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Characterization of virulence genes, serogroups and antimicrobial susceptibility of Shiga toxin producing *Escherichia coli* isolated from bovine mastitic milk in Tehran, Iran

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Abstract. Clinical mastitis caused by Enterobacteriaceae accounts for significant economic loss in dairy herds. One of the important pathogens that causes mastitis is Shiga toxin-producing *Escherichia coli* (STEC). Moreover, mastitis caused by STEC can be considered as a source of transmission of STEC strains to humans through unpasteurized milk. The aim of the current study was to determine of the prevalence, the identification of serogroups, the molecular characterization of virulence factors, and the antibiotic resistance properties of STEC isolates from bovine mastitic milk in dairy cattle in Tehran. A total of 325 milk samples from dairy cattle with clinical signs of mastitis were collected. All *E. coli* isolates (n: 87, 26.7%) were subjected to multiplex PCR for the detection of *stx1*, *stx2*, *eaeA*, and *ehly* genes and serogroups. Antibiotic susceptibility testing was carried out by the disc diffusion method for all the STEC isolates. Eighty-seven (26.8%) *E. coli* and 9 (2.8%) STEC strains were isolated from the bovine mastitic milk samples. Shiga-like toxin genes (*stx1* and *stx2* or one of them), *eaeA* and *ehly* were detected in 100%, 66.6%, and 33.3% of STEC isolates, respectively. O26 (22.2%) and O111 (22.2%) were the most commonly detected STEC serogroups. Other serogroups included O145, O121, O128, O157 and O113. High resistance rate to ampicillin and tetracycline (100%) was observed, followed by trimethoprim/sulfamethoxazole (66.6%) and chloramphenicol (55.5%). STEC isolates were found in bovine mastitic milk in Tehran and most of the STEC isolates in our study were non-O157 strains.

INTRODUCTION

Clinical mastitis caused by Enterobacteriaceae accounts for significant economic loss in dairy herds. Mastitis is one of the most economically important disease that causes reduced production of milk and discarded milk, high costs for veterinary services and treatment, and sometimes animal suffering (Lira *et al.*, 2004, Salwa *et al.*, 2011). *Escherichia coli* mastitis accounts for a considerable proportion of clinical mastitis cases among dairy cattle and could be associated with a wide range of systemic diseases (Wenz *et al.*, 2001).

E. coli is part of the normal flora of the human and animal gastrointestinal tract, but certain *E. coli* strains such as enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC) and entero-

toxigenic *E. coli* (ETEC) are associated with gastroenteritis and diarrhea in humans and domestic animals (Franzin & Sircili, 2015, Jayamani & Mylonakis, 2014, Wang *et al.*, 2010, Shafaati *et al.*, 2016). The term Shiga toxin-producing *E. coli* (STEC), also referred to as verocytotoxin-producing *E. coli* (VTEC), refers to *E. coli* strains capable of producing bacteriophage-encoded Shiga toxin 1 (Stx1), Shiga toxin 2 (Stx2) or both, encoded by *stx1* and *stx2* genes, respectively (Gyles, 2007). The Stx1 and Stx2 groups contain several subtypes. However, Stx2 comprises a category that is significantly more heterogeneous and involves an expanding number of subtypes such as Stx2vha, Stx2vhb, Stx2g, Stx2dact, Stx2e, Stx2f and Stx2O118 (Prager *et al.*, 2011, Fernandez *et al.*, 2013). Virulent STEC strains may also express several accessory virulence factors that cause severe human illnesses, including intimin (encoded by *eaeA*), enterohaemolysin (encoded by *ehly*) and autoagglutinating protein (encoded by *saa*) (Fernandez *et al.*, 2013, Herold *et al.*, 2009). Concretely, STEC strains producing Stx belong to certain serotypes, including O157:H7, O26:H11, O103:H2, O111:H8, O121:H19, and O145:H28 (Bugarel *et al.*, 2010).

STEC are important foodborne pathogens associated with a broad spectrum of diseases in humans, ranging from mild gastroenteritis and diarrhea to severe syndromes, such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). Due to the extremely low infective dose of STEC and the high intensity of the associated disorders especially in children, the pathogens have been categorized as foodborne pathogens that pose high risk to public health (Pizarro *et al.*, 2013). Cattle are considered a primary reservoir for STEC. For STEC, significant differences have been reported in herd incidences that range from 0.3 to 56% in beef cattle and 0.2 to 74% in dairy cattle (Singh *et al.*, 2015). The prevalence of STEC strains, as well as the presence of virulence genes and antibiotic resistance phenotypes vary according to different studies in Iran (Kargar & Homayoon, 2015). The main aim of the present study was to investigate the presence of STEC serotypes and specific virulence markers including *stx1*, *stx2*, *eaeA* and *ehly* in bovine mastitic milk collected from traditional dairy cattle in Tehran. Antibiotic susceptibility testing was also performed to further characterize the STEC isolates.

MATERIALS AND METHODS

Bacterial isolation and identification

Milk samples from dairy cows with clinical manifestation of mastitis such swelling and inflammation were obtained aseptically by expert veterinarians with laboratory training from twelve traditional dairy cattle farms in Tehran and surrounding areas from November 2014 to October 2015. California Mastitis Test (CMT) (Schalm & Noorlander, 1957) were performed by the veterinarians for identification of subclinical mastitis within the collection day. About 5 ml of confirmed mastitic milk samples(n=325) were collected in sterile screwed tubes for further microbial examination. Samples were kept in ice packs during the transfer to the microbiology laboratory for culture. Samples were streaked on MacConkey and EMB agar (Merck, Germany). Then agar plates were incubated at 37ºC and bacterial growth was assessed after 24 and 48 hr. Up to three suspect lactose-positive colonies were sub-cultured onto MacConkey agar to achieve pure cultures. Determination of *E. coli* strains was carried out by conventional biochemical tests including Gram staining, triple sugar iron agar (TSI), cytochrome oxidase, citrate, indole, and motility tests.

Antibiotic Susceptibility Test

Antibiotic susceptibility testing was carried out by the Kirby-Bauer disc diffusion method on Mueller Hinton agar (Merck, Germany) and the results were evaluated according to the 2014 Clinical Laboratory Standards Institute (CLSI) guidelines. The isolated strains were assayed against the following antibiotics: ampicillin (10 µg), cefotaxime (30 µg), ceftriaxone (30 µg), trimethoprim / sulfamethoxazole (1.25/23.75 µg), gentamicin (10 µg), kanamycin (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg), chloram-

phenicol (30 µg) and imipenem (10 µg) (Mast Co., UK). *E. coli* ATCC 25922 was used as a quality control for antibiotic susceptibility testing.

Extraction of total genomic DNA

Total genomic DNA of the isolates was extracted from overnight trypticase soy agar (TSA) cultures by the boiling method. Concisely, a loopful of pure colonies was picked from the TSA agar culture and they was suspended in 250µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and then boiled for 10 min. Then, samples were centrifuged at 10,000 × g for 5 min, and the supernatant was stored at -20ºC for later analysis.

Detection of virulence genes by multiplex PCR

The DNA templates were subjected to multiplex PCR assays with specific primers (Table 1), for the detection of *stx1*, *stx2*, *eae* and *ehly* virulence genes, according to Paton and Paton (Paton & Paton, 2002). Multiplex PCR reactions was performed in a total volume of 25 µl amplification mixture consisting of 3 µl template DNA, 1 unit Taq DNA polymerase (Cinnagen, Iran), 0.3 µM of each oligonucleotide primer, 0.2 mM dNTP mix, 2 mM $MgCl_2$, 2.5 µl of 10× PCR buffer and PCR grade water. Samples were subjected to 35 cycles of touch-down PCR, each involving 1 min denaturation at 95ºC, 2 min annealing at 65ºC for the first 10 cycles, decreasing to 60ºC by cycle 15 and 1.5 min elongation at 72ºC, incrementing to 2.5 min from cycles 25–35. The PCR products were electrophoresed on 1.5% agarose gel for 90 min at 85 v and visualized by staining with ethidium bromide. Positive controls (*E. coli* O157:H7 reference strain EDL933) and negative controls (sterile water) were included in all PCR reactions. Only isolates that harbored *stx1* or *stx2*, or both of the genes, by multiplex PCR scheme were included in the study.

Detection of serotypes by multiplex PCR

The extracted genomic DNA, was amplified by three reactions of multiplex PCR (Table 2) for identification of STEC serotypes as recently described by Sánchez *et al.* (Sanchez *et al.*, 2015). Specific primer pairs targeting *wzx* or *wzy* genes of 15 STEC serogroups were designed corresponding to previous studies (Paton & Paton, 1998, Monday *et al.*, 2007).

First reaction contained specific primers for detection of O26, O103, O145, O121 and O111, second reaction contained O45, O113, O76, O128 and O146 primers, and third reaction contained O15, O157, O123, O172 and O165 primers. The PCR amplification mixture was as described above for the detection of virulence genes. Amplification was performed as follows: denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, repeated 25 times. The PCR products were analyzed as described above.

RESULTS

Out of 325 samples, 87 isolates (26.7%) were identified as *E. coli* by using culture media and conventional biochemical tests. Among the 87 *E. coli* isolates analyzed, nine isolates were identified as STEC strains by PCR as Shiga-like toxin genes (*stx1* and/or *stx2*) were detected from nine (10.3%) milk samples. Our findings revealed that nine STEC strains carried one or more of *stx1*, *stx2 eaeA*, *ehly* virulence genes, with different frequency.

Table 1. Primers used for detection of virulence genes in STEC isolated from bovine mastitis

Target Gene	Primer sequence (5´–3´)	PCR Product Size (bp)	References
stx1	ATAAATCGCCATTCG TTGACTACAGAACGCCCACTGAGATCATC	180	
stx2	GGCTCGCCAGTTATCTGACATTCT TCGCCAGTTATCTGACATTCTG	255	Paton and
eaeA	GACCCGGCACAAGCATAAGCCCACCTGCAGCAACAAGAGG	384	Paton (15)
ehly	GCATCATCAAGCGTACGTTCCAATGAGCCAAGCTGGTTAAGCT	534	

Table 2. Multiplex reactions used for identification of serogroups of STEC isolated from bovine mastitis

Multiplex	O type	Gene	Primer sequence (5´–3´)	PCR product (bp)	References
Reaction 1	O26	*wzx*	ACTCTTGCTTCGCCTGTT CAGCGATACTTTGAACCTTAT	268	Monday *et al.* (18).
	O103	*wzx*	TATCCTTCATAGCCTGTTGTT TTATAATAGTAATAAGCCAGACACC	327	Monday *et al.* (18).
	O111		GTTGCGAGGAATAATTCTTCA CCATAGATATTGCATAAAGGC	829	Monday *et al.* (18).
	O121		GTAGCGAAAGGTTAGACTGG ATGGGAAAGCTGATACTGC	651	Monday *et al.* (18).
	O145		TTGAGCACTTATCACAAGAGATT GATTGAATAGCTGAAGTCATACTAAC	418	Monday *et al.* (18).
Reaction 2	O45	*wzx*	GACTTTCGTTGCGTTGTG CTGCAAGTGTAGCGAAAAC	608	Sánchez *et al.* (16).
	O76	*wzx*	CATATGCAGATTGAAGGTAG GAAAGCCATAAAGTGCC	550	Sánchez *et al.* (16).
	O113	*wzy*	TAACGGGATTAGAAGTGGAT ATATAAGGCAGAAATGAGAGG	294	Sánchez *et al.* (16).
	O128	*wzx*	TTTCGATCGTCTTGTTCAGG CAATGGGCAATTAACACAGAG	193	Sánchez *et al.* (16).
	O146	*wzy*	ATCAGTTCATGGGTTGTATTC AGGAACATGGATGAAAGAAG	390	Sánchez *et al.* (16).
Reaction 3	O15	*wzx*	GCGTTGCCTACTTACTTATTATC ATGCAAGTCCAGCCAAAC	225	Sánchez *et al.* (16).

Table 3. Virulence gene profile and serogroups of STEC isolates from mastitic milk in Tehran

Isolate number	Virulence gene	Serogroups
84	*stx2*	O121
99	*stx1*, *stx2*, *eaeA*,	O157
123	*stx2*, *eaeA*	O145
178	*stx2*, *eaeA*, *ehly*	O128
203	*stx2*, *eaeA*, *ehly*	O26
214	*stx2*, *eaeA*	O113
276	*stx1*, *ehly*	O111
307	*stx2*, *eaeA*	O111
317	*stx1*, *stx2*	O26

The presence of the virulence genes among these nine STEC isolates are summarized in Table 3. Six of the nine (66.6%) STEC isolates carried the *eaeA* gene, mainly associated with *stx2* gene. One (11.1%) of the STEC isolates was positive only for *stx2* gene. Two isolates (22.2%) was positive for *stx1* and *stx2* simultaneously. *ehly* was detected in three (33.3%) STEC isolates.

We also found that O26 (22.2%) and O111 (22.2%) were the most commonly detected STEC O serogroups in the samples of bovine mastitic milk. Other serogroups included O145, O121, O128, O157 and O113 (Table 3).

Table 4. Antimicrobial resistance patterns of nine STEC strains from mastitic milk in Tehran

Isolate No.	AM	CTX	CRO	TMP-SMX	GM	K	TET	CP	C	IMP
84	R	S	S	R	S	S	R	S	R	S
99	R	S	S	S	S	S	R	S	S	S
123	R	S	S	S	S	S	R	R	R	S
178	R	S	S	R	S	S	R	S	S	S
203	R	S	S	S	S	S	R	S	S	S
214	R	R	R	R	R	R	R	R	R	S
276	R	S	S	R	R	R	R	R	R	S
307	R	S	S	R	S	S	S	S	R	S
317	R	S	S	R	S	S	R	S	S	S

Antibiotic susceptibility testing revealed that all of the isolates were resistant to ampicillin and tetracycline. High resistance frequency was observed for trimethoprim / sulfamethoxazole (66.6%) followed by chloramphenicol (55.5%). All the isolates were susceptible to imipenem. The results from antimicrobial susceptibility testing are presented in Table 4.

DISCUSSION

In the current study, we found less than 3% of the mastitic raw-milk samples tested contained STEC strains. Higher STEC isolation frequencies from diarrheic calves were reported in previous investigations in some provinces of Iran, including Isfahan, Fars, Chaharmahal, and Khuzestan (Shahrani *et al.*, 2014). This is important given that transmission of STEC from cattle to humans occurs via consumption of contaminated foods, such as unpasteurized milk, ground beef, water, vegetables and fruits (Amezquita-Montes *et al.*, 2015). Various authors around the world reported that the STEC strains can be isolated from raw milk and raw-milk dairy products (Hussein & Sakuma, 2005, Solomakos *et al.*, 2009, Stephan *et al.*, 2008). Several factors in traditional dairies can be important in milk contamination with STEC, including unhygienic practices during milk collection such as the use of old or unsanitized equipment and unsanitary methods of milking, and the contamination of raw milk by external sources such as dust, insects and laborers contaminated with bovine feces (Fremaux *et al.*, 2008).

In the present study, 87 *E. coli* isolates were recovered from 325 bovine mastitic milk samples collected from traditional dairy farms in Tehran and 10.3% of them considered as STEC. This estimate is lower than that reported by Momtaz *et al*. in 2012 in Shahrekord (Momtaz & Jamshidi, 2013), as out of 268 bovine mastitic milk samples tested, they detected 11 (15.1%) STEC isolates. In the other study in Kermanshah in 2013, Mohammadi *et al.* (Mohammadi *et al.*, 2013) reported that about 16% of raw-milk samples were contaminated with STEC. This difference can be due to variations in geographic area, number of cows on dairy farms, sampling, hygiene of farms, and the collection of samples in dissimilar seasons. Unfortunately, adequate studies about the prevalence of STEC in dairy products are not available in Iran and thus, the incidence of bovine mastitis due to STEC in the different regions of Iran is not known. Some investigators in the rest of the world reported the presence of STEC in raw milk. The reported prevalence estimates in raw milk varied from 0.87% to 21% in different countries such as Canada, Germany and France (Klie *et al.*, 1997, Perelle *et al.*, 2007, Steele *et al.*, 1997).

In this study multiplex-PCR was used to detect the *stx1*, *stx2*, *eaeA* and *ehly* virulence genes. Our findings indicated that the *stx2* gene was more prevalent than the *stx1* gene, which is in line with results of other authors (Mohammadi *et al.*, 2013, Momtaz & Jamshidi, 2013). However, these results

contrast with those reported by Askari *et al.* who found a predominance of the *stx1* gene in STEC isolates (Askari Badouei *et al.*, 2015). There is some conclusive evidence that *stx2* (particularly the subtypes *stx2a*, *stx2c*, and *stx2d*) is more associated with the development of HUS in humans (Brandal *et al.*, 2015). Moreover, we found that two STEC isolates (22.2%) harbored both *stx1* and *stx2* genes simultaneously, and almost the co-detection frequency of these two genes (16%) was reported by Askari *et al.* (2015).

The *eaeA* gene is located in in the LEE (locus of enterocyte effacement) pathogenicity island and encodes a 94-kDa outer membrane protein, intimin, which is a bacterial adhesin (Melton-Celsa *et al.*, 2012). Previously, it has been proposed that *stx*-positive *E. coli* isolates which lack *eaeA* may be less virulent for humans than *eaeA*-positive STEC strains (Beutin *et al.*, 1995). The presence of *eaeA*-positive STEC in raw milk has been reported in a few investigations in Iran. A high frequency (100%) of *eaeA*-positive STEC isolated from raw milk has been reported in the study by Momtaz *et al.* (2012). On the other hand, earlier data obtained by Mohammadi *et al.* indicated that all of 36 isolated STEC strains were *eae*-negative (Mohammadi *et al.*, 2013). In the current study, the majority of the STEC isolates (66.6%) from mastitic raw-milk samples harbored the *eaeA* gene.

The enterohaemolysin (*ehly* gene) was detected in 33.3% of the examined STEC isolates. The *ehly* gene was detected in 87.3% of the *E.coli* isolated from fecal samples of diarrheic children, sheep, and cattle in a survey conducted by Askari *et al.* in Garmsar (Askari Badouei *et al.*, 2015). Investigations by Momtaz *et al.* in Shahrekord & Shahrani *et al.* in Isfahan, Chaharmahal, Fars and Khuzestan provinces indicated that all of the STEC isolates harbored *ehly* (Momtaz *et al.*, 2012, Shahrani *et al.*, 2014). However, in the study conducted by Kargar *et al.* in Fars none of the STEC isolates were identified as *ehly*-positive (Kargar & Homayoon, 2015).

Since the prevalence of *stx1*, *stx2*, *eaeA* and *ehly* virulence genes was found to be different in various studies in Iran, it can be hypothesized that the prevalence of STEC strains in raw milk is affected by geographical area, sample type, seasonal variations during the sampling period and even differences between cattle species. It should also be noted that since the genes encoding Stx are thought to be generally encoded in the genome of lambdoid prophages, their prevalence can vary from strain to strain.

Our findings revealed higher isolation frequencies of serogroups O26 (22.22%) and O111 (22.22%). A high incidence of serogroups O157 (26%), O26 (12%) and O128 (8%) among 50 STEC isolated from dairy products was reported previously by Dehkordi *et al.* (2014). Momtaz and his colleagues also reported that O157 (26%) and O26 (12%) were the predominant STEC serogroups isolated from bovine mastitic milk samples (Momtaz *et al.*, 2012). These studies demonstrated that the majority of STEC strains isolated from dairy products belonged to the O26 and O157 serogroups. Even though no O111 was detected in the samples tested by these authors, in our study, O111 was one of the most frequently detected serogroups. In the present study 88.8% of the STEC isolates were non-O157 strains. However, serogroup distribution may vary from region to region, between different cities and within different parts of the country.

In our study, all of the STEC isolates were resistant to ampicillin and tetracycline. Moreover, high frequency of resistance was detected for trimethoprim /sulfamethoxazole (66.6%) and chloramphenicol (55.5%). Previous studies showed that tetracycline and beta-lactams were the most widely used antimicrobials to treat dairy cattle in Iran (Khaniki, 2007). Meanwhile, our research and previous investigations demonstrated the high antibiotic resistance frequency of STEC isolated from milk (Momtaz *et al.*, 2012, Rahimi *et al.*, 2011). Thus, in these circumstances, prescription of beta-lactams and tetracycline may not effective for the treatment of coliform bovine mastitis.

The high antimicrobial resistance found among some of the STEC isolates indicate a grave hazard towards the control of serious STEC infections in the future. Previously,

multidrug resistant STEC isolates have been reported by some authors around the world (Iweriebor *et al.*, 2015, Meng *et al.*, 2014). Unfortunately, only rare surveys on the antibiotic resistance among STEC strains isolated from mastic milk samples has been performed in Iran so far. Multi-drug resistant strains were detected in 65% of STEC isolated from raw milk in the survey conducted by Momtaz *et al.* (2012). Shahrani *et al.* reported antibiotic resistance to penicillin (100%), ampicillin (71.11%), tetracycline (98.09%), streptomycin (98.25%), gentamycin (79.68%), sulfamethoxazol (90.31%), trimethoprim (62.22%), lincomycin (92.69%), enrofloxacin (61.42%), ciprofloxacin (60.31%) and chloramphenicol (73.8%) among STEC isolated from diarrheic calves (Shahrani *et al.*, 2014). Dissemination and spread of antibiotic resistance among STEC strains can lead to problematic clinical implications, while the diarrheal phase of the disease associated with STEC is usually self-limiting and the role of early antibiotic therapy in the prevention of HUS is still imprecise. Continuous exposure to antibiotic agents used in livestock for the prophylaxis and disease treatment of dairy cattle can be an important factor in the spread of antibiotic resistance, especially of transmissible plasmid-mediated resistance genes. Therefore, cattle that are frequently treated with antibiotics are thought to act as a reservoir of resistance genes that can be transferred to other zoonotic and food-borne pathogens. These situations are frequently seen in developing countries. While in some developed countries, such as Switzerland, there was no increasing incidence and widespread dissemination of antibiotic resistance among mastitis pathogens during the last 20 years, signifying different points of view about this theme (Roesch *et al.*, 2006). Research done in the United States demonstrate that there is no correlation between increased antibiotic resistance and antibiotics that are frequently used in dairy cattle for treatment of mastitis (Erskine *et al.*, 2002).

CONCLUSIONS

In this study, we have detected the presence of STEC strains harbouring one or more of the *stx1*, *stx2*, *eaeA* and *ehly* genes in mastitic bovine milk. The prevalence of Shiga toxin-producing *E. coli* obtained in the study was less than those reported in other parts of Iran.

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CONFLICT INTERESTS

The authors declare that they have no conflict interests.

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