Antimicrobial Pattern and Clonal Dissemination of Extended-Spectrum Beta-Lactamase Producing \textit{Klebsiella Spp} Isolates

\textsuperscript{1}Sobhan Ghafourian, \textsuperscript{2}Nourkhoda Sadeghifard, \textsuperscript{1}Zamberi bin Sekawi, \textsuperscript{1}Vasantha Kumari Neela, \textsuperscript{1}Mariana Nor Shamsudin, \textsuperscript{2}Reza Mohebi, \textsuperscript{3}Mohammad Rahbar and \textsuperscript{4}Mohammad Raftari

\textsuperscript{1}Department of Medical Microbiology and Parasitology, Faculty of Medicine and Science Health, University Putra, Malaysia
\textsuperscript{2}Department of Medical Microbiology, Ilam university of Medical Sciences, Iran
\textsuperscript{3}Reference laboratory of Iran
\textsuperscript{4}Faculty of Food Science and Technology, University Putra Malaysia, Malaysia

\textbf{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 \textit{Klebsiella spp} producing ESBLs towards non-beta-lactam antibiotics in the different seasons, identify the various clonal types of ESBL-producing \textit{K. pneumoniae} and detect the dominant ESBL clonal types.\textbf{Approach:} Clinical isolates of \textit{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 \textit{K. pneumoniae}. \textbf{Results:} The findings showed that 51.6\% of \textit{K. pneumoniae} were produces ESBLs. 35.8, 21.2 and 38.7\% of \textit{K. pneumoniae} producing ESBLs were resistant to amikacin, ciprofloxacin and cotrimoxazol, respectively. It was found that 40 and 27.3\% of \textit{K. oxytoca} producing ESBLs were resistant to cotrimoxazol and amikacin, respectively. The findings reflected that ESBLs existed in 73\% of \textit{K. oxytoca}. The results showed that the frequency of bla\textit{SHV}, bla\textit{TEM} and bla\textit{CTX-M} due to \textit{K. pneumoniae} producing ESBLs were 87.5, 12.4 and 24.8\%, respectively. Of the eleven \textit{K. oxytoca} producing ESBLs, 100\% bla\textit{SHV} were obtained. Based on the nucleotide variations of the five genetic loci, twenty-five different STs could be identified among thirty \textit{K. pneumoniae} producing ESBLs isolates. Among the STs shared by multiple isolates, the most frequently encountered were 14, 16 and ST18. \textbf{Conclusion:} In conclusion, the percentage of \textit{K. oxytoca} producing ESBLs was higher than \textit{K. pneumoniae} producing ESBLs. Generally, \textit{K. pneumoniae} produces more ESBLs in winter and fall than in the other seasons.

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

\textbf{INTRODUCTION}

In recent years, extended-spectrum beta-lactamase-producing \textit{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 \textit{K. pneumoniae} in Milad Hospital in Tehran of Iran, as Iran is a four-seasoned country. \textit{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 \textit{Klebsiella spp}, investigate the susceptibility of \textit{Klebsiella spp} producing ESBLs towards non-beta-lactam antibiotics in the different seasons, identify the various clonal types of ESBL-producing \textit{K. pneumoniae} and detect the dominant ESBL clonal types.

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial isolates:} Clinical isolates of \textit{Klebsiella spp} were identified during the period March 2007-April
Table 1: Primers for PCR

| Primers   | Sequence of primers | Size of amplicon | References        |
|-----------|---------------------|------------------|-------------------|
| Blatem    | F: 5-GAGTATCAACATTTCCGTGTC-3 R: 5-TAACGATGAGGCACACATCCTTC-3 | 848bp            | (Nasehi et al. 2010) |
| Blashv    | F: 5-AGAGATCCACTATTGCCTGCCCACAGCAG-3 R: 5-ATTCAGTTCCGTGTCATAGGCACACAGCAG-3 | 231bp            | (Nasehi et al. 2010) |
| Blactx-m  | F: 5-ACGCTGTTGTTAGGAAGTG-3 R: 5-TTGAGGCTGGGTGAAGT-3 | 759bp            | Mansouri and Ramazanadeh (2009) |

Table 2: Primers for MLST

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

2008 in Milad hospital in Tehran. The strains were isolated from urinary tract infections, ICUs, surgery wards, lesions 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).

**PCR amplification of blaTEM, blaSHV and blaCTX-M:** The Polymerase Chain Reaction (PCR) was carried out by using primers in Table 1. *K. pneumoniae* ATCC 700603 was used as a control 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* (glyceraldyes 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 unique sequence.
allelic profile, represented as a series of five integers corresponding to the alleles at each of the loci, in the order of \textit{rpoB}, \textit{gyrA}, \textit{gapA}, \textit{groEL} and \textit{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 \textit{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 \textit{Klebsiella} spp. Two hundred and sixty-five \textit{K. pneumoniae} and fifteen \textit{K. oxytoca} were obtained.

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

Screening stage: Of the two hundred and sixty-five \textit{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 \textit{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 \textit{K. pneumoniae} showed the highest resistance to cefteriaxone (59.4\%) and cefazidime (56.25\%). Generally, thirteen \textit{K. pneumoniae} were suspected of being able to produce ESBLs (Table 6). Twenty-six \textit{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 \textit{K. pneumoniae} were prone to ESBLs production (Table 7). Twenty-two \textit{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 \textit{K. pneumoniae} (Table 9).

Confirming stage: All \textit{K pneumoniae} suspected to produce ESBLs had confirmed as showed in the Tables 8-12 in the confirming stage.

| Table 3: Screening stage of all \textit{K.pneumoniae} in all parts of Milad hospital |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | \textit{K.pneumoniae} | Cefazidime | Cefotaxime | Cefteriaxone | Cefpodoxime | Aztreonam |
| Total            | 265              | 170         | 125         | 163           | 138           | 137          |
|                  | (65.8\%)         | (46.4\%)    | (60.4\%)    | (52\%)        | (51\%)        | (51\%)      |

| Table 4: Effect of non-beta-lactam antibiotics against \textit{K.pneumoniae} producing ESBLs in all parts of Milad hospital |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | \textit{K.pneumoniae} producing ESBLs | Amikacin | Ciprofloxacin | Cotrimoxazol |
| Total            | 137             | 49             | 29             | 53             |
|                  | (35.8\%)        | (21.2\%)      | (38.7\%)      |

| Table 5: Screening stage of \textit{K.pneumoniae} isolated from patients with UTI |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | \textit{K.pneumoniae} | Cefazidime | Cefotaxime | Cefteriaxone | Cefpodoxime | Aztreonam |
| Spring          | 31              | 11            | 19            | 18             | 16             | 16           |
|                  | (23.14\%)       | (35.48\%)    | (61.29\%)    | (58.06\%)     | (51.61\%)     | (51.61\%)   |
| Summer          | 21              | 9             | 2             | 8              | 5              | 5            |
|                  | (15.67\%)       | (42.85\%)    | (9.52\%)     | (38.09\%)     | (23.8\%)      | (23.8\%)    |
| Fall            | 39              | 21            | 15            | 19             | 18             | 18           |
|                  | (29.1\%)        | (53.85\%)    | (38.46\%)    | (48.71\%)     | (46.15\%)     | (46.15\%)   |
| Winter          | 43              | 32            | 18            | 32             | 29             | 29           |
|                  | (32.08\%)       | (74.41\%)    | (41.86\%)    | (74.41\%)     | (67.44\%)     | (67.44\%)   |
| Total           | 134             | 73            | 54            | 77             | 68             | 68           |
|                  | (100\%)         | (54.4\%)     | (40.3\%)     | (57.5\%)      | (50.74\%)     | (50.74\%)   |
Table 6: Screening stage of K. pneumoniae of admitted patients in ICUs ward

| ICUs | K.pneumoniae | Ceftazidime | Cefotaxime | Cefteraxone | 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%)       | (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%)     | (100%)      | (71.4%)    | (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         | 19          | 14          | 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 | Cefteraxone | 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 | Ceftriaxone | 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. pneumoniae producing ESBLs from patients with UTI

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

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

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

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

| Surgery ward | K. pneumoniae / clavulanic acid | Ceftazidime / clavulanic acid | Cefotaxime / clavulanic acid | Cefpodoxime | Amikacine | Ciprofloxacin | Cotrimoxazol | Imipenem |
|--------------|--------------------------------|-------------------------------|-------------------------------|-------------|-----------|---------------|--------------|----------|
| Spring       | 1                              | 1                             | 0                             | 1           | 0         | 0             | 0            | 0        |
|              | (9.1%)                         | (100%)                        | (100%)                        | (100%)      | (0%)      | (0%)          | (0%)         | (0)      |
| Summer       | 4                              | 4                             | 1                             | 4            | 1         | 0             | 2            | 0        |
|              | (36.4%)                        | (100%)                        | (25%)                         | (100%)      | (25%)    | (50%)         | (50%)        | (0)      |
| Fall         | 2                              | 2                             | 1                             | 2            | 1         | 1             | 1            | 0        |
|              | (18.2%)                        | (100%)                        | (50%)                         | (100%)      | (50%)    | (50%)         | (50%)        | (0)      |
| Winter       | 4                              | 4                             | 2                             | 4            | 2         | 1             | 2            | 0        |
|              | (36.4%)                        | (100%)                        | (50%)                         | (100%)      | (50%)    | (25%)         | (50%)        | (0)      |
| Total        | 11                             | 11                            | 4                             | 11            | 4         | 2             | 5            | 0        |
|              | (100%)                         | (100%)                        | (36.6%)                       | (100%)      | (36.6%)  | (18.1%)       | (45.5%)      | (0)      |
Table 13: Confirming stage and effect of non-beta-lactam antibiotics toward K.pneumoniae producing ESBLs isolated from patients with lesion infection

| K.pneumoniae suspected to produce ESBLs | Cefazidime /clavulanic acid | Cefotaxime /clavulanic acid | Cefpodoxime /clavulanic acid | Amikacine | Ciprofloxacine | Cotrimoxazol | Imipenem |
|----------------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------|----------------|-------------|----------|
| Spring                                 | 2 (25%)                     | 1 (50%)                     | 2 (100%)                    | 0         | 0              | 0           | 0        |
| Summer                                 | 1 (12.5%)                   | 0 (100%)                    | 1 (100%)                    | 0         | 0              | 0           | 0        |
| Fall                                   | 2 (25%)                     | 2 (100%)                    | 1 (100%)                    | 1 (50%)   | 1 (50%)        | 1 (50%)     | 1 (50%)  |
| Winter                                 | 3 (37.5%)                   | 2 (100%)                    | 3 (100%)                    | 0         | 0              | 0           | 0        |
| Total                                  | 8 (100%)                    | 8 (100%)                    | 4 (100%)                    | 0         | 0              | 0           | 0        |

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

| K.pneumoniae suspected to produce ESBLs | Cefazidime /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%)   | 0 (42.85%)|
| Summer                                 | 2 (5.4%)                    | 2 (100%)                    | 1 (50%)                     | 2 (100%)  | 1 (50%)        | 1 (50%)     | 0 (50%)  |
| Fall                                   | 8 (21.6%)                   | 8 (100%)                    | 2 (50%)                     | 8 (100%)  | 2 (50%)        | 2 (50%)     | 0 (50%)  |
| Winter                                 | 20 (54.1%)                  | 16 (80%)                    | 8 (40%)                     | 20 (100%) | 7 (35%)        | 4 (20%)     | 8 (40%)  |
| Total                                  | 37 (100%)                   | 32 (86.5%)                  | 14 (37.9%)                  | 37 (100%) | 13 (35.1%)     | 8 (21.6%)   | 14 (37.9%)|

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, 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 spring, summer, fall and winter, respectively. Of the four K. pneumoniae with blaSHV from patients with lesions infections, 25% (n = 2), 12.5% (n = 1), 12.5% (n =1) 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 electrophoresis 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 electrophoresis 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 Colonial 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 | DLV | 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 | DLV | 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 | DLV | 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    | 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, 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 winter and both were suspected of being able to produce ESBLs. They were confirmed by ceftazidime/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 cefpodoxim/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 cefpodoxim/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 cefpodoxim/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  | ICUs | 3 | 5 | 12 | - | |
| 2 1-3-1-5-4    | UTI | 23 | 12 | 12 | 15 | |
| 3 4-4-2-4-3    | rti | 16 | 12 | 12 | - | |
| 4 2-2-6-3-10   | uti | 1 | 12 | 12 | 15 | |
| 5 4-2-2-4-3    | rti | 17 | 5 | 12 | 12 | - | |
| 6 4-4-2-4-3    | rti | 16 | 12 | 12 | 15 | |
| 7 1-2-6-3-10   | UTI | 4 | 12 | 12 | 15 | |
| 8 2-1-2-4-2    | rti | 14 | 5 | 12 | 12 | 15 | |
| 9 2-1-2-4-2    | rti | 14 | 5 | 12 | 12 | 15 | |
| 10 2-2-5-3-9   | uti | 5 | 12 | 12 | - | |
| 11 5-4-2-4-1   | rti | 21 | 5 | 12 | - | |
| 12 2-7-4-1-10  | surgery | 13 | 4 | 12 | 15 | |
| 13 2-9-4-1-10  | rti | 12 | 4 | 12 | 15 | |
| 14 2-2-4-4-7   | rti | 11 | 2 | 12 | - | 15 | |
| 15 2-2-4-4-8   | surgery | 9 | 2 | 12 | - | 15 | |
| 16 2-2-3-4-8   | rti | 10 | 2 | 12 | - | |
| 17 2-2-3-4-5   | icus | 20 | - | 12 | 12 | - | |
| 18 1-10-2-4-6  | lesion | 19 | 5 | 12 | - | |
| 19 2-4-6-3-10  | uti | 3 | 12 | 12 | - | |
| 20 6-11-5-6-9  | rti | 6 | 12 | 12 | - | |
| 21 4-1-1-1-4   | surgery | 18 | - | 12 | - | |
| 22 4-1-1-1-4   | surgery | 18 | - | 12 | - | |
| 23 6-2-5-6-9   | icus | 8 | 3 | 12 | 12 | - | |
| 24 1-1-3-4-2   | rti | 25 | - | 12 | - | |
| 25 2-6-2-2-3   | rti | 24 | - | 12 | - | |
| 26 2-2-5-3-10  | uti | 2 | 12 | 12 | - | |
| 27 2-1-5-4-2   | rti | 15 | 5 | 12 | - | |
| 28 2-3-3-4-2   | uti | 22 | - | 5 | - | |
| 29 2-1-2-4-2   | rti | 14 | 5 | 12 | - | |
| 30 2-1-2-4-2   | rti | 14 | 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%)      | 6 (54.5%)    |

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

| Surgery ward | K. oxytoca | Cefazidime | Cefotaxime | Cefteraxone | 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

| Fall | K.oxytoca | Cefazidime | Cefotaxime | Cefotyroxone | Cefpodoxime | Aztreonam |
|------|------------|------------|------------|--------------|-------------|-----------|
| 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 | Cefazidime | Cefotaxime | Cefotyroxone | Cefpodoxime | Aztreonam |
|-----|------------|------------|------------|--------------|-------------|-----------|
| Fall | 2 (16.6%) | 2 (100%) | 0 | 2 (100%) | 2 (100%) | 2 (100%) |
| Winter | 10 (83.3%) | 8 (80%) | 4 | 7 (70%) | 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 | Cefazidime | Cefotaxime | Cefpodoxime | Amikacin | Ciprofloxacine | Cotrimoxazol | Imipenem |
|--------------|------------|------------|------------|--------------|----------|---------------|--------------|----------|
| Winter | 2 (100%) | 2 (100%) | 0 | 2 (100%) | 0 | 1 | 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 | Cefazidime | Cefotaxime | Cefpodoxime | Amikacin | Ciprofloxacine | Cotrimoxazol | Imipenem |
|------------------|------------|------------|------------|--------------|----------|---------------|--------------|----------|
| Fall | 1 (100%) | 1 (100%) | 0 | 1 (100%) | 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 | Cefazidime | Cefotaxime | Cefpodoxime | Amikacin | Ciprofloxacine | Cotrimoxazol | Imipenem |
|-----|------------|------------|------------|--------------|----------|---------------|--------------|----------|
| Fall | 2 (25%) | 2 (100%) | 0 | 2 (100%) | 0 | 1 | 0 |
| Winter | 6 (75%) | 6 (100%) | 0 | 6 (100%) | 2 | 6 (33.3%) | 4 | 0 |
| Total | 8 (100%) | 8 (100%) | 0 | 8 (100%) | 3 | 3 (37.5%) | 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 was in 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 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 colonial type by MLST. The dominant Sequence Type (ST) was ST14. The findings of this study showed different colonial 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. pneumoniae* produces more ESBLs in winter and fall than in the other seasons. The dominant gene responsible for ESBLs production was *bla*SHV. 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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