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Original Article

Microbiology Section

Phenotypic and Molecular Characterization of Plasmid Mediated AmpC among Clinical Isolates of *Klebsiella pneumoniae* Isolated from Different Hospitals in Tehran

LEILA AZIMI[1], GHOLAMREZA ERAJIYAN[2], MALIHE TALEBI[3], PARVIZ OWLIA[4], MAHSA BINA[5], ALI SHOJAIE[6], ABDOLAZIZ RASTEGAR LARI[7]

ABSTRACT

Introduction: *Klebsiella pneumoniae* is one of the main opportunistic pathogens which can cause different types of infections. Production of beta-lactamases like AmpC and ESBL mostly lead to beta-lactam resistance in these Gram-Negative bacteria. The aim of this study was the detection of AmpC-producing *K. pneumoniae* in clinical isolates.

Materials and Methods: Three hundred and three isolates of *K. pneumoniae* were identified. Double disc method including cefoxitin with cefepime and using boronic acid with cloxacillin were performed as two phenotypic methods for detection of AmpC. Amplification of *AmpC* gene was performed by PCR.

Results: Eight and three isolates showed positive results in double disc method and by using boronic acid with cloxacillin, respectively. Five isolates had specific band for *AmpC* gene after electrophoresis.

Conclusion: Our results were indicated the low prevalence of AmpC-producer-*K. pnemoniae* in Iran. On the other hand these two tested phenotypic methods showed low sensitivity for detection of AmpC.

Keywords: Beta-lactamases, Boronic acid, Cloxacillin, Double disc method, *Enterobactriaceae,* Iran

INTRODUCTION

K. pneumoniae is the third common gram negative bacteria of nosocomial infections in burn patients after *P. aeruginnosa* and *A. baumannii* [1-3]. This microorganism can cause diverse type of infection like pneumonia, urinary tract infection, and septicemia etc [3,4]. These infections cause increasing morbidity and mortality among patients [3,5]. *K. pneumoniae* can persist on hospital environmental and can colonize human skin [4]. Therefore, it can easily transmit among patients via hands of health care personnel [4]. On the other hand over use of antibiotic and/or inappropriate antibiotic therapy are the major causes for the emergence of antibiotic resistant *K. pneumoniae* [3,5,6]. Resistance to antibiotics can contribute by different mechanism like efflux-pump, production of different enzymes, etc [4]. One of the main mechanism for resistance to beta-lactam antibiotics is the production of beta-lactamases including AmpC betalactamases (AmpC) and extended-spectrum beta-lactamases (ESBLs) [7,8]. AmpC beta-lactamases are group I cephalosporinases and can cause resistance to wide variety of beta-lactam antibiotics including third generation of cephalosporins and aztreonam [6,9]. Even AmpC may also mediate resistance to carbapenems in combination with porin loss [6]. One of the main characterizations of this enzyme is related to poor inhibition by beta-lactamases inhibitor like clavulanic acid [10]. AmpC is originally mediated chromosomally in *Enterobactriaceae*; however, plasmid-mediated AmpC beta-lactamases has arisen through the transfer from chromosome to plasmid and can lead to spread among different bacteria population including *Klebsiella* spp. which typically do not have chromosomal AmpC [6,10]. The increasing frequency of plasmid mediated AmpC isolates posing therapeutic challenges for health care worldwide by causing resistance to a variety type of beta-lactam antibiotics [6]. The aim of this study was to determine the AmpC-producer -*K. pneumoniae* isolated from different clinical isolates.

MATERIALS AND METHODS

Bacterial Isolations

This cross-sectional study was conducted from August to December 2013. Three hundred and three *K. pneumoniae* were collected from different clinical sources in different wards of hospital including, burn wound, pneumonia, septicemia and urinary tract infections, inpatients 17% (52) and 83% (251) outpatients, from three teaching hospital in Tehran. The plate of bacteria sent to Antimicrobial Resistance Research Center, Iran University of Medical Sciences for future examines.

These isolates were identified with conventional biochemical and microbiological tests. Like, TSI, urea, SIM and citrate etc.

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was done by disc diffusion agar method according to CLSI guide line [11]. Standard antibiotic discs were prepared from MAST Company (Mast Diagnostics, UK) and include:

Cefoxitin (30µg), ceftriaxone (30µg), cefotaxime (30µg), cefepime (30µg), imipenem (10µg), meropenem (10µg), amoxicillin-clavulanic acid (20/10µg), aztreonam (30µg), tobramycin (10µg), gentamicin (10µg), amikacin (30µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), chloramphenicol (30µg) and tetracycline (30µg), ceftazidime (30 µg), cefoxitin, ceftriaxone), piperacillin (100µg).

Phenotypic detection of AmpC

Two phenotypic tests were used for identification of AmpC:

1. The increasing inhibition zone at least 5mm around meropenem (10µg) plus 600 µg/disc boronic acid and meropenem (10µg) plus 750 µg/disc cloxacillin simultaneously in comparison to meropenem (10µg) alone. The stock of boronic acid was prepared 30mg/ml and the stock of clocacillin was prepared 37.5 mg/ml [12].
2. Double disc methods with cefoxitine (30µg) and cefepime

Antibiotics	CRO	FOX	CTX	CAZ	CEF	IMI	MEM	AT	TO	GM	AK	SXT	PRL	C	T
Percentage of resistance	20.8	50	55	50	43	23	23	54	100	37	79	91	65	97	91
	(63)	(151)	(167)	(163)	(130)	(69)	(69)	(164)	(303)	(112)	(239)	(276)	(197)	(294)	(276)

[Table/Fig-1]: (Number) Percentage of resistance to tested antibiotics. CRO: ceftriaxone, FOX: cefoxitin, CTX: cefotaxime, CAZ: ceftazidime, CEF: cefepime, IMI : imipenem, AT, aztreonam, MEM: meropenem, TO: tobramycin, GM: gentamicin, AK: amikacin, SXT: trimethoprim sulfamethoxazole, PRL: piperacillin-Tazobactam, , C: chloramphenicol, T: tetracycline

(30µg). The isolates which susceptible to cefepime and resistant to cefoxitine were consider as an AmpC-producer isolate [13].

Genotypic detection of AmpC

PCR was used as a molecular test for detection of *AmpC* plasmid mediate (*cmy*) gene. The forward primer was 5-ATTCCGGGTATG GCCGT-3 and the reverse was 3-5-GGGTTTACCTCAACGGC [14].

The polymerase chain reaction was performed in following condition:

The first denaturation at 94°C for 5 min and 30 cycles of 94°C for 60 s, annealing at 58°C for 60 sec, extension at 72°C for 60 sec and at last the final extension at 72°C for 5 min.

Isolates which showed specific band after PCR and electrophoresis have been sent for sequencing to Genfanavaran, Macrogen, Seoul, Korea.

RESULTS

Three hundred and three isolates of *K. pneumoniae* were confirmed according to the results of biochemical and microbiological tests. The percentage of antibiotic resistance was showed in [Table/Fig-1].

Eight isolates had less than 14mm inhibition zone around cefepime and more than 18 mm around cefoxitin according to CLSI table 2013. On the other hand, only three isolates showed at least 5 mm in diameter of inhibition zone around meropenem plus boronic acid and meropenem plus cloxacillin simultaneously in comparison to meropenem alone. None of the isolates showed positive results in both of detection phenotypic tests. Three positive PCR isolates were positive in at least one of phenotypic methods that were used in this study (two out of eight double disc method positive and one out of three when use of boronic cid and cloxcillin combined with meropenem). Two isolates were negative in both of phenotypic methods with positive results in PCR [Table/Fig- 2].

Phenotypic methods	No. of strains	PCR
Meropenem+boronic acid/ Meropenem+cloxacillin	3	1
Cefoxitine/ cefepime	8	2
Strains without positive results in both phenotypic methods	292	2
Total	303	5

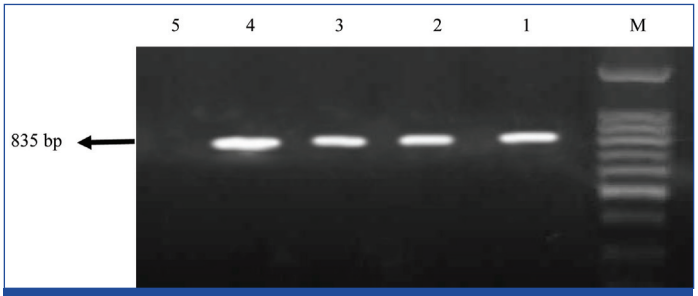
[Table/Fig- 2]: Number of isolates showing positive AmpC by all three methods

Number of confirmed AmpC positive strains with PCR according to source of isolated bacteria was showed in [Table/Fig-3].

Source of specimens	Burns infection	Ventilator association pneumonia
No. of confirmed AmpC positive strains	4	1

[Table/Fig- 3]: Number of confirmed AmpC positive strains with PCR according to source of isolated bacteria

Totally, in five strains *AmpC* specific band was observed after PCR [Table/Fig- 4] and gel electrophoresis in agaros 1.5%. Direct sequencing of PCR amplified products was carried out using ABI 3730X capillary sequencer (Genfanavaran, Macrogen, Seoul, Korea).



[Table/Fig-4]: PCR amplification fragments for detection of *AmpC* agene among *K.pneumoniae* isolates M: 1kb DNA size marker; lane 1; Psitive Control of *AmpC*; lane 2-4; Positive strains; lane 5; Negative Control of *AmpC* gene

DISCUSSION

K. pneumoniae is one of the important Gram - negative bacteria in nosocomial infections [1-3,15]. Antibiotic resistant isolates make therapeutic challenge for physicians [3]. *K. pnemoniae* can produce variety of beta – lactamase enzymes [3,5]. Recent study was conducted in Iran reported the prevalence of OXA-48-producer *K. pneumoniae* [15]. Also, this study indicated the AmpC – producer *K. pneumoniae* but in low prevalence with the rate of 1.6%. The results of study was conducted in Turkey in west-north of Iran were showed the prevalence of 8% AmpC-producer *K. pneumoniae* isolated from blood culture [16]. This prevalence is more than our results because *K. pneumoniae* were isolated from hospitalized patients in Turkey but in our study most of specimens were isolated from outpatients. Despite of the low prevalence of AmpC producer *K. pneumoniae* in our tested isolates, it is notable that the different number of isolates was isolated from different clinical specimens. Other studies in Iran have indicated the higher resistance in *K. pneumoniae* which isolated from burn patients [3]. This can be due to the expand using of beta-lactam antibiotics in patients whome were hospitalized in burn ward in Iran [3,5, 6]. Since the AmpC is the cephalosporinase, this low prevalence of AmpC producer *K. pneumoniae* in our tested isolates can related to use of other broad spectrum antibiotics for empirical treatment except cephalosporins and pressure of the overuse of antibiotics for selection of this mechanism of antibiotic resistant is low. On the other hand, we can inhibit the increasing prevalence of AmpC producer *K. pneumonia* by prevention of broad spectrum cephalosporins misuse.

In Pakistan that is in east – south border of Iran the rate of AmpC – producer – *K. pneuminiae* was 12% [7]. The results of study in China were showed 12% AmpC – producer – *K. pneuminiae* [4]. In a study that was conducted in India six out of 44 *Klebsiella* spp. (13.6%) were positive for AmpC [17].These isolates were collected

from different clinical specimens [17]. In other study that was done in India five out of 120 *K. pneumonia* (94.1%) were positive for detection of AmpC [18]. This difference between two studies that were conducted in the same country (India) can be related to different centers that were selected for study and also, source of specimens. The influence of antibiotic treatment and their pressure for resistant bacteria selection can be different in different health care settings. In this regard, six of 22 *K. pneumonia* (27.2%) were positive for AmpC [19]. These results indicated the relation with the source of isolated bacteria, because these isolates were collected from blood specimens. Generally, broad spectrum antibiotics are using for treatment of septicemia and the pressure of antibiotic over use can lead to select more resistance bacteria. These results are equal to our finding from burn patients in current study. On the other hand, this subject should be considered that *AmpC* gene is plasmid mediated and can transfer to other bacteria and make complication in treatment of patients [10].

CONCLUSION

According to finding of this study it seems that both phenotypic tests have low sensitivity. Thus the phenotypic method can be useful for primary screening. On the other hand, phenotypic methods are more economical than molecular methods such as PCR and also, phenotypic methods do not require expertise and professionals. But it is notable that PCR is more reliable test for detection of AmpC-producing strains and can use for critical and important cases.

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PARTICULARS OF CONTRIBUTORS:
1. Faculty, Antimicrobial Resistance Research Center, Iran University of Medical sciences, Tehran, Iran. And Department of Microbiology, Iran University of Medical sciences, Tehran, Iran.
2. Faculty, Department of Microbiology, Iran University of Medical sciences, Tehran, Iran.
3. Faculty, Department of Microbiology, Iran University of Medical sciences, Tehran, Iran.
4. Faculty, Molecular Microbiology Research Center, Shahed University, Tehran, Iran.
5. Faculty, Department of Microbiology, Iran University of Medical sciences, Tehran, Iran.
6. Faculty, Tehran Heart center, Tehran university of Medical Sciences, Tehran, Iran.
7. Faculty, Antimicrobial Resistance Research Center, Iran University of Medical sciences, Tehran, Iran. And Department of Microbiology, Iran University of Medical sciences, Tehran, Iran.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Abdolaziz Rastegar Lari,
P.O. Box 14515-717, Tehran, Iran.
E-mail: azizlari@gmail.com

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