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CHARACTERIZATION OF EXTENDED-SPECTRUM Β-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 cefoxitin. 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 **(Bauernfeind *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-lactemase found in *Enterobactereceae* **(Fonze *et al.*, 1995)**. The CTX-M family, first described in 1992 **(Bauernfeind *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 cefotaxime rather than ceftazidime **(Bonnet, 2004)**. CTX-M group The latter is a small, but growing, family of plasmid encoded ESBLs that hydrolyze cefotaxime 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 **(Barthelemy *et al.*, 1992; Bauernfeind *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 *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 amplicons 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 antibiogram standard methods. The following antimicrobial agents were tested: ampicillin (10 µg), gentamicin (10 µg), cefatoxime (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), imipenem (10 µg), tetracycline (30 µg), cephalotin (30 µg), sulfamethoxazole-Trimethoprim (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), amoxicillin/clavulanic acid (20/10 µg), cefepime (30µg) and aztreonam (30 mg). 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. Discs containing aztreonam (30 mg), ceftazidime (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 mg). 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 vortexing, 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 $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$), (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 collected. Amplified products were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide. Visualization was undertaken using a UV transilluminator (BTS-20, Japan) and The 100 bp DNA ladder was used as molecular size marker.

Statistical analysis

Statistical analysis of the occurrence of the genes and phenotypic properties of the isolates was performed using SPSS version 12.0.1. Discrete variables were expressed as percentages and proportions were compared using the Chi-square test with the significance level defined at $P < 0.05$.

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

Name of primer	Sequence (5` to 3`)	Target gene	Annealing temperature	Product size (bp)	Reference
CTX-un-1-F CTX-un-1-R	CATGTGCAGYACCAGTAA CCGCRATATCRTTGGTGGTG	*blaCTX-M-1-cluster*	42ºC	544	**(Saladin *et al*., 2002)**
PERF PERR	ATGAATGTCATTATAAAAGC AATTTGGGCTTAGGGCAGAA	*blaPER*	47ºC	925	**(Girlich *et al*., 2001)**
VEBF VEBR	CGACTTCCATTTCCCGATGC GGACTCTGCAACAAATACGC	*blaVEB*	51ºC	643	**(Naas *et al*., 2001)**
OT3 OT4	ATGAGTATTCAACATTTCCG CCAATGCTTAATCAGTGAGG	*blaTEM*	46ºC	850	**(Eckert *et al*., 2006)**

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. Isolates showed high susceptibility to imipenem and cefoxitin and high resistance to amoxicillin-clavulanic acid, aztreonam, cefepime, cephalothin, tetracycline and ampicillin. Statistical analysis declared that ESBL-producing strains significantly ($p<0.05$) showed high resistance to caphalotin 54/57 (94.7%) and cefepime (100%). 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 beta-lactamse 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 beta-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; Canton *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 Peerayeh, 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 **(Canton *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 bla_{CTX-M} was 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% (17/57) 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, **Tabbouche *et al*. (2011)** showed 22.2% occurrence of the bla_{TEM} and Eslami **and Najar Peerayeh (2012)** showed 44% presence of the bla_{TEM} but they could not detect the *PER* and *VEB* genes. **Kiratisin *et al*. (2008)** showed 77.0%, 3.8%, 99.6% and 8.5% prevalence of *bla*TEM, *bla*SHV, *bla*CTX-M and *bla*VEB 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 in isolates in Turkey, France and Italy; VEB-1 and VEB-2 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 epidemic strains **(Ruppe´, 2010)**. Isolates showed 10%, 17.3%, 36% and 47.3% resistance to imipenem, cefoxitin, cefotaxime, 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 β-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 outermembrane 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-Lactamse (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; Reinert *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.

Origin	No	Antibiotic resistance (%)													
		FOX	**CAZ**	**CTX**	**AUG**	**AZM**	**FEP**	**CF**	**GM**	**TE**	**SXT**	**IPM**	**NA**	**CP**	**AM**
Milk	**100**	17 (17)	50 (50)	43 (43)	78 (78)	99 (99)	45 (45)	93 (93)	77 (77)	89 (89)	67 (67)	9 (9)	70 (70)	40 (40)	80 (80)
Cheese	**50**	9 (18)	21 (42)	11 (22)	30 (60)	48 (96)	19 (34)	31 (62)	28 (56)	34 (68)	30 (60)	6 (12)	27 (54)	17 (34)	32 (64)
Total	**150**	26 (17.3)	71 (47.3)	54 (36)	108 (72)	147 (98)	64 (42.6)	124 (82.6)	105 (70)	123 (82)	97 (64.6)	15 (10)	97 (64.6)	57 (38)	112 (74.6)

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

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

Origin	Number of isolates	ESBL producing (%)	Positives for ESBL genes (%)				
			VEB	CTX-M	CTX-M+VEB	CTX-M+TEM	TEM
Milk	100	43 (43)	3(6.9)	26(60.4)	2(4.6)	8(18.6)	4(9.3)
Cheese	50	14 (28)	1(7.1)	8(57.1)	0(0)	2(14.2)	3(21.4)
Total	150	57 (38)	4(7)	34(59.6)	2(3.5)	10(17.5)	7(12.2)

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 *bla*$_{CTX-M}$ 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 cephalotin and aztreonam.

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