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Original Scientific Article

VIRULENCE PROPERTIES OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* ISOLATED FROM IRANIAN RAW MILK AND DAIRY PRODUCTS

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Summary: Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains are a diverse group of food-borne pathogens with various levels of virulence for humans. The main object of the present study was to determine virulence properties of STEC isolated from Iranian raw milk and dairy products. A total number of 300 samples, including sheep's (35), goat's (46), cow's (106), and buffalo's milk (21), soft cheese (42), butter (32) and ice cream (18), was obtained from farm bulk tanks, milk collection centres, and various supermarkets and retailer shops in different regions of Iran. Biochemical and molecular (PCR) method proved 26% of the samples to be *E. coli* positive, and among them, *Stx1* and *Stx2* genes were detected in 33 and 52 samples, respectively. But, *eaeA* and *sfaS* genes were not found in any of the sample. Beside *Stx2*, cnfI had the highest prevalence (42 isolates), and beside *eaeA* and *sfaS*, *fyuA* had the lowest prevalence of virulence genes. The results of this study demonstrate that there is a widespread distribution of potentially virulent *E. coli* strains in the environment and food that may be a cause of concern for human health.

Key words: STEC; *Escherichia coli*; virulence factors; raw milk

Introduction

Raw, unpasteurized milk is consumed directly by a large number of people in rural areas, and indirectly by a much larger segment of the population via consumption of several types of cheese. One of the main reasons why people prefer raw milk and its products may be their belief in their advantages or higher value compared to pasteurized milk (1). Many microorganisms, coliforms among them, can have access to milk and dairy products, and *Escherichia coli* are often used as marker organisms. Detecting and counting of *E. coli* is used as a reliable indicator of faecal contamination and indicates a possible presence of enteropathogenic and/or toxigenic microorganisms which constitute a public health hazard (2). *E. coli*, a gram negative bacillus, is naturally present in the gastrointestinal tracts of humans and animals as a part of natural microflora (3). While *E. coli* typically harmlessly colonizes the intestinal tract, several *E. coli* clones have evolved the ability to cause a variety of diseases within the intestinal tract and elsewhere in the host (4). Enterohemorrhagic *Escherichia coli* (EHEC) strains are a subset of Shiga toxin (*Stx*)-producing *E. coli* (STEC) strains that are

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isolated from human patients and are responsible for severe clinical symptoms, including diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome. These diseases are directly related to the virulence genes present in the causative agent (5,6). Four of the most commonly assayed virulence factors of STEC are the two phage-encoded cytotoxins, called Shiga toxin 1 and 2 (encoded by the *Stx1* and *Stx2* genes, respectively), the protein intimin (encoded by the chromosomal gene *eae*), which is responsible for the intimate attachment of the bacteria to intestinal epithelial cells, and the plasmid-encoded enterohaemolysin, also called enterohaemorrhagic *E. coli* haemolysin (EHEC-HlyA), encoded by the *ehxA* gene (3,7). Shiga toxin-producing *E. coli* can be transmitted through different routes, including food and water, person-to-person contact, and animal-to-person contact. Most human infections are caused by consumption of contaminated food. Domestic animals and wild ruminants, in particular cattle, are considered the main reservoir of STEC and the main source of contamination of the food supply (8,9). The presumed route of *E. coli* contamination of raw milk is via faecal contamination during milking (10). However, direct excretion of the organisms from the infected udder has also been reported (11).

There is a lack of information on virulence properties of Shiga Toxin-producing *Escherichia coli* contaminating Iranian milk and dairy products. Therefore, the main goal of the present study was to evaluate these properties.

Material and methods

Sample collection and identification of E. coli

From March 2010 to March 2011, raw bovine (n= 106), caprine (n= 46), ovine (n= 35) and buffalo (n= 21) milk samples were collected from farm bulk tanks and milk collection centres from several regions throughout Iran. Bovine and buffalo milk samples were collected in the above mentioned time period, whereas ovine and caprine milk samples were only available in the lactating periods of ewes and goats (i.e. from March through May, and from September to November of the subsequent year) within the same time frame. At each site, sampling of milk was performed according to the International Dairy Federation guidelines (IDF) (12). Samples (100 mL each, in sterile glass containers) were transported to the laboratory at ca. +4°C within 6–12 h after sampling.

Among dairy products, 42 samples of soft cheese, 32 samples of soft butter and 18 samples of soft ice-cream, all made of raw milk, were purchased at the supermarkets in the city of Shiraz, Isfahan and Shahrekord. All samples were kept under refrigeration and in plastic bags. Their information about dates of production and of assigned shelf-life was not presented. Dairy product samples were collected over a period of six months between May and November 2010, and were analyzed on the day of acquisition. Samples were transported under refrigeration (+4 to +6°C) in thermal boxes containing ice packs and were tested immediately after collection. A 25g portion of each sample was blended with 225 mL of nutrient broth (Merck, Germany) for two minutes, and at normal speed, using a Stomacher lab blender, and incubated at +37°C for 24h. A 1 mL sample of the nutrient broth culture was mixed with 9 mL of MacConkey broth (Merck, Germany) and further incubated at +37°C for 24h. One loop of each tube was streaked on MacConkey agar (Merck, Germany).

Four colonies from each plate with typical *E. coli* morphology were selected and examined by biochemical tests, including hydrogen sulphide, citrate, urease and indole.

All *E. coli* colonies were confirmed by molecular (PCR) method, determined by amplification of 16S rRNA gene region of *E. coli* as described by Sabat et al. (13). The identification of *E. coli* O157: H7 was also performed with the method described by Fode-Vaughan et al. (14). PCR conditions were optimized for DPCR by recommendations reported previously. The PCR conditions for amplification of *Stx1* and *Stx2* were the same as used for *pmoA*.

DNA isolation

Bacterial strains were grown overnight and at +37°C in the trypticase soy agar (TSA– Merck, German). One colony was suspended in 100 µL of sterile distilled water. The suspension was being boiled for 13 min, then frozen and subsequently centrifuged at 14,000 rpm for 15 min to pellet the cell debris (15). The supernatant was used as a template for amplification reaction.

Polymerase chain reaction (PCR)

The PCR assays, specific primer sequences and the predicted size of the amplified products for different pathogenic gene coding regions were employed as previously described (16-20). Details are shown in Table 1. For cycling, a DNA thermocycler (Eppendorf Mastercycler, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) was used. The amplified products were visualized by ethidium bromide staining after gel electrophoresis of 10 µL of the final reaction mixture in 1.5% agarose. Strains of *E. coli* O157:K88ac:H19, CAPM 5933 and *E. coli* O159:H20, CAPM 6006 were used as positive controls.

Table 1: Sequences and predicted lengths of PCR amplification products of the oligonucleotide primers used in this study

Reference	Product size (bp)	Primer sequences (5'–3')	Primers name	Pathogenic factor
(16)	1105	ATCTTATACTGGATGGGATCATCTTGG GCAGAACGACGTTCTTCATAAGTATC	CNF1a CNF1b	Cytotoxic necrotizing factor 1 (*cnf1*)
(17)	543	AATCTAATTAAAGAGAAC CATGCTTTGTATATCTA	CNF2f CNF2r	Cytotoxic necrotizing factor 2 (*cnf2*)
(18)	366	AAATCGCCATTCGTTGACTACTTCT TGCCATTCTGGCAACTCGCGATGCA	Stx1f Stx1r	Shiga toxin 1 (*stx1*)
(18)	282	CGATCGTCACTCACTGGTTTCATCA GGATATTCTCCCCACTCTGACACC	Stx2f Stx2r	Shiga toxin 2 (*stx2*)
(19)	629	TGCGGCACAACAGGCGGCGA CGGTCGCCGCACCAGGATTC	EAE1 EAE2	Enteropathogenic attachment & effacement (*eaeA*)
(16)	430	AAATCACCAAGAATCATCCAGTTA AAATCTCCTGCAATCATCCAGTTTA	Cdt 1 Cdt 2	Cytolethal distending factor (*cdtB*)
(16)	720	ATGGCAGTGGTGTCTTTGGTG CGTCCCACCATACGTGCTCTTC	PapA-f PapA-r	P-Fimbriae (*papA*)
(16)	240	GTGGATACGACGATTACTGTG CCGCCAGCATTCCCTGTATTC	SfaS-f SfaS-r	S-Fimbriae adhesion (*sfaS*)
(16)	880	TGATTAACCCCGCGACGGGAA CGCAGTAGGCACGATGTTGTA	fyuA-f fyuA-r	Yersiniabactin (*fyuA*)
(16)	300	GGCTGGACATCATGGGAACTGG CGTCGGGAACGGGTAGAATCG	AerJ-f AerJ-r	Aerobactin (*iutA*)
(16)	290	GGTGTGGTGCGATGAGCACAG CACGGTTCAGCCATCCCTGAG	TraT-f TraT-r	Serum survival (*traT*)
(20)	432	CAATGCAGATGCAGATACCG CAGAGATGTCGTTGCAGCAG	Hly F Hly R	Haemolysin (*hlyA*)

Results

Eventually, 78 *E. coli* strains (representative of 78 different colony morphologies) (26%) were isolated from 300 samples (Table 2). Colony confirmation was performed by biochemical and molecular (PCR) methods. According to the obtained results, most pollution is related to cow's milk.

PCR assays were successfully developed for detection of twelve different virulence genes of genomic DNA of *E. coli* isolates from milk and dairy products. Agarose gel electrophoresis of PCR products revealed various bands that represent each virulence gene. In our research, 33 and 52 of *E. coli* isolates had *Stx1* and *Stx2*, respectively. By colony counting, 93.5% of independent isolates of *Stx*-positive *E. coli* was detected among 78 *E. coli* isolates; and, *Stx1* and *Stx2* genes were detected in 33 and 52 isolates, respectively. But, *eaeA* gene was not found in any of the samples.

The prevalence of samples containing *Stx*-positive and other virulence genes-positive *E. coli* is shown in Table 2. The prevalence of virulence genes was evaluated. 38.4% of the isolates contained only one virulence gene, 26.9% contained two, and, 34.6% contained three or more virulence genes. None of the samples in this study was positive on *E. coli* O157:H7 (the prevalence of *eaeA* gene was 0.0%). Our study showed that cow's milk (41.5%), cheese and ice cream (16.6%) had the highest prevalence of STEC. PCR-amplification and individually amplified fragments of gene detection were subjected to agarose gel electrophoresis (Figure 1 and 2).

Table 2: Distribution of virulence factor genes in E. coli isolated from raw milk and dairy products

Sample	Sample No.	*E.coli* positive (%)	*stxI*	*stxII*	*eaeA*	*cnf1*	*cnf2*	*iutA*	*cdtB*	*papA*	*hlyA*	*traT*	*sfaS*	*fyuA*
Sheep milk	35	6 (17.1)	3	4	-	3	3	3	1	3	1	4	-	2
Goat milk	46	7 (15.21)	3	4	-	2	2	2	1	4	1	5	-	1
Cow milk	106	44 (41.5)	16	35	-	26	12	12	9	18	5	14	-	6
Buffalo milk	21	6 (28.5)	1	3	-	4	2	2	1	1	2	1	-	-
Cheese	42	7 (16.6)	6	5	-	2	1	1	1	4	2	1	-	1
Butter	32	5 (15.6)	3	1	-	4	3	3	-	-	1	4	-	-
Ice cream	18	3 (16.6)	1	-	-	1	-	-	1	-	-	-	-	-
Total	300	78 (26.0)	33	52	-	42	23	23	14	30	12	29	-	10

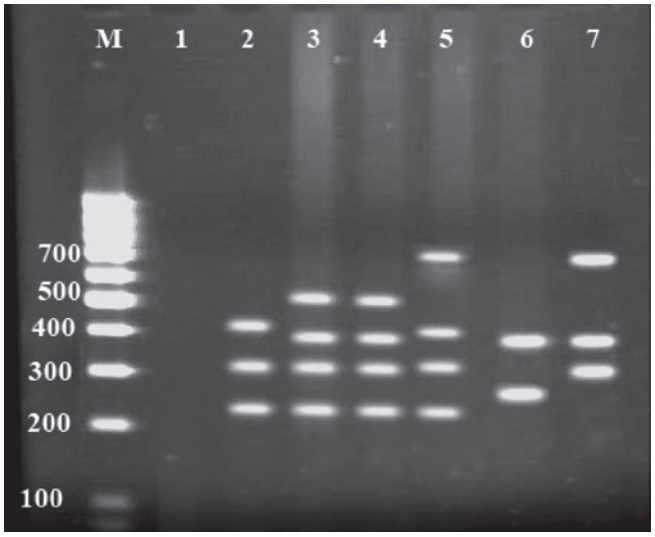

Figure 1: Agarose gel electrophoresis of PCR products amplified with PCR method for the *cnf2* (543 bp), *Stx1* (366 bp), *Stx2* (282 bp), cdtB (430 bp), papa (720 bp), *iutA* (300 bp) and *traT* (290 bp) genes in *E. coli* isolated from raw milk and dairy products

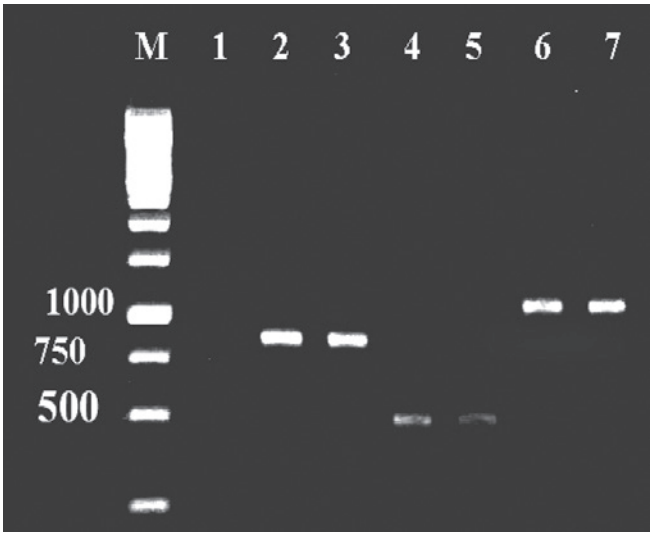

Figure 2: Agarose gel electrophoresis of PCR products amplified with PCR method for the *cnf1* (1105 bp), *fyuA* (880 bp) and *hlyA* (432 bp) genes in *E. coli* isolated from raw milk and dairy products

Discussion

Milk and dairy products can harbor a variety of microorganisms and can be an important source of food-borne pathogens. The presence of food-borne pathogens in milk is due to direct contact with contaminated sources in the dairy farm environment and to udder excretion of an infected animal. There are several reasons to be concerned about the microbial quality of dairy products: first, outbreaks of disease in humans have been traced back to the consumption of unpasteurized milk, and also to pasteurized milk; second, unpasteurized milk is consumed directly by dairy producers, farm employees, their families and neighbours, and raw milk advocates; third, unpasteurized milk is consumed directly by a large segment of population via consumption of several types of cheese produced from unpasteurized milk (21,22).

Nowadays, public health concern associated with microbial food safety is on the increase since *E. coli* is not only regarded as an indicator of faecal contamination but more likely as an indicator of poor hygiene and insufficient sanitary practices during milking. Furthermore, Shiga toxin-producing strains of *Escherichia coli* (STEC) are now recognized as an important agent of diarrhea and other foodborne-related illnesses through ingestion of contaminated food (23,24). Hence, the prevalence of Shiga toxin-producing *E. coli* isolated from milk and dairy products is evaluated in the present study. Our results indicate that 78 (26%) of 300 Iranian milk and dairy samples were contaminated with *E. coli*. 93.5% were found to be positive on two target genes, *Stx1* and *Stx2*. To our knowledge, the main reason for such high prevalence of E. *coli* and their virulence factors in milk and dairy products arises from contamination with faeces. Yet other reasons are: failure to comply with relevant rules, unsanitary milking, failure to control the staff of milking room and factories and/or workshops producing butter, ice cream and cheese, and finally, the use of raw animal milk for the production of dairy products.

The data of this study is older than the recent survey conducted for detection of prevalence and antimicrobial resistance of *E. coli* O157 isolated from Iranian traditional cheese, ice cream and yoghurt. The study reports about *E. coli* O157 being detected in 9 (3.1%) of the 290 samples tested, 5 isolated from traditional cheese and 4 isolated from traditional ice cream samples, whereas *E. coli* O157: H7 was not detected in any of the samples (25). One reason for this difference may be due to differences in the areas under study and the number of samples. Yet another study was conducted to investigate the presence of *E. coli* O157 and *E. coli* O157: H7 strains, and the presence of virulence genes *Stx1, Stx2, eaeA* and *ehlyA* isolates derived from some Iranian traditional dairy products and minced beef meat. *E. coli* non-O157, *E. coli* O157: NM and *E. coli* O157:H7 were isolated from 7%, 1.5% and 0.5% of the traditional butter, cream and kashk (a dairy product, similar to sour cream), respectively. Also, all *E. coli* O157:H7/NM isolates were positive on *eaeA* and *Stx1* and/or *Stx2*, and one *E. coli* O157:H7 isolate was positive on *ehlyA*. Of the 3 *Stx*-positive isolates, one and two isolates contained *Stx1* and *Stx2*, respectively (26).

Globally, several other studies reported the prevalence of *E. coli* and virulence properties of dairy products. For example in Italy, a polyphasic approach was evaluated for the detection of eight staphylococcal enterotoxin (SE)-encoding genes (*sea*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej*, *sel*) and the *Escherichia coli* genes were most commonly associated with the virulence factors (*eae*, *elt*, *ipaH*, *Stx*) in traditional soft cheese, produced artisanally from raw milk in the Lombardy region. The results indicate that some of the artisanal cheese examined may constitute a potential hazard for the consumer health (27). According to the study of raw milk cheese production in Kerman, Iran, *Stx* genes were detected in 6.4% of the samples, but STEC strains were isolated in only 5 of them (4%) (28). Another study from Iran shows that about 21.8% of *E. coli* isolates from cattle were positive on *ehxA, Stx1*, and/or *Stx2* genes (29), but our study indicates that *Stx1*and *cnf1* with incidences of 42.3% and 53.84% had the highest frequency rate of virulence genes of *E. coli* isolates from raw milk samples. The results offer an answer to why food contamination from animal faecal sources is so common in Iran.

Yet another study of lamb food chain shows that all three virulence genes, *eae*, *Stx1*, and *Stx2* were the most prevalent genes in slaughterhouses (69%), processing plants (32%) and butcheries (9–10%) (30), but our results indicate that *Stx2* and *cnf1* had the highest prevalence of *E. coli* virulence genes in raw milk and dairy products.

Detection of bacteria in milk samples is still important, especially in more rural areas as it is easily available and economical. Due to bad climate conditions in some geographical places of Iran, oxen, ewes and goats have unsatisfactory survival conditions, so buffaloes present the main milk source for people. Buffalo's milk hygiene is therefore essential for human health, especially in arid and desert areas.

In United States, Van Kessel et al. (31) detected Shiga toxin genes enrichments in 15.2% of the bulk tank milk samples and in 51.0% filters by real-time PCR. These data confirm those from earlier studies showing significant contamination of bulk tank milk by zoonotic bacterial pathogens, and also, that the consumption of raw milk and raw milk products present a health risk. In Spain, occurrence of STEC in 'Castellano' cheese, a non-cooked and hard or semi-hard Spanish cheese made from ewe's milk, was reported. According to the report, three STEC strains were detected in two samples (2.4%) of 'Castellano' cheese, one with 2.5 and the other one with 12 month-ripening period. From those STEC isolates, two were identified as *E. coli* O14 and one third presented an O-specific polysaccharide not-groupable serologically (ONG) (32). In Bangladesh, the prevalence of Shiga toxin (*Stx*)–producing *Escherichia coli* was found in different types of food samples, and their genetic relation to STEC strains previously isolated from animal sources was investigated. In this study, approximately 10% of raw milk and 8% of fresh juice samples were positive for *Stx* (33).

All these data lead to conclusion that milk and dairy products represent a potential hazard for consumers, due to the potential presence of Shiga-toxin-producing *E. coli*, because of neglected sanitary measures during manufacture, handling and distribution of such fresh foods. Consequently, food manufacturers and specialists should design comprehensive programmes as good manufacturing practices (GMP) and implementation of HACCP system to ensure food free from the pathogens. In addition, effective heat treatments of food, providing information to food handlers and consumers, as well as implementation of strict hygienic measures during manufacture, storage and sale of these products are of significant importance in improving the quality of food and safeguarding the consumers against infections with such organisms. On the other hand, since many people still drink raw milk, especially in rural areas, this emphasises the need for educational efforts about health risks associated with consumption of raw, unpasteurized milk.

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VIRULENTNOST SEVA BAKTERIJE *ESCHERICHIA COLI*, KI IZLOČA ŠIGA TOKSIN, IZOLIRANE IZ SUROVEGA MLEKA IN MLEČNIH IZDELKOV

R. Farzan, E. Rahimi, H. Momtaz

Povzetek: Sevi bakterije *Escherichia coli*, ki izločajo šiga (Stx) toksin (STEC), so raznolika skupina patogenov, ki jih lahko najdemo v hrani in so različno nevarni za človeka. Glavni cilj raziskave je bil določiti virulentnost STEC, izoliranih iz surovega mleka in mlečnih izdelkov v Iranu.

Skupno število 300 vzorcev, ki so vključevali mleko ovac (35), koz (46), krav (106) in bivolov (21), mehki sir (42), maslo (32) in sladoled (18), je bilo pridobljenih iz zbiralnih rezervoarjev na kmetijah, zbirnih centrov za mleko, različnih trgovin in od trgovcev na drobno iz različnih področij Irana. Biokemijske in molekularne metode (PCR) so pokazale, da je bilo 26 % vzorcev pozitivnih na *E. coli* in med njimi smo gene *Stx1* in *Stx2* odkrili v 33 oziroma 52 vzorcih. Genov *eaeA* in *sfaS* nismo našli v nobenem vzorcu. Poleg gena *Stx2* je imel najvišjo prevalenco (42 izolatov) gen cnfI, poleg genov *eaeA* in *sfaS* pa je imel najnižjo prevalenco izmed virulentnih genov gen *fyuA*. Rezultati raziskave kažejo na to, da so potencialno virulentni sevi *E. coli* v okolju zelo razširjeni in da je hrana lahko vzrok za okužbo ljudi.

Ključne besede: STEC; *Escherichia coli*, virulentni faktorji; surovo mleko