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Phenotypic and Genotypic Assay for Detection of Extended Spectrum β-lactamases Production by *Klebsiella pneumoniae* Isolates in Emam Reza Hospital in Tabriz, Iran

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Objectives of this study were to investigate the prevalence of *K. pneumoniae* producing ESBLs, to evaluate the susceptibility of *K. pneumoniae* producing ESBLs towards non-beta-lactam antibiotics and to study the dominant ESBLs gene in Emam Reza hospital. *K. pneumoniae* producing ESBLs identified by phenotypic and genotypic methods. Polymerase Chain Reaction (PCR) performed for detection of blaSHV, TEM and CTX-M. The findings showed that 43.69%, 13.59%, 7.77%, 11.65% and 23.3% were from UTI, ICUs, surgery ward, lesion infections and RTI, respectively. The results showed that 43.7% of isolates were ESBLs produces. The findings revealed that 26.7%, 6.7%, 20% and 0% of K.pneumoniae producing ESBLs were resistant to amikacin, ciprofloxacin, cotrimoxazol and imipenem, respectively. Thirty-nine blaSHV, seven blaTEM and seven blaCTX-M identified among *K.pneumoniae* producing ESBLs. The results reflected in cold month resistant to third generation cephalosporins were more than warm months. Generally, frequency of blaSHV was more than blaCTX-M and blaTEM.

Key words: Phenotypic, Genotypic, *K. pneumoniae* and Tabriz.

Abbreviations: RTI= Respiratory tract infection, UTI= Urinary tract infection, KPSPE=*Klebsiella pneumoniae* suspected to produce ESBL, Cac= ceftazidime/clavulanic, Cec= cefotaxime /clavulanic acid, Cep=cefpodoxim/clavulanic acid, Ak=Amikacin, Co=cotrimoxazol, Cf=ciprofloxacin, I=imipenem, Ca=ceftazidime, Ce=cefotaxime, Ci=cefteriaxone, Cep= cefpodoxime and Ao=Aztreonam.

Extended spectrum beta-lactamases (ESBLs) are defined as beta-lactamases capable of hydrolyzing oxyimino cephalosporins and are inhibited by beta-lactamase inhibitors. These enzymes are bacterial enzymes that are encoded by chromosomal or plasmid borne genes, and confer multiple drug resistance. There are three main types of ESBLs: TEM, SHV and CTX-M[1]. ESBLs are clinically important because they destroy cephalosporins, workhorse hospital antibiotics, given as first-line agents to many severely ill patients, including those with intra-abdominal infections, community acquired pneumonias and bacteraemias. Delayed

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recognition and inappropriate treatment of severe infections caused by ESBL producers with cephalosporins has been associated with increased mortality[2].

Objectives of this study were to investigate the prevalence of *K. pneumoniae* producing ESBLs, to investigate the susceptibility of *K. pneumoniae* producing ESBLs towards non-beta-lactam antibiotics and to investigate the dominant ESBLs gene in Emam Reza hospital.

MATERIAL AND METHODS

Bacterial isolates

Clinical isolates of *K. pneumoniae* were identified during Mar. 2007 to Apr. 2008 in Emam Reza hospital in Tabriz in the northwest of Iran. The strains were isolated from UTI, ICUs ward, surgery ward, lesion and respiratory tract infections.

Identification of *K. pneumoniae*

Procedure of identification: Gram staining by Modified Preston-Morrel method.Oxidase and Catalase .biochemical tests including motility,indol ,SH2(SIM medium), citrate reaction(Simon Citrate medium), methyl red and vogsprasgoer tests(MR-VP medium), decarboxcilation and deamination of lysine(Lysine Iron Agar Medium), lactose, SH2 and gas producing (Kligler iron Agar),phenylalanine reaction(Phenylalanin Agar Medium), Urea reaction (Urea Agar Medium), Blood Agar, Maccanky Agar[3].

All of the media were from MERCK Company (Germany).

Antimicrobial susceptibility testing and screening for ESBL enzyme production

Antimicrobial susceptibilities were initially determined with the cephalosporins, Amikacin, cotrimoxazol, ciprofloxacine and carbapenems. The screening for ESBLs was determined using the double disk synergy test[4] and phenotypic confirmatory disk diffusion test[5] according to the Clinical Laboratory Standards Institute (CLSI) guidelines. *E.coli* ATCC 25922 was used as a negative control and *K. pneumoniae* ATCC 700603 as an ESBLpositive Control.

Molecular method

DNA extraction

K. pneumonia. Producing ESBLs were cultured in LB broth at 37°C overnight and DNA was extracted by DNA extraction KIT (Fermenrtase). The primers of this study have showed in Table 1.

blaSHV gene was amplified under the following condition: initially denaturation at 94°C for 3 minute, following by35 cycles of denaturation 95c for 30 second annealing 56°C for 1 minute, and 72°C for 1 minute, with a final extension of 72°C for 10 minute, BlaTEM gene was amplified under the following condition: initially denaturation at 94°C for 3 minute, following by35 cycles of denaturation 95c for 30 second annealing 45°C for 1 minute, and 72°C for 1 minute, with a final extension of 72°C for 10 minute. The *K. pneumonia* 7881 strain contains blaSHV and blaTEM genes were used as a positive control and BlaCTX-m gene was amplified under the following condition: initially denaturation at 94°C for 3 minute, following by35 cycles of denaturation 95c for 30 second annealing 48°C for 1 minute, and 72°C for 1 minute, with a final extension of 72°C for 10 minute. The amplicons were run in 1% agarose gel. The gels were stained with ethidium bromide and a band observed at desired position was photographed on an ultraviolet light transilluminator.

RESULTS

Of the one hundred and three clinical isolates of *K. pneumoniae* collected in Emam Reza Hospital, Tabriz, 43.69% (n=45) ,13.59% (n=14), 7.77% (n=8) ,11.65% (n=12) and 23.3% (n=24) were from UTI, ICUs , surgery wards, lesion infections and RTI, respectively. Generally, 57.3%, 38.8%, 62.1%, 45.6 and 43.7% of *K. pneumoniae* showed resistant to ceftazidime, cefotaxime, cefteriaxone, cefpodoxime and azteronam, respectively (Table 2). The results showed that 43.3% of isolates produce ESBLs.

The results showed that 26.7%, 6.7%, 20% and 0% of *K. pneumoniae* producing ESBLs were resistant to amikacin, ciprofloxacin, cotrimoxazol and imipenem, respectively (Table 3).

Of the forty five *K. pneumoniae* isolated from patients with UTI, 11.11% (n=5), 4.44% (n=2), 44.44% (n=20) and 40% (n=18) were obtained in spring, summer, fall and winter, respectively. Of the five *K. pneumoniae* isolated in spring, 20% (n=1), 20% (n=1), 40% (n=2), 0.00% (n=0) and 40%

(n=2) were found resistant to aztreonam, cefpodoxime, cefteriaxone, cefotaxime and ceftazidime, respectively. Therefore, at the screening stage in spring, 20% (n=1) were suspected of being able to produce ESBLs. Of the two *K. pneumoniae* isolated in summer, only 50% (n=1) were resistant to ceftazidime, while no resistance to other antibiotics were observed. Therefore, no *K. pneumoniae* was suspected of producing ESBLs in this season. Of the twenty *K. pneumoniae* isolated in fall, 45% (n=9), 45% (n=9), 50% (n=10), 55 % (n=11) and 45% (n=9) were found resistant to aztreonam, cefpodoxime, cefteriaxone, cefotaxime and ceftazidime, respectively. Thus, at the screening stage in fall, 45% (n=9) were suspected of being able to produce ESBLs. Of the eighteen *K. pneumoniae* isolated in winter, 61.11% (n=11), 61.11% (n=11), 88.88% (n=16), 50% (n=9), and 77.77% (n=14) were resistant to azteronam, cefpodoxime, cefteriaxone, cefotaxime and ceftazidime, respectively. Therefore, at the screening stage in winter, 61.11% (n=11) were

Table 1. Primers of PCR amplification of ESBLs genes

primers	Sequence of primers	Size of amplicon	References
blaTEM	F: 5-GAGTATCAACATTTCCGTGTC-3 R: 5-TAATCAGTGAGGCACCTTCTC-3	885bp	6
blaSHV	F: 5–AAGATCCACTATCGCCCAGCAG-3 R: 5-ATTCAGTTCCGTTTCCCAGCGG-3	235bp	6
blaCTX-M	F:5-ACGCTGTTGTTAGGAAGTG-3 R:5-TTGAGGCTGGGTGAAGT-3	759bp	7

Table 2. Antibiotic resistance of third-generation of cephalosporins and aztreonam

Emam Reza Hospital	*K.pneumoniae*	Ca	Ce	Ci	Cep	Ao
Total	103 (100%)	59 (57.3%)	40 (38.8%)	64 (62.1%)	47 (45.6%)	45 (43.7%)

Table 3. Effect of non-beta lactam antibiotics on *K.pneumoniae* producing ESBLs

	KPPE	Ak	Cf	Co
Total	45 (100%)	12 (26.7%)	3 (6.7%)	9 (20%)

two *K. pneumoniae* isolated in fall, 50% (n=1), 50% (n=1), 100% (n=2), 50% (n=1) and 50% (n=1) were resistant to azteronam, cefpodoxime, cefteriaxone, cefotaxime and ceftazidime, respectively. Therefore, at the screening stage in fall, 50% (n=9) were suspected of being able to produce ESBLs. Of the eighteen *K. pneumoniae* isolated in winter, 66.7%

Table 4. Confirming stage and antibiogram panel of *K.pneumoniae* producing ESBLs from patients with UTI

	KPSPE	Cac	Cec	Cepc	Ak	Cf	Co	I
Spring	1 (4.8%)	1 (100%)	0	1 (100%)	0	0	0	0
Fall	9 (42.8%)	8 (88.9%)	1 (11.1%)	9 (100%)	3 (33.3%)	0	2 (22.2%)	
Winter	11 (52.4%)	9 (81.8%)	3 (27.3%)	11 (100%)	4 (36.4%)	1 (9%)	2 (18.8%)	0
Total	21	18	4	21	7	1	4	

suspected of being able to produce ESBLs.

Of the fourteen *K. pneumoniae* isolated from patients in ICUs, 21.42% (n=3), 21.42% (n=3), 14.3% (n=2) and 42.85% (n=6) were obtained in spring, summer, fall and winter, respectively. Of the three *K. pneumoniae* isolated in spring, 33.33% (n=1), 66.66% (n=2), 66.66% (n=2), 33.33% (n=1) and 33.33% (n=1) showed resistance to aztreonam, cefpodoxime, cefteriaxone, cefotaxime and ceftazidime, respectively. Therefore, at the screening stage in spring, 33.33% (n=1) were suspected of being able to produce ESBLs. Of the three *K. pneumoniae* isolated in summer, 33.33% (n=1), 33.33% (n=1), 33.33% (n=1) and 66.66% (n=2) were found to be resistant to aztreonam, cefpodoxim, cefteriaxone and ceftazidime, respectively. The results showed that all isolates were susceptible to cefotaxime. Thus, atthe screening stage in summer, 33.33 % (n=1) were suspected of being able to produce ESBLs. Of the

Table 5. Confirming stage and antibiogram panel of *K. pneumoniae* producing ESBLs isolated from patients in ICUs

	KPSPE	Cac	Cec	Cepc	Ak	Cf	Co	I
Spring	1 (14.3%)	1 (100%)	0	1 (100%)	0	0	0	0
Summer	1 (14.3%)	1 (100%)	1 (100%)	1 (100%)	0	0	1 (100%)	0
Fall	1 (14.3%)	1 (100%)	0	1 (100%)	1 (100%)	0	0	0
Winter	4 (57.1%)	4 (100%)	0	4 (100%)	0	0	1 (25%)	0
Total	7 (100%)	7 (100%)	1 (14.2%)	7 (100%)	1 (14.2%)	0	2 (28.5%)	0

Table 6. Confirming stage and antibiogram panel of *K. pneumoniae* producing ESBLs isolated from patients in surgery ward

	KPSPE	Cac	Cec	Cepc	Ak	Cf	Co	I
summer	1 (50%)	1 (100%)	1 (100%)	1 (100%)	0	0	1 (100%)	0
winter	1 (50%)	1 (100%)	0	1 (100%)	0	0	0	0

Table 7. Confirming stage and antibiogram panel of *K.pneumoniae* producing ESBLs isolated from patients with lesion Infections

	KPSPE	Cac	Cec	Cepc	Ak	Cf	Co	I
Spring	1 (33.3%)	1 (100%)	0	1 (100%)	0	0	0	0
Summer	1 (33.3%)	0	0	0	0	0	0	0
Winter	1 (33.3%)	1 (100%)	0	1 (100%)	0	0	0	0
Total	3 (100%)	2 (66.6%)	0	2 (66.6%)	0	0	0	0

(n=4), 66.7% (n=4), 83.4% (n=5), 50% (n=3) and 83.4% (n=5) were resistant to aztreonam, cefpodoxime, cefteriaxone, cefotaxime and ceftazidime, respectively. So, at the screening stage in winter, 66.7% (n=11) were suspected of being able to produce ESBLs.

Of the eight *K. pneumoniae* isolated from patients admitted in surgery wards, 25% (n=2), 37.5% (n=3), 12.5% (n=1) and 25% (n=2) were obtained in spring, summer, fall and winter, respectively. Of the three *K. pneumoniae* isolated in spring, no *K. pneumoniae* were observed to be resistant to any antibiotics. Of the three *K. pneumoniae* isolated in summer, 33.33% (n=1), 33.33% (n=1), 66.66% (n=2), 33.33% (n=1) and 66.66% (n=2) were found resistant to aztreonam, cefpodoxime, cefteriaxone, cefotaxime and ceftazidime, respectively. Thus, at the screening stage in summer, 33.33 % (n=1) were suspected of being able to produce ESBLs. The results showed that there was only one *K. pneumoniae* isolated in fall which showed no resistance to any antibiotics. Of the two *K. pneumoniae* isolated in winter, 50% (n=1), 50% (n=1), 100% (n=2), 50% (n=1) and 100% (n=2) were resistant to aztreonam, cefpodoxime, cefteriaxone, cefotaxime and ceftazidime, respectively. Therefore, at the screening stage in winter, 50 % (n=1) were suspected of being able to produce ESBLs.

Of the twelve *K. pneumoniae* isolated from patients with lesion infections, 33.3% (n=4), 25% (n=3), 16.7% (n=2) and 25% (n=3) were obtained in spring, summer, fall and winter, respectively. Of the four *K. pneumoniae* isolated in spring, 25% (n=1), 25% (n=1), 25% (n=1) and 25% (n=1) were found to be resistant to aztreonam, cefpodoxime, cefteriaxone and ceftazidime, respectively. The results showed there was no resistance to cefotaxime. Therefore, at the screening stage in spring, 25% (n=1) were suspected of be able to produce ESBLs. Of the three *K. pneumoniae* isolated in summer, 33.33% (n=1) showed resistance to aztreonam and 33.33% (n=1) to ceftazidime. All of them were susceptible to cefpodoxime, cefteriaxone and cefotaxime. Of the two *K.pneumoniae* isolated in fall, resistance was only seen to ceftazidime. Of the two *K.pneumoniae* isolated in winter, 33.3% (n=1), 33.3% (n=1), 66.7% (n=2), 33.3% (n=1) and 66.7% (n=2) were resistant to aztreonam, cefpodoxime, cefteriaxone, cefotaxime and ceftazidime, respectively. Thus, at the screening stage in winter, 33.3 % (n=1) were suspected of being able to produce ESBLs.

Of the twelve *K. pneumoniae* isolated from patients with RTIs, 33.4% (n=8), 20.83% (n=5), 20.83% (n=5) and 25% (n=6) were found in spring, summer, fall and winter respectively. Of the eight *K. pneumoniae* isolated in spring, 50% (n=4), 62.5% (n=5), 87.5% (n=7), 75% (n=6) and 100% (n=8) were resistant to aztreonam, cefpodoxime, cefteriaxone, cefotaxime and ceftazidime, respectively. Therefore, at the screening stage in spring, 50% (n=4) were suspected of being able to produce ESBLs. Of the five *K. pneumonia* isolated in summer, 20% (n=1), 20% (n=1), 20% (n=1), 40% (n=2) and 40% (n=2)

Table 8. Confirming stage and antibiogram panel of *K.pneumoniae* producing ESBLs isolated from patients with RTI

	KPSPE	Cac	Cec	Cepc	Ak	Cf	Co	I
Spring	4 (30.8%) (100%)	4 (25%)	1 (100%)	4 (25%)	1	0 (25%)	1	0
Summer	1 (7.7%)	1 (100%)	0	1 (100%)	0	0	0	0
Fall	3 (23%)	3 (100%)	0	3 (100%)	1 (33.3%)	1 (33.3%)	1 (33.3%)	0
Winter	5 (38.5%)	5 (100%)	0	5 (100%)	1 (20%)	0	1 (20%)	0
Total	13 (100%)	13 (100%)	1 (7.7%)	13 (100%)	3 (23%)	1 (7.7%)	3 (23%)	1 (7.7%)

were resistant to aztreonam, cefpodoxime, cefteriaxone, cefotaxime and ceftazidime, respectively. Thus, at the screening stage in summer, 20 % (n=1) were suspected of being able to produce ESBLs. Of the five *K. pneumoniae* isolated in fall, 60% (n=3), 60% (n=3), 80% (n=4), 60% (n=3) and 60% (n=3) were resistant to aztreonam, cefpodoxime, cefteriaxone, cefotaxime and ceftazidime, respectively. So, at the screening stage in fall, 60% (n=3) of the isolates were suspected of being able to produce ESBLs. Of the six *K. pneumoniae* isolated in winter, 83.3% (n=5), 83.3% (n=5), 100% (n=6), 83.3% (n=5) and 100% (n=6) were resistant to aztreonam, cefpodoxime, cefteriaxone, cefotaxime and ceftazidime, respectively. Therefore, at the screening stage in winter, 83.3% (n=5) were suspected of being able to produce ESBLs.

Confirming stage

Of the forty-five *K. pneumoniae* collected from patients with UTI, 46.7% were suspected of being able to produce ESBLs at the screening stage. Of these 4.8% (n=1), 42.8% (n=9) and 52.4% (n=11) were obtained in spring, fall and winter. One *K. pneumoniae* suspected of being able to produce ESBL was obtained in spring and confirmed by ceftazidime/clavulanic acid and cefpodoxime/ clavulanic acid at the confirming stage. Of the nine *K. pneumoniae* suspected of producing ESBLs in fall, 88.9% (n=8), 11.1% (n=1) and 100% (n=9) were confirmed by ceftazidime/clavulanic acid, cefotaxime/clavulanic acid and cefpodoxime/ clavulanic acid respectively, at the confirming stage. Of the eleven *K. pneumoniae* suspected of being able to produce ESBLs in winter, 81.8% (n=9), 27.3% (n=3) and 100% (n=11) were confirmed by ceftazidime/clavulanic acid, cefotaxime/clavulanic acid and cefpodoxime/clavulanic acid, respectively, at the confirming stage (Table 4). Of the seventeen *K. pneumoniae* collected from patients admitted in ICUs, 50% were suspected of being able to produce ESBLs at the screening stage. Of these, 14.3% (n=1), 14.3% (n=1), 14.3% (n=1) and 57.1% (n=4) were found in spring, summer, fall and winter, respectively. One *K. pneumoniae* suspected of producing ESBL was obtained in spring and confirmed by ceftazidime/clavulanic acid and cefpodoxime/clavulanic acid. One *K. pneumoniae* suspected of producing ESBLs was found in summer and confirmed by ceftazidime/clavulanic acid, cefotaxime/clavulanic acid and cefpodoxime/ clavulanic acid. One *K. pneumoniae* suspected of being able to produce ESBL was obtained in autumn and confirmed by ceftazidime/clavulanic acid as well as cefpodoxime/clavulanic acid at the confirming stage. All the four *K. pneumoniae* suspected of being able to produce ESBLs in winter, were confirmed by ceftazidime/clavulanic acid and cefpodoxime/clavulanic acid (Table 5).

Of the eight *K. pneumoniae* collected from patients in surgery wards, 25% (n=2) were suspected of being able to produce ESBLs at the screening stage. Of these 50% (n=1) were obtained in summer and 50% (n=1) in winter. Both were confirmed by ceftazidime/clavulanic acid and cefpodoxime/clavulanic acid (Table 6).

Of the twelve *K. pneumoniae* collected from patients with lesion infections, 25% (n=3) were suspected of producing ESBLs at the screening stage, of which 33.3% (n=1), 33.3% (n=1) and 33.3% (n=1) were found in spring, summer, and winter, respectively. The *K. pneumoniae* suspected of producing ESBLs in spring and winter was confirmed by ceftazidime/clavulanic acid and cefpodoxime/clavulanic acid. However, one *K. pneumoniae* obtained in summer showed that it was prone to producing ESBL. However it was resistant to ceftazidime and aztreonam and susceptible to cefpodoxime. Therefore it was not confirmed at the confirming stage (Table 7).

Of the twenty five *K. pneumoniae* collected from patients with RTIs, 54.1% (n=13) were suspected of producing ESBLs at the screening stage, of which 30.8% (n=4), 7.7% (n=1), 23% (n=3) and 38.5% (n=5) were obtained in spring, summer, fall and winter, respectively. The results showed that all of them were confirmed by ceftazidime/clavulanic acid and cefpodoxime/ clavulanic acid, except for *K. pneumoniae* collected in spring, which was confirmed by ceftazidime/clavulanic acid, cefotaxime /clavulanic acid and cefpodoxime/clavulanic acid (Table 8).

Antimicrobial panel

The results showed that in Emam Reza Hospital, the *K. pneumoniae* isolated from patients with UTI were found to be able to produce ESBLs Of the nine *K. pneumoniae* producing ESBLs in fall, 33.3% (n=3) were resistant to amikacin and 22.2% (n=2) cotrimoxazol . Of the eleven *K. pneumoniae* producing ESBLs in winter, 36.4%

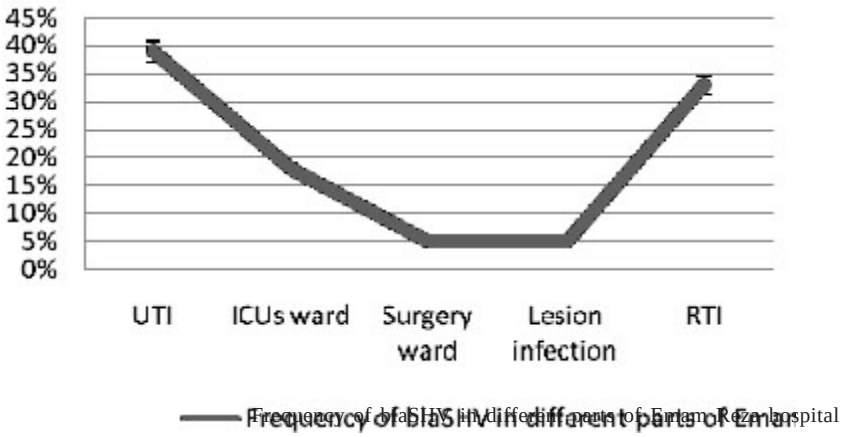

Fig. 1. Frequency of blaSHV

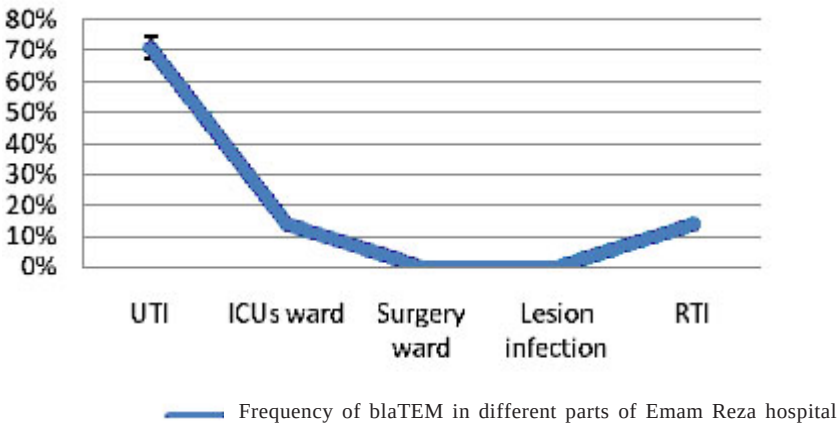

Fig. 2. Frequency of blaTEM

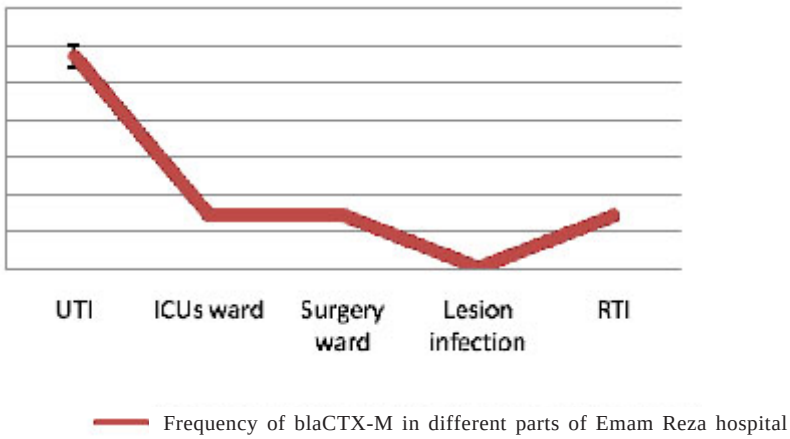

Fig. 3. Frequency of blaCTX-M

(n=4), 9% (n=1) and 18.8% (n=2) of isolates were resistant to amikacin, ciprofloxacin, and cotrimoxazol, respectively (Table 4). In the ICUs, one *K. pneumoniae* producing ESBLs was isolated in each season, except spring, which was found to be resistant to cotrimoxazol in summer and winter. The *K. pneumoniae* producing ESBLs isolated in fall, showed resistance to amikacin (Table 5). Among the patients in the surgery wards, only one *K. pneumoniae* producing ESBLs in summer was found resistant to cotrimoxazol (Table 6). No resistance to non-beta-lactam antibiotics was observed among all the patients with lesion infections. Among the patients with RTI, of the four *K. pneumoniae* producing ESBLs in spring, 25% (n=1) were found resistant to amikacin and 25% (n=1) to cotrimoxazol. In summer, no resistance to any antibiotics was seen. Of the three *K. pneumoniae* producing ESBLs in fall, 33.3% (n=1), 33.3% (n=1) and 33.3% (n=1) were found to be resistant to amikacin, ciprofloxacin and cotrimoxazol, respectively. Of the five *K. pneumoniae* producing ESBLs in winter, 20% (n=1) were resistant to amikacin and 33.3% (n=1) to cotrimoxazol (Table 8).

PCR results

Of the thirty nine *K. pneumoniae* with blaSHV, 38.5% (n=15), 18% (n=7), 5.1% (n=2), 5.1% (n=2) and 33.3% (n=13) were from patients with UTI, patients in ICUs, surgery wards, patients with lesion infections and patients with RTIs, respectively (Fig. 1).

Of the seven *K. pneumoniae* with blaTEM, 71.4% (n=5), 14.3% (n=1) and 14.3% (n=1) were obtained from the patients with UTI, patients in ICUs and patients with RTIs, respectively (Figure 2). Of the seven *K. pneumoniae* with blaCTX-M, 57.1% (n=4), 14.3% (n=1), 14.3% (n=1) and 14.3% (n=1) were from patients with UTIs, patients in ICUs, surgery wards and the patients with RTIs, respectively (Fig. 3).

Of the fifteen *K. pneumoniae* with blaSHV from patients with UTIs, 100% (n=1), 88.9% (n=8) and 55.4% (n=6) were obtained in spring, fall and winter, respectively. Five *K. pneumoniae* with blaTEM were observed, of which 22.3% (n=2) were obtained in fall and 27.3% (n=3) in winter. Of the four *K. pneumoniae* with blaCTX-M from the patients with UTI, 11.1% (n=1) and 27.3% (n=3) were obtained in fall and winter, respectively. Of the seven *K. pneumoniae* with blaSHV from patients admitted in ICUs, 100% (n=1), 100% (n=1), 100% (n=1) and 100% (n=4) were obtained in spring, summer, fall and winter, respectively. One *K. pneumoniae* with blaTEM was isolated in spring and another *K.pneumoniae* with blaCTX-M was isolated in summer.

Of the two *K. pneumoniae* with blaSHV from patients admitted in surgery wards, 100% (n=1) and 100% (n=1) were obtained in summer and winter, respectively. The results showed that only one *K.pneumoniae* with blaCTX-M was isolated in summer. Of the two *K. pneumoniae* with blaSHV from lesion infections, 100% (n=1) were found in spring and 100% (n=1) in winter. Of the thirteen *K. pneumoniae* with blaSHV from patients with RTIs, 100% (n=4), 100% (n=1), 100% (n=3) and 100% (n=5) were obtained in spring, summer, fall and winter, respectively. Only one *K.pneumoniae* with blaTEM and blaCTX-M was isolated in spring. The results showed that 6.7% of *K.pneumoniae* positive ESBLs harbored two or three ESBLs genes, and frequency of blaSHV-TEM, blaSHV-CTX-M, and balSHV-TEM-CTX-M were 1%, 4.8% and 1%, respectively. These statistical analyses also indicated 86.7%, 15.6% and 15.6% of *K.pneumoniae* producing ESBLs as being positive for blaSHV, blaTEM and blaCTX-M, respectively

DISCUSSION

The development of extended-spectrum cephalosporins in the early 1980s was regarded as major addition to our therapeutic armamentarium in the fight against beta-lactamase-mediated bacterial resistance[8]. Regrettably, the emergence of *K. pneumoniae* resistant to ceftazidime and other cephalosporins seriously compromised the efficacy of these life saving antibiotics. The new bacterial beta-lactamases present in these common enteric bacilli (the parent TEM-1 and SHV-1 enzymes) demonstrated unique hydrolytic properties. Point mutations in the *bla*SHV and *bla*TEM genes that resulted in single amino acid changes (Gly238 '!Ser, Glu240'!Lys, Arg164'!Ser, Arg164'!His, Asp179'!Asn, and Glu (Asp) 104 '!Lys) formed the basis of this remarkable resistance phenotype[9]. Currently, ESBLs are becoming a major threat for patients in the hospitals, long-term care facilities and community.

Patients with infection due to ESBL-producing enterobacteria tend to have less satisfactory outcomes than those infected by pathogens that do not produce ESBLs (Marra *et al.,* 2006). In a prospective multinational study analyzing bloodstream infections due to ESBL-producing *K.pneumoniae* isolates, cephalosporin monotherapy was found to be associated with a 40% 14-day mortality rate[10].

Generally, ESBLs production in winter was more than the other seasons, our finding also had signed winter as a dominant season for resistance toward non-beta-lactam antibiotics. The highest resistance to third-generation of cephalosporins at the Emam Reza Hospital, was to cefteriaxone (62.1%) Table 2. The resistance to non-beta-lactam antibiotic was for amikacin (26.7%) (Table 3).

In patients with UTI, the highest resistance at the screening stage was found in cefteriaxone (88.8%) in winter. The ESBLs production by *K.pneumoniae* was 52.4%, while resistance to amikacin, 36.4% and to ciprofloxacine, 9% in winter. Cotrimoxazol resistance was 22.2% in fall which was higher than in the other seasons (Table 4).

At the screening stage of patients admitted in ICUs in fall, we observed greater resistance to cefteriaxone (100%). The results showed that ESBLs production by *K.pneumoniae* in winter was higher than in the other seasons. Ciprofloxacin and imipenem were found to be the most effective antibiotics in this study. The highest resistance to cotrimoxazol (100%) and amikacin (100%) was observed in summer and fall respectively (Table 5).

The results indicated that the highest percentage of antibiotic resistance was observed in patients admitted to the surgery wards during winter, who had been given ceftazidime and cefteriaxone at the screening stage (100%). It was found that except for cotrimoxazol in summer, the rest of non-beta-lactam antibiotics were effective antibiotics (Table 6). In patients with lesion infections, the most antibiotic resistant at the screening stage was obtained for ceftazidime (66.7%) in winter. The results showed that all non-beta-lactam antibiotics were good choices for prescription. In patients with RTIs, the findings showed that resistance to ceftazidime in spring and winter (100%) were higher than other antibiotics in different seasons at the screening stage. *K.pneumoniae* producing ESBLs were observed to be more in winter (38.5%). The highest resistance to cotrimoxazol, amikacin and ciprofloxacin resistance was seen in fall (33.3%) (Table 8).

In an investigation in Madrid (Spain) for analyzing ESBLs and comparing the *Klebsiella spp*. producing ESBL during 2001-04 with that of 1989-2000, an increase of *Klebsiella spp* producing ESBL from 2.5% to 4.8% during 2001–4 (3.2% mean) and from 0.4% to 18.2% (4.8% mean) during 1989–2000 was obtained. The increased diversity of ESBLs during 2001–04 was due to the appearance of new enzymes in their geographical area (TEM-110, SHV-11, SHV-12, CTX-M-14 and CTXM-15) with persistence of previously identified enzymes (TEM-4, SHV-2, CTX-M-9 and CTX-M-10). Distribution of different ESBL groups during 2001–4 was as follows: SHV-type 44%, TEM-type 26%, CTX-M-1 cluster 25%, and CTX-M-9 cluster 5%. The appearance of CTX-M-15-producing isolates in 2002 and the increase in their rate during the last year of the search was noteable. Moreover, very few isolates expressed CTX-M-9 or CTX-M-14 enzymes. A polyclonal structure, including epidemic clones with specific ESBLs (TEM-4, SHV-12 and CTX-M-15), was observed. Phylogenetic analysis showed that most the isolates (74.6%) belonged to KpI-type with a clear relationship between KpIII-type and CTX-M-10 producers. Persistence of specific plasmids associated with specific ESBLs (TEM-4, SHV-12, CTX-M-10 and CTX-M-15) had observed. A co-resistance analysis revealed an increment in resistance to trimethoprim (41.5% versus 10.3%), sulphonamide (54.7% versus 29.3%) and nalidixic acid (34% versus 6.9%) when compared with that of 1989–2000[11]. In a study in Milad hospital in Tehran between March and June 2009, of one hundered and fifteen strains of *K.pneumoniae* from urine specimens of patients admitted to Milad Hospital of Tehran 12% isolates of *K. pneumoniae* were positive for ESBLS[12].

These studies showed different percentage of *K.pneumoniae* producing ESBLs in different regions in the world. Our results revealed frequency of ESBLs amongst *K. pneumoniae* was 43.7% that was more than Milad hospital in 2009 and Madrid.

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