapenemase-producing isolates varied widely, ranging in sensitivity from 30% to 100% and in specificity from 90.8% to 100%.
Conclusion: This is the first report of *MOX*-type AmpC β-lactamase and bla_{GES} in *K. pneumoniae* in Iran. A comparison of the phenotypic methods showed that a combination of cefoxitin plus boronic acid is optimal for detecting plasmid-

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mediated AmpC enzymes in *K. pneumoniae*, whereas the implementation of molecular methods is often complex, requires specially trained personnel, and is associated with higher costs.

1. Introduction

AmpC β-lactamases are clinically important cephalosporinases encoded on the chromosome of many organisms such as *Citrobacter* spp., *Enterobacter* spp., *Morganella* spp., *Hafnia* spp., and *Serratia* spp. in which they mediate resistance to cephalothin, cefazolin, cefoxitin, most penicillins, and all β-lactamase inhibitor antibiotics except cefepime and imipenem [1]. AmpC β-lactamase hydrolyzes cephamycins and its activity is not inhibited by clavulanic acid. These features distinguish AmpC β-lactamase from extended-spectrum β-lactamases. Since 1989, plasmid-mediated AmpC β-lactamases (PMABLs) have been detected worldwide in the strains of *Escherichia coli*, *Klebsiella* spp., *Proteus mirabilis*, and *Salmonella* spp. This is because transmissible plasmids have acquired genes for AmpC enzymes, which consequently can appear in bacteria lacking (e.g., *Klebsiella pneumoniae*) or poorly expressing a chromosomal *ampC* gene (e.g., *E. coli*).

Although many phenotypic tests are available for AmpC detection, none of these is validated by the Clinical and Laboratory Standards Institute (CLSI). Therefore, it is important to develop methods that can reliably detect PMABLs [2,3]. Significant spread of carbapenem-resistant *K. pneumoniae* was observed in the United States, Europe, and some Asian countries [4,5]. Spreading of different types of carbapenemases, such as *K. pneumoniae* carbapenemase and the New Delhi metallo-β-lactamase-1, was reported in Iran and elsewhere [6,7]. Therefore, we evaluated the prevalence of PMABLs and carbapenemases in Iranain clinical isolates of *K. pneumoniae* and compared the performance of phenotypic tests for detection of these enzymes.

2. Materials and methods

2.1. Bacterial strains and susceptibility testing

In this cross-sectional study, 100 nonduplicate *K. pneumoniae* isolates were collected from clinical specimens obtained in Valiasr Hospital in the central province of Iran (March−September 2011).

The identification of all isolates was confirmed by the API 20E system (bioMerieux, Marcy-l'Etoile, France). Susceptibilities to 21 different antibiotics (MAST, Bootle, Merseyside, UK) were defined by disk diffusion according to the CLSI guidelines (Figure 1) [8].

2.2. Polymerase chain reaction for detecting PMABLs and carbapenemases genes

DNA extraction was performed based on the method described by Pérez-Pérez and Hanson [9]. Polymerase chain reaction (PCR) was used for amplification of genes encoding PMABLs (bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{ACC}, bla_{EBC}, and bla_{FOX}), Ambler class A (bla_{KPC} and bla_{GES}), metallo-β-lactamases (MBLs; bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{SPM}, bla_{SIM}, and bla_{GIM}), and class D carbapenemase (bla_{OXA48}) [9−11]. PCR products were sequenced and analyzed by the Basic Local Alignment Search Tool algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Phenotypic detection of PMABLs and carbapenemases

AmpC production in all isolates was determined by the following: AmpC Etest (Figure 2B), cephamycin Hodge test (CHT; Figure 2C), AmpC disk test (Figures 2E and 2F), and combination-disk test with boronic acid (Figure 2H) [12−14]. In addition, carbapenemase production was determined using the following: modified Hodge test (MHT; Figure 2D), MBL Etest (Figure 2A), EDTA disk synergy (EDS) testing (Figure 2I), and combination-disk testing with boronic acid (Figure 2G). The performances of these test methods were compared with PCR as the gold standard [15−17].

3. Results

Clinical samples were from surgical wounds ($n = 36$; 36%), urines ($n = 41$; 41%), blood ($n = 13$; 13%), and respiratory secretions ($n = 10$; 10%) obtained in surgery and neurology and neurosurgery intensive care units. Antimicrobial resistances of the isolates are shown in Figure 1.

Of the 100 isolates tested, 19 (19%) harbored PMABLs as shown by multiplex PCR; of these 19 isolates, eight (42.2%) carried *bla* genes of the *CIT* group (*CMY-2* genes), seven (36.8%) carried genes of the *MOX* group, whereas three (15.7%) and one (5.2%) carried genes belonging to the *EBC* and *DHA* groups (*DHA-1*; Table 1), respectively.

The PCR result showed that of the 100 isolates, 12 (12%) carried a carbapenemase gene, the most common being bla_{VIM} ($n = 10$, 10%) and bla_{GES} ($n = 2$; 2%). One isolate (1%) contained both bla_{VIM} and bla_{AmpC} (*CIT* group; Table 2), whereas bla_{FOX}, bla_{ACC}, bla_{IMP}, bla_{SIM}, bla_{NDM}, bla_{GIM}, bla_{SPM}, bla_{KPC}, and bla_{OXA48} were not detected in our isolates.

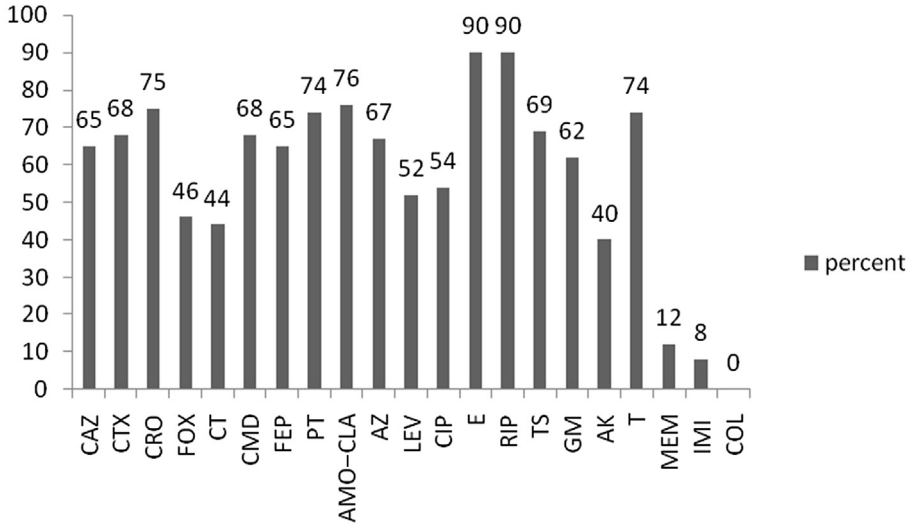

Figure 1. Resistance percent of isolates to the 21 antibiotics used in this study. AK = amikacin; AMO–CLA = amoxicillin–clavulanic acid; AZ = aztreonam; CAZ = ceftazidime; CIP = ciprofloxacin; CMD = cefamandole; COL = colistin; CRO = ceftriaxone; CT = cefotetan; CTX = cefotaxime; E = erythromycin; FEP = cefepime; FOX = cefoxitin; GM = gentamicin; IMI = imipenem; LEV = levofloxacin; MEM = meropenem; PT = piperacillin–tazobactam; RIP = rifampicin; T = tetracycline; TS = trimethoprim/sulfamethoxazole.

The antimicrobial-susceptibility profiles of the 19 AmpC-producing isolates showed 100% (n = 19) resistance to rifampicin and erythromycin; 79% (n = 15) to cefamandole, ceftazidime, and cefotaxime; 73.6% (n = 14) to tetracycline, ceftriaxone, and aztreonam; 68.5% (n = 13) to piperacillin–tazobactam and amoxicillin–clavulanic acid; 63% (n = 12) to cefoxitin; 57.8% (n = 11) to cefepime; 52.6% (n = 10) to cefotetan, ciprofloxacin, levofloxacin, gentamicin, and trimethoprim/sulfamethoxazole; 31.5% (n = 6) to amikacin; 5.2% (n = 1) to imipenem and meropenem. None of the isolates was resistant to colistin in this study.

The results of phenotypic methods for detection of AmpC- and carbapenemase-producing isolates are shown in Tables 1 and 2, respectively.

All carbapenemase-producing isolates were resistant to rifampicin, erythromycin, ciprofloxacin, aztreonam, meropenem, piperacillin–tazobactam, amoxicillin–clavulanic acid, cefamandole, ceftazidime, cefoxitin, cefotaxime, and ceftriaxone. The frequency of resistance is as follows: 11/12 (91.6%) to tetracycline and amikacin; 10/12 (83.3%) to trimethoprim/sulfamethoxazole, gentamicin, and levofloxacin; 9/12 (75%) to cefotetan; and 8/12 (66.6%) to imipenem. The most effective antimicrobial agent against carbapenemase-producing *K. pneumoniae* in this study was colistin with 100% susceptibility. Eight of the 12 carbapenemase-producing isolates were AmpC positive in the MHT; AmpC was not detected in the other four isolates. Five noncarbapenemase-producing isolates were falsely positive in MHT. Of these five isolates, four were AmpC producers.

4. Discussion

The prevalence of PMABLs evaluated in this study (19%) is much higher than described in several other parts of the world. In an earlier study from Iran, the prevalence of PMABLs was 8.4% in *K. pneumoniae*. In Japan, the prevalence of PMABLs was 13% in *K. pneumoniae* [18]. In China, the rate was 10.1% in *K. pneumoniae* and the lowest rates of AmpC genes were reported from Switzerland (0.4%) [19]. High prevalence of AmpC genes was reported from Korea (77%) and Singapore (26%) [19]. In this study, similar to other reports, *CIT*- (*CMY-2*) and *MOX*-type genes were predominantly present in *K. pneumoniae,* followed by the *EBC* and *DHA* types, whereas the *ACC* and *FOX* genes were not detected. The *CMY-2* is one of the most prevalent and most widely distributed PMABLs and has been previously found in several countries [1].

The rate of resistance to cefoxitin in our isolates was 46% (n = 46); only 12 isolates were AmpC producers and 34 isolates were negative. Cefoxitin resistance in these nonproducers could be due to some other resistance mechanism such as alterations in outer membrane permeability [2,20]. In this study, 63% (12/19 isolates) and 68.4% (13/19) of AmpC producers were found to be resistant to cefoxitin and amoxicillin–clavulanic acid, respectively.

Cefotetan has a lower sensitivity than cefoxitin concerning the detection of AmpC production (52.6% vs. 63%). These data indicate that although screening methods using cefoxitin and amoxicillin–clavulanic acid for detection of AmpC-harboring isolates are useful, they are not perfect [20,21]. Therefore, in contrast to

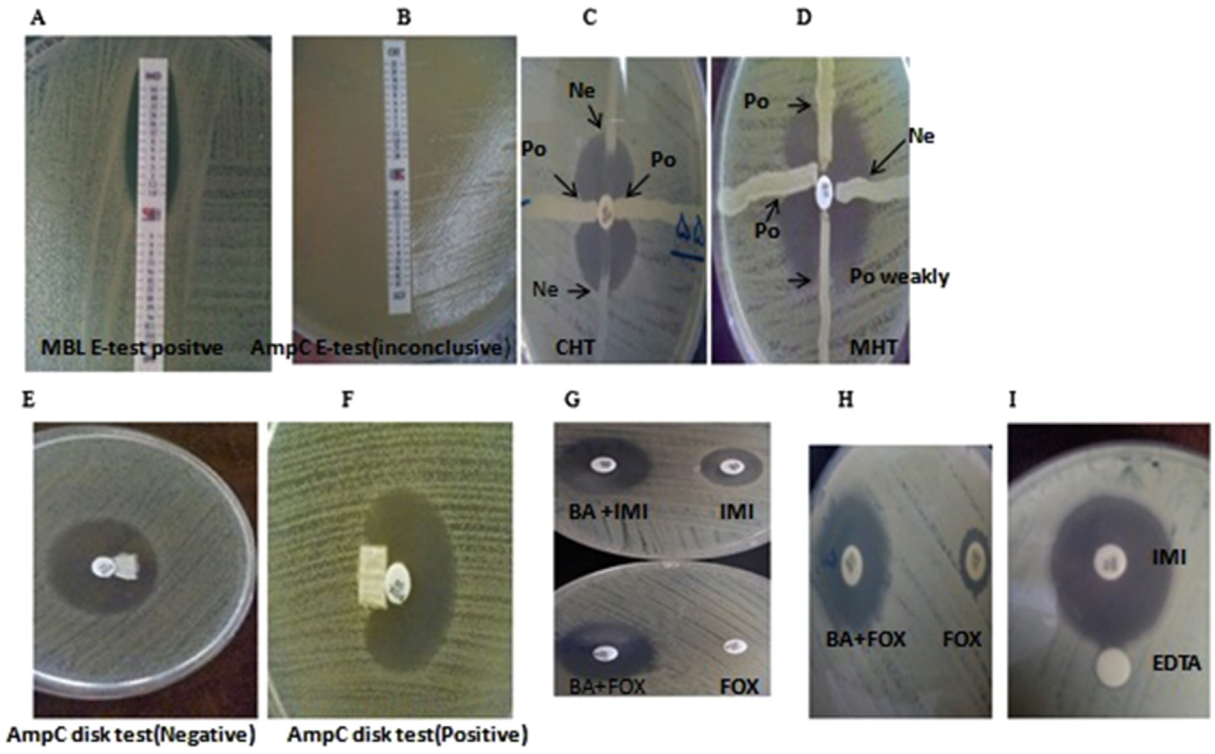

Figure 2. Results of the phenotypic methods for detection of plasmid-mediated AmpC and carbapenemase enzymes in *Klebsiella pneumoniae* isolates. (A) MBL-positive isolate, (B) AmpC Etest, inconclusive result in isolate, (C) cephamycin Hodge test (CHT), AmpC-negative and AmpC-positive isolates, (D) modified Hodge test (MHT), (E and F) AmpC-negative and AmpC-positive isolates, (G) a positive result obtained from bla_{GES} using the imipenem + boronic acid disk test (top) and a positive result obtained from AmpC using the cefoxitin + boronic acid disk test (bottom), (H) cefoxitin + boronic acid disk test (positive isolate), (I) positive result obtained from MBL-producer isolate using the EDTA disk synergy (EDS) test. BA = boronic acid; CHT = cephamycin Hodge test; FOX = cefoxitin; IMI = imipenem; MBL = metallo-β-lactamase; MHT = modified Hodge test; Ne = negative; Po = positive.

Ingram et al [22], our study cannot recommend cefoxitin, cefotetan, and amoxicillin−clavulanic-acid-susceptibility testing for initial AmpC screening. Resistance to cefepime among 11/19 (58%) AmpC-positive *K. pneumoniae* isolates is another important finding. No synergy was found between expanded-spectrum cephalosporins and clavulanic acid using the double-disk synergy test (data not shown). Only 1/11 cefepime-resistant isolates in this study had carbapenemase genes (bla_{VIM}) and thus, resistance to cefepime may be mediated by structurally modified AmpC β-lactamases [23].

We found that the cefoxitin ± boronic acid disk test detected 19/19 AmpC-positive *K. pneumoniae* isolates and gave one false-positive result. The AmpC disk test and CHT detected 7/19 and 5/19 AmpC-positive strains (12 and 14 false negatives), respectively. Possibly, inhibitory compounds exhibit different levels of inhibition among heterogeneous groups of AmpCs [24].

The AmpC disk test and CHT gave five false-positive results. Of these five isolates, three were MBL (bla_{VIM}) producers and the two others were bla_{GES} positive.

The AmpC Etest was easy to use, but was unsuitable for AmpC detection. Overall, 17/19 AmpC-producing isolates were not detected by this method. Test performance could be improved by developing an Etest strip with a broader minimum inhibitory concentration range. Inconclusive results using this test have been previously reported [21,22]. However, we indicate that combination-disk testing with boronic acid is potentially useful for clinical laboratories for the detection of PMABLs [13].

The prevalence of carbapenemases among our isolates was found to be 12%. The sensitivity and specificity of disk diffusion for their identification using imipenem disk were 66.6% and 100%, respectively, whereas meropenem disk demonstrated 100% sensitivity and specificity. Apparently, application of meropenem disk is superior for screening, which is in contrast to the study carried out by Benenson et al [25].

This is the first report of bla_{GES} and bla_{MOX} in Iran. Guiana extended-spectrum β-lactamase (GES β-lactamase) has been reported in Europe [26]. In our region, carbapenem resistance has emerged in *K. pneumoniae*, mostly as *VIM*-type MBLs. *VIM*-MBL producers have been reported in Greece, Kuwait, and Korea [27].

Four *VIM*-producing isolates were not detected by MHT (Table 2). It has been suggested that addition of

Table 1. Characteristics of the 19 AmpC-positive isolates in this study.[a]

No. of isolates	AmpC group	Cephalosporin phenotype					AmpC disk test	Cephamycin Hodge test	AmpC Etest	Cefoxitin/cefoxitin + boronic acid
		CAZ	CTX	FOX	CT	FEP				
5	CIT	S	S	S	S	S	−	−	−	+
7	CIT	S	S	S	S	S	−	−	−	+
9	MOX	R	R	R	R	R	−	−	IR	+
11	MOX	R	R	R	R	R	+	−	IR	+
12	MOX	R	R	R	R	R	−	−	IR	+
17	MOX	R	R	R	R	R	−	−	+	+
1	MOX	R	R	S	S	R	−	−	−	+
21	MOX	S	S	S	S	S	−	−	−	+
22	MOX	R	R	R	S	S	+	+	−	+
3	DHA	R	R	R	R	S	+	−	IR	+
6	EBC	S	S	S	S	S	−	−	−	+
51	EBC	R	R	S	S	R	−	−	−	+
49	CIT	R	R	S	S	R	−	−	−	+
55	CIT	R	R	R	R	R	+	+	IR	+
60	CIT	R	R	R	S	R	+	+	−	+
68	CIT	R	R	R	R	R	−	−	IR	+
75	CIT	R	R	R	R	S	+	+	IR	+
89	CIT	R	R	R	R	R	−	−	IR	+
27	EBC	R	R	R	R	S	+	+	+	+

[a]*Results were rated inconclusive if the minimum inhibitory concentrations exceeded the scale of the Etest for cefotetan alone and/or cefotetan in combination with cloxacillin (Etest AmpC).* CAZ = ceftazidime; CT = cefotetan; CTX = cefotaxime; FEP = cefepime; FOX = cefoxitin; IMI = imipenem; IR = inconclusive result.

zinc sulfate or the use of MacConkey agar may improve the sensitivity of the MHT for MBL producers [28]. In addition, the MHT gave five false positives. The overall sensitivity and specificity of the MHT were 66% and 94.3%, respectively. The sensitivity and specificity of the imipenem ± boronic acid disk test were 100% and 90.8%, respectively.

Although two GES-producing isolates were detected using the MHT, there were also nine false-positive results. Of these nine isolates, six were MBL (bla_{VIM}) producers and three were AmpC producers. It is possible that other AmpC families, not detected by the multiplex PCR, were present in the six *VIM*-producer isolates in our study.

The MBL Etest was the most specific test (100%) for MBL detection in this study. In the EDS test, the EDTA−ceftazidime combination detected additional MBL producers, which were not identified by the EDTA−imipenem combination (90% sensitivity vs. 30% sensitivity). Therefore, ceftazidime appears to be

Table 2. Characteristics of 12 carbapenemase-positive isolates in this study.

Isolates	Carbapenem phenotype		β-Lactamases	IMI/IMI + boronic acid	MHT	IMI/IMI + EDTA	CAZ/CAZ + EDTA	MBL Etest
	IMI	MEM						
15	R	R	VIM	+	−	+	+	+
18	R	R	VIM	+	+	+	+	+
20	R	R	VIM	−	−	+	+	+
39	S	R	VIM	+	+[a]	−	+	+
43	S	R	VIM	−	−	−	+	+
47	S	R	VIM	−	−	−	+	+
55	R	R	VIM and CIT	+	+	−	−	−
69	S	R	VIM	+	+	−	+	+
70	R	R	VIM	+	+	−	+	+
74	R	R	VIM	+	+	−	+	+
23	R	R	GES	+	+	+	+	−
73	R	R	GES	+	+	+	+	−

[a]*Weakly positive.* CAZ = ceftazidime; IMI = imipenem; MBL = metallo-β-lactamases; MEM = meropenem; MHT = modified Hodge test.

the better substrate for the EDS test. Similar results were observed by Noyal et al [29].

In conclusion, this study confirmed the prevalence of AmpC-producing *K. pneumoniae* and carbapenemase (bla_{VIM} and bla_{GES}) in the central hospital of Arak (Iran) to be very high. By understanding the resistance pattern and prevalence of the β-lactamase-producing organisms, especially *K. pneumoniae*, we would be able to manage infection-control policy as well as proper and rational antibiotics prescription. The results indicate that a cefoxitin plus boronic acid is potential method for clinical laboratories to detect emerging AmpC-plasmid-mediated enzymes in *K. pneumoniae*, whereas the implementation of molecular methods is often complex, requires specially trained personnel, and is associated with higher costs.

Conflicts of interest

The authors declare no conflicts of interest.

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