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Antimicrobial Pattern and Clonal Dissemination of Extended-Spectrum Beta-Lactamase Producing *Klebsiella Spp* Isolates

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Abstract: Problem statement: Gram-negative pathogens harboring ESBLs have caused numerous outbreaks of infections and are becoming an increasing therapeutic problem in many countries. The incidence of ESBL-producing strains among clinical isolates has been steadily increasing over the past years resulting in limitations of therapeutic option. The focus of this study was to examine the molecular epidemiology of ESBL-producing *Klebsiella spp*, investigate the susceptibility of *Klebsiellae spp* producing ESBLs towards non-beta-lactam antibiotics in the different seasons, identify the various clonal types of ESBL-producing *K.pneumoniae* and detect the dominant ESBL clonal types. **Approach:** Clinical isolates of *Klebsiella spp* were identified during the period March 2007-April 2008. ESBLs production identified by phenotypic and genotypic methods. MLST was performed for dissemination of ESBLs producing *K. pneumoniae.* **Results:** The findings showed that 51.6% of *K.pneumoniae* were produces ESBLs. 35.8, 21.2 and 38.7% of *K. pneumoniae* producing ESBLs were resistant to amikacin, ciprofloxacin and cotrimoxazol, respectively. It was found that 40 and 27.3% of *K.oxytoca* producing ESBLs were resistant to cotrimoxazol and amikacin, respectively. The findings reflected that ESBLs existed in 73% of *K. oxytoca*. The results showed that the frequency of blaSHV, blaTEM and blaCTX-M due to *K.pneumoniae* producing ESBLs were 87.5, 12.4 and 24.8%, respectively. Of the eleven *K. oxytoca* producing ESBLs, 100% blaSHV were obtained. Based on the nucleotide variations of the five genetic loci, twenty-five different STs could be identified among thirty *K.pneumoniae* producing ESBLs isolates. Among the STs shared by multiple isolates, the most frequently encountered were 14, 16 and ST18. **Conclusion:** In conclusion, the percentage of *K.oxytoca* producing ESBLs was higher than *K.pneumoniae* producing ESBLs. Generally, *K.penomoniae* produces more ESBLs in winter and fall than in the other seasons.

Key words: Antimicrobial pattern, *Klebsiella spp*, Extended Spectrum Beta-Lactamases (ESBL), *Klebsiella pneumoniae*, Urinary Tract Infection (UTI), Respiratory Tract Infection (RTI), Intensive Care Units (ICUs), Single Locus Variant (SLV)

INTRODUCTION

In recent years, extended-spectrum beta-lactamase-producing *K.pneumoniae* strains have become important pathogens in hospital-acquired infections, showing multi-resistance and causing more and more outbreaks in hospitals (Kamatchi *et al*., 2009). It is important to study the prevalence of *K. pneumoniae* in Milad Hospital in Tehran of Iran, as Iran is a four-seasoned country. *K. pneumoniae* is frequently isolated in the winter season and responsible for majority of the nosocomial infections.

The focus of this study was to examine the molecular epidemiology of ESBL-producing *Klebsiella spp*, investigate the susceptibility of *Klebsiellae spp* producing ESBLs towards non-beta-lactam antibiotics in the different seasons, identify the various clonal types of ESBL-producing *K.pneumoniae* and detect the dominant ESBL clonal types.

MATERIALS AND METHODS

Bacterial isolates: Clinical isolates of *Klebsiella spp* were identified during the period March 2007-April

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Table 1: Primers for PCR

Primers	Sequence of primers	Size of amplicon	References
Blatem	F: 5-GAGTATCAACATTTCCGTGTC-3 R: 5-TAATCAGTGAGGCACCTTCTC-3	848bp	(Nasehi *et al*. (2010)
Blashv	F: 5–AAGATCCACTATCGCCCAGCAG-3 R: 5-ATTCAGTTCCGTTTCCCAGCGG-3	231bp	(Nasehi *et al*. (2010)
Blactx-m	F:5-ACGCTGTTGTTAGGAAGTG-3 R:5-TTGAGGCTGGGTGAAGT-3	759bp	Mansouri and Ramazanzadeh (2009)

Table 2: Primers for MLST

Primers	Sequences	Refrences	Size
rpoB	RPOB1 CAG TTC CGC GTT GGC CTG RPOB2 CGG AAC GGC CTG ACG TTG CAT	Mollet *et al*. (1997)	687 bp
gyrA	gyrA1 ATG AGC GAC CTT GCG AGA GAA AT gyrA2 CTC GTC ACG CAG CGC GCT GAT GCC	Wertz *et al*. (2003)	752 bp
gapA	gapA1 AGA ACA TCA TCC CGT CCT CTA CC gapA2 CCA GAA CTT TGT TGG AGT AAC C	Wertz *et al*. (2003)	366 bp
gyrB	gyrB1 GCC TCG AAA CCT TCA CCA gyrB2 CGC GAC GTG CGG CCT CAC GG	Wertz *et al*. (2003)	648 bp
groEL groEL2	groEL1 GAC GCT CGY GTR AAA ATG CTS C GCA GTG CAA CTT TGA TAC CCA CG	Wertz *et al*. (2003)	786 bp

2008 in Milad hospital in tehran. The strains were isolated from urinary tract infections, ICUs, surgery wards, lesion and respiratory tract infection.

Detection of *Klebsiella spp*. producing ESBLs: The methods for the laboratory detection of ESBLs were based on recommendations from the Clinical and Laboratory Standard Institute (CLSI) and the Canadian External Quality Assessment Advisory Group for Antibiotic Resistance. However, there have been minor variations from these guidelines to suit the operations of laboratories in our settings. All the clinically significant isolates of *Klebsiella* spp. had to be tested against beta lactam drugs using a disc diffusion method. Any decrease in the zone sizes for the third - generation of cephalosporins had to be used as a criterion to test for ESBLs identification (CLSI, 2010).

ESBL screening methods: *In-vitro* sensitivity testing was performed using established CLSI procedure with ceftazidime (Ca) (30 ug), cefotaxime (Ce) (30 ug), ceftriaxone (Ci) (30 ug), azteronam (Ao) (30 ug) and cefpodoxime (Cep) (30 ug). The zone diameters were read using the revised NCCLS. Any zone diameter within the "grey zone" was considered a probable ESBL producing strain requiring phenotypic confirmatory testing (CLSI, 2010).

Phenotypic confirmatory method: Ceftazidime (30ug) versus ceftazidime/clavulanic (Cac) (30 10 ug^{-1}), cefotaxime (30 ug) versus cefotaxime /clavulanic acid (Cec) (30 10 ug^{-1}) and cefpodoxim versus cefpodoxim/clavulanic acid (Cep) (30 10 ug^{-1}) were placed into a Muller-Hinton agar plate lawned with the test organism and incubated as described above. Regardless of the zone diameters, a > 5mm increase in a zone diameter for an antimicrobial agent tested in combination with clavulanic acid versus its zone size when tested alone, indicated a probable ESBL production (CLSI, 2010). *K.pneumoniae* ATCC 700603 was used as a control for ESBL tests.

PCR amplification of blaTEM, blaSHV and blaCTX-M: The Polymerase Chain Reaction (PCR) was carried out by using primers in Table 1. *K.pneumoniae* 7881 was used as a control for blagenes responsible for ESBLs production.

Effect of non beta-lactame antibiotics against *K. pneumonia* Producing ESBLs: Amikacin (Ak) (30 ug), cotrimoxazol (Co) (30 ug) ciprofloxacin (Cf) (30 ug), imipenem (I) (30 ug) were used among *Klebsiella spp* producing ESBLs towards non beta-lactam antibiotics (Sabrina *et al*., 2009 and Erum *et al*., 2010).

Multilocus Sequence Typing (MLST): Five housekeeping genes were selected to establish the MLST scheme for thirty *K. pneumoniae* in our study; *rpoB* (RNA polymerase -subunit), *gyrA* (DNA gyrase subunit A), *gapA (*glyceraldehydes 3-phosphate dehydrogenase A), *groEL* (GroEL protein) and *gyrB* (DNA gyrase subunit B) (Table 2). PCR amplifications were carried out under the following conditions: 35 cycles of denaturation at 94c for 30 sec, annealing at 50-55c for 30 second extension at 72c for 1 min; the process was preceded by a 5 min denaturation at 94c, followed by a 5min final extension at 72c. The PCR products were purified using a PCR purification kit (fermentase); then, the products were sequenced. The raw sequences were concatenated and edited by using the EditSeq and MegAlign programs. For each locus, distinct allele sequences were assigned as an arbitrary allele number. Each isolate was characterized by its

allelic profile, represented as a series of five integers corresponding to the alleles at each of the loci, in the order of *rpoB*, *gyrA*, *gapA*, *groEL* and *gyrB*. The Sequence Type (ST) was designated for each unique allelic profile as a DNA start program. URL: (www.pubmlst.com).

Detection of bla TEM, SHV and CTX-M: To specify the subtype of the *bla* genes, the amplified PCR products were sequenced on both the strands as done in the MLST analysis. The amino acid sequences were deduced from the nucleotide sequences using the MegAlign program. They were compared with the database of the website (http://www.lahey.Org/ Studies/).

RESULTS

Of two hundred and eighty *Klebsiella spp.* Two hundred and sixty-five *K. pneumoniae* and fifteen *K.oxytoca* were obtained.

K.pneumoniae: In this study, generally, 65.8, 46.4, 60.4, 52 and 51.7% of the isolates were resistant to ceftazidime, cefotaxime, cefteriaxon, cefpodoxime and aztreonam, respectively (Table 3). The findings showed that 51.6% of *K.pneumoniae* produces ESBLs. Of the one hundred and thirty-seven *K. pneumoniae* producing ESBLs, 35.8, 21.2 and 38.7% were resistant to amikacin, ciprofloxacin and cotrimoxazol, respectively (Table 4). Of the *K.pneumoniae* suspected of producing ESBLs, 86.9%, were confirmed by Ceftazidime/clavulanic acid, 31.4% by Cefotaxime/clavulanic acid and 100% by Cefpodoxime/ clavulanic acid.

Screening stage: Of the two hundred and sixty-five *K. pneumoniae* collected in Milad hospital, 50.56% (n = 134), 12.07% (n = 32), 9.82% (n=26), 8.68% (n = 23) and 18.86% (n = 50) were from UTI, ICUs, surgery wards, lesion and respiratory tract infections, respectively.

As you can see in the Table 5, of the one hundred and thirty-four *K.pneumoniae* isolated from patients with UTI, it was found that there was more resistance to cefteriaxone than to the other beta-lactam antibiotics. The results also showed that sixty-eight isolates were suspected of being able to produce ESBLs. In the ICUs, thirty two *K. pneumoniae* showed the highest resistance to cefteriaxone (59.4%) and cefatzidime (56.25%). Generally, thirteen *K. pneumoniae* were suspected of being able to produce ESBLs (Table 6). Twenty six *K. pneumoniae* were obtained from patients admitted in surgery wards. Generally, resistance to ceftazidime (80.4%) was not comparable to the other antibiotics. The findings revealed that eleven *K. pneumoniae* were prone to ESBLs production (Table 7). Twenty-two *K. pneumoniae* were obtained from patients with lesion infections,of which 34.8% were suspected of being able to produce ESBLs (Table 8). RTI had specified fifty *K. pneumoniae* (Table 9).

Confirming stage: All *K. pnemoniae* suspected to produce ESBLs had confirmed as showed in the Tables 8-12 in the confirming stage.

Table 3: Screening stage of all K.pneumoniae in all parts of Milad hospital

Milad hospital	K.pneumoniae	Ceftazidime	Cefotaxime	Cefteriaxone	Cefpodoxime	Aztreonam
Total	265	170 (65.8%)	125 (46.4%)	163 (60.4%)	138 (52%)	137 (51%)

Table 4: Effect of non-beta-lactam antibiotics against K.pneumoniae producing ESBLs in all parts of Milad hospital

	K.pneumoniae producing ESBLs	Amikacine	Ciprofloxacine	Cotrimoxazol
Total	137	49 (35.8%)	29 (21.2%)	53 (38.7%)

Table 5: Screening stage of K.pneumoniae isolated from patients with UTI

UTI	K.pneumoniae	Ceftazidime	Cefotaxime	Cefteryaxone	Cefpodoxime	Aztreonam
Spring	31 (23.14%)	11 (35.48%)	19 (61.29%)	18 (58.06%)	16 (51.61%)	16 (51.61%)
Summer	21 (15.67%)	9 (42.85%)	2 (9.52%)	8 (38.09%)	5 (23.8%)	5 (23.8%)
Fall	39 (29.1%)	21 (53.85%)	15 (38.46%)	19 (48.71%)	18 (46.15%)	18 (46.15%)
Winter	43 (32.08%)	32 (74.41%)	18 (41.86%)	32 (74.41%)	29 (67.44%)	29 (67.44%)
Total	134 (100%)	73 (54.4%)	54 (40.3%)	77 (57.5%)	68 (50.74%)	68 (50.74%)

Table 6: Screening stage of K. pneumoniae of admitted patients in ICUs ward

ICUs	K.pneumoniae	Ceftazidime	Cefotaxime	Cefteryaxone	Cefpodoxime	Aztreonam
Ward						
Spring	6	3	5	4	2	2
	(18.75%)	(50%)	(83.3%)	(66.7%)	(33.3%)	(33.3%)
Summer	7	2	1	3	3	2
	(21.88%)	(28.57%)	(14.28%)	(42.85%)	(42.85%)	(28.57%)
Fall	10	7	3	6	4	4
	(31.25%)	(70%)	(30%)	(60%)	(40%)	(40%)
Winter	9	6	4	6	5	5
	(28.1%)	(66.7%)	(44.5%)	(66.7%)	(55.5%)	(55.5%)
Total	32	18	13	19	14	13
	(100%)	(56.25%)	(40.62%)	(59.4%)	(43.75%)	(40.62%)

Table 7: Screening stage of K. pneumoniae collected of admitted patients in surgery ward

Surgery ward	K.pneumoniae	Ceftazidime	Cefotaxime	Cefteriaxone	Cefpodoxime	Aztreonam
Spring	5	2	0	1	1	1
	(19.23%)	(40%)		(20%)	(20%)	(20%)
Summer	7	7	5	6	4	4
	(29.92%)	(100%)	(71.4%)	(85.74%)	(57.14%)	(57.14%)
Fall	7	5	4	4	2	2
	(29.92%)	(71.4%)	(57.14%)	(57.14%)	(28.57%)	(28.57%)
Winter	7	7	4	5	4	4
	(29.92%)	(100%)	(57.14%)	(71.4%)	(57.14%)	(57.14%)
Total	26	21	13	16	11	11
	(100%)	(80.4%)	(50%)	(61.5%)	(42.3%)	(42.3%)

Table 8: Screening stage of K.pneumoniae isolated of patients with lesion infection

Lesion infection	K.Pneumoniae	Ceftazidime	Cefotaxime	Cefteryaxone	Cefpodoxime	Aztreonam
Spring	5	3	1	3	2	2
	(21.7%)	(60%)	(20%)	(60%)	(40%)	(40%)
Summer	4	3	1	2	1	1
	(17.8%)	(75%)	(25%)	(50%)	(25%)	(25%)
Fall	6	4	2	4	2	2
	(26.1%)	(66.7%)	(33.4%)	(66.7%)	(33.4%)	(33.4%)
Winter	8	7	3	4	3	3
	(34.78%)	(87.5%)	(37.5%)	(50%)	(37.5%)	(37.5%)
Total	23	17	7	13	8	8
	(100%)	(73.9%)	(30.4%)	(56.52%)	(34.8%)	(34.8%)

Effects of non-beta-lactam antibiotics against *K. pneumoniae* producing ESBLs: In all the seasons, we found that *K. pneumoniae* producing ESBLs isolated from patients with UTI were resistant towards non-beta lactam antibiotics, but there were no resistance for imipenem (Table 10). In the ICUs, no resistance was observed in summer. In this part, also cotrimoxazol was found to be the most resistant antibiotics (Table 11). The findings showed that the *K. pneumoniae* producing ESBLs isolated from patients admitted in the surgery ward were not resistant to non-beta-lactam antibiotics in spring. The results also showed that there were more resistance to cotrimoxazol than to ciprofloxacin. Imipenem, as in the other parts was found to be an effective antibiotic (Table 12). Lesion infections did not show any resistance to imipenem and ciprofloxacin. It was also observed that there was no resistance in summer (Table 13). The results showed that ESBLs, produced by *K.pneumoniae* isolated from patients with RTI, were resistant to non-beta-lactam antibiotics except imipenem, in all the seasons (Table 14).

PCR results: Of the one hundred and twenty *K. pneumoniae* with blaSHV, 47.5% (n = 57), 9.1% (n = 11), 9.1% (n = 11), 6.7% (n = 8) and 27.5% (n = 33) were from patients with urinary tract infections, patients admitted in ICUs, surgery ward, patients with lesion infections and patients with respiratory tract infections, respectively**.** Of the seventeen *K. pneumoniae* with blaTEM, 23.5% (n = 4), 11.7% (n = 2), 11.7% (n = 2), 5.9% (n = 1) and 47% (n = 8) were from patients with UTI, patients admitted in ICUs, surgery ward, patients with lesion infections and patients with RTIs, respectively.

Table 9: Screening Stage of K. pneumoniae isolated from patients with RTI

RTI	K.Pneumonia	Ceftazidime	Cefotaxime	Cefteryaxone	Cefpodoxime	Aztreonam
Spring	9	8	8	7	7	7
	(18%)	(88.9%)	(88.9%)	(77.8%)	(77.8%)	(77.8%)
Summer	5	2	3	3	2	2
	(10%)	(40%)	(60%)	(60%)	(40%)	(40%)
Fall	12	11	9	9	8	8
	(24%)	(91.7%)	(75%)	(75%)	(66.7%)	(66.7%)
Winter	24	20	18	19	20	20
	(48%)	(83.3%)	(75%)	(79.1%)	(83.3%)	(83.3%)
Total	50	41	38	38	37	37
	(100%)	(82%)	(76%)	(76%)	(74%)	(74%)

Table 10: Confirming stage and effect of non-beta-lactam antibiotics toward K.pnemoniae producing ESBLs from patients with UTI

UTI	K.pneumoniae Suspected toproduce ESBLs	Ceftazidime/ clavulanic acid	Cefotaxime/ clavulanicacid	Cefpodoxime clavulanicacid	Amikacine	Ciproflox acine	Cotrimoxazol	Imipenem
Spring	16	16	4	16	5	4	6	0
	(23.5%)	(100%)	(25%)	(100%)	(31.25%)	(25%)	(37.5%)	
Summer	5	5	2	5	2	1	2	0
	(7.35%)	(100%)	(40%)	(100%)	(40%)	(20%)	(40%)	
Fall	18	16	3	18	6	5	9	0
	(26.4%)	(88.9%)	(16.7%)	(100%)	(33.3%)	(27.8%)	(50%)	
Winter	29	21	9	29	12	6	9	0
	(42.65%)	(72%)	(31%)	(100%)	(41.4%)	(20.7%)	(31%)	
Total	68	58	18	68	25	16	26	0
	(100%)	(85.5%)	(26.47%)	(100%)	(36.7%)	(23.5%)	(38.2%)	

Table 11: Confirming stage and effect of non-beta-lactam antibiotics toward K.pnemoniae producing ESBLs isolated from patients in ICUs ward

ICUS Ward	K.pneumoniae suspected to produce ESBLs	Ceftazidime/ clavulanic acid	Cefotaxime/ clavulanicacid	Cefpodoxime/ clavulanicacid	Amikacine	Ciprofloxacine	Cotrimoxazol	Imipenem
Spring	2	2	1	2	1	1	1	0
	(15.4%)	(100%)	(50%)	(100%)	(50%)	(50%)	(50%)	
Summer	2	1	1	2	0	0	0	0
	(15.4%)	(50%)	(50%)	(100%)				
Fall	4	3	1	4	1	1	1	0
	(30.8%)	(75%)	(25%)	(100%)	(25%)	(25%)	(25%)	
Winter	5	5	2	5	2	1	3	0
	(38.4%)	(100%)	(40%)	(100%)	(40%)	(20%)	(60%)	
Total	13	11	5	13	4	3	5	0
	(100%)	(84.6%)	(38.46%)	(100%)	(30.7%)	(23%)	(38.46%)	

Table 12: Confirming stage and effect of non-beta-lactam antibiotics toward K.pnemoniae producing ESBLs isolated from patients in surgery ward

Surgery ward	K.pneumoniae /clavulanic acid	Ceftazidime /clavulanicacid	Cefotaxime /clavulanicacid	Cefpodoxime	Amikacine	Ciprofloxacine	Cotrimoxazol	Imipenem
Spring	1	1	0	1	0	0	0	0
	(9.1%)	(100%)	(100%)					
Summer	4	4	1	4	1	0	2	0
	(36.4%)	(100%)	(25%)	(100%)	(25%)	(50%)		
Fall	2	2	1	2	1	1	1	0
	(18.2%)	(100%)	(50%)	(100%)	(50%)	(50%)	(50%)	
Winter	4	4	2	4	2	1	2	0
	(36.4%)	(100%)	(50%)	(100%)	(50%)	(25%)	(50%)	
Total	11	11	4	11	4	2	5	0
	(100%)	(100%)	(36.6%)	(100%)	(36.6%)	(18.1%)	(45.5%)	

Table 13: Confirming stage and effect of non-beta-lactam antibiotics toward K.pnemoniae producing ESBLs isolated from patients with lesion infection

Lesion infection	K.pneumoniae suspected to produceESBLs	Ceftazidime /clavulanic acid	Cefotaxime /clavulanic acid	Cefpodoxime /clavulanic acid	Amikacine	Ciprofloxacine	Cotrimoxazol	Imipenem
Spring	2 (25%)	2 (100%)	1 (50%)	2 (100%)	1 (50%)	0 (50%)	1	0
Summer	1 (12.5%)	1 (100%)	0	1 (100%)	0	0	0	0
Fall	2 (25%)	2 (100%)	1 (50%)	2 (100%)	1 (50%)	0	1 (50%)	0
Winter	3 (37.5%)	3 (100%)	2 (66.7%)	3 (100%)	1 (33.3%)	0	1 (33.3%)	0
Total	8 (100%)	8 (100%)	4 (50%)	8 (100%)	3 (37.5%)	0	3 (37.5%)	0

Table 14: Confirming stage and effect of non-beta-lactam antibiotics toward K.pnemoniae producing ESBLs isolated from patients with RTI

RTI	K.pneumoniae suspected to produce ESBLs	Ceftazidime/ clavulanic acid	Cefotaxime/ clavulanic acid	Cefpodoxime/ clavulanic acid	Amikacine	Ciprofloxacine	Cotrimoxazol	Imipenem
Spring	7 (18.9%)	6 (85.7%)	3 (42.85%)	7 (100%)	3 (42.85%)	2 (28.6%)	3 (42.85%)	0
Summer	2 (5.4%)	2 (100%)	1 (50%)	2 (100%)	1 (50%)	1 (50%)	1 (50%)	0
Fall	8 (21.6%)	8 (100%)	2 (25%)	8 (100%)	2 (25%)	1 (12.5%)	2 (25%)	0
Winter	20 (54.1%)	16 (80%)	8 (40%)	20 (100%)	7 (35%)	4 (20%)	8 (40%)	0
Total	37 (100%)	32 (86.5%)	14 (37.9%)	37 (100%)	13 (35.1%)	8 (21.6%)	14 (37.9%)	0

Of the thirty four *K. pneumoniae* with blaCTX-M , 41.2% (n =14), 11.8% (n = 4), 11.8% (n = 4), 5.9% (n = 2) and 29.5% (n = 10) were obtained from patients with UTI, in ICUs ward, in surgery ward, patients with lesions and respiratory tract infections, respectively. In patients with UTI, fifty seven *K. pneumoniae* with blaSHV were observed. Of these 28.1% (n = 16), 8.7% (n = 5), 28.1% (n =16) and 35.1% (n = 20) were obtained in spring, summer, fall and winter, respectively. Of the four *K. pneumoniae* with blaTEM, 25% (n = 1), 25% (n = 1) and 50% (n = 2) were found in spring, fall and winter, respectively. Our findings also showed that of the fourteen *K. pneumoniae* with blaCTX-M from the patients with UTIs, 7.1% (n=1) were obtained in spring, 21.5% (n = 3) in fall and 71.4% (n = 10) in winter. Of the eleven *K. pneumoniae* with blaSHV from patients admitted in ICUs, 18.2% (n = 2), 9.1% (n = 1), 27.2% (n = 3) and 45.5% (n = 5) were found in spring, summer, fall and winter, respectively. Two *K. pneumoniae* with blaTEM were isolated in fall and winter. Four *K. pneumoniae* with blaCTX-M were observed, of which 25% (n = 1), 25% (n = 1) and 50% (n = 2) were obtained in summer, fall and winter, respectively. Of the eleven *K. pneumoniae* with blaSHV from patients admitted in the surgery ward, 9.1% (n = 1), 36.4% (n = 4), 18.2% (n = 2) and 36.4% (n = 4) were obtained in spring, summer, fall and winter, respectively. Two *K. pneumoniae* with blaTEM were isolated, of which one was obtained in fall and the other in winter. Of the four *K. pneumoniae* with blaCTX-M from the surgery ward, 25% (n = 1) were obtained in summer, 25% (n = 1) in fall and 50% (n = 2) in winter. The results showed that of the eight *K. pneumoniae* with blaSHV, from patients with lesion infections, 25% (n = 2), 12.5% (n = 1), 25% (n = 2) and 37.5% (n = 3) were found in spring, summer, fall and winter, respectively. One *K.pneumoniae* with blaTEM was isolated in winter. Of the two *K. pneumoniae* with blaCTX-M, from the patients with lesion infections, 50% (n = 1) were obtained in spring and 50% (n = 1) in winter.

The results indicated that of the thirty-three *K. pneumoniae* with blaSHV, from patients with RTIs, 18.8% (n = 6), 9% (n = 3), 24.2% (n = 8) and 48.5% (n=16) were obtained in spring, summer, fall and winter, respectively. Of the eight *K. pneumoniae* with blaTEM from RTIs, 37.5% (n = 3), 12.5% (n = 1), 12.5% (n =1) and 37.5% (n = 3) were obtained in spring, summer, fall and winter, respectively. Of the ten *K. pneumoniae* with blaCTX-M, from patients with RTIs, 30% (n=3) were obtained in spring, 10% (n = 1) in summer, 10% (n = 1) in fall and 50% (n = 5), in winter.

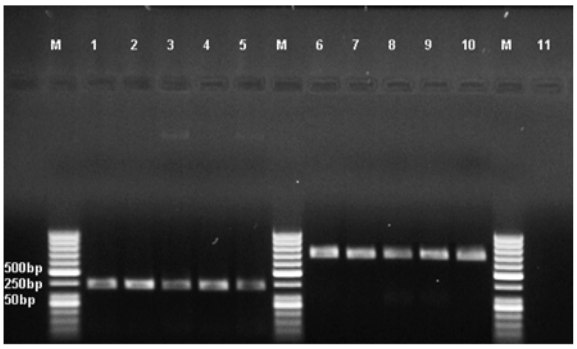

Fig. 1: Agarose gel electropherosis of amplified fragment obtained by PCR for blaSHV and blaCTX-M. Lanes: M= Molecular weight marker (50bp) 1 = *K.pneumoniae* 7881 (positive control), 2-5 blaSHV, 6-10 BlaCTX-M, 11 = negative control

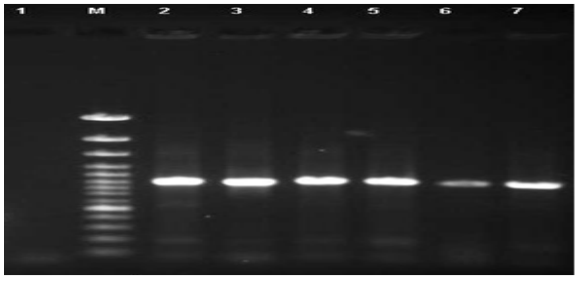

Fig. 2: Agarose gel electropherosis of amplified fragment obtained by PCR for blaTEM. Lanes: 1 = Negative control M= Molecular weight marker (100bp) 2 = *K.pneumoniae* 7881 (positive control), 3-7 blaTEM.

The results reflected the frequency of blaSHV, blaTEM and blaCTX-M among *K.pneumoniae* producing ESBLs were 87.5, 12.4 and 24.8%, respectively. Further, 1.5, 7.9, 0.4 and 1.5% of *K.pneumoniae* producing ESBLs were found positive for blaSHV-TEM, blaSHV-CTX-M, blaTEM-CTX-M and blaSHV-TEM-CTX-M, respectively.

blaSHV, TEM and CTX-M showed in Fig. 1-2.

MLST results: MLST was assessed for thirty *K. pneumoniae* producing ESBLS in Milad hospital, of which thirteen had blaTEM, BlaSHV, seven blaSHV and blaCTX-M, two blaSHV and the other eight blaTEM, BlaSHV and blaCTX-M. The MLST data calculated by eBURST were the Sequence Types (STs) and their associated allelic profiles. The different allelic profiles are showed in Table 16. The Sequence Type (ST) was designated for each unique allelic profile. The definition of the group is to identify groups of related STs using the most conservative definition, where all members assigned to the same group share identical alleles at = 4 of the 5 loci, with at least one other member of the group.

Clonal complex is a set of STs that are all believed to have descended from the same founding genotype. Using the stringent group definition (4/5 shared alleles) isolates in the group defined by eBURST will be considered to belong to a single clonal complex.

SLV=all STs must be a Single Locus Variant (SLV) of at least one other ST in the group

DLV= double locus variant of at least one other ST in the group.

TLV= three loci (TLVs)and those that are more distantly related (satellites).

The bootstrap values shown for each ST are the percentage of times the ST was predicted to be the primary founder of the group in the bootstrap re-samplings. As a ST cannot be the predicted founder if it is not present in a re-sampled dataset, the calculation of the percentage of times each ST is predicted to be the primary founder omits those re-samplings in which that ST is absent. In larger eBURST groups there may be several STs besides the predicted primary founder that have a number of SLVs of their own. A ST that appears to have diversified to produce multiple SLVs was called a subgroup founder (eBURST program).

Based on the nucleotide variations of the five genetic loci, twenty-five different STs could be identified among thirty *K.pneumoniae* producing ESBLs isolates. The majority of these (5 out of 25 STs) were represented by a single isolate. Among the STs shared by multiple isolates, the most frequently encountered were ST14 (four isolates) ST16 (two isolates), ST18 (two isolates). Six Colonal Complexes (CCs) were identified. The predicted founder was defined in the eBURST results. CC1 included five STs (st1,5,4,3,2), CC2 three STs (ST9,10,11), CC3 three STs (ST8,7,6), CC4 two STs (st12,13),CC5 two STs (ST15,14) and CC6 two STs (ST16,17) (Table 15).

K.oxytoca: The results showed that fifteen clinical isolates of *K.oxytoca* were obtained in the surgery ward, patients with lesion and respiratory tract infections. It was found that 40% (n = 6) and 27.3% (n = 3) of *K.oxytoca* producing ESBLs were resistant to cotrimoxazol and amikacin, respectively. Resistance to the other non-beta-lactam antibiotics were not observed (Table 17). The findings showed 73.3% of *K.oxytoca* produces ESBLs.

eBURST results was:

eBURST Report
No. isolates = 25 | No. STs = 25 | No. re-samplings for bootstrapping = 1000
No. loci per isolate = 5 | No. identical loci for group def = 4 | No. groups = 6
Group 1: No. Isolates = 5 | No. STs = 5 | Predicted Founder = 3

ST	FREQ	SLV	Average DLV	ST Bootstrap TLV	SAT	Distance	Group	Subgrp
k4	1	3	1	0	0	1.25	60%	16%
k26	1	2	2	0	0	1.5	19%	0%
k19	1	1	2	1	0	2.0	0%	0%
k7	1	1	2	1	0	2.0	0%	0%
k10	1	1	1	2	0	2.25	0%	0%

Group 2: No. Isolates = 3 |No. STs = 3| Predicted Founder = Multiple Candidates

ST	FREQ	SLV	Average DLV	ST Bootstrap TLV	SAT	Distance	Group	Subgrp
k20	1	2	0	0	0	1.0	6%	0%
k1	1	2	0	0	0	1.0	8%	0%
k23	1	2	0	0	0	1.0	15%	0%

Group 3: No. Isolates = 3 | No. STs = 3 | Predicted Founder = 13

ST	FREQ	SLV	Average DLV	ST Bootstrap TLV	SAT	Distance	Group	Subgrp
k15	1	2	0	0	0	1.0	32%	0%
k16	1	1	1	0	0	1.5	0%	0%
k14	1	1	1	0	0	1.5	0%	0%

Group 4: No. Isolates = 2 | No. STs = 2 | Predicted Founder = None

ST	FREQ	SLV	DLV	TLV	SAT	Distance
k13	1	1	0	0	0	1.0
k12	1	1	0	0	0	1.0

Group 5: No. Isolates = 2 | No. STs = 2 | Predicted Founder = None

ST	FREQ	SLV	DLV	TLV	SAT	Distance
k8	1	1	0	0	0	1.0
k27 1	1	0	0	0	1.0	

Group 6: No. Isolates = 2 | No. STs = 2 | Predicted Founder = None

ST	FREQ	SLV	DLV	TLV	SAT	Distance
k6	1	1	0	0	0	1.0
k5	1	1	0	0	0	1.0

Singletons: size 8
k21, k18, k17, k11, k28, k2, k25, k24

Screening stage: *K. oxytoca* was only found in winter from patients admitted in the surgery ward **(**Table 18). The findings indicated that one *K. oxytoca* obtained from patients with lesion infections in autumn, was resistant to all the third-generation of cephalosprines, except cefotaxime (Table 19). Of the twelve *K. oxytoca* collected from patients with RTIs, 16.6% (n = 2) were obtained in fall and 83.3% (n = 10) in winter (Table 20).

Confirming stage: Two *K. oxytoca* were isolated from patients admitted in surgery wards in winterand both were suspected of being able to produce ESBLs. They were confirmed by ceftazidim/clavulanic acid and cefpodoxim/clavulanic acid (Table 21). One *K. oxytoca*, collected from patients with lesion infections in fall, was confirmed by ceftazidime/clavulanic acid as well as cefpodoxime /clavulanic acid, at the confirming stage, to be prone to produce ESBLs (Table 22). The two *K. oxytoca* collected from patients with RTIs in fall, were confirmed by ceftazidime/clavulanic acid and cefpodoxime/clavulanic acid, to produce ESBLs. Of the ten *K.oxytoca* collected from patients with RTIs in winter, 60% (n = 6) were suspected of being able to produce ESBLs. They were confirmed by ceftazidime/clavulanic acid and cefpodoxime/clavulanic acid, at the confirming stage (Table 23).

PCR results: Of the eleven *K. oxytoca* producing ESBLs at the phenotypic stage, 100 % (n = 11) blaSHV were obtained by the PCR method. The results that showed blaTEM and blaCTX-M were negative.

Table 15: Characteristics of 30 ESBL-producing K. pneumoniae isolates from different parts of Milad hospital

	Allelic profile	St	Part of hospital	cc	blaSHV	blaTEM	BlaCTX-M
1	1-6-3- 5-6-9						
	7	ICUs	3	5	12	-	
2	1-3-1-5-4	23	UTI	-	12	12	15
3	4-4-2-4-3						
	16	LESION	6	12	12	-	
4	2-2-6-3-10	1	UTI	1	12	12	15
5	4-2-2-4-3	17	RTI	5	12	12	-
6	4-4-2-4-3						
	16	SURGERY	6	12	12	15	
7	1-2-6-3-10						
	4	UTI	1	12	12	15	
8	2-1-2- 4-2	14	RTI	5	12	12	15
9	2-1-2- 4-2	14	RTI	5	12	12	15
10	2-2-5-3-9						
	5	UTI	1	12	12	-	
11	5- 4-2-4-1	21	RTI	-	5	12	-
12	2-7- 4-1-10	13	SURGERY	4	12	12	15
13	2- 9-4-1-10	12	RTI	4	12	12	15
14	2-2-4-4-7	11	RTI	2	12	-	15
15	2-2-4-4-8						
	9	SURGERY	2	12	-	15	
16	2-2-3-4-8	10	RTI	2	12	-	-
17	2-8-3-4-5	20	ICUs	-	12	12	-
18	1-10-2-4-6						
	19	LESION	-	5	12	-	
19	2- 4-6-3-10	3	UTI	1	12	-	-
20	6-11-5-6-9						
	6	RTI	3	12	12	-	
21	4-1-1-1- 4	18	SURGERY	-	12	-	-
22	4-1-1-1- 4	18	SURGERY	-	12	12	-
23	6-2-5-6-9	8	ICUs	3	12	12	-
24	1- 1-3-4-2						
	25	RTI	-	12	-	-	
25	2-6-2-2-3						
	24	RTI	-	12	12	-	
26	2- 2-5-3-10	2	UTI	1	12	12	-
27	2-1-5-4-2						
	15	RTI	5	12	12	-	
28	2-3-3-4-2						
	22	UTI	-	5		-	
29	2-1-2- 4-2	14	RTI	5	12		-
30	2-1-2- 4-2	14	RTI	5	12		-

Table 16: Variation in loci used in the present K. pneumoniae MLST scheme

Locus	Size	No. Allele
Gapa	366bp	6
Gyra	752bp	11
Gyrb	648bp	5
Rpob	687bp	6
Groel	786bp	10

Table 17: Effect of non-beta-lactam antibiotics against K.oxytoca producing ESBLs

	K.oxytoca producing ESBLs	Amikacine	Ciprofloxacine	Cotrimoxazol
Total	11 (100%)	3 (27.3%)	0	6 (54.5%)

Table 18: Screening stage for detection of K.oxytoca producing ESBLs from patients in surgery ward

Surgery ward	K.oxytoca	Ceftazidime	Cefotaxime	Cefteryaxone	Cefpodoxime	Aztreonam
Winter	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)

Table 19: Screening stage for detection of K.oxytoca producing ESBLs from patients with lesion infection

Lesion infection	K.oxytoca	Ceftazidime	Cefotaxime	Cefteryaxone	Cefpodoxime	Aztreoname
Fall	1 (100%)	1 (100%)	0	1 (100%)	1 (100%)	1 (100%)

Table 20: Screening stage for detection of K.oxytoca producing ESBLs from patients with RTI

RTI	K.Oxytoca	Ceftazidime	Cefotaxime	Cefteryaxone	Cefpodoxime	Aztreonam
Fall	2 (16.6%)	2 (100%)	0	2 (100%)	2 (100%)	2 (100%)
Winter	10 (83.3%)	8 (80%)	4 (40%)	7 (70%)	6 (60%)	6 (60%)
Total	12 (100%)	10 (83.3%)	4 (33.3%)	9 (75%)	8 (66.7%)	8 (66.7%)

Table 21: Confirming stage and effect of non-beta-lactam antibiotics toward K.oxytoca producing ESBLs isolated from patients in surgery ward

Surgery ward	K.oxytoca suspectedto produce ESBLs	Ceftazidime /clavulanic acid	Cefotaxime /clavulanic acid	Cefpodoxime /clavulanic acid	Amikacine	Ciprofloxacine	Cotrimoxazol	Imipenem
Winter	2 (100%)	2 (100%)	0 0	2 (100%)	0 0	0 0	1 (50%)	0

Table 22: Confirming stage and effect of non-beta-lactam antibiotics toward K.oxytoca producing ESBLs isolated from patients with Lesion infection

Lesion infection	K.oxytoca suspected to produce ESBLS	Ceftazidime /clavulanic acid	Cefotaxime /clavulanic acid	Cefpodoxime /clavulanic acid	Amikacine	Ciprofloxacine	Cotrimoxazol	Imipenem
Fall	1 (100%)	1 (100%)	0 0	1 (100%)	0 0	0 0	0 0	0 0

Table 23: Confirming stage and effect of non-beta-lactam antibiotics toward K.oxytoca producing ESBLs isolated from patients with RTI

RTI	K.oxytoca suspected to produce ESBLs	Ceftazidime /clavulanic acid	Cefotaxime /clavulanic acid	Cefpodoxime /clavulanic acid	Amikacine	Ciprofloxacine	Cotrimoxazol	Imipenem
Fall	2 (25%)	2 (100%)	0	2 (100%)	1 (50%)	0	1 (50%)	0
Winter	6 (75%)	6 (100%)	0	6 (100%)	2 (33.3%)	0	4 (66.6%)	0
Total	8 (100%)	8 (100%)	0	8 (100%)	3 (37.5%)	0	5 (62.5%)	0

DISCUSSION

Gram-negative pathogens harboring ESBLs have caused numerous outbreaks of infections and are becoming an increasing therapeutic problem in many countries. The incidence of ESBL-producing strains among clinical isolates has been steadily increasing over the past years resulting in limitations of therapeutic option (AL-Haj *et al*., 2010). ESBLs are now a significant problem in hospitalized patients throughout the world. The prevalence of ESBLs among clinical isolates varies worldwide and patterns are rapidly changing over time (Shayanfar *et al*., 2010).

These findings showed high level of antibiotic resistance, especially, for third-generation of cephalosporins. Interestingly, we found Imipenem to be effective in this study and could still be used for treatment. However our findings revealed the urgency for controlling irregular consumption of antibiotics in Iran.

K.pneumoniae: The highest resistance was found for ceftazidime (65.8%) at the screening stage (Table 3) and The ESBLs production by *K.pneumoniae* from patients with UTI (49.6%) was more than in the others. Cotrimoxazol resistance was the highest due to non-beta-lactam antibiotics (Table 4). Ceftazidime and cefteriaxone resistance were the highest in patients with UTI at the screening stage (Table 5). In these patients, the highest *K.pneumoniae* producing ESBLs (42.65%) and amikacin resistance was found in winter (41.4%) while resistance to ciprofloxacin (27.8%) and cotrimoxazol (50%) was more in fall than in the other seasons (Table 10). The results showed that blaSHV was the dominant gene responsible for ESBLs production, followed by blaSHV and blaCTX-M. Frequency of all the three genes in winter was more than in the other seasons while the lowest frequency wasin summer. Our findings showed resistance to

antibiotics and *K.pneumoniae* producing ESBLs in the cold seasons was higher than during the warm seasons.

In ICUs, the highest resistance to third-generation of cephalosporins was observed for cefotaxime (83.4%) in spring (Table 6). *K.pneumoniae* producing ESBLs was more in winter than in the other seasons (38.4%). %). Also the highest ciprofloxacin resistance was reported in winter (60%) while amikacin and cotrimoxazol resistance was more in spring than in the other seasons (50%) (Table 11). The results indicated that the dominant gene was blaSHV and the highest blaSHV was observed in winter, followed by blaSHV and blaCTX-M, which showed greater frequency than blaTEM (Table 7). Our results from patients admitted in surgery wards indicated that ceftazidime resistance in summer and winter (100%) was the highest compared to other antibiotics at the screening stage. The findings of this study also clarified that there were more *K.pneumoniae* producing ESBLs in winter than in the other seasons (42.65%). We found cotrimoxazol (50%) and ciprofloxacin (27.8%) resistance in fall to be more than in the other seasons while the highest amikacin resistance occurred in winter (41.4%) (Table 12). BlaSHV was the dominant gene responsible for ESBLs production but it showed the same frequency in winter and summer while blaTEM in fall and winter.

K.pneumoniae isolated from patients with lesion infections were more resistant to ceftazidime in winter (87.5%) at the screening stage (Table 8). We also found winter as a season responsible for the highest ESBLs production (37.5%). Cotrimoxazol and amikacin resistance in spring and fall (50%) were more than in the other seasons (Table 13). The results further showed that the dominant gene responsible for ESBLs production was blaSHV. The results showed that for patients with RTIs, the highest antibiotic resistance at the screening stage was observed for ceftazidime (91.7%) in fall (Table 9). However ESBLs production in winter (54.1%) was greater than in the other seasons. Amikacin, cotrimoxazol and ciprofloxacin resistance were observed to be more in summer (50%) (Table 14). According to the findings of this study, the dominant gene responsible for ESBLs production was BlaSHV and the highest frequency of this gene was found in winter. The frequency of blaTEM and blaCTX-M in winter were also more than in the other seasons.

The highest resistance to cotrimoxazol was found in *K.oxytoca* producing ESBLs isolated from patients with RTIs in winter (Table 17). Ciprofloxacin and imipenem were found to be effective antibiotics in this study. Our study showed that antimicrobial resistance in *K. pneumoniae* was higher than in *K. oxytoca* but frequency of ESBLs due to *K. oxytoca* (73.3%) was more than *K. pneumoniae* (51.6%). Frequency of the blaSHV was more in comparison with the other genes responsible for ESBLs production. The results showed that *K. oxytoca* only had frequency in cold seasons. Our findings showed the dominant gene responsible for ESBLs production was blaSHV and it had the highest frequency in winter. Generally, resistance to third-generation cephalosporins and aztreonam and ESBLs production in cold seasons was greater than during the warm seasons. These results were valid for resistance of *K. pneumoniae* producing ESBLs to non-beta-lactam antibiotics. It also showed that all isolates from all parts were susceptible to imipenem.

Molecular typing is a pre-requisite for elucidating the epidemiology and population structure of bacterial pathogens. Of the molecular typing methods, MLST is becoming more popular, owing to its several advantages, which have been discussed repeatedly elsewhere (like PFGE, RFFLP). It enjoys a high level of discrimination, unambiguity, reproducibility and scalability due to application of nucleotide sequences and also has an electronic portability via the Internet, so that it can easily analyze the generated data with a wider applicability (Andrade *et al*., 2010).

The objectives of this study were to detect the dominant colonal type by MLST. The dominant Sequence Type (ST) was ST14. The findings of this study showed different colonal complex (CC). Interestingly, most STs (ST14) were observed in RTIs. In UTI, there were no CC and the isolates were from different STs. Our findings showed a different ST and reduced epidemiology of the ESBLs in Milad Hospital. This was the first study of MLST scheme of *K.pneumoniae* producing ESBLs in Iran. The results of this study indicated that the most genes were BlaSHV-12. The highest allelic variation occurred in GyrA (11allele).

One hundred and sixty eight clinical isolates of *K.pneumoniae* were collected in a survey during the period September 2006 to February 2007, from three general hospitals in Tehran, Iran. It was found that 69% of the one hundred and sixty eight clinical isolates were positive and fifty-one isolates (31%) were negative for ESBLs (Bameri *et al*., 2010). Our results showed lower frequency of ESBL production in *K.pneumoniae* and higher for *K.oxytoca* in comparison with the study by Bameri in Milad hospital. However it was at least double when compared with Irajian *et al* in Semnan with 28.9% ESBL production. (Irajian and Moghadas, 2010). In a research in Netherlands ESBLs were found in 2.4% of *K.oxytoca* (Strum *et al*., 2010) that was not

comparable with the 73.4% ESBls positive *K.oxytoca* in this study. All the findings in our research showed high levels of ESBLs production by *K.oxytoca.*

CONCLUSION

In conclusion, the percentage of *K.oxytoca* producing ESBLs was higher than *K.pneumoniae* producing ESBLs. Generally, *K.penomoniae* produces more ESBLs in winter and fall than in the other seasons. The dominant gene responsible for ESBLs production was blaSHV. MLST due to *K.pneumoniae* producing ESBLs released different ST and different CC, which showed that the source of ESBLs production among *K.pneumoniae* was not the same.

In general, resistance to any antibiotics used in this study during winter and fall were higher than in the other seasons.

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