Molecular Identification of Extended-Spectrum β -lactamase and Integron Genes in Klebsiella pneumonia

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ABSTRACT

Introduction: Infections caused by Gram negative bacteria, producing extended-spectrum β -lactamase, including Klebsiella pneumoniae are increasing all over the world with high morbidity and mortality. The aim of the present study was determined antimicrobial profile susceptibility and the prevalence of antibiotic resistance genes by multiplex PCR.

Methods: In the present study, we obtained one-hundred isolates of K. pneumoniae from different clinical samples. The antibiotic susceptibility testing was done in thirteen antibiotic and, therefore, M-PCRs were conducted using the DNA amplification for detection of ESBLs (bla_{TEM}, bla_{CTX-M}, bla_{SHV}) and int (I, II, III) genes.

Results: The results of resistance to amoxicillin/clavulanate, ciprofloxacin, amikacin, trimethoprimsulfamethoxazole, cefotaxime, ampicillin, aztreonam, imipenem, gentamicin, ceftazidime, Cefepime, ceftriaxone and levofloxacin were obtained 37%, 37%, 93%, 84%, 52%, 87%, 59%, 8%, 24%, 67%, 52%, 43% and 26%, respectively. The frequency of the extended-spectrum β -lactamase K. pneumoniae was obtained 37%. The prevalence of resistance genes of ESBLs in the M-PCR method showed that the bla_{TEM} bla_{CTX} and bla_{SHV} were 38%, 24% and 19%, respectively, however, only 8 (8%) out of 100 isolates were found to have positive outcomes for the existence of class 1 integrons and there were no detected class 2 or class 3 integrons.

Conclusions: Our results recommend the likely co-carriage of some ESBLs genes and antibiotic resistance integrons on the same plasmids harboring the MDR genes.

Keywords: Klebsiella pneumonia; integrons; drug resistance.

INTRODUCTION

Klebsiella pneumoniae (K. pneumoniae) is one of the most important causative agents both in form of community-acquired and hospital acquired infections such as urinary tract infections, pneumonia, septicemia and meningitis.¹ Multidrug resistance has been developing trouble clinically, particularly for elderly and immunocompromised patients, and moreover infant with immature body physiology have the least

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capability against infection from MDR pathogens.² The release of extended-spectrum β -lactamases (ESBLs) are unique of the significant mechanisms of antimicrobial agents resistance.3 The appearance of ESBLs in Enterobacteriaceae groups has been a problem of medical concern. ESBLs producing K. pneumonia can degrade a wide-ranging of beta-lactam groups such as penicillins, cephalosporins, carbapenems and cephamycins.^{2,4} In general, ESBLs are distributed into four classes A, B, C and D. Temoneira (TEM), cefotaximase (CTX-M) and sulfhydryl variable (SHV) are present in class A ESBLs.⁵ Some researchers have described various occurrences of ESBLs between 6% and 88% in difference health care locations particularly between K. pneumoniae and E. coli.⁶ SHV type β -lactamases relate to high level resistance to ceftazidime, but not to Cefazolin and cefotaxime, while CTX-M β-lactamases are more an effective against cefotaxime. In contrast, TEM *β*-lactamases confer resistance to oxyimino-\beta-lactams groups, such as ceftazidime, cefotaxime, and aztreonam. In addition to ESBLs, the transportable genetic elements, such as integrons, provide for the evolution and distribution of MDR genes (blaCTX-M, blaIMP, and blaGES) in K. pneumoniae by vertical or horizontal transmission.7 According to the variation aminoacid sequence of the Intl protein, 5 classes of integrons have been identified. Three classes of antibiotic resistance integrons (ARIs) (classes 1, 2, and 3) have been generally complexed in MDR phenotypes criteria and are recognized depending on their particular integrase genes.8 The transportable class 1 integrons are related to transposon Tn21 and have been commonly occur in ESBL producing clinical isolates of K. pneumoniae. However, Class 2 integrons found less frequently in ESBL-producing bacteria like K. pneumoniae and Escherichia coli, and class 3 integrons are infrequently in ESBL-producing K. pneumoniae.¹⁰ In previous reports have confirmed the relations among ESBL producing bacteria and resistance to several groups of antibiotics, as well bla_{FSBL} with ARI gene carriage in clinical isolates of K. pneumoniae.11 Unfortunately, in the last decades, the incidence of ESBL-producing K. pneumoniae and their resistance is raising.¹² The identification of ESBLs genes, involving CTX-M, TEM and SHV, by molecular methods in bacteria that producing ESBL and their antimicrobial susceptibility profile can provide appropriate evidence about their high risk factors and epidemiology related to their infections.13,14 A few studies have been conducted to identify the types of ESBLs producing Enterobacteriaceae in Iranian hospitals.^{15,16} The aim of the present study was detection of bla_{TEM} , bla_{CTX-M} , bla_{SHV} , and int genes (I,II,III) in the clinical K. pneumonia strains isolated from two large urban teaching general hospitals in Tehran, Iran by multiplex PCR (M-PCR) and their antibiotic resistance profile.

METHODS

This cross-sectional study was directed during a one year period of time from April 2014 till March, 2015, in two teaching hospitals in Tehran, Iran. Generally, 100 non-repetitive K. pneumoniae strains were obtained from different clinical specimen including blood, skin lesions, broncho-alveolar lavage (BAL), urine, sputum, cerebrospinal fluid (CSF), Pus/swap, pleural effusion, ascites and catheter. Each sample was cultured on the Mac Conkey agar (Merck Co., Germany) and incubated in 37 °C for 24 h. Then, all suspected grown colonies were recognized as K. pneumoniae by standard biochemical and microbiological tests such as, urease, Gram staining, oxidase, motility, citrate utilization, urease production, TSI, KIA, MR-VP, SIM and confirmed API 20E system. (Analytab, Inc., New York).

Antibiotic susceptibility test was achieved by disc diffusion method on the Mueller- Hinton Agar (Merck Co., Germany) plates agreeing with Clinical and Laboratory Standards Institute (CLSI) guideline for the following antibiotics: amoxicillin/clavulanate (Aug; 20/10 μ g), ciprofloxacin (CIP: 5 μ g), amikacin (AK: 30 μ g), trimethoprim-sulfamethoxazole (TS, 2.5 μ g), cefotaxime (CTX: 30 μ g), Ampicillin (AMP: 10 μ g), Aztreonam (AZT: 30 μ g), imipenem (IPM: 10 μ g), gentamicin (GEN: 10 μ g), ceftazidime (CAZ: 30 μ g), cefepime (FEP: 30 μ g), ceftriaxone(CRO: 30 μ g), impeenem (IMP: $10\mu g$) and levofloxacin (LEV: $5\mu g$) (Mast, Merseyside, UK). Briefly, a bacterial suspension was achieved from fresh cultures. The turbidity of each bacterial suspension was adjusted equivalent to a no. 0.5 McFarland standard and then injected on Mueller-Hinton agar (Oxoid, UK). Diameter of inhibition zones was dignified after incubation at 37°C for 18-24 hours, and results were reported as susceptible, intermediate, and resistant. The standard strain of K. pneumoniae ATCC 1029 was used as a quality control.¹¹

M-PCRs were performed using the DNA amplification instrument master cycler gradient (Eppendorf Co., Germany) for detection of ESBLs genes (bla_{TEM}, bla_{CTX-M} and bla_{SHV}) and int genes (I,II,III). Genomic DNA was acquired from K. pneumoniae colonies grown overnight on blood agar (Merck Co., Germany) plates by the boiling lysis method.²³ Briefly, a loopful of bacterial colonies was suspended in the 700 μ l sterile distilled water and boiled for 10 min and centrifuged at 7000 × g for 4 min at 4 °C and then cooling in ice for 10 minutes and centrifugation for 3 min at 8000 × g. The concentration and the quality of the extracted cellular DNA were assessed using a Nanodrop spectrophotometer (ND-1000; Thermo Scientific; Wilmington, DE, USA). The genes encoding carbapenemases were amplified using the primer sequences, which were presented in Table 1, for m-PCR. M-PCR was done for amplification of ESBLs genes (bla_{TEM}, bla_{CTX-M}, bla_{SHV}) and in a volume of 1.5 μ l of extracted genomic DNA was added to a total volume of 25 μ l PCR reaction mixture including 2.5 μ l of 10 × PCR buffer, 1.5 µl MgCl₂ (50 mM), 0.5 µl dNTPs (10 mM), 1.25 μ l of each primer, 0.5 μ l of Tag DNA polymerase (5 U/ μ I) (Amplicon Co., Denmark) and 8.5 μ I sterile distilled water. The reaction mixture was performed with the following PCR procedure: Denaturation at 94°C for 1 min, 35 cycles with denaturation at 94°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 1min and final extension at 72°C for 6 min. So, amplification of int genes (I,II,III), the reaction mixture was completed in a thermal gradient cycler (Eppendorf Co., Germany) with the following PCR protocol: The cycling conditions were one cycle of 5 min at 95 °C; 30 cycles of 1 min at 95°C, 1min at 65°C, and 1min at 72°C; and one cycle of 10min at 72°C (11).

RESULTS

The mean age of the population studied was 47 ± 1.5 years, with a range of 14 to 76 years. The isolates were collected from patients belonging to various age groups: 10- 25 years No; 23, 26- 40 No; 28, 41 - 55 years No; 36, 56-60 years No; 8 and 60-76 years No; 5. Sixty seven (67%) patients were male and 33 (33%) patients were female.

Out of 374 different clinical samples, 100 (26.7%)

K. pneumoniae isolates were obtained. The specimens included the blood (n = 7, 7%), skin lesions (n = 9, 9%), BAL (n = 5, 5%), urine (n = 62, 62%), sputum (n = 6, 6%), CSF (n = 3, 3%), Pus/swap (n = 2, 2%), pleural effusion (n = 1, 1%), ascites (n = 2, 2%), and catheter (n = 3, 3%). The distribution analysis of K. pneumoniae strains showed that the most isolates (62%) were obtained from the urine and the lowest (1%) found to be isolated from pleural effusion.

The antibiotic susceptibility testing by antibiogram method showed that the percentage of resistance to amoxicillin/clavulanate, ciprofloxacin, amikacin, trimethoprim-sulfamethoxazole, cefotaxime, ampicillin, aztreonam, imipenem, gentamicin, ceftazidime, cefepime, ceftriaxone and levofloxacin were 37%, 37%, 93%, 84%, 52%, 87%, 59%, 8%, 24%, 67%,52%, 43% and 26%, respectively (Table 2). The antibiotics susceptibility profile of non-ESBL producing K. pneumoniae strains and ESBL-producing K. pneumoniae strains are existing in Table 3.

ESBLs genes amplification test showed that the prevalence of bla_{TEM} , bla_{CTX} and bla_{SHV} were 38%, 24% and 19%, respectively. M-PCR simultaneously amplified and identified the existence of bla_{TEM} (445 bp), bla_{CTX-M} (593 bp) and bla_{SHV} (747 bp), respectively (Figure 1). Molecular distribution analysis of integrons genes showed that, only 8 (8%) out of 100 isolates were found to have positive outcomes for the existence of class 1 integrons and no class 2 or class 3 integrons were identified between isolates (Figure 2).

Table 1. The nucleotide sequences of the primers used for the M-PCR.				
Genes	Oligonucleotide sequence (5□→3□)	Size of amplicon (bp)		
bla-SHV	F = 5'-ATGCGTTATATTCGCCTGTG-3' R = 5'-TGCTTTGTTATTCGGGCCAA-3'	747		
TEM-1	F = 5'-TCGCCGCATACACTATTCTCAGAATGA-3' R = 5'-ACGCTCACCGGCTCCAGATTTAT-3'	445		
CTX-M	F = 5'-ATGTGCAGCACCAGTAAAGTGATGGC-3' R = 5'-TGGGTAAAGTAAGTGACCAGAATCAGCGG-3'	593		
intl	F = 5'-GCCTTGCTGTTCTTCTACGG -3' R = 5'-GATGCCTGCTTGTTCTACGG -3'	558		
intll	F = 5D-CACGGATATGCGACAAAAAGGT-3' R = 5D-GTAGCAAACGAGTGACGAAATG-3'	789		
intIII	F = 5D-GCCTCCGGCAGCGACTTTCAG-3' R = 5D-ACGGATCTGCCAAACCTGACT-3'	979		





marker.

Table2.AntibioticsresistancepatterninK. pneumoniae isolates.					
Antibiotic	R	I	S		
	No (%)	No (%)	No (%)		
amoxicillin/ clavulanate (Aug)	37 (37)	0 (0)	63 (63)		
ciprofloxacin (CIP)	37 (37)	5 (5)	58 (58)		
amikacin (AK)	93 (93)	3 (3)	4 (4)		
trimethoprim- sulfamethoxazole (TS)	84 (84)	4 (4)	12 (12)		
cefotaxime (CTX)	52 (52)	1 (1)	47 (47)		
ampicillin (AMP)	87 (87)	2 (2)	11 (11)		
aztreonam(AZT)	59 (59)	1 (1)	40 (40)		
imipenem (IPM)	8 (8)	9 (9)	83 (83)		
gentamicin (GEN)	24 (24)	6 (6)	70 (70)		
ceftazidime (CAZ)	67 (67)	2 (2)	31 (31)		
cefepime (FEP)	52 (52)	0 (0)	48 (48)		
ceftriaxone (CRO)	43 (43)	1 (1)	56 (56)		
levofloxacin (LEV)	26 (26)	0(0)	74 (74)		
^t I= Intermediate, *R= Resistance, *S= Susceptible.					

Table 3. Antibioteic resistance rates among Non-ESBL producing K. pneumoniae strains and ESBL-producing K. pneumoniae strains

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	ESBL	Non-ESBL
Antibiotic	producing K.	producing K.
Antibiotic	pneumoniae	pneumoniae
	strains (%)	strains (%)
amoxicillin/	27	63
clavulanate (Aug)	57	05
ciprofloxacin (CIP)	21	42
amikacin (AK)	18	45
trimethoprim-		
sulfamethoxazole	32	31
(TS)		
cefotaxime (CTX)	14	49
ampicillin (AMP)	35	28
aztreonam(AZT)	12	51
imipenem (IPM)	6	57
gentamicin (GEN)	31	32
ceftazidime (CAZ)	33	30
cefepime (FEP)	24	39
ceftriaxone (CRO)	15	48
levofloxacin (LEV)	7	56

DISCUSSION

At first, ESBLs-producing K.pneumoniae was discovered in 1983.¹⁷ Infections caused by K. pneumoniae that ESBL-producing are raising in several countries such as Iran,¹⁸ India^{19,20} and Italy.²¹ Resistance to various antibiotic is related to extent of transmissible plasmids

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and integrons which can be integrated on the plasmids or chromosome.²² In order to, we evaluated antibiotic susceptibility of 100 strain of K. pneumoniae to thirteen antibiotic. The high resistance rate were related to AK (93%), TS (84%), AMP (87%), AZT (59%), GEN (67%) and FEP (52%). Amiri et al, (2016) reported resistance to ampicillin, ceftazidime, ceftriaxone, aztreonam and cefotaxime was (92%), (67%), (65%), (64%) and (59%), respectively,23 which is approximately similar to our results. Our results indicated only eight ESBL-producing isolates were shown to be resistance to imipenem in disk diffusion method. This result of high susceptibility (83%) to imipenem was agreement to Mansury et al, (2016), Ahmad et al, (2009), Amiri et al, (2016) and Edelstein et al., (2003) studies²³⁻²⁶ According to disk diffusion results, only three isolates of K. pneumoniae were resistant to all antibiotics. In present study, MDR isolates are resistant to three classes of antimicrobial agents,²⁷ therefore, 31% MDR isolates were obtained. This finding is mismatch with Mansury et al (2016) study.²⁴ A total of 52% and 67% isolates were resistant to the third generation cephalosporins (ceftazidime and cefotaxime), this outcomes is compatible with Ullah et al., (2009), Amiri et al., (2016) and Jalalpoor et al., (2011) studies.^{23,28,29} Of these MDR-isolates, 28 strains were positive ESBL production (28%). The present results were correlated with the Shukla et al., (2004) and Sarojamma et al, (2011) reported that 28% and 32% ESBL production, respectively.^{30,31} Nowadays, a critical increase in ESBL-producing Klebsiella spp. was also reported from 4.2-44% (USA), 32-34 4.9% (Canada),35 20.8% (Spain),36 28.4% (Taiwan)(37), 78.6% (Turkey) (38), 20% (Algeria),³⁹ and 51% (China).40 Our results indicated the high incidence of positive ESBL production was showed in urine (14%) samples. This research was conducted to identify the existence of bla_{TEM} , bla_{SHV} , bla_{CTX-M} and integrons genes (I, II, III) in ESBL producing K. pneumoniae. The M-PCR results was for each resistance genes as follows: the bla_{TEM} gene only, detected in 38% (14/37); the bla_{CTY} gene in 24% (9/37) and the bla_{SHV} gene in 19% (7/37); $bla_{_{TEM}}$ and $bla_{_{SHV}}$ detected in 8% (3/37); $bla_{_{TFM}}$ and bla_{CTX-M} detected in 16% (6/37). Bora et al., (2014) reported between three ESBLs profile genotypes, the most predominant genotype was observe to be bla_{TEM} in K. pneumoniae (77.58%) ESBL producing strains.41 Monstein et al, (2007) reported the bla_{SHV} distinguished

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in 8% (3/37), bla_{SHV} and bla_{TEM} discovered in 2.7% (1/37), and bla_{TEM} , bla_{SHV} and bla_{CTX-M} discovered in 8% (3/37) strains of K. pneumoniae.⁴² Hassan and Abdalhamid (2014) indicated increase in the bla_{CTX-M} (97.4%) in compared with to prevalence of $\mathsf{bla}_{_{\mathsf{SHV}}}$ (23.1%) in K. pneumoniae.43 In contrast, in European countries, East Asia and Latin America, in current study the bla variations have displaced $\mathsf{bla}_{\mathsf{CTX}}$ and $\mathsf{bla}_{\mathsf{SHV}}$ enzymes as the major beta-lactamases produced by isolates of K. pneumoniae. This outcome reflects significant raise in the bla_{TEM} genotype in Iran. In Arab countries, the principal ESBL was bla_{CTX-M}.⁴⁴⁻⁴⁶ In addition ESBLs genes, integrons genes was evaluated in 100 isolates of K. pneumoniae. Our results was indicated eight isolates were positive outcomes for the existence of class 1 integrons and no class 2 or class 3 integrons were identified between isolates. This finding is comparable with lima et al, and Ashayeri et al., studies.^{11,47} Since, only a rare studies about class 3 integron has been described and categorized^{10,48,50} Mobarak-Qamsari et al, described 22 (44%) integron class 1, only 3 (6%) isolates of K. pneumoniae carried integron class 2 and none of them contained of integon class 3.51 Possible critical role of integrons genes in distribution of ESBL-encoding bla_{TEM} gene among ESBL-producing K. pneumoniae clinical isolates may be inferred. This highpoints the increasing complication of antimicrobial agent resistance problems, and the explanations for this condition need further investigation.

CONCLUSIONS

In conclusion, this project approves that a high level of bla_{TEM} and class 1 integrons-positive ESBL K. pneumoniae is circulating in two hospitals of Tehran, Iran. The trend of MDR profiles related with the recovery of the bla_{TEM} and lass 1 integron genes is worrying. This highpoints values is require for establish an antibiotic susceptibility surveillance network for monitoring of Enterobacteriaceae spp., infection in Iran.

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