IJM Iranian Journal of Microbiology

Volume 15 Number 3 (June 2023) 373-382

ORIGINAL ARTICLE

Detection of plasmid-mediated AmpC β-lactamases in *Klebsiella pneumoniae* clinical isolates from Bushehr province, Iran

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Received: December 2022**, Accepted:** March 2023

ABSTRACT

Background and Objectives: Plasmid-mediated AmpC producers are considered an increasing concern. The aim of this study was to investigate the prevalence of plasmid-mediated AmpC β-lactamases (pAmpCs) in *Klebsiella pneumoniae* isolates.

Materials and Methods: A total of 228 clinical isolates of *K. pneumoniae* were collected in Bushehr province, Iran, from December 2017 to February 2019. Cefoxitin disks were applied for screening AmpC-producing isolates. Furthermore, 3 phe- notypic confirmatory tests including combine disk test (CDT), double disk synergy test (DDST) and modified three dimen- sional test (M3DT) were used. Finally, the presence of pAmpC genes was tested by multiplex PCR.

Results: We identified 18 pAmpC-KP isolates among the 228 isolates (7.9%): 12 DHA (66.6%) and 6 CMY (33.3%). In the present study only 47% of cefoxitin-resistant isolates were pAmpC producers. The sensitivity of CDT, DDST, and M3DT was 89%, 67% and 100% and the specificity was 90%, 90% and 85%, respectively. In addition, M3DT displayed a higher rate of efficiency (92%) than CDT (89%) and DDST (79%) in detecting plasmid-meditated AmpC producers.

Conclusion: DHA was the most prevalent pAmpC beta-lactamase in this study. DDST and CDT tests proved inefficient to detect two and six pAmpC producers, respectively, while M3DT represented the best overall performance.

Keywords: AmpC; Beta-lactamase; Modified three dimensional test; *Klebsiella pneumoniae*

INTRODUCTION

The spread of multidrug-resistant Gam-negative bacteria has been increasing in the last few years (1). Production of β-lactamases is predominantly the main mechanism for resistance to β-lactam antibiotics in Gram-negative bacteria and among the β-lactamases the production of ESBLs and AmpC β-lactamases are the most frequent (2-4).

Organisms overexpressing AmpC β-lactamases are of grave concern because these organisms are typically capable of showing great resistance to all β-lactam drugs, including penicillins, cephalosporins, and monobactams, except for cefepime, cefpirome,

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and the carbapenems (5, 6). Unlike ESBLs, these enzymes hydrolyze cephamycins and are not impacted by the present β-lactamase inhibitors. Moreover, they can also produce resistance to carbapenems in association with the loss of outer membrane porins (7). In the Ambler structural classification of β-lactamases, AmpC enzymes belong to class C, while in the functional classification scheme of Bush et al. they pertain to group 1 (8). Constitutive overexpression of AmpC β-lactamases in Gram-negative organisms happens either by deregulation of the *ampC* chromosomal gene or by acquisition of a transferable ampC gene on a plasmid or other transferable elements. The transferable *ampC* gene products are normally referred to plasmid-mediated AmpC β-lactamases (pAmpC) (5). Based on sequence similarities, pAmpC variants are classified into five evolutionary groups: the *Citrobacter freundii* CIT variants, the *Morganella morganii* DHA variants, the *Enterobacter* sp. EBC variants, the *Hafnia alvei* ACC variants, and the *Aeromonas* sp. FOX and MOX variants (9). These pAmpC β-lactamases were found among *Enterobacteriaceae*, especially in *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Proteus mirabilis, Salmonella enterica*, as well as *C. freundii, Enterobacter cloacae* and, *Enterobacter aerogenes* which are natural AmpC-producer species (10-12). Plasmids with these genes which can spread among other members of the *Enterobacteriaceae* family (13), consequently leading to nosocomial outbreaks in many countries (7).

In addition, treatment failures of infection caused by pAmpC-producer with broad-spectrum cephalosporins have been documented (7). Pai et al. reported a high rate of clinical failure of blood infection caused by pAmpC-β-lactamase-producing *Klebsiella pneumoniae* in initial antimicrobial therapy with cefotaxime and ceftazidime (14). It is notable that pAmpCs are associated with potentially deadly errors of false susceptibility in routine antimicrobial susceptibility tests. According to the report of Pai et al. AmpC-producing *K. pneumoniae* isolates were susceptible to cephalosporins agents in vitro but some of them did not respond to definitive treatment with these agents and treatment failure was associated with death until therapy was changed to a carbapenem (2).

The majority of plasmid-mediated *ampC* genes can be found in nosocomial isolates of *Escherichia coli* and *Klebsiella pneumoniae* (15). Many clinical laboratories currently test these organisms for the production of ESBLs but the search for plasmid mediated AmpC B-lactamases goes unnoticed (2). Indeed, the detection of AmpC β-lactamases remains a hurdle for clinical laboratories to overcome (7) because no Clinical and Laboratory Standards Institute (CLSI) guidelines for the detection of plasmid-mediated AmpC-producing organisms are available (14). Currently phenotypic tests are either inconvenient, subjective, lack sensitivity and/or specificity, or require reagents which are not readily accessible; however, it is still necessary that clinical laboratories accurately detect isolates producing a pAmpC beta-lactamase (2).

This study aimed to evaluate the prevalence rate of plasmid-mediated AmpCs among *K. pneumoniae* clinical isolates in Bushehr province, Iran by three phenotypic methods and multiplex PCR.

MATERIALS AND METHODS

Bacterial isolates. This project was approved by the Ethical Committee of Bushehr University of Medical Sciences with reference number IR.BPUMS.REC.1397.117. A total of 228 *K. pneumoniae* clinical isolates were collected from six hospitals located in Bushehr province, in the south of Iran, during a period spanning from December 2017 to February 2019. Various clinical specimens formed these isolates. Identification of the grown colonies was conducted by conventional biochemical tests such as Gram staining (+), oxidase (-), A/A reaction on triple sugar iron (TSI) agar, indole production (-), methyl red (-), voges proskauer (+), Simmon's citrate (+), urease (+), motility (-) and confirmed by PCR to target malate dehydrogenase (*mdh*) gene (Fig. 2a) (16).

Antimicrobial susceptibility testing. Antimicrobial susceptibilities were determined by disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (17). The following antibiotic disks (MastGroup Ltd., Merseyside, United Kingdom) were applied: amoxicillin–clavulanic acid (20/10 μg), piperacillin-tazobactam (100/10 μg), cefoxitin (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), cefepime (30 μg), aztreonam (30 μg), amikacin (30 μg), gentamicin (10 μg), tobramycin (30 μg), trimethoprim- sulfamethoxazole (25 μg), fosmomycin (200 μg), imipenem (10 μg), ertapenem (10 μg), ciprofloxacin (5 μg),

and tigecycline (15 µg). In addition, MICs of cefoxitin were determined using E-tests (Liofilchem, Italy) on Muller-Hinton agar (Biolife, Italy). *Escherichia coli* ATCC 25922 was used as the control strain for the antibiotic susceptibility tests.

Screening for AmpC production. Cefoxitin disks (30 µg) were used for screening AmpC-producing isolates, as described by Polsfuss et al. (18). Isolates displaying inhibition zones with a diameter <18 mm were assumed positive for AmpC screening; therefore being subject to further phenotypic confirmatory tests.

Phenotypic AmpC confirmation testing. All the screen positive isolates were subjected to three different phenotypic tests including combination-disk test (CDT) (19, 20), double disk synergy test (DDST) (19) and, modified three dimensional test (M3DT) (19).

Combination-disk test (CDT). Boronic acid (BA) solution was prepared by dissolving 12 mg of 3-Aminophenylboronic acid (Sigma-Aldrich, USA) in 3 ml of dimethyl sulfoxide and 3 ml of sterile distilled water was added to this solution (14). A total of 20 µl (400 µg) of the BA solution was dispensed onto disks containing cefoxitin (FOX). A test strain was inoculated on Mueller Hinton agar (MHA) plates according to the CLSI guideline. Disks containing cefoxitin (FOX) and cefoxitin plus BA (FOX/BA) were placed on the MHA plate and incubated at 37°C overnight. An increase in the zone size of ≥5 mm for cefoxitin in the presence of BA compared with that of cefoxitin alone was considered as positive result (20, 21).

Double disk synergy test (DDST). Double disk synergy test was done using cloxacillin 500 µg (10 µl) as inhibitor of AmpC β lactamases. Using MHA plate, a lawn culture of each test organism was made, the disk of the inhibitor was placed in the center of the plate between cefotaxime (30 µg) and ceftazidime (30 µg), with a distance of 15 mm from center to center. After overnight incubation at 37°C, expansion of inhibitory zone of either one or both, cefotaxime and ceftazidime, towards the cloxacillin disk was interpreted as positive results for the production of AmpC β lactamases by the isolates (19).

Modified three dimensional tests (M3DT). Lawn culture from the growth of non-AmpC producing *E. coli* ATCC 25922 with turbidity of 0.5 McFarland was prepared on MHA plate and cefoxitin 30 µg disk was placed in the center of the plate. A linear slit of 3cm length, 3 mm away from the cefoxitin disk, was cut using sterile surgical blade. Small circular wells were made on the slits at 5 mm distance, inside the outer edge of the slit, by stabbing with a sterile pasture pipette on the agar surface. The extract of the AmpC β-lactamases enzyme was prepared by freezing and thawing the test organism 8 times and then subjecting it to centrifugation at 2000 rpm for 15 min. A total of 30 µl of the supernatant containing the AmpC β-lactamases enzyme was then loaded in the prepared well. The plates were kept upright for 5-10 min until the solution dried, and were then incubated at 37°C overnight. Extension of growth into the zone of inhibition due to neutralization of cefoxitin by the enzyme at the junction of the slit along the line of inhibition was considered a positive M3D test and interpreted as evidence for the presence of AmpC β-lactamases (19).

Molecular characterization of plasmid-mediated AmpC genes. The presence of plasmid-mediated AmpC genes was investigated using a multiplex PCR assay according to Pérez-Pérez and Hanson (5). Specific primers targeting the genes of six different phylogenetic groups (MOX, CIT, DHA, ACC, EBC, and FOX) are listed in Table 1. A single colony of each isolate was inoculated from a blood agar plate into 5 ml of Muller Hinton broth (Merck, Germany) and incubated for 18 h at 37°C with shaking. Cells from 1.5 ml of the overnight culture were harvested by centrifugation at 12,000 × g for 5 min. Total DNA was extracted using an extraction kit (GeneAll, Korea) as recommended by manufacturer. PCR was performed with a final volume of 50 µl PCR mixture containing 25 µ Taq DNA polymerase 2× Master mix with 1.5mM $MgCl_2$ (Ampliqon, Odense, Denmark) 25 µl 2× master mix1.5 mM $MgCl_2$, 16.6 µl nuclease free water, 2 µl of DNA template, 0.6 µM primers MOXMF, MOXMR, CITMF, CITMR, DHAMF, and DHAMR; 0.5 µM primers ACCMF, ACCMR, EBCMF, and EBCMR; 0.4 µM primers FOXMF and FOXMR. The PCR program consisted of an initial denaturation step at 94°C for 3 min, followed by 25 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 64°C for 30 s, and primer extension at 72°C for 1 min with a final extension step at 72°C for 7 min. Amplified products were then analyzed by electrophoresis on a 2% agarose gel at 80 V for

Table 1. The sequences of primers used in PCR amplification of plasmid-mediated AmpC genes (5).

Target (s)	Primer	Sequence (5' to 3')	Amplicon size (bp)
MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11	MOXMF	GCT GCT CAA GGA GCA CAG GAT	520
	MOXMR	CAC ATT GAC ATA GGT GTG GTG C	
LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITMF	TGG CCA GAA CTG ACA GGC AAA	462
	CITMR	TTT CTC CTG AAC GTG GCT GGC	
DHA-1, DHA-2	DHAMF	AAC TTT CAC AGG TGT GCT GGG T	405
	DHAMR	CCG TAC GCA TAC TGG CTT TGC	
ACC	ACCMF	AAC AGC CTC AGC AGC CGG TTA	346
	ACCMR	TTC GCC GCA ATC ATC CCT AGC	
MIR-1T ACT-1	EBCMF	TCG GTA AAG CCG ATG TTG CGG	302
	EBCMR	CTT CCA CTG CGG CTG CCA GTT	
FOX-1 to FOX-5b	FOXMF	AAC ATG GGG TAT CAG GGA GAT G	190
	FOXMR	CAA AGC GCG TAA CCG GAT TGG	

45 min, stained with safe dye (Yekta Tajhiz Azma, Iran), and the image was captured using gel documentation system (Upland, CA, USA). This multiplex AmpC PCR methodology was used as the gold standard AmpC detection methodology. Multiplex PCR positive isolates were further tested using specific singleplex AmpC PCRs under the same reaction conditions to ensure that the PCR products found in the multiplex PCR positive isolates were correct. Purified PCR products were subjected to sequencing. BLAST analysis was performed to search for homologous sequences in the GenBank database.

Data analysis. Statistical analyses were done by Chi-square test and Fisher's exact test using SPSS, version 24.0. Sensitivity and specificity of the screening and confirmation methods were calculated using multiplex AmpC PCR results as the gold standard.

RESULTS

Antimicrobial susceptibilities of *K. pneumoniae* isolates. Results of antimicrobial susceptibility testing in 38 cefoxitin-resistant *Klebsiella pneumoniae* clinical isolates were shown in Table 2. The most effective antibiotic on cefoxitin-resistant isolates was amikacin, and fosmomycin, to which 79% of isolates were susceptible, followed by imipenem (68.4%) and ertapenem (55.2%).

Screening for AmpC production. Thirty-eight (16.6%) out of 228 isolates were positive for AmpC enzyme production in the screening method by the use of cefoxitin disks. As shown in Table 3, the majority (n=27) of these isolates were isolated from urine samples.

Table 2. *In vitro* activities of antimicrobial agents against 38 cefoxitin –resistant *K. pneumoniae* clinical isolates by using disk diffusion method.

	Cefoxitin -resistant isolates (38) N:%		
Antibiotic Disk	Resistant	Intermediate	Susceptible
Cefepime	22 (57.9)	1 (2.6)	15 (39.5)
Ceftazidime	31 (81.6)	2 (5.3)	5 (13.1)
Cefotaxime	31 (81.6)	1 (2.6)	6 (15.8)
Ceftriaxone	28 (73.7)	2 (5.3)	8 (21)
Cefoxitin	38 (100)	0	0
Aztreonam	29 (76.4)	1 (2.6)	8 (21)
Imipenem	12 (31.6)	0	26 (68.4)
Ertapenem	15 (39.5)	2 (5.3)	21 (55.2)
Ciprofloxacin	24 (63.2)	5 (13.1)	9 (23.7)
Trimethoprim-sulfamethoxazole	22 (57.8)	4 (10.6)	12 (31.6)
Fosmomycin	2 (5.3)	6 (15.7)	30 (79)
Piperacillin-tazobactam	18 (47.4)	8 (21)	12 (31.6)
Amoxicillin–clavulanic acid	32 (84.2)	4 (10.5)	2 (5.3)
Amikacin	8 (21)	0	30 (79)
Gentamicin	18 (47.4)	1 (2.6)	19 (50)
Tobramycin	21 (100)	2	15
Tigecycline	2 (5.3)	16 (42.1)	20 (52.6)

Table 3. Phenotypic and genotypic characterization of all 38 cefoxitin-resistant *Klebsiella pneumoniae* clinical isolates.

			FOX Screening Test		Phenotypic Confirmatory Tests					Antimicrobial agents			
Sample ID	**Source**	**G/A**	**Disk Zone (mm)**	**MIC (µg/ml)**	**CDT**	**DDST**	**M3DT**	**PCR**	**ESBL**	**CPM**	**ETP**	**IMI**	**FO**
Kp4	Urine	F/82Y	0	128	-	+	-	-	-	S	S	S	S
Kp5	Urine	*/*	0	≥ 256	-	-	-	-	+	R	R	S	R
Kp6	Urine	F/32Y	10	4	-	-	-	-	+	R	S	S	S
Kp9	Urine	F/62Y	7	6	-	-	-	-	+	R	R	S	S
Kp11	Urine	F/49Y	0	≥ 256	-	-	+	CMY	+	R	R	R	I
Kp14	Urine	F/22Y	8	≥ 256	+	+	+	DHA	-	S	S	S	S
Kp21	Urine	F/49Y	0	≥ 256	-	-	+	CMY	+	R	R	R	I
Kp34	Urine	F/23Y	0	≥ 256	+	+	+	DHA	+	S	R	S	S
Kp46	Urine	F/3 M	13	48	+	+	-	-	-	S	S	S	S
Kp65	ETT	M/59Y	0	≥ 256	-	-	-	-	+	R	R	R	S
Kp71	Urine	M/52Y	12	8	+	-	+	CMY	+	R	S	S	S
Kp73	Urine	M/46Y	8	128	+	+	+	CMY	+	S	S	S	S
Kp74	Urine	F/41Y	8	≥ 256	+	+	+	CMY	+	S	S	S	S
Kp83	Urine	F/5Y	0	≥ 256	+	-	+	DHA	+	R	S	S	S
Kp89	Blood	M/48Y	0	≥ 256	-	-	-	-	+	R	R	R	S
Kp91	Urine	F/49Y	0	≥ 256	+	+	+	DHA	+	S	S	S	S
Kp92	Wound	M/60Y	0	≥ 256	-	-	-	-	+	R	R	R	I
Kp97	Blood	M/48Y	0	≥ 256	-	-	-	-	+	R	R	R	S
Kp102	ETT	M/50Y	0	≥ 256	-	-	+	-	+	R	R	R	S
Kp106	Blood	F/30Y	0	≥ 256	-	-	-	-	+	R	R	R	I
Kp113	Urine	F/48Y	0	≥ 256	-	-	-	-	-	S	S	S	S
Kp121	Wound	M/58Y	12	64	+	-	+	CMY	+	R	S	S	S
Kp122	Shaldon	F/*	0	≥ 256	+	-	+	DHA	+	R	S	S	S
Kp127	Urine	M/10Y	8	≥ 256	+	-	+	-	-	S	S	S	S
Kp128	Urine	F/23D	0	≥ 256	-	-	+	-	+	R	R	R	R
Kp144	Urine	*/*	0	16	-	-	-	-	+	R	S	S	S
Kp147	Urine	M/53Y	7	≥ 256	-	-	-	-	+	R	R	R	I
Kp150	Urine	F/27Y	0	≥ 256	-	-	-	-	+	R	R	R	S
Kp162	Urine	M/42Y	0	≥ 256	-	-	-	-	+	R	R	S	S
Kp172	Urine	M/40Y	0	≥ 256	+	+	+	DHA	+	S	S	S	S
Kp180	Urine	M/57Y	8	≥ 256	+	+	+	DHA	+	S	S	S	S
Kp181	ETT	M/45Y	0	≥ 256	+	+	+	DHA	-	S	S	S	S
Kp184	Urine	F/17Y	10	≥ 256	-	-	-	-	+	R	R	S	S
Kp200	Nephrectomy	M/12Y	0	≥ 256	+	+	+	DHA	-	S	S	S	S
Kp208	Urine	F/51Y	0	≥ 256	+	+	+	DHA	-	S	S	S	S
Kp215	Urine	M/32Y	0	≥ 256	+	+	+	DHA	+	S	S	S	S
Kp217	Wound	M/74Y	13	≥ 256	-	-	-	-	+	R	S	S	S
Kp228	Urine	F/3Y	0	≥ 256	+	+	+	DHA	-	S	S	S	S

ETT: Endotracheal tube, G: Gender, A: age, Y: Year, M: Month, D: Day, *: Not defined, CDT: Combination-disk test, DDST: Double Disk Synergy Test, M3DT: Modified Three Dimensional Test, FOX: Cefoxitin, CPE: Cefepime, ETP: Ertapenem, IMI:.Imipenem, FO: Fosmomycin , S: Susceptible, R: Resistant.

Phenotypic AmpC confirmation testing. A total of 16 pAmpC-producing strains were positive with the CDT with BA (Fig.1a), but 2 pAmpC-producing strains were not detected by this method and 2 non pAmpC-producer were also positive by this method. The overall sensitivity, specificity, and efficiency of the CDT were 89%, 90%, and 89%, respectively. By DDST, 12 strains were determined as pAmpC-producers and 2 non pAmpC-producer were also positive by this method (Fig. 1b), while 6 strains were false-negatives. The overall sensitivity, specificity, and efficiency of the DDST were 67%, 90%, and 79%, respectively. M3DT detected all 18 pAmpC-producers (Fig.1c), but 3 non pAmpC-producers were also positive by this method. The overall sensitivity, specificity, and efficiency of the M3DT were 100%, 85%, and 92%, respectively (Table 4).

Molecular characterization of *mdh* and plasmid-mediated AmpC genes. All tested isolates were positive for malate dehydrogenase (*mdh*) housekeeping gene gene (Fig. 2a). Among 228 isolates plasmid-mediated AmpC genes were detected in 18 (7.9%) cefoxcitin-resistant isolates by multiplex PCR and sequencing. DHA group genes were predominantly observed in 12 (66.6%) of pAmpC producers and CIT group genes (CMYlike) were detected in 6 (33.3%) pAmpC producer isolates (Fig. 2b).

The sequences of 12 DHA group genes were submitted to the GenBank database under accession numbers QII80479- QII80490, and the sequences of six CIT group genes were submitted to the GenBank database under accession numbers QII80473- QII80478.

DISCUSSION

Plasmid-mediated AmpC producers are considered an increasing concern because they cannot only cause outbreaks but also limit therapeutic choices and treatment failures (22-25). So considering the spread of pAmpC producing strains around the world, it is essential that we become fully aware of these strains in our hospitals. To improve the clinical management of infections and provide correct epidemiological data, it is highly important to have the capability to detect pAmpC, but at present, lack of standardized phenotypic screening methods in microbiology labo-

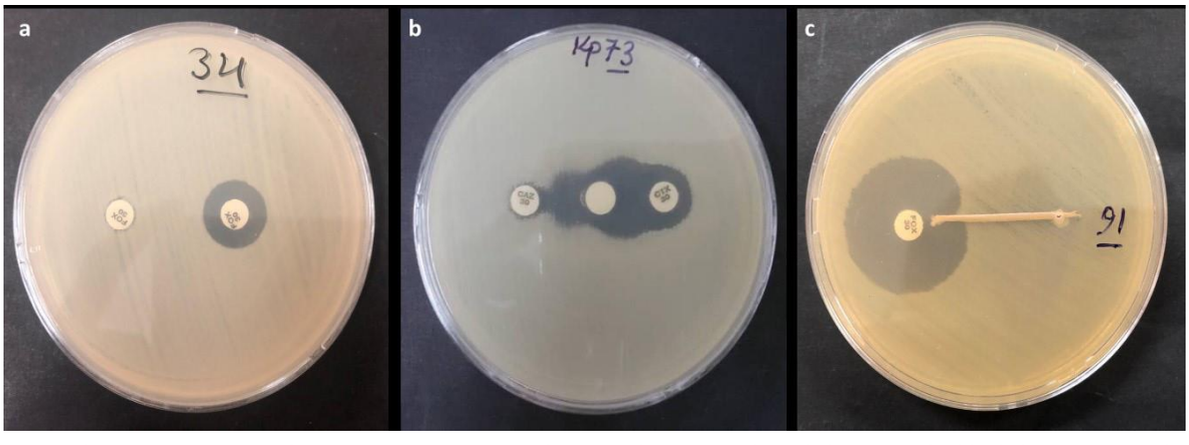

Fig. 1. Phenotypic AmpC confirmatory tests. AmpC-producing isolates in combine disk test (a), double disk synergy test (b) and modified three dimensional test (c).

Table 4. Frequency, sensitivity, specificity and efficiency of phenotypic confirmatory tests.

Phenotypic confirmatory test	Frequency N (%)	Sensitivity	Specificity	Efficiency	PPV	NPV	*p value
CDT	18 (47.4)	89%	90%	89%	88%	90%	p < 0.0001
DDST	14 (36.8)	67%	90%	79%	85%	75%	p < 0.0001
M3DT	21(55.3)	100%	85%	92%	86%	100%	p < 0.0001

PPV: Positive Predictive Value, NPV: Negative Predictive Value

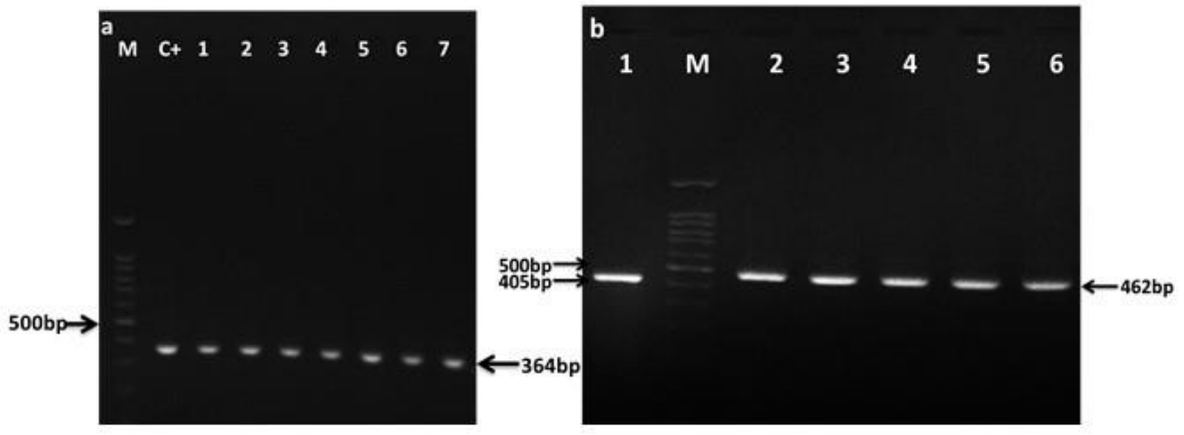

Fig. 2. a: Agarose gel electrophoresis of *mdh* gene (364 bp) of *K. pneumoniae* clinical isolates, lanes 1-7: *K. pneumoniae* carrying *mdh* gene, C+: *K. pneumoniae* ATCC 700603, M: 100 bp DNA ladder. b: Agarose gel electrophoresis of plasmid-mediated AmpC genes. Lane 1: *K. pneumoniae* carrying bla_{DHA} gene (405 bp), M: 100 bp DNA ladder, lanes 2-6 : *K. pneumoniae* harboring bla_{CMY} gene (462 bp).

ratories is the main deterrent. Reduced susceptibility to cefoxitin in the *Enterobacteriaceae* may be an indicator of AmpC activity (26). Cefoxitin resistance is a sensitive test; however, many investigators have reported that cefoxitin resistance in general was a nonspecific indicator of AmpC production, mostly because of a reduced permeability of the bacterial outer membrane as well as the expression of some carbapenemase enzymes which might also result in cefoxitin resistance (25), as seen in our isolates 9 out of 20 pAmpC PCR-negative cefoxitin-resistant isolates were carbapenemase producers (NDM/OXA-48) (27). In the present study only 47% (18/38) of cefoxitin-resistant isolates were pAmpC producers. Different percentages were reported by Helmy et al. (88.4%) (28), Palmieri et al. (79.4%) (29), Gupta et al. (66%) (30), Manoharan et al. (36%) (21), Reuland et al. (27%) (25), Coudron et al. (20%) (14) and, Yilmaz et al. (19%) (31).

Another downside attributed to using cefoxitin resistance as a phenotypic screening methodology is the susceptibility of ACC-1-type enzymes to cefoxitin, which means that isolates harboring these genes will be considered as pAmpC negative. This is an important point to be taken into consideration because ACC-type enzymes have been found in several different countries in Europe, including a large outbreak in a teaching hospital in Garches, France (25).

Combination of reduced sensitivity to the third generation cephalosporins (cefotaxime and/or ceftazidime) and reduced susceptibility to cefoxitin is regarded a good indicator for the presence of pAmpC gene expression. Reuland et al. reported that applying these combined methodologies would not yet fill the void for the presence of ACC-1 type AmpC (25). That's why PCR was done on all *Klebsiella pneumoniae* isolates in the present study.

Nowadays, using molecular testing strategies such as multiplex AmpC PCRs is currently the gold standard for pAmpC detection (25). Our results revealed that the prevalence of pAmpC-producing *Klebsiella pneumoniae* isolates in Bushehr province was 7.9% by using multiplex AmpC PCR. The reported prevalence of pAmpC-KP differed from city to city in Iran, where the prevalence was 19% in Arak (32), 16.7% in Mashhad (33) 14.6% in Kerman (34) and, 5.4% in Qazvin (35). Comparing the prevalence of pAmpC-KP isolates in Bushehr with other regions of Iran, it can be concluded that the prevalence of pAmpC-KP in Bushehr is relatively lower than that of the nation average. The prevalence of pAmpC-KP differs in other countries, as well. For instance, Nepal (36), Turkey (31), China (37), the Netherlands (25), and Libya (38) reported the least amount of pAmpC-KP isolates with 2.7%, 3.6%, 4.2%, 4.8% and 7.9%, respectively, while the higher prevalence of pAmpC was reported in Singapore (49%) (26) and India (36.5%) (21).

In the current study the majority of *ampC*-positive strains were derived from urine cultures (n = 14), but there were also *ampC*-positive isolates obtained from other sample types. In addition, DHA-like enzymes

(originally derived from *Morganella morganii*) dominated our clinical isolates (n = 12) but also enzymes from the CIT group (CMY-like enzymes derived from *C. freundii*) were also in six cefoxitin-resistant *Klebsiella pneumoniae* clinical isolates. No enzymes belonging to the ACC, FOX, MOX, or EBC family were detected. Tan et al. reported similar findings (26). Contrary to our study, in the studies conducted by Li (37) Gupta (N:32,12 CMY,8 DHA) (30) Reuland (N:13, 9 CMY-2, 3 DHA-1,1 ACC-1) (25) and El-Hady (N:22, 15 CMY-1, 12 CMY-2, 9 FOX-1) (39) on *Klebsiella pneumoniae* clinical isolates, the most frequently reported plasmid mediated AmpC β-lactamases was CMY-like enzymes followed by DHA-like enzymes.

It is notable that in the studies conducted in the other regions of Iran similar to other countries and in contrast to the present study the most commonly reported pAmpC was CIT group followed by EBC, DHA, MOX and, FOX (32, 34, 40). It also indicated that some plasmid-mediated AmpC β-lactamases may be clinically more harmful than others. Black et al. reported that the inducible DHA-1 enzyme was associated with a crude mortality rate of 46%, whereas the constitutively produced CMY-1-like enzyme was associated with only a 14.3% mortality rate (2). So it is essential that clinical laboratories accurately detect isolates producing a plasmid-mediated AmpC β-lactamase.

The present study revealed no significant difference ($p < 0.0001$) between the results reached by the three phenotypic confirmatory methods, but M3DT displayed the best performance as phenotypic confirmation method for AmpC detection; while it is too time-consuming and laborious for routine use. M3DT detected all 18 pAmpC-producers (Fig. 1c), however three *K. pneumoniae* isolates also yielded false-positive results by this test. Two (Kp102 and Kp128) of them were resistant to cefepime, ertapenem and, imipenem and also harbored bla_{CTX-M} but the other false-positive isolate (Kp127) was non-ESBL-porducer and susceptible to all the mentioned antimicrobial agents; therefore leaving the cause of resistance for the single remaining isolate unexplained. It is also notable that among cefoxitin-resistant isolates 76.3 % were also positive for ESBL production (16). The overall sensitivity, specificity, and efficiency of the M3DT were 100%, 85%, and 92%, respectively. Moreover, positive extended-spectrum-β-lactamase (ESBL) screening or confirmatory phenotypic results may be due to AmpC β-lactamases, as seen in the present study four ESBL-confirmatory-positive isolates did not harbor any bla_{CTX-M} genes but were positive for AmpC genes. Therefore, positive ESBL confirmatory results may be due to ESBL genes other than CTX-M groups or AmpC production.

Reuland et al. reported the DDST cloxacillin as the best AmpC confirmatory test, with the best sensitivity and specificity (25). In contrast to these authors, we found that the sensitivity and specificity of DDST in detecting pAmpC in our isolates was 67% and 90%, respectively. In addition in the current study, DDST demonstrated the least efficiency among three AmpC confirmatory tests.

Out of 38 cefoxitin-resistant isolates, 16 of them failed to exhibit positive results in any of the phenotypic confirmatory tests. In all such isolates, cefoxitin resistance may be due to mechanism other than AmpC production.

It is also worth noting that detection of AmpC-producing organisms is essential to ensure effective therapeutic intervention and optimal clinical outcome. Treatment of infections caused by pAmpC-producing strains with cephalosporins is associated with adverse clinical outcomes, which is a major concern (25). Pai et al. reported therapeutic failures with cefotaxime and ceftazidime among 28 bacteremia cases caused by DHA-1-like or CMY-1-like β-lactamases producers (41). Moreover, these authors emphasized the importance of using imipenem as definitive therapy of pAmpC-producing isolates. In accordance with their findings in the present study antimicrobial susceptibility test revealed 88.8% and 83.3% of pAmpC-KP isolates were resistant to ceftazidime and cefotaxime, respectively (Data not shown). In addition, the most effective antibiotic on our pAmpC-KP isolates was imipenem, to which 88.9% of isolates were susceptible, followed by ertapenem (83.3%) and cefepime (66.6%).

CONCLUSION

In the present study, M3DT showed the best performance as phenotypic confirmation method for pAmpC production and DHA was the most prevalent pAmpC beta-lactamase. In addition, 72.2% of PCR-positive isolates harbored both AmpC and ESBL β-lactamases. Coexistence of pAmpC resistance genes in ESBL-producing of *Klebsiella*

pneumoniae may become a worrying public health issue, since the plasmid carrying these genes may have caused the acceleration of the simultaneous and quick spread of both pAmpC and ESBL genes. Therefore, there is a vital need to keep the spread of these clinical isolates under surveillance.

ACKNOWLEDGEMENTS

This article was from the postgraduate MSc thesis of Khatereh Faghihi and was supported by the Vice-Chancellor of Research of Bushehr University of Medical Sciences, Bushehr, Iran (Grant no. 1455). We would like to thank Clinical Research Development Center, The Persian Gulf Martyrs Hospital, Bushehr University of Medical Sciences, Bushehr, Iran for facilitating the process of sampling.

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