CHARACTERIZATION OF EXTENDED-SPECTRUM B-LACTAMASE-PRODUCING ESCHERICHIA COLI STRAINS ISOLATED FROM DAIRY PRODUCTS

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ABSTRACT
Extended-spectrum β-lactamases (ESBLs) are enzymes that hydrolyze the β-lactam ring, and ESBL-producing E. coli has rapidly spread worldwide with pose a serious hazard for humans. The aim of this study was to determine the prevalence of ESBL producing E. coli and molecular evaluation of four ESBL-associated genes among E. coli strains isolated from milk and cheese in southern Iran. Antibiotic susceptibility test was carried out for a total of 150 isolates of E. coli, previously collected from dairy products. ESBL production was screened using a double-disc synergy test (DDST) and presence of four ESBL genes (PER, VEB, TEM and CTX-M) was tested using PCR. Among 150 E. coli strains 57 (38%) isolates were identified as ESBL-producing strains. All ESBL positive isolates could be typed for one or more genes and the most prevalent ESBL-associated gene was CTX-M (80.7%). The PER gene was not present among isolates. Isolates showed high susceptibility to imipenem and cefotaxin. The results showed the high prevalence of ESBL producing E. coli strains among dairy products and high occurrence of CTX-M-associated ESBL activity among isolates indicating the hazards of increasing the strains with antibiotic resistance which can transfer to human trough the dairy food products.

Keywords: ESBL, Escherichia coli, antibiotic susceptibility, dairy products

INTRODUCTION
Extended-spectrum β-lactamases (ESBLs) are enzymes that compromise the efficacy of all β-lactams, apart from cephamycins and carbapenems, by hydrolysis of the β-lactam ring, and are inhibited by β-lactamase inhibitors (Coque et al., 2008). The most common cause of resistance to expanded-spectrum cephalosporins in Escherichia coli is the production of ESBLs (Paterson, 2006) and ESBL-producing E. coli have rapidly spread worldwide with pose a serious hazard for health care-associated (HA) infection. ESBLs have been reported from all parts of the world. However, prevalence varies widely even in closely related regions. Most of the clavulanic acid-inhibited ESBLs are either derivatives of narrow-spectrum TEM and SHV-type β-lactamases or CTX-M, PER, VEB, and GES/IBC-type β-lactamases (Bauerfeind et al., 1996; Nordmann, 1998; Pitout et al., 2005). ESBL producing organisms are often resistant to several other classes of antibiotics, as the plasmids with the gene encoding ESBLs often carry other resistance determinants. Initially ESBL producing organisms were isolated from nosocomial infections but these organisms are now also being isolated from community (Pitout and Laupland, 2008). The TEM-1 enzyme was first reported from an E.coli isolate in 1965 and is now the most common β-lactamase found in Enterobacteraceae (Fonzi et al., 1995). The CTX-M family, first described in 1992 (Bauerfeind et al., 1992), is known to be the most dominant non-TEM, non-SHV ESBL among Enterobacteriaceae and is recognized as a rapidly growing family of ESBLs that selectively prefer to hydrolyze cephalosporins rather than cepazidime (Bonnet, 2004). CTX-M group The latter is a small, but growing, family of plasmid encoded ESBLs that hydrolyze cephalosporins and although 20 CTX-M enzymes have been described between 1989 and 2001 in various enterobacterial species but mostly clinical isolates of Salmonella typhimurium, Escherichia coli and Klebsiella pneumonia carry CTX-M-1 (Barthelmy et al., 1992; Bauerfeind et al., 1996). VEB-1 ESBL was identified among Enterobacteriaceae and Pseudomonas aeruginosa isolates and was previously reported from Thailand (Girlich et al., 2001; Girlich et al., 2002). The widespread incidence of VEB-1 in Enterobacteriaceae and Pseudomonas aeruginosa suggests that this ESBL gene is prevalent in numerous gram-negative species (Girlich et al., 2001; Girlich et al., 2002). The PER family, first identified in P. aeruginosa, is also among β-lactamase family exhibiting ESBL like activities. Nevertheless, epidemiologic data on this less common ESBLs are very limited (Kiratisin et al., 2008). A zoonotic contribution to the spread of extended-spectrum β-lactamase (ESBL)-producing Escherichia coli at the community level has been proposed repeatedly (Carattoli, 2008). Until now, no data were available regarding the types and frequency of ESBLs in food origin isolated E. coli strains in Iran. In this study, we investigated the prevalence and antibiotic susceptibility of ESBL producing E. coli strains in southern Iran. In this study, we investigated the prevalence and antibiotic susceptibility of ESBL producing E. coli strains in southern Iran. In this study, we investigated the prevalence and antibiotic susceptibility of ESBL producing E. coli strains and occurrence of four ESBL-associated genes (TEM, CTX-M, VEB and PER) among raw dairy samples including milk and cheese in Shiraz, southern Iran.

MATERIAL AND METHODS
Bacterial strains
A total of 150 isolates of E. coli, previously collected in the department of Public Health and Food Hygiene, School of Veterinary Medicine, Shiraz University, were used in present study. Each isolate obtained from one sample separately and cheese and milk samples were collected from different milk and cheese sources referred to the school of veterinary medicine from cattle mastitis milk, rural dairt products or traditional marketing of dairy products around Shiraz. Strains were obtained from raw dairy products (milk and cheese) by conventional cultivation methods during June 2012 to September 2013. Positive control for PCR method was conducted using the reference strains Pseudomonas aeruginosa U2A1125 (for PER gene) and Acinetobacter baumannii U2A2026 (for VEB gene). The amplicoms of the positive samples were used as positive control for the TEM and CTX-M genes after the reaction was set up.

Antimicrobial drug susceptibility testing and ESBL detection
Antimicrobial drug susceptibility was determined by a disc-diffusion method on Mueller-Hinton (MH) agar plates (Merck, Germany), according to the antibigram standard methods. The following antimicrobial agents were tested: ampicillin (10 µg), gentamicin (10 µg), cefotaxime (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), imipenem (10 µg), tetracycline (30 µg), cephaparin (30 µg),
sulfamethoxazole-Trimethoprim (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), amoxicillin/clavulanic acid (20/10 µg), cefepime (30µg) and aztreonam (30 µg). Quality control was conducted using the reference strain E. coli ATCC 25922. ESBL production was screened using a double-disc synergy test (DDST) as a standard disc-diffusion assay on MH agar. Disks containing aztreonam (30 mg), cefazidime (30 mg), cefepime (30 mg) and cefotaxime (30 mg) were placed at a distance of 30 mm (centre to centre) around a disc containing amoxicillin/clavulanic acid (20/10 µg). Isolates that were DDST negative and resistant to third-generation cephalosporins were screened for an ESBL phenotype. All antibiotic disks were from Merck, Germany.

DNA preparation

A loopful colony of each isolate on agar plate was picked and suspended in 200 µL of distilled water. After suspending, the suspension was boiled for 5 min, and 50 µL of the supernatant was collected after spinning for 10 min at 14,000 rpm in a microcentrifuge. The DNA concentration of boiled extracts was determined with spectrophotometer (Lin et al., 1996).

PCR assay

PCR amplifications were performed in a final volume of 25 µL in PCR tubes. The reaction mixtures consisted of 2 µL of the DNA template, 2.5 µL 10x PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM MgCl₂, 50 mM KCl, 20 mM (NH₄)₂SO₄), (CinnaGen, Iran), 1 µL dNTPs (50 µM), (CinnaGen, Iran), 1 µL (1U Ampli Taq DNA polymerase), (CinnaGen, Iran), 1 µL (25 pmol) from the forward and reverse primers (CinnaGen, Iran), of both primer pairs (Table 1) and the volume of the reaction mixture was completed to 25 µL using distilled deionized water. The thermal cycler (MJ mini, BioRad, USA) was adjusted under the following conditions: Initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing as shown in Table 1 for 1 min and extension at 72°C for 1 min. Final extension was carried out at 72°C for 7 min and the PCR products were stored in the thermal cycler at 4°C until they were undertaken using a UV transilluminator (BTS-20, Japan) and The 100 bp DNA ladder was used as molecular size marker.

Table 1 Nucleotide sequences used as primers in the PCR reaction of four ESBL-associated genes in E. coli strains.

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Target gene</th>
<th>Annealing temperature</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX-un-1-F</td>
<td>CAGTGCCGAYACCAGCTA</td>
<td>blaCTX-M-1-cluster</td>
<td>42°C</td>
<td>544</td>
<td>(Saladin et al., 2002)</td>
</tr>
<tr>
<td>CTX-un-1-R</td>
<td>CGCCRTATGCTTGGTGTTG</td>
<td>blaCTX-M-1-cluster</td>
<td>43°C</td>
<td>569</td>
<td>(Saladin et al., 2002)</td>
</tr>
<tr>
<td>PER</td>
<td>ATGAATGTCATTAAAAAGC</td>
<td>blaPER</td>
<td>47°C</td>
<td>925</td>
<td>(Girlich et al., 2001)</td>
</tr>
<tr>
<td>PERR</td>
<td>AATTTGGGCTTGGGCAGAA</td>
<td>blaPER</td>
<td>47°C</td>
<td>925</td>
<td>(Girlich et al., 2001)</td>
</tr>
<tr>
<td>VEBF</td>
<td>CGACTCTCCATTCCCGATGC</td>
<td>blaVEB</td>
<td>51°C</td>
<td>643</td>
<td>(Naas et al., 2001)</td>
</tr>
<tr>
<td>VEBR</td>
<td>GGACTCTGCAACAAATACGC</td>
<td>blaVEB</td>
<td>51°C</td>
<td>643</td>
<td>(Naas et al., 2001)</td>
</tr>
<tr>
<td>OT3</td>
<td>ATGGAAGTTCCACATTGCC</td>
<td>blaTEM</td>
<td>46°C</td>
<td>850</td>
<td>(Eckert et al., 2006)</td>
</tr>
<tr>
<td>OT4</td>
<td>CCAAAGTGTCAATGCTGAG</td>
<td>blaTEM</td>
<td>46°C</td>
<td>850</td>
<td>(Eckert et al., 2006)</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

Among 150 E. coli strain isolated from dairy products 57 (38%) isolates were identified as ESBL-producing strains using DDST method. All ESBL positive isolates could be typed for one or more genes. The most prevalent ESBL-associated gene was CTX-M which was present among 46 of 57 (80.7%) strains. The PER gene was not present among isolates. Among ESBL-strains 12 (21.1%) showed multiple presence of ESBL-associated genes (CTX-M+VEB and CTX-M+TEM) but 45 (78.9%) strains showed solitary occurrence of the genes. All CTX-M producing strains were susceptible to imipenem. There were no significant (p>0.05) differences in presence of ESBL genes between milk and cheese isolates but some antibiotics (CTX, CF, NA) showed significantly (p<0.05) high resistant level in milk isolates compared with cheese isolates. Detailed results of antibiotic susceptibility testing and prevalence of ESBL-associated genes were listed in tables 2 and 3.

Among gram-negative pathogens and Enterobacteriaceae family, particularly E. coli, resistance to an extended spectrum ß-lactamase is increasingly associated with ESBLs. The accurate recognition of ESBL producing microorganisms is a challenge for the clinical associated laboratories, requiring not only phenotypic tests, but also genotypic tests for genes related with ß-lactamase production (Kaftandzieva et al., 2011). The prevalence of ESBL-producing E. coli in Asia is reported differ from 5% in Japan to 20-50% in other countries and in Europe, the prevalence varies from 3% in Sweden to 34% in Portugal (Babini et al., 2000; Cantorn et al., 2008; Winokur et al., 2001). In other study conducted in Iran Eslami and Najar Peerayeh (2012) showed that 47% of strains were ESBL producing E. coli. However, epidemiologic data and characterization of ESBL-producing E. coli in Iran are still rarely documented. Some studies were conducted on ESBL strains obtained from humans (Eslami and Najar Peerayah, 2012). To our knowledge, this is the first description of ESBL producing E. coli in dairy productions in Iran. Occurrence of ESBL E. coli strains in present study was 38% (58/150) which is high prevalence compared with other similar studies (Cantorn et al., 2008; Winokur et al., 2001). Dissimilarities in occurrence of ESBL strains between our study and others may be due to the different origins. It seems that investigations on prevalence of these organisms among food animals and their products show higher presence as Ilse et al. (2011) showed that 76.8% of chicken meat samples contained ESBL-producing E. coli which the genotype blaCTX-M, the most frequent drug resistance gene in the samples. Present study reports a widespread distribution (80.7%) of CTX-M gene in ESBL E. coli strains isolated from milk and cheese dairy products in accordance with other studies which previously reported the increase in occurrence of this gene compared with other ESBL-associated genes (Baudry et al., 2009; Arpin et al., 2009). The genotypic methods help us to confirm the genes responsible for ESBL production. The PER gene was not present among isolates and TEM gene was present among 29.7% (15/51) of ESBL producing isolates. The VEB gene showed 10.5% (6/57) occurrence which was less than CTX-M and TEM genes. In similar studies, Tabbouch et al. (2011) showed 22.2% occurrence of the blaCTX-M and Eslami and Najar Peerayeh (2012) showed 44% presence of the blaCTX-M but they could not detect the PER and VEB genes. Kirafins et al. (2008) showed 77.0%, 3.8%, 99.6% and 8.5% prevalence of blavirus, BlaSHV, blaCTX-M and blaVEB groups respectively. We did not investigate the presence of the SHV gene in due to less importance and clear distribution of this gene between previous above studies. Plasmid-mediated ESBLs, such as PER and VEB beta-lactamases, are uncommon and have been found mainly in P. aeruginosa at a limited number of geographic sites. PER-1 isolates in Turkey, France and Italy; VEB-1 and VEB-2 isolates in strains from Southeast Asia but CTX-M enzymes, the most dominant non-TEM, non-SHV ESBL among Enterobacteriaceae, have been involved in various epidemiological situations and have disseminated throughout all continents as a result of epidemic plasmids and/or particular epidemiic strains (Ruppe, 2010). Isolates showed 10%, 17.3%, 36% and 47.3% resistance to imipenem, cefotaxin, cefepime, and ceftazidime respectively. Statistical analysis declared that ESBL-producing strains significantly (p<0.05) showed high resistance to cephalotin 54/57 (94.7%) and cefepime (100%). Fifteen (10%) of isolates were resistant to Imipenem, which 11 of them were ESBL. Carbapenems belong to the ß-lactam group of antibacterial agents. They are not inactivated by extended-spectrum ß-lactamases and Carbapenem resistance is emerging in ESBL-producing Enterobacteriaceae (Woodford et al., 2007). Carbapenem resistance has been rarely reported in E. coli. The occurrence of an outermembran porin deficiency and the expression of a plasmid-mediated class C ß-lactamase were reported to be responsible for carbapenem resistance in E. coli (Stapleton et al., 1999). We did not investigate the presence of carbapenem resistance genes (KPC, OXA, MBL). The results showed the presence and emerging existence of carbapenem resistant isolates among ESBL E.coli. In our study, we found 54.6% (82/150) of E. coli isolates tested to be resistant to different third-generation cephalosporins, but only 57 (38%) isolates
showed ESBLs producing phenotype. Milk isolates showed significantly higher resistance to Cefotaxime and Nalidixic acid. β-Lactamase (mainly extended-spectrum cephalosporins and carbapenems) constitute the main therapeutic choices to treat infections caused by Enterobacteriaceae microorganisms. However, resistance to these compounds has been reported increasingly from different parts of the world in recent years (Canto’n et al., 2008; Reinet et al., 2007) and therapeutic options for infections due to ESBL producers have also become increasingly limited indicating that continuous monitoring systems and effective infection control measures are absolutely required. It is indistinct whether ESBL genes in different types of food samples are related to a reservoir in food-production animals or contamination at processing facilities and more studies are necessitate describing the diversities among E. coli isolates obtained from various origins.

### Table 2 Antimicrobial resistance of E. coli strains isolated from dairy products.

<table>
<thead>
<tr>
<th>Origin</th>
<th>No</th>
<th>Antibiotic resistance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>100</td>
<td>FOX CAZ CTX AUG AZM FEP CF GM TE SXT IPM NA CP AM</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>(17) 50 43 78 99 45 93 (93) 77 89 67 70 40 80</td>
</tr>
<tr>
<td>Cheese</td>
<td>50</td>
<td>(18) 21 11 30 48 19 31 28 34 30 6 27 17 32</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>(17.3) 71 54 108 147 64 124 105 123 97 15 97 57 112</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>(47.3) 36 (72) 98 (42.6) 82.6 105 82 (10) 64.6 (10) 64.6 (38) 74.6</td>
</tr>
</tbody>
</table>

Abbreviations: AM, Ampicillin; CP, Ciprofloxacin; NA, Nalidixic acid; IPM, Imipenem; SXT, Sulfamethoxazole-trimethoprim; TE, Tetracyclin; GM, Gentamicin; CF, Cephalothin; FEP, Cefepime; AZM, Aztreonam; AUG, Ampicillin-clavulanic acid; CTX, Cefotaxime; CAZ, Ceftazidime; FOX, Cefoxitin.

### Table 3 Occurrence of ESBL producing E. coli and ESBL-associated genes among isolates.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Number of isolates</th>
<th>ESBL producing (%)</th>
<th>Positive for ESBL genes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>100</td>
<td>43(43)</td>
<td>3(6.9)</td>
</tr>
<tr>
<td>Cheese</td>
<td>50</td>
<td>14(28)</td>
<td>1(7.1)</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>57(38)</td>
<td>4(7)</td>
</tr>
</tbody>
</table>

Abbreviations: VEB, TEM, CTX-M, CTX-M+VEB, CTX-M+TEM.

### CONCLUSION

In conclusion, we report the first study regarding the prevalence and molecular characterization of ESBL genes and the epidemiology of ESBL-producing E. coli isolates recovered from dairy products in Iran. Present study showed that blaCTX-M is the most prevalent ESBL gene among dairy isolates including milk and cheese. The results of antibiotic susceptibility revealed high rates of resistance against the cephalothin and aztreonam.

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**REFERENCES**


