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FIRST REPORT OF COEXISTENCE OF AmpC BETA-LACTAMASE GENES IN *KLEBSIELLA PNEUMONIAE* STRAINS ISOLATED FROM BURN PATIENTS

ROYA GHANAVATI[1], DAVOOD DARBAN-SAROKHALIL[1], FATEME NAVAB-MOGHADAM[1], HOSSEIN KAZEMIAN[2,3], GHOLAMREZA IRAJIAN[1] and SHABNAM RAZAVI[1]*

[1]Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[2]Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran
[3]Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

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Klebsiella spp. are among the most frequently isolated bacteria from burn wounds. These organisms are among the most important opportunistic pathogens, causing hospital-acquired and healthcare-associated infections worldwide. Limited information is available about prevalence of AmpC-producing *Klebsiella pneumoniae* from burn patients. Therefore, the aim of this study was to determine the characterization of AmpC beta-lactamase among *K. pneumoniae* isolated from burn patients. Samples were collected from wound specimens of patients with burn injury from a burn hospital in Tehran during 18 months (March 2015 to August 2016). For phenotypic detection of AmpC beta-lactamase, disk diffusion method with cefoxitin was used for screening, AmpC disk test and boronic acid inhibitor-based method were used as confirmatory tests. Polymerase chain reaction (PCR) was performed to screen all isolates with AmpC genes including ACCM, DHAM, EBCM, FOXM, MOXM, and CITM. Finally, PCR products were validated using sequencing. During this study, 102 isolates of *K. pneumoniae* were collected. Among these isolates, 52.9% suspected as AmpC producer by disk agar diffusion cefoxitin screening method. By confirmatory phenotypic methods, 19.6% of isolates considered as AmpC producer. Molecular analysis revealed 43.1% of cefoxitin-resistant isolates harbored at least one of the AmpC genes including CITM (22.5%), EBCM (21.5%), DHAM (7.8%), and FOXM (0.98%). In addition, 5.8% of isolates harbored two AmpC genes and 2.9% harbored three AmpC genes. In conclusion, *K. pneumoniae* is becoming a serious problem in burn patients. Accurate and precise methods and guidelines should be designed for detection of antibiotic-resistant mechanisms. Our data showed the high rate of AmpC

*Corresponding author; E-mail: razavi.sh@iums.ac.ir

beta-lactamase among *K. pneumoniae* isolated from burn patients, which limit the treatment options. Therefore, the results of this study can provide evidence to help for appropriate treatment of burn patients.

Keywords: *K. pneumoniae*, beta-lactamase, AmpC, burn wounds

Introduction

In comparison with different type of nosocomial infections among hospitalized patients, burn patients are more susceptible to nosocomial infections because of their immunocompromised state. In this situation, antibacterial treatments of these patients are important in burn wards. For example, choosing the best antibiotic regimens is a crucial means of infections prevention and control. Unfortunately, during recent decades the widespread use of antibiotics led to the emergence of drug-resistant bacteria, such as multidrug and extensively drug-resistant organisms. Thus in that area, precise knowledge of the bacterial resistance patterns is essential for determining the best antibiotic regimens or empirical therapy in critical conditions, for treatment of burn patients [1].

Klebsiella spp. are among the most frequently isolated bacteria from burning wounds [2]. This organism is one of the most important opportunistic pathogens, causing hospital-acquired and healthcare-associated infections worldwide [3]. In recent decades, due to its tendency to develop antibiotic resistance and increasing virulence factor, lead to emerging this bacterium as clinically important organism [4, 5]. *Klebsiella pneumoniae* strains commonly have different types of beta-lactamase enzymes such as extended-spectrum beta-lactamases (ESBLs), AmpC beta-lactamases, and carbapenemases, which confer resistance to different types of beta-lactam antibiotics [6]. The AmpC beta-lactamases are particularly troublesome group because can easily be transmitted by conjugation to other bacteria, which has increased incidence worldwide [7]. According to the Ambler classification, AmpCs belong to Class C beta-lactamase. AmpC genes may be located on chromosome or plasmids. In addition to resistance to penicillins and most cephalosporins, unlike ESBLs, AmpC-producing organisms can degrade cephamycins [8]. AmpC beta-lactamase-producing organisms show more extensive antibiotic resistance features in comparison with other ESBLs-producing organisms [9]. It has been reported that AmpC beta-lactamase can confer resistance to cefepime (fourth-generation cephalosporin antibiotic) and also can increase the minimum inhibitory concentration (MIC) value of carbapenems [9, 10].

With respect to clinically important AmpC-producing strains, standard guidelines do not introduce the ideal test for AmpC detection. However, polymerase chain reaction (PCR) remains the gold standard for the detection of AmpC beta-lactamases [11].

Thus, due to the lack of standard guiding principle for the revealing of AmpC, little is known about the prevalence of AmpC-producing *K. pneumoniae* among burn patients, which early detection and identification of AmpC lead to implementation of the ideal antimicrobial therapy, and also may allow infectious control measures to be introduced to prevent spread of drug-resistant organisms. Therefore, the aim of this study was to determine the characterization of AmpC beta-lactamase among *K. pneumoniae* isolated from burn patients by phenotypic and genotypic methods.

Materials and Methods

Bacterial strains

This study was a cross-sectional study. Samples were collected from wound specimens of patients with burn injury from a burn hospital in Tehran, Iran during 18 months (March 2015 to August 2016). Phenotypic identification and biochemical validations performed for *K. pneumoniae* detection according to medical laboratory guidelines.

Phenotypic detection of AmpC beta-lactamase

For screening of AmpC beta-lactamase disk diffusion method with cefoxitin, amoxicillin–clavulanate, cefotaxime, and ceftazidime antibiotic disks was performed according to the guidelines of Clinical and Laboratory Standards Institute [12]. The isolates, which were resistant to all of the disks considered as AmpC producer. Then confirmatory tests, such as AmpC disk test, boronic acid inhibitor-based method were performed [13, 14]. *K. pneumoniae* ATCC 700603 was used as standard strain.

Molecular analysis of AmpC

For molecular evaluation, the boiling method was used for extracting DNA. PCR was performed to screen all isolates with AmpC genes including ACCM, DHAM, EBCM, FOXM, MOXM, and CITM. Specific primers were used for each gene (Table I) [15]. PCR was performed by thermal cycler (Eppendorf, Mastercycler gradient). PCR was carried out in a total volume of 25 µl containing 2 mM $MgCl_2$, 1 µl PCR buffer, 2 mM dNTPs, 1 pmol of primers, 0.25 U Taq DNA polymerase (CinnaGen Co., Iran), and 5 µl of template DNA. PCR products were

Table I. Specific primers for detection of AmpC genes

Gene name	Primers	Annealing temperature (°C)	Product size (bp)
ACCM	F-AAC AGC CTC AGC AGC CGG TTA R-TTC GCC GCA ATC ATC CCT AGC	58.6	346
DHAM	F-AAC TTT CAC AGG TGT GCT GGGT R-CCG TAC GCA TAC TGG CTT TGC	60	405
EBCM	F-TCG GTA AAG CCG ATG TTG CGG R-CTT CCA CTG CGG CTG CCA GTT	63	302
FOXM	F-AAC ATG GGG TAT CAG GGA GATG R-CAA AGC GCG TAA CCG GAT TGG	59	190
MOXM	F-GCT GCT CAA GGA GCA CAG GAT R-CACATTGAC ATA GGTGTGGTGC	63	520
CITM	F-TGG CCA GAA CTG ACA GGC AAA R-TTT CTC CTG AAC GTG GCT GGC	63	426

analyzed by electrophoresis on 1% (w/v) agarose gel (Merck, Germany) containing ethidium bromide and the gels were visualized under UV light irradiation (Gel Doc^{TM} XR+, USA). Finally, PCR products were validated using sequencing (Bioscience Co., UK).

Results

During this study, 102 isolates of *K. pneumoniae* were collected from burn wound infections. Among these isolates, 52.9% ($n = 54$) suspected as AmpC producer by disk agar diffusion (DAD) cefoxitin screening method. By confirmatory phenotypic methods for detection of AmpC, 19.6% ($n = 20$) of isolate considered as AmpC producer (Figure 1).

Molecular analysis revealed 43.1% ($n = 44$) of isolates harbored at least one of the AmpC genes including CITM 22.5% ($n = 23$), EBCM 21.5% ($n = 22$), DHAM 7.8% ($n = 8$), and FOXM 0.98% ($n = 1$) (Table II). In addition, 5.8% ($n = 6$) of isolates harbored two AmpC genes and 2.9% ($n = 3$) harbored three AmpC genes (Table III).

Discussion

In this study, the prevalence of AmpC-producing *K. pneumoniae* isolates among burn wound infections was evaluated by phenotypic and genotypic methods. By cefoxitin screening method, 52% of isolates suspected as AmpC

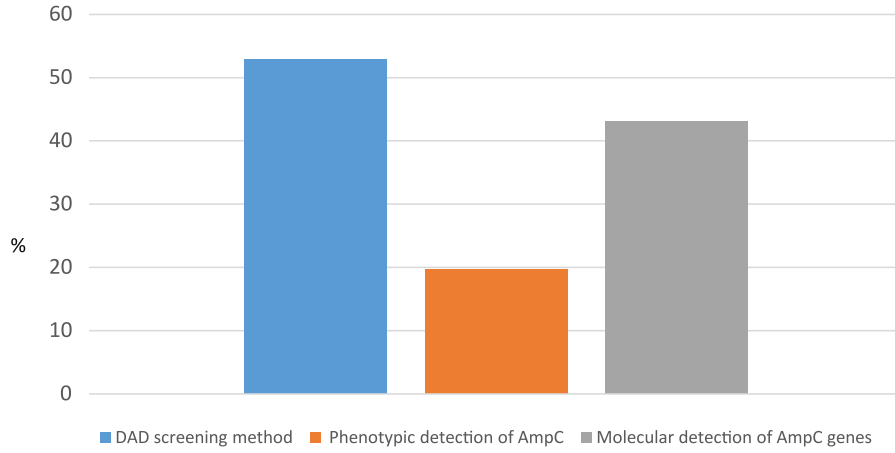

Figure 1. Compression of different methods for AmpC beta-lactamase detection

Table II. Distribution of AmpC genes among clinical isolates of *K. pneumoniae*

Distribution of AmpC genes	CITM	EBCM	DHAM	FOXM
% of isolates (*N*)	22.5 (23)	21.5 (22)	7.8 (8)	0.98 (1)
% of isolates among AmpC producer (*N*)	52.2 (23)	50 (22)	18.1 (8)	2.2 (1)

Table III. Coexistence of AmpC genes among clinical isolates of *K. pneumoniae*

Coexistence of AmpC genes	CITM and DHAM	CITM and EBCM	EBCM and DHAM	CITM, DHAM, and EBCM
% of isolates (*N*)	1.9 (2)	2.9 (3)	0.98 (1)	1.9 (2)
% of isolates among AmpC producer (*N*)	4.5 (2)	6.8 (3)	18.1 (8)	4.5 (2)

producer, but by confirmatory methods just 19% of isolates considered as AmpC producer. Molecular analysis revealed that 43% of isolates harbor AmpC beta-lactamase genes. In our analysis, high rate of false negative results was seen in phenotypic detection methods. But, cefoxitin was more sensitive than phenotypic methods. Any AmpC genes were not detected in the ten cefoxitin-resistant strains. One reason for this phenotypic resistance is due to loss of outer membrane porins [16].

Our analysis revealed the high prevalence of AmpC beta-lactamase among clinical isolates of *K. pneumoniae* isolated from burn patients. According to Azimi et al.'s study during 2013 in Iran, the prevalence of AmpC beta-lactamase among clinical isolates of *K. pneumoniae* was reported 1.6% [17]. Our data showed the sharp rise of AmpC beta-lactamase incidence over the past 3 years. Some studies have showed that AmpC beta-lactamases are produced in upward of 50% of clinical isolates [18–20]. A limited number of antibiotic treatment options are available in infections caused by AmpC-producing *K. pneumoniae*. Carbapenems have emerged as a primary agent for management of infections due to AmpC-producing *K. pneumoniae* [9]. However, it has been reported that AmpC beta-lactamase can increase the MIC value of carbapenems and confer resistance to this class of antibiotics [10, 11].

Japoni-Nejad et al. [21] during 2014 in Iran showed that 19% of clinical isolates of *K. pneumoniae* harbor AmpC genes. Of this 19%, 42.2% carried CITM gene, 36.8% carried MOX gene, whereas 15.7% and 5.2% carried EBCM and DHAM genes, respectively. According to our data, the most AmpC genes prevalent among the *K. pneumoniae* isolated from burn infections are CITM and EBCM, respectively. Our data reveal that shifting the prevalence of AmpC genes occurred during the short time in our country. In addition, according to Jean et al.'s [22] study, the most abundant AmpC beta-lactamase variant among clinical isolates of *K. pneumoniae* in Asia-Pacific region was DHAM.

In our analysis, eight strains were found which coharbored at least two AmpC genes. Two of these isolates were coharbored three AmpC genes. Therefore, this study is the first report of coexistence of AmpC genes in clinical isolates of *K. pneumoniae*.

In conclusion, *K. pneumoniae* is becoming a serious problem in burn patients. Accurate and precise methods and guidelines should be designed for detection of antibiotic resistance mechanisms. Our data showed high rate of AmpC beta-lactamase among *K. pneumoniae* isolated from burn patients, which limit the treatment options. Therefore, the results of this study provide evidence to help for appropriate treatment of burn patients. Infection control programs and strict antimicrobial stewardship policies should be applied to reduce the prevalence of these high-risk strains. Finally, using the antibacterial activities of medicinal plants with low level of toxicity can improve treatment of infection due to drug-resistant organisms [23, 24].

Conflict of Interest

The authors declare that there is no conflict of interest.

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