ORIGINAL ARTICLE

Extended-spectrum β-lactamase and carbapenemase production among burn and non-burn clinical isolates of *Klebsiella pneumoniae*

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ABSTRACT

Background and Objectives: *Klebsiella pneumoniae* is an opportunistic pathogen responsible for up to 10% of nosocomial infections. The emergence and spread of multidrug resistant *K. pneumoniae*, mostly due to the production of extended-spectrum β-lactamases (ESBL) and carbapenemases, is often responsible for antibiotic treatment failure of these infections. We compared the antibiotic resistance profiles, ESBL and carbapenemase production as well as presence of KPC-type genes in burn and non-burn clinical isolates of *K. pneumoniae*.

Materials and Methods: Fifty five clinical isolates were collected from Shahid Motahari (25 burn isolates) and Shariati (30 non-burn isolates) hospitals between August 2011 to January 2012. Antibiotic susceptibility was determined to 12 antibiotics using disc diffusion. The phenotypic confirmatory test (PCT) was used to screen for ESBL production. Carbapenemase activity was measured by the modified Hodge test (MHT) and KPC-type carbapenemases were further sought by PCR using specific primers.

Results: Both groups were highly resistant to cefotaxime and ceftazidime (>92%). Burn isolates were significantly more resistant to cefepime, amoxiclav, imipenem, meropenem, gentamicin and ciprofloxacin compared to the non-burn strains (p<0.05). No significant differences were observed in ESBL production between the two groups. Carbapenem resistance was only observed among the burn isolates (n=5, 9.1%). Five carbapenem-resistant isolates produced carbapenemases. However, none of the isolates harbored the KPC-type genes.

Conclusion: Higher rates of drug resistance were observed in burn isolates of *K. pneumoniae* compared to the non-burn strains. Carbapenemase phenotype was only observed among the burn isolates but KPC-type gene was not detected.

Keywords: *Klebsiella pneumoniae,* ESBL, extended-spectrum β-lactamase, carbapenemase, KPC

INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen which can cause severe infections such as septicemia, pneumonia, urinary tract (UTI) and soft tissue infections in hospitalized and immune-compromised patients with underlying diseases (1). Emergence and spread of multidrug resistant *K. pneumoniae*, specifically the extended spectrum β-lactamase (ESBL) producing strains, is often responsible for the failure of antibiotic treatment in hospital settings (2, 3). ESBLs confer bacterial resistance to extended-spectrum penicillins, cephalosporins, and aztreonam which are inhibited by β-lactamase inhibitors such as clavulanic acid, but not cephamycins or carbapenems (2). ESBL producing bacteria are typically associated with multidrug resistance and are most often encoded on

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plasmids, which can easily be transferred between isolates (3). Carbapenems such as imipenem and meropenem have been used for treatment of Gram-negative multidrug resistant infections caused by *Pseudomonas aeruginosa*, *Acinetobacter spp.* and ESBL producing *Enterobacteriaceae* including *K. pneumoniae* (4, 5). However, carbapenem resistance mechanisms have developed among Gram-negative clinical isolates worldwide (4, 6). Among these, production of carbapenem hydrolyzing enzymes, carbapenemases, is a major threat which limits the therapeutic use of these valuable drugs. A number of carbapenemases have been reported including KPC, GES, SME, NMC-A and IMI types (Amber class A), IMP, VIM and NDM type (Amber class B) metallo-β-lactamases and OXA type (Amber class D) oxacillinases (7, 8). KPC-type carbapenemase was first isolated from a carbapenem-resistant strain of *Klebsiella pneumoniae* in the United States and soon after, a rapid rise and extensive dissemination of KPC producing *K. pneumoniae* occurred in US Northeastern parts during the first decade of the 21st century (9, 10). During the same period, KPC producing *K. pneumoniae* was reported to emerge in Latin America, Israel, China and Greece (11). At least 12 KPC types have been observed and their fast spread worldwide shows that their related genes are usually located on plasmids and can easily disseminate among Gram-negative pathogens (11-13). The aims of this study were to compare antibiotic resistance profiles, ESBL and KPC-type carbapenemase production in burn and bon-burn clinical isolates of *K. pneumoniae* collected from two major hospitals in Tehran

MATERIALS AND METHODS

Bacterial strains. Fifty five isolates of *K. pneumoniae* were collected from patients admitted to Shariati Hospital (30 non-burn) and Motahari Burn Hospital (25 burn) in Tehran from August 2011 to January 2012. The identity of the isolates was further confirmed by standard biochemical tests. The specimens were from wounds (n= 26, 47.3%), urine (n=15, 27.3%), blood (n= 6, 10.9%), sputum (n= 2, 3.6%), throat (n= 2, 3.6%), ascetic fluid (n= 1, 1.8%), discharge (n= 2, 3.6%), and pleural fluid (n= 1, 1.8%). The gender distribution among patients was 32 (58%) females and 23 (42%) males.

Antibacterial susceptibility. Antibiotic susceptibility testing was performed according to the CLSI recommendations using disc diffusion method (14). The antibiotic discs (Himedia, India) were: imipenem (IMP: 10 μg), meropenem (MEM, 10 μg), aztreonam (ATM, 30 μg), gentamicin (GM, 10 μg), amikacin (AK, 30 μg), cefepime (CPM, 30 μg), ciprofloxacin (CIP, 5 μg), piperacillin (PRL, 100 μg), piperacillin-tazobactam (PTZ, 100/10 μg), amoxiclav (AUG, 20/10 μg) (MAST, UK), ceftazidime (CAZ, 30 μg) and cefotaxime (CTX, 30 μg). *Escherichia coli* ATCC 25922 was used as the susceptibility control.

Screening for ESBL production. ESBL production was screened in isolates showing resistance to at least one of the third generation cephalosporins or aztreonam by the phenotypic confirmatory test (PCT) as instructed by the CLSI (15). In brief, pairs of discs containing cefotaxime (30 μg) and ceftazidime with or without clavulanic acid (30 + 10 μg) were placed 20 mm apart on the surface of a Muller Hinton agar (MHA) (Liofilchem, Italy) plate previously inoculated with the test organism. A positive test result was defined as an increase of ≥ 5 mm in the inhibition zone diameter of the antibiotic combination with clavulanic acid compared to the antibiotic alone.

Screening for MBL production. Screening for MBL was performed by the double disc synergy test (DDST). Briefly an overnight broth culture of the test strain was inoculated on MHA. A sterile blank disc containing 10μl of a 0.5 M EDTA solution was placed on the bacterial lawn and an imipenem disc (10 μg) was placed at a distance of 15 mm from the EDTA disc. Presence of an enlarged zone of inhibition towards the EDTA disc after overnight incubation was interpreted as positive for the putative MBL production (15).

Phenotypic detection of carbapenemase activity. The modified Hodge test (MHT) was employed for detection of carbapenemases (16). Briefly, the surface of MHA plate was inoculated evenly with a suspension of *E. coli* ATCC 25922 (1:10 dilution of turbidity adjusted to McFarland no. 0.5). After brief drying, discs containing 10 μg imipenem, meropenem or ertapenem were placed at the center of the plate and carbapenem-resistant test isolates from overnight culture plates were streaked from the

edge of the disc to the plate periphery. The test was considered positive if a distorted inhibition zone was observed around the imipenem disc after overnight incubation at 37°C.

PCR detection of KPC-type carbapenemases. Bacterial DNA was extracted using a rapid phenol-chloroform method (17). PCR amplification of bla_{KPC} genes was carried out using two sets of primers (Bioneer, South Korea): set1F: 5'-ATGTCACTGTATCGCCGTCTAGT-3' and set1R: 5'-CGTTGACGCCCAATCC-3' with an amplicon size of 870 bps (18). Set2F: 5'-TGTCACTGTATCGCCGTC-3' and set2R: 5'-CTCAGTGCTCTACAGAAAACC-3' with an amplicon size of 879 bps (9). PCR reaction mixtures (25 μl) contained: 1 μl DNA template, 1.5 mM MgCl₂, 0.4 mM of each dNTP, 1 pM of each primer and 1 unit of DSF Taq DNA Polymerase (Cinnagen, Iran). PCR amplifications were performed in a thermal cycler (Peqlab, Germany) using the following program for primer 1: initial denaturation at 95°C for 4 min followed by 30 cycles of 1 min at 94°C, 1 min annealing at 56°C and 45s extension at 72°C with a final extension of 7 min at 72°C. For primer 2, amplification was carried out with an initial denaturation at 95°C for 5 min followed by 35 cycles of 1 min at 95°C, 30s annealing at 58°C and 90s extension at 72°C with a final extension of 10 min at 72°C. PCR products were run on 1% agarose gels, stained with Red safe (Intronbio, Korea) and were visualized using an image analysis system (UVLtec, St John's Innovation Centre).

Statistical analysis. Comparison of antimicrobial susceptibility patterns and ESBL production among the burn and non-burn isolates was carried out by the two tailed Mann-Whitney U-test allowing for continuous variables, independent groups and non-normal distribution using SPSS software version 21.0.

RESULTS

Antibiotic susceptibility patterns of burn and non-burn isolates are shown in Fig 1. The rate of drug resistance in burn isolates was: 92% to piperacillin, aztreonam, cefepime, ceftazidime, cefotaxime and amoxiclav, 84% to piperacillin-tazobactam, gentamicin and ciprofloxacin, 52% to amikacin and 20% to imipenem and meropenem. The non-burn isolates were 96.66% resistant to cefotaxime, 93.33% to ceftazidime, 83.33% to piperacillin, 73.3% to aztreonam and amoxiclav, 66.7% to piperacillin-tazobactam and cefepime, 60% to ciprofloxacin, 56.7% to gentamicin, 33.3% to amikacin and 0.0% imipenem and meropenem. The highest resistance rate in both groups was observed to cefotaxime and ceftazidime. Compared to the non-burn strains, antibiotic resistance rates were generally higher in burn isolates and significant for cefepime, amoxiclav (*P<0.01*), imipenem, meropenem, gentamicin and ciprofloxacin (*P<0.05*) (Table 1). Carbapenem resistant isolates (n=5) were from burn wounds and resistant to all tested antibiotics. Four of the 5 carbapenem resistant strains were from female patients.
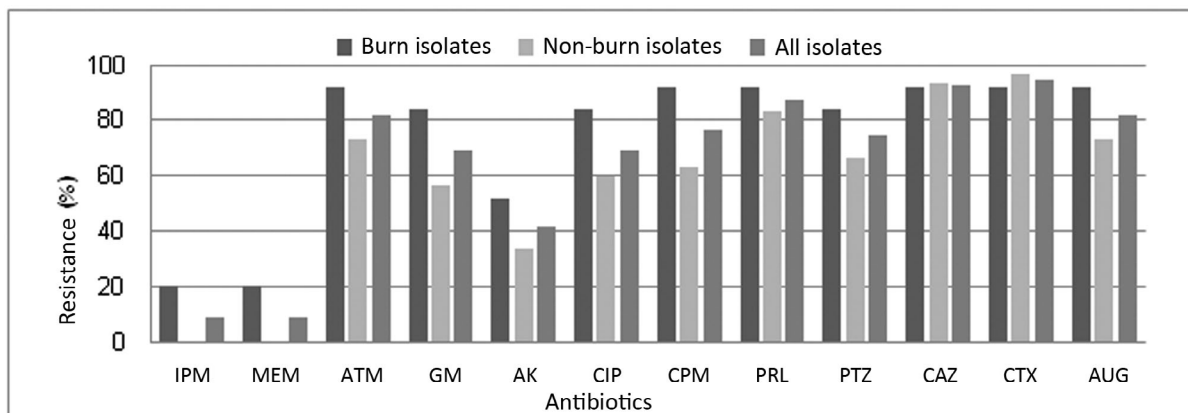

Fig. 1. Antibiotic resistance profiles of burn and non-burn clinical isolates of *Klebsiella pneumoniae*.

Table 1. Comparison of antibiotic susceptibility between burn and non-burn clinical isolates of *Klebsiella pneumoniae.*

| Antibiotic | Antibiotic resistance | | Level of significance |
	non-burn isolates (n= 30)	burn isolates (n=25)	
Piperacillin	83.33%	92%	NS
Piperacillin-Tazobactam	66.66%	84%	NS
Amoxiclav	73.33%	92%	p<0.01
Aztreonam	73.33%	92%	NS
Imipenem	0.00%	20%	p<0.05
Meropenem	0.00%	20%	p<0.05
Cefepime	63.33%	92%	p<0.01
Ceftazidime	93.33%	92%	NS
Cefotaxime	96.66%	92%	NS
Ciprofloxacin	60%	84%	p<0.05
Amikacin	33.33%	52%	NS
Gentamicin	56.66%	84%	p<0.05

ESBL production was shown in 83.3% (n=25) of non-burn and 72% (n=18) of the burn isolates. No significant differences were observed in ESBL production between the two groups and there was no relationship between ESBL production and the specimen type. Phenotypic tests for MBL production showed that none of the isolates were MBL producers. MHT results for imipenem/meropenem resistant isolates showed that 4/5 carbapenem resistant strains (90%) had the carbapenemase phenotype of which, two were ESBL producers. However, the PCR results were negative for KPC-type carbapenemases and no amplified KPC gene was observed.

DISCUSSION

Several outbreaks of nosocomial infections caused by ESBL producing isolates of *K. pneumoniae* have been reported throughout Europe, United States and Asia (7-9). In Iran, high levels of ESBL production by *K. pneumoniae* have been shown to occur (19-21). Overall, we found that 78.2% of our test strains produced ESBL with no significant difference in the rate of ESBL production between the two groups of isolates.

Multidrug resistant, ESBL producing *Klebsiella pneumoniae* are major causes of nosocomial

infections (1). We found high levels of resistance to almost all tested antibiotics except for imipenem and meropenem. Carbapenem resistance was observed in 9.1% of our burn isolates and none of the non-burn strains. The most commonly reported carbapenem resistance mechanism in *K. pneumoniae* is the production of carbapenemases, called *K. pneumoniae* carbapenemases or KPC among which, KPC-2 type is of extreme clinical importance (11). The most extensively used phenotypic method for detection of carbapenemase activity, and the only test recommended by the CLSI for carbapenemase screening is the MHT (16). However, the assay does not distinguish between the types of carbapenemases. More importantly, false-positive results occur with isolates producing CTX-M type ESBLs or high levels of AmpC β-lactamases (11, 22). Another recommended phenotypic method for detection of KPC production is based on susceptibility of KPCs to boronic acid and its derivatives which preferentially inhibit KPC-type carbapenemases (23). However, since boronic acid derivatives are also potent inhibitors of AmpC-type β-lactamases, specificity problems may arise with isolates producing these enzymes (11).

Overall, it is suggested that carbapenem resistant enteric isolates should be tested by the MHT or a boronic acid screening test for carbapenemase phenotype and if the results are positive, a

confirmatory polymerase chain reaction should be employed for identification of the KPC-type (11, 24). Surprisingly, some investigators have used the MHT results as an indication for KPC-type carbapenemase production and based on that, high levels of KPC producers have been reported (25, 26). However, other carbapenemase such as metallo-β-lactamases and OXA type carbapenemases can also produce positive MHT results (27). We believe that care should be taken in the interpretation of MHT and identification of KPC-type carbapenemases should be based on gene amplification and further sequencing of the amplified gene product. Although KPCs do not represent the sole mechanism of carbapenem resistance, they are often not detected by routine susceptibility screening tests and possess an exceptional potential for dissemination (11-13). In addition, due to the limited antibiotic options, treatment of infections caused by these organisms present serious challenges. Active surveillance programs and early *in vitro* identification of KPC producing bacteria is important for the success of infection control measures as well as preventing their horizontal spread.

ACKNOWLEDGMENTS

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