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THE PREVALENCE OF CHECK NDM–1 GENE CAUSING BETA-LACTAM ANTIBIOTIC RESISTANCE IN KLEBSIELLA PNEUMONIAE ISOLATES FROM CLINICAL SAMPLES AND PLASMID CURING

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ABSTRACT

Existence of extended spectrum B-lactamase (ESBL) genes plays an important role in causing B-lactam antibiotic resistance in the producing strains of these anzymes. The resistance of gram-negative bacteria, such as klebsiella pneumonia, to different antimicrobial agents, especially B-lactam and carbapenem, has increasingly been reported. This study was conducted to determine the prevalence of NDM-1 beta-lactamases in klebsiella pneumonia isolates through PCR method. Materials and methods: In this descriptive-analytic study, 120 klebsiella pneumonia isolates collected from patients with Lung infection and UTI were subjected to bacteriological tests. The samples were cultured and identified according to standard methods. Then, frequency of the strains producing extended spectrum beta-lactamases was determined with Disk diffusion method. Using kite method, DNA was extracted and examined for the existence of NDM-1 gene by PCR. Result: out of the 120 klebsiella pneumonia isolates, 13 (10.83 %) isolates were ESBL positive, 7.5 % of which were positive for NDM-1 B-lactamases resistance gene. Conclusion: considering the increasing rate of the ESBLs producing strains, using the appropriate treatment protocol based on the antibiogram pattern of the strains is highly recommended.

KEYWORDS: Extended-spectrum B-lactamases, ESBL, klebsiella pneumonia, NDM-1

INTRODUCTION

Antimicrobial resistance has been regarded as a serious problem for health of human (1) and afflicts patients in all hospitals of the world (2, 3). Change in microbial flora with antibiotics leads to attack of opportunistic bacteria and fungi (4). For this reason, World Health Organization introduced 2011 as year of antibiotic resistance. This Organization has recommended considering important cases such as assessment of antibiotic resistance, using antibiotics correctly, selling antibiotics only with physician's prescription and preventing and controlling infections to control and prevent antibiotic resistance to governments(5). Klebsiella pneumonia is a Gram Negative opportunistic pathogen and one of the prevalent factors of hospital infections (6). Increase in manifestation of multiple drug resistance among the hospital isolates has therapeutic options for infections caused with bacteria (7, 8). Today, most of Klebsiella pneumonia isolates are resistant to several drugs (9, 12). These bacteria are among the important causes of acquired infections in society and hospital (9). Klebsiella pneumonia is one of the most prevalent hospital pathogens which has high mortality rate (13). Enterobacteriaceae family particularly Escherichia coli and klebsiella pneumonia cause all types of infections in different persons particularly neonates (14) including pneumonia (15), Septicemia (10), dysentery (16), abscess in liver, Endophthalmitis, meningitis (6) and Bacteremia and urinary infections (13).

4 million neonates die due to bacterial infections every year (17) and the highest mortality rate which has been reported relates to pneumonia, Septicemia, meningitis and dysentery and it seems that neonates are more vulnerable due to lack of a full immunity system (18). Today, treatment of infection in neonates with organisms resistant to several drugs has been converted into an important global problem (10).

B-lactamases

B-lactamases have been recognized as the main defense of Gram Negative bacteria against antibiotics (19). B-lactamases are divided into four groups according to Bush-Jacoby, Amnler which are presented in Table 1(20).

Table 1
***Classification of bacterial* B-lactamases**
NDM-1(New-delhi-methallo-B-lactamase-1

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros' group (1995)	Molecular class (subclass)	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
				CA or TZB[a]	EDTA		
1	1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	*E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	NI[b]	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-β-lactams	GC1, CMY-37
2a	2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10
2ber	NI	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino-β-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c	2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	NI	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	NI	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino-β-lactams	OXA-11, OXA-15
2df	NI	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	3	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
		B (B3)					L1, CAU-1, GOB-1, FEZ-1
3b	3	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1
NI	4	Unknown					

[a] CA, clavulanic acid; TZB, tazobactam.
[b] NI, not included.

NDM-1 is a new enzyme which makes bacteria resistant to many antibiotics. For this reason, it has caused concerns in hospitals as a threatening factor and problem of the global health (21-23) (24). Another name of this enzyme is PMC (Plasmid Encoding Carbapenem Resistant Metallo Beta-lactamase). Different bacteria which have this enzyme can be resistant to many antibiotics (25). NDM-1 is an extended spectrum Beta-lactamase which is able to inactivate all antibiotics of Beta-lactam (26-30) such as penicillin, cephalosporins(29.31,32) and Carbapenems(10,27, 32). NDM-1 encoding gene is placed on moving plasmids (2,22,24,28) including A/C, FII,Inc1/M and two other plasmids which haven't been typed (33,34). This plasmid has been also observed in klebsiella pneumonia and is able to be transferred to other different bacterial strains causing spread of drug resistance all over the world (1, 26, 28, 35, and 36). Mortality rate resulting from infections caused by bacteria containing NDM-1 enzyme has been reported about 18-67 %(12, 26) (Figure 1). In 2011, NDM-1 enzyme was introduced as an expanding global problem and their importance was assessed for AIDS, tuberculosis and malaria (11).

Figure 1
Geographical distribution of bacteria producing NDM-1 enzyme

Due to effect of mutation on NDM-1 gene, a new type of NDM called NDM-5 was created recently which caused resistance of bacteria to carbapenems and cephalosporins. 6 types of NDMs have been identified all over the world (Table 2).

Table 2-
Types of Beta-lactamase, type NDM

reference	nucleotide	Enzyme
AAC 53:5046-5054, 2009	FN396876	NDM-1
JAC 66:1260-1262, 2011	JF703135	NDM-2
	Assigned	NDM-3
AAC 56:2184-2186, 2012	JQ348841	NDM-4
	JN104597	NDM-5
	JN967644	NDM-6

(Reprinted from www.lahey.org/studies/)

Beta-lactamases identified in Iran

In the previous studies conducted in Iran, TEM, SHV and PER enzymes were diagnosed with phenotype methods (35, 36) and the gene which can produce them can be identified with molecular methods (34). Figure 2 shows distribution of Beta-lactamase genes in Iran (37-38). Due to failure to study NDM-1 gene in Iran, there is no careful statistics about the presence of this gene and its dispersion is not available and in this research, attempt has been made to achieve the careful statistic by studying some provinces of the country and identifying this gene.

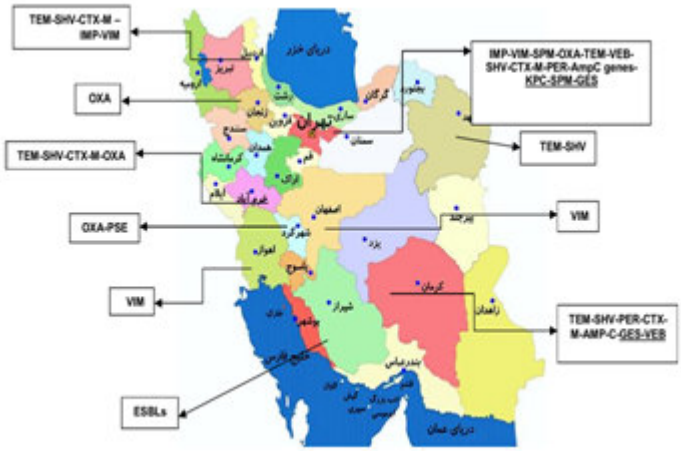
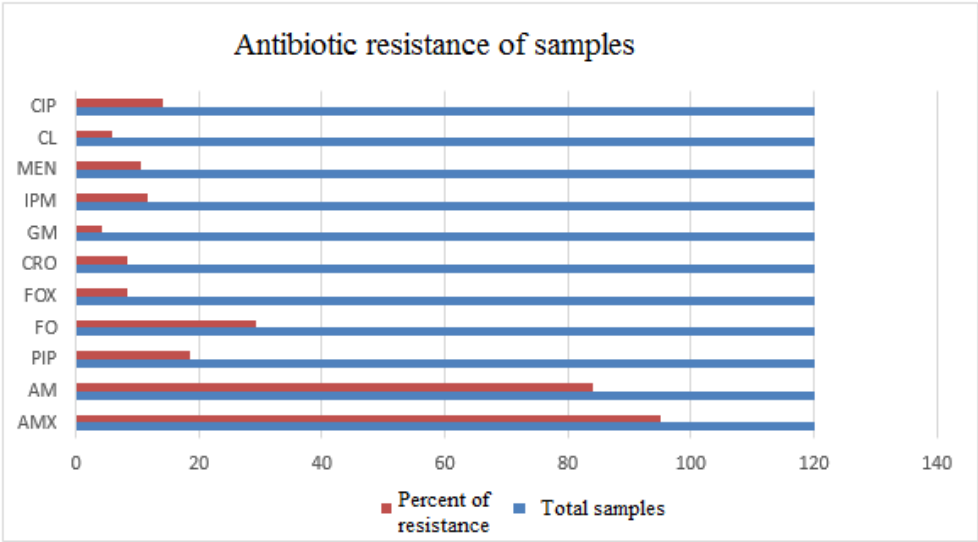


Figure 2
Distribution of Beta-lactamase genes in Iran

MATERIALS AND METHODS

In this study, there were 120 isolates of klebsiella pneumonia from the clinical samples including urine, mucus and blood based on creation of Eosin methylen-blue agar medium (EMB) and also diffenretial tests and non-fermentative condition in TSI(Triple Sugar Iron Agar) medium . Isolates of klebsiella pneumonia were studied based on Disk Diffusion method for the presence of extended-spectrum Beta-lactamase. In this method, 11 antibiotic disks including cefoxitin(30 µg) , Ceftriaxone(30 µg), Colistin(10 µg), Meropenem(10 µg), Imipenem(10 µg), gentamicin(10 µg), Ampicillin(10 µg), Ciprofloxacin(5 µg), Fosfomycin(200 µg), Piperacillin(100 µg) and Amoxicillin (25 µg), prepared from Padtan Teb Company were placed in Mueller hinton agar medium in distance of 15 mm . In case the inhibition zone diameter around the antibiotic disk particularly imipenem and meropenem disks exceeds 5 mm, its resistant bacteria is regarded as antibiotic and is a part of bacteria producing extended-spectrum Beta-lactamase. Percentage of each of the antibiotics is given in Table 3. We studied the isolates which were identified as the strain producing extended-spectrum Beta-lactamase in this stage with PCR method for the presence of NDM-1 gene.

Table 3
Resistance percent of antibiotics



For this purpose, one colony of the resistant bacteria was placed in 5 ml of the sterile TSB culture medium containing 50 to 100 µg/ml of ampicilin antibiotic (for protection of plasmid) and was cultured for 24 hours

and then the resistant bacteria were isolated with Plasmid extraction kit of Sina Clon Company and then plasmid extraction is done for 13 isolates with electrophoresis loading buffer for more assurance. Now, PCR (polymeras chain reaction) test was used to determine NDM-1 genotype. In this method, a specific primer synthesized by Sina Clon Company relating to NDM-1 gene has been used in Klebsiella pneumoniae bacteria and its sequence and temperatures used in PCR are given in Table 4.

Table 4
Sequence of primer and temperatures used in PCR

gene (base pair)	number of cycle	stages of reaction PCR			Sequence of primer	Primer
		extension	Annealing	Denatoration		
295 bp	30	72 C° 30 sec	52 C° 30 sec	94C° 30 sec	CAACTGGATCAAGCAGGAGA	NDM-1(F)
					TCGATCCCAACGGTGATATT	NDM-1(R)

After performing PCR, plasmid curing test has been done for studying the presence of Beta-lactamase genes. Plasmid curing is the process which loses its plasmid with different compounds such as ethidium-bromide or acridine orange or with physical conditions such as high temperatures and by performing centrifuge. For this purpose, antibiogram was prepared from the resistant bacteria to ensure that our bacteria contain plasmid and antibiotic resistant genes. Then, 400 µg/ml was added to TSB culture medium which contains resistant bacteria with ethidium bromide poisonous paint which is a very poisonous matter and cultured for 24 hours at 35 to 37 °C. After 24 hours, antiobiogram is done with sterile cotton swap on Mueller hinton agar medium and heated for 18 to 24 hours at 35 to 37°C and we can see the results. In case the bacteria lose its plasmid, bacteria will be found sensitive to antibiotics and inhibition zone will be created around antibiotic disks.

RESULTS

In this study, 120 klebsiella pneumonia isolates collected from different clinical samples including 63 urinary samples, 45 mucus samples, and 12 blood samples are shown in Figure 3.

Figure 3- percent of different clinical samples

After 13 isolates (10.83%) of extended-spectrum Beta-lactamase were positive after determining 120 isolates from the clinical samples with disk diffusion method and 107 isolates (89.17%) were reported as negative phenotype.

Figure 4: confirmatory phonotypical test with disk diffusion method

Assessment of PCR results among 13 strains of extended-spectrum Beta-lactamase shows that 9 isolates (7.5%) have NDM-1 gene.

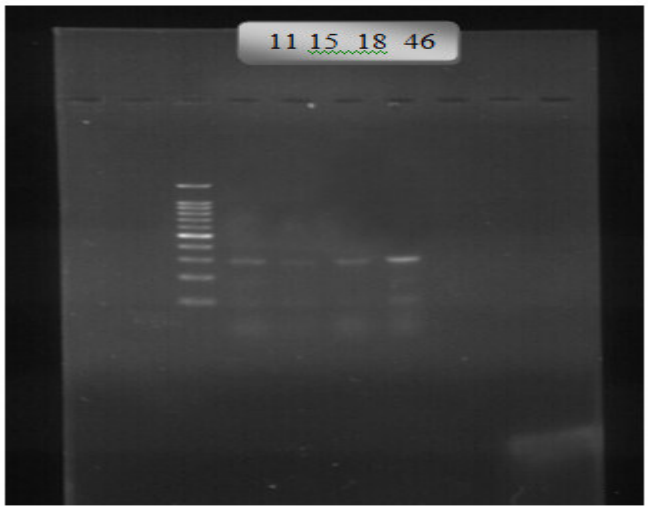


Figure 3
PCR of samples 11, 15, 18 and 46

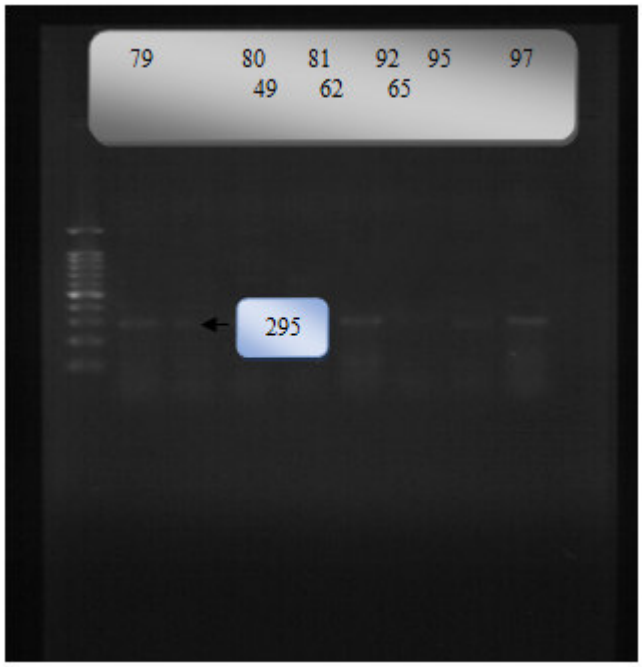


Figure 4
PCR of other 9 isolates

After obtaining PCR results , plasmid curing test was performed and the related bacteria which had plasmid containing extended-spectrum Beta-lactamase genes lost its plasmid and had become sensitive to all antibiotics.

DISCUSSION

In this study, 120 klebsiella pneumonia isolates were studied. Among them, there were 63 isolates from urinary culture, 14 isolates from mucus culture and 12 isolates from blood culture. In this study, 10.83% of the isolated strains produced extended-spectrum Beta-lactamase (NDM-1). Dr Deborah Williamson et al. identified and studied NDM-1 in 2011 which they had obtained from Enterobacteriaceaes isolated from a hospital in New Zealand. The above gene causes Carbapenem Resistance Gene. The above research group identified and reported NDM-1 gene in 4 bacteria i.e. E.coli, Klebsiella pneumonia, Proteus mirabilis etc. it has been also specified that a point mutation will produce another antibiotic resistance gene i.e. NDM-6 in position 698(C→T) of NDM-1 gene. In another research, Dr. Shern Shoma et al. conducted extensive studies on NDM-1 gene available in klebsiella pneumonia bacteria in Australia in 2013. In 2009, a research was conducted on NDM-1 gene by Danjion Yung et al. in India who described this gene and how resistance was created.

CONCLUSION

Considering importance of klebsiella pneumonia in hospital infections and also high prevalence of strains producing extended-spectrum Beta-lactamase particularly NDM-1 Beta-lactamase, rapid diagnostic methods should be used for determination of these strains in laboratories routinely because these results can be regarded as a strategy for physicians to use extended-spectrum cephalosporins in treatment of infections.

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