

Virulence Genes, Antimicrobial Resistance and Clonality of *Escherichia coli* O157:H7 Isolated from Mastitic Bovine Milk

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ABSTRACT

This study was carried out to determine the prevalence of *E. coli* O157:H7 in mastitic bovine milk, to investigate the major virulence genes [shiga toxins (*stx1* and *stx2*), intimin (*eae*), enterohaemolysin (*ehlyA*), and traslocated intimin receptor (*tir*)], antibiotic resistance profiles and clonality of the isolates. A total of 484 bovine mastitic milk samples collected from dairy farms in Aydin is a county situated by Aegean Sea at the west part of Turkey. The serotype of the isolates was confirmed by the presence of specific genes using polymerase chain reaction (PCR). Presence of virulence genes was also tested by PCR. Resistance to 13 antimicrobial agents was investigated by the disk diffusion method. The clonality was determined by enterobacterial repetitive intergenic consensus (ERIC) PCR. From 484 bovine milk with mastitis a total of 8 (1.65%) *E. coli* O157:H7 was isolated from 5 farms. Among virulence genes tested, *ehlyA* was positive for all 8 (100.0%) isolates, 7 isolates carried (87.5%) *eae*, *tir* and *stx2* genes. Isolates were all susceptible to the antimicrobials tested except to tetracycline for two (25.0%) and to streptomycin for one (12.5%). The clonal spread was detected among the animals with mastitis by ERIC PCR. Among 8 strains tested, a total of 6 profiles were detected. There were two couples of "identical" isolates. The four isolates from the same farm appeared to be closely related, and distantly related to the isolates from other farms. Presence of *E. coli* O157:H7 strains in mastitic bovine milks may cause public health problems. We suggest that there is a need for improving the hygienic conditions of the farms which may help reduce transmission of *E. coli* O157:H7 to humans.

Keywords: Mastitis; *E. coli* O157:H7; Virulence Genes; Antimicrobial Resistance; Clonality.

INTRODUCTION

Although healthy domestic ruminants are reported to be the natural reservoirs of *Escherichia coli* O157:H7, this serotype is important for serious acute illness and long term side effects in humans (1). *E. coli* O157:H7 has several virulence factors [shiga toxins (*stx1* and *stx2*), intimin (*eae*), enterohaemolysin (*ehlyA*) and traslocated intimin receptor (*Tir*)] that appear to play a major role in the pathogenesis of infections. Two potent phage-encoded cytotoxins called as shiga toxins inactivate ribosomal RNA, inhibit protein synthesis and cause the death of host cells (2). Intimin is responsible for intimate at-

tachment of the agent to the intestinal epithelial cells, causing attaching and effacing (A/E) lesions in the intestinal mucosa (3). Enterohaemolysin has been considered as the cause of enterocyte and leukocyte lysis (4). A secreted protein encoded by the genes of the shiga toxin-producing *E. coli* (STEC) chromosomal pathogenicity island called locus of enterocyte effacement (LEE) is *Tir*. The adhesin is an intimin, and its receptor is *Tir*. Intimin and *Tir* promoted initial adherence and colonization, but haemolysin did not influence colonization at the bovine rectal mucosa (5).

The most important feature of *E. coli* is its ability to colo-

nize and expand clonally (6). Enterobacterial repetitive intergenic consensus (ERIC) polymerase chain reaction (PCR) has been widely explored as a genomic DNA fingerprinting technique since its first introduction for use in molecular identification and classification of bacteria at the subspecies level (7). Typing of *E. coli* strains with ERIC PCR have been carried out successfully in medical research (8), as well as in research on bovine mastitis (9) isolates.

There are few reports available on human related *E. coli* O157:H7 in Turkey (10). Similarly, there are several studies from the veterinary field which indicate that ruminants are the most important reservoir of *E. coli* O157:H7 (11, 12). To the best of our knowledge, there is no study on the virulence genes, antibiotic resistance, and clonality traits of *E. coli* O157:H7 strains isolated from mastitic milk samples from dairy cattle in Turkey. This study was carried out to investigate the prevalence of *E. coli* O157:H7 in mastitic bovine milk, to investigate the major virulence genes (*stx1*, *stx2*, *eae*, *ehlyA*, *tir*), antibiotic resistance profiles and clonality of the isolates.

MATERIAL AND METHODS

Origin and collection of milk samples

A total of 484 clinical and subclinical mastitic milk samples from 212 Holstein cows were aseptically collected from 35 dairy farms (average 4-7 bovine from each farm) in recent two years period. Cows were chosen which had not taken any treatment at least in two months. The age of the cows that at least have one calve were varied between 3 to 9 years. The milk samples were collected from small scale dairy farms in Aydin. Aydin is a county of 8007 km² by Aegean Sea at the west part of Turkey. It was mentioned by the owner of the farms that they have had some mastitis problems in their recent production history.

For collection of milk samples, teat ends were cleaned using 70% alcohol moistened swabs and allowed to dry. After discarding the first few streams, 2-5 ml of the milk samples were collected into sterile 10 ml glass flasks. Clinical mastitis was diagnosed by changes in the udder and milk compositions. Changes in the milk were clotting and discoloration of the milk and in the udder swelling, heat, reddening, pain and hardening of the udder. Cows that did not have clinical mastitis were subjected to further investigation for subclinical mastitis by using the California Mastitis Test (CMT). CMT,

a qualitative measurement of the somatic cell counts (SCC) in milk, is a screening test for mastitis that can be used easily. From each quarter of the udder, a squirt of milk sample was placed in each of the cups on the CMT paddle and an equal amount of 3% CMT reagent was added to each cup and mixed. The CMT reaction of each quarter was noted in an ordered scale as either, 0, 1, 2, or 3, with 0 showing no reaction, and 1 being a trace and a slight positive reaction (13).

Isolation and identification of *E. coli* O157:H7

A total of 484 mastitic milk samples was cultured on Sorbitol MacConkey agar (SMAC) (Oxoid Ltd, Hampshire, England) and incubated in aerobic conditions at 37°C for 24 h. After incubation, non-sorbitol fermenting colorless colonies were accepted *E. coli* O157:H7 presumptive bacteria. One isolate were recovered from each plate. There were no sorbitol positive and sorbitol negative mix cultures from the same quarter. All sorbitol negative (*E. coli* O157:H7) and sorbitol positive (red *E. coli* colonies) colonies were sub-cultured on the *E. coli* O157:H7 ID agar (bioMerieux SA, Marcy-l'Etoile, France) and incubated overnight at 37°C. *E. coli* O157:H7 is usually sorbitol negative (14), for this reason bluish green colonies on *E. coli* O157:H7 ID agars were regarded as suspect *E. coli* O157:H7. These colonies were confirmed serologically using *E. coli* O157:H7 monovalent (Denka Seiken Co., Tokyo, Japan) antiserum (15). All colonies were subcultured on Eosin Methylene Blue Agar (Merck, Darmstadt, Germany) agar and stored at -80°C in Brain Heart Infusion Broth (Oxoid Ltd, Hampshire, England) with 20% glycerol until use for molecular confirmation.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility tests were performed by the Kirby-Bauer disc diffusion method using Mueller-Hinton agar (Oxoid Ltd, Hampshire, England), according to the Clinical and Laboratory Standards Institute guidelines (16). The following 13 antimicrobial agents (Oxoid Ltd, Hampshire, England) were tested: ampicillin (AMP 10µg), amoxicillin-clavulanic acid (AMC 20/10µg), gentamicin (GEN 10µg), streptomycin (STR 10µg), kanamycin (K 30µg), tetracycline (TET 30µg), cefoperazone (CFP 75µg), cefotaxim (C 30µg), ceftizoxim (MCG 30µg), ceftriaxon (CRO 30µg), nalidixic acid (NA 30µg), chloramphenicol (C 30µg), trimethoprim sulfamethoxazole (SXT 25µg). The *E. coli* ATCC 25922 was used as a reference strain for quality control.

PCR

Genomic DNA of *E. coli* isolates were extracted with InstaGene matrix™ (Bio-Rad Laboratories, Mississauga, Ontario, Canada) according to the manufacturer's instructions. Reference strains such as *E. coli* ATCC 35150 (*stx1*, *stx2*, *eae*, *ehlyA* genes positive *E. coli* O157:H7 strain) and *E. coli* ATCC 25922 were used as positive and negative control in PCR, respectively. There was no positive control for *tir* gene. Positive samples were identified based on the presence of bands of the expected sizes. The oligonucleotide primers described by previously for *rfb*_{O157} (17), for *fli*_{CH7} (18), for *stx1*, *stx2*, *eae* (19), for *ehlyA* (20), for *tir* (5), for ERIC (7) were used. PCR conditions used for this study are given in Table 1.

Two multiplex (m) PCR and two uniplex (u) PCR were used to detect the serotype specific genes (*rfb*_{O157}, which encodes the *E. coli* O157 serotype and *fli*_{CH7}, which encodes the *E. coli* flagellum H7 serotype) and five virulence genes (*stx1*, *stx2*, *eae*, *ehlyA*, *tir*). The first mPCR were performed for the detection of *rfb*_{O157}, *ehlyA*, *fli*_{CH7} genes, second for

stx1, *stx2*, *eae*, and uPCR for *tir* genes. Another uPCR was used to detect the clonality by ERIC PCR. The mixtures for the amplification reactions consisted of 10X Taq buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2 mM MgCl₂, 0.2 mM dNTP, 0.4 μmol of each primers, 2.5 U Taq polymerase (Fermentas), and 2 μl of template sample DNA in a final volume of 30 μl. For ERIC PCR, with the Bio-1D++ (Vilber Lourmat, Marne-la-Vallée Cedex 1, France) program, relying on the existence of bands, genetic relations were calculated and a dendrogram was produced.

The appearance of the gels was digitized, and PC assisted examination of genomic fingerprints was made with the BIO-GENE software programme (Version 11.02, Vilber Lourmat, Marne-la-Vallée Cedex 1, France). Similarity matrices of the complete densitometric curves of the gel tracks were calculated using the Dice coefficient. Cluster analysis of similarity matrices was made by the UPGMA algorithm. All The amplification products were inspected by electrophoresis on 1.5% agarose gel at 100 V for 45 min in Tris-acetate-EDTA buffer and shown using ethidium bromide (20 μg/ml).

Table 1: PCR conditions for the detection of virulence genes and clonality

Action	uPCR ¹			mPCR ²			ERIC PCR		
	Temperature (°C)	Duration (min)	Cycle	Temperature (°C)	Duration (min)	Cycle	Temperature (°C)	Duration (min)	Cycle
Initial denaturation	94	5	1	94	5	1	94	5	1
Denaturation	94	1	35	94	1	35	94	1	35
Annealing	60	1	35	55	1	35	43	1	35
Extension	72	1	35	72	2	35	72	3	35
Elongation	72	10	1	72	10	1	72	10	3
Cooling	4	infinite		4	infinite		4	infinite	

¹ Presence of genes *tir* were tested by uPCR

² Presence of genes *rfb*_{O157}, *ehlyA*, *fli*_{CH7} and *stx1*, *stx2*, *eae* were tested by mPCR

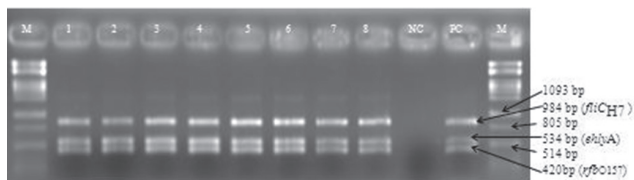


Figure 1: mPCR I (*rfbE*, *ehlyA*, *fliC*) M1: Marker (Lambda phage DNA restricted with *Pst*I enzyme) 1-8: *E. coli* O157:H7 isolates PC: Positive Control (ATCC 35150), NC: Negative Control (master mix without DNA) (ATCC 25952).

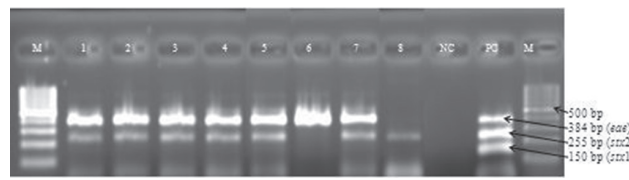


Figure 2: mPCR II (*eae*, *stx1*, *stx2*) M: Marker (100 bp DNA ladder) 1-8: *E. coli* O157:H7 isolates PC: Positive Control (ATCC 35150), NC: Negative Control (master mix without DNA) (ATCC 25952)

RESULTS

A total of 484 milk samples was taken from 35 farms and tested for the presence of *E. coli* O157:H7. A total of 8 (1.65%) of the 51 (10.53%) *E. coli* were identified as O157:H7. These results were confirmed by PCR, which showed that all 8 (100.0%) isolates carried the *rfb*_{O157} and *fli*_{H7} genes, indicators of O157 and H7, respectively (Figure 1). The *E. coli* O157:H7 strains were isolated from five different (14.3%) farms.

The presence of genes coding for the virulence genes, *ehlyA*, *eae*, *tir*, *stx1*, and *stx2* was tested by PCR using specific primers. The PCR results indicated that all 8 (100.0%) isolates carried *ehlyA* gene which encodes for haemolysin (Figure 1). Except one isolate the remaining 7 (87.5%) had *stx2*, *eae* (Figure 2), and *tir* (Figure 3). None of the isolates tested carried the *stx1* gene. One of the *E. coli* O157:H7 strain (6. isolate) investigated was positive for *eae*, *ehlyA*, *tir* genes but negative for both genes tested coding for shigatoxin (Table 2).

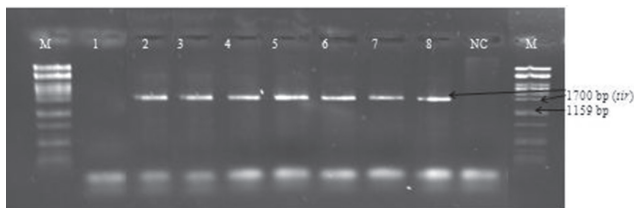


Figure 3: uPCR (*tir*) M: Marker (Lambda phage DNA restricted with *Pst*I enzyme) 1-8: *E. coli* O157:H7 isolates NC: Negative Control (master mix without DNA) (ATCC 25952)

Susceptibilities of *E. coli* O157:H7 isolates were tested for 13 antimicrobial agents by disk diffusion method. The isolates were all susceptible to ampicillin, amoxicillin-clavulanic acid, gentamicin, kanamycin, cefoperazone, cefotaxim, ceftizoxim, ceftriaxon, nalidixic acid, chloramphenicol, trimethoprim sulfamethoxazole. Among the isolates tested, 2 were resistant to tetracycline (25.0%) and 1 isolate was resistant to streptomycin (12.5%) (Table 2, Figure 4).

Of the 8 *E. coli* O157:H7 isolates subjected to ERIC PCR all isolates had amplicons ranging from approximately 150 to 1700 bp. The clonal spread was detected among the animals with mastitis. According to the dendrogram, 2 groups with 10-20% genetic approximately were determined. Among 8 strains tested, a total of 6 profiles were detected. Four strains had distinct profiles while 2 profiles were common for 2 strains. There were two couples of “identical” isolates. The four isolates from the same farm seem to be closely related, and distantly related to the isolates from other farms (Figure 5).

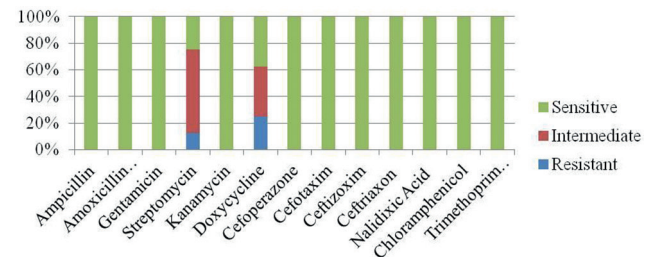


Figure 4: Antimicrobial resistance patterns against 13 antimicrobial agents in 8 *E. coli* O157:H7 isolates from mastitic bovine milk.

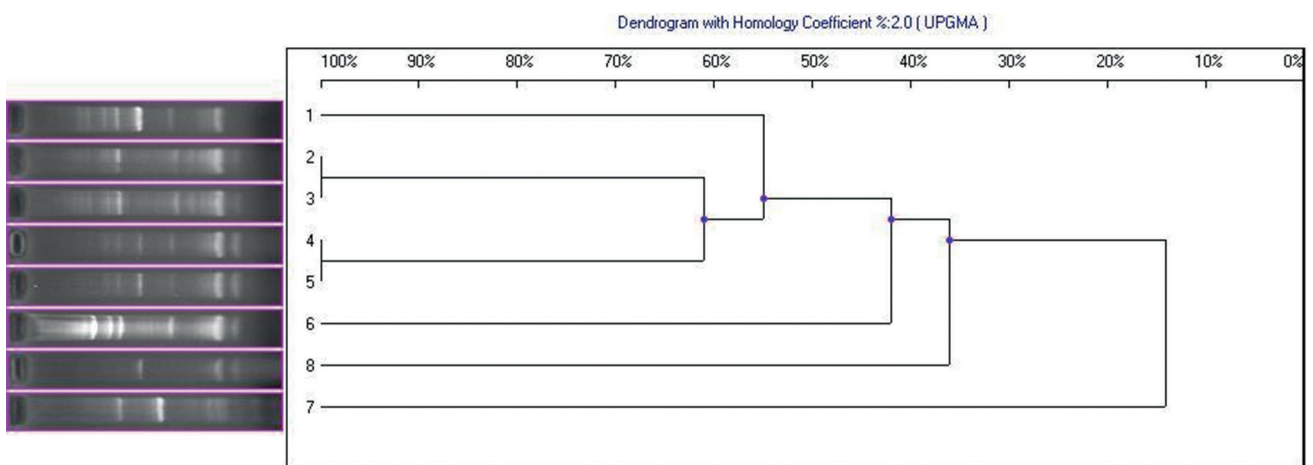


Figure 5: Dendrogram of *E. coli* O157:H7 strains from mastitic bovine milk. ERIC PCR results showed presence of 6 profiles among 8 isolates tested.

Virulence genes, antibiotic resistance phenotype, ERIC types of *E. coli* O157:H7 isolates are presented in Table 2 according to farms from which the samples were derived.

DISCUSSION

Cattle are the principal reservoir of *E. coli* O157:H7 (21) which is frequently excreted in their feces (22) and responsible for an increasing number of food based infection in Turkey (11,12) and other countries (14).

Studies have been carried out from mastitic cow milk samples for the determination of both *E. coli* and *E. coli* O157:H7 strains in Turkey (23,24) and in the world (25-30). In these studies, it was reported that the isolation ratio of *E. coli* was high (5.8%-45.5%), while generally *E. coli* O157:H7 isolation ratio was low (0%-8.8%), except one study in Lebanon (30) (Table 3). There are 3 studies from Brazil that reported distinct isolation rates of *E. coli*: two of them, Kobori *et al.* (25) and Lira *et al.* (26) reported lower isolation rates (5.8 and 8.4%, respectively), however in reports published by Rangel and Marin (30) where the isolation rate (34.4%) was higher than our study (10.53%). Results of Turutoglu and Mudul (23) from Turkey, Daood (27) from Lebanon indicated a higher *E. coli* rates in raw milks than our findings. The isolation rate of *E. coli* was similar in our study to that of Momtaz (28) in Bulgaria (Table 3).

A few studies have been reported to deal with the rates of *E. coli* O157:H7 from raw milk. *E. coli* O157:H7 isolation rate in our study (1.65%) was found to be higher than findings of Turutoglu and Mudul (23) and Aydin *et al.* (24) in Turkey

and but found to be lower than Daood (27) in Lebanon and Onono *et al.* (29) in Kenya. The differences in the *E. coli* O157:H7 isolation rates may be due to variations of hygienic conditions during the milking process, regional prevalence, or to methodology used.

Testing multiple genes by means of one PCR is also used for determination of the presence of virulence genes in *E. coli* isolates (19,31) and mPCR is considered as a convenient and practical method. In this study, the first mPCR was used for the determination of flagellary and somatic antigen genes with enterohaemolysin, a virulence gene causing the lysis of host erythrocytes. The second mPCR was carried out to determine the shiga toxins with intimin gene. Flagellary and somatic antibodies can also be determined by serological tests, but mPCR can be useful for the determination of isolates with masked O antigen in rough isolates (32).

The shiga toxin genes are well known to be the primary virulence factors of *E. coli* O157:H7, but the *stx2* gene is usually more widespread in *E. coli* O157:H7 strains (1). In the present study, a larger proportion of the isolates 7 (87.5%) possessed the *stx2* gene compared to *stx1*. The result contrasts with reports showing that most enterohaemorrhagic (EHEC) strains from cattle harbor the *stx1* gene (25), but parallel to another study (26) which had been carried out in Brazil reporting that mostly the *stx2* gene was isolated from *E. coli* O157:H7 isolates from mastitic bovine milk. Jenkins *et al.* (33) reported that among healthy cattle prevalence of EHEC strains may vary depending on the season. They found that the rates of *stx2* were higher among cattle except during the winter season.

Table 2: Virulence genes, antibiotic resistance phenotype, ERIC types of *E. coli* O157:H7 isolates according to farms

Isolate No	Virulence genes	Antibiotic Resistance Phenotype	ERIC type	Farm
1	<i>stx2, eaeA, ehlyA</i>	*	I	1
2	<i>stx2, eaeA, ehlyA, tir</i>	Tetracycline	II	2
3	<i>stx2, eaeA, ehlyA, tir</i>	Tetracycline	II	2
4	<i>stx2, eaeA, ehlyA, tir</i>	*	III	2
5	<i>stx2, eaeA, ehlyA, tir</i>	*	III	2
6	<i>eaeA, ehlyA, tir</i>	Streptomycin	IV	3
7	<i>stx2, eaeA, ehlyA, tir</i>	*	V	4
8	<i>stx2, ehlyA, tir</i>	*	VI	5

* The susceptible isolates

Table 3. *E. coli* and *E. coli* O157:H7 isolation ratios in the total samples tested in mastitic bovine

Country, (Year)	Isolation rate (%)	
	<i>E. coli</i>	<i>E. coli</i> O157:H7
Turkey, (2002)	13.0	0
Brasil, (2004)	5.8	*
Brasil, (2004)	8.4	*
Lebanon, (2007)	45.45	30.90
Brasil, (2009)	34.4	
Bulgarian, (2010)	10.5	*
Kenya, (2010)	*	8.8
Turkey, (2010)	*	0
Present study	10.53	1.65

* Not done

The majority of EHEC strains produce shiga toxins making *E. coli* O157:H7 particularly virulent. This serotype is regarded as serious human diseases which may result in the hemolytic uremic syndrome (HUS) (34). Similar to our study, *stx* genes negative *E. coli* O157:H7 isolates have been reported to be isolated from ruminant feces (11, 12). Parallel the above results an *E. coli* O157:H7 isolate has been isolated without that shiga toxin genes; its capacity to perform HUS and diarrhea has not studied. The results were found to be similar to studies reporting that the presence of *ehlyA* (35, 36) and *eae* (28,37) in EHEC strains and isolates having no *eae* genes (38). According to the knowledge of the authors, there are few reports available where the *tir* gene was investigated in mastitic cattle milk (39).

Antimicrobial resistance to bovine EHEC strains may spread to persons through food. The problem of drug resistance is increasingly receiving close attention in veterinary medicine (40). It is therefore imperative in clinical practice to recognize the need to use drugs that are effective in treating the particular problem. It is known that the development of antibiotic resistance in microorganisms is a risk to human health (40). A study from Spain showed that the resistance levels of tetracycline and streptomycin was elevated among *E. coli* O157:H7 isolated from cattle (40). Another study from Lebanon reported to find 16.9% tetracycline resistance from raw milk samples (27). On the other hand, other authors (27, 28) have concluded that there are increases in resistance of *E. coli* O157:H7 isolates to sulphonamides. This differs from our study for trimethoprim sulfamethoxazole, since high susceptibility to trimethoprim sulfamethoxazole was found among our isolates. High resistance rates to tetracycline and streptomycin in our study may be due to the extensive use of these antimicrobials for treatment in veterinary cases in our region.

It has been reported that ERIC PCR is a useful method to show the clonality between *E. coli* strains (8,9). In this study it was seen that some strains (number 2-3, 4-5 and 7) isolated from different farms were actually different clones, although they have the same virulence genes. Wieczorek *et al.* (41) also used ERIC PCR for clonality testing and reported that among isolates with the same virulence genes, genetic relatedness was low.

In Turkey, it is traditional practice that non-pasteurized milk is often sold in farmer's markets. While sporadic *E. coli* O157:H7 cases originating from Turkey and small

epidemics in humans have been identified, this serotype has not been usually been examined in most of the diagnostic microbiology laboratories and studies related to this subject are inadequate (10). In previous studies on cattle in different areas of Turkey, it has been reported that *E. coli* O157 was identified in cow's feces with rates varying between 0.6% and 25% (11, 42-44). Geographic variations may be the reason of the discrepancy in the isolation rates. While there are few reports available from animal's relating to *E. coli* O157, to the best of our knowledge, there is no study on the *E. coli* O157:H7 strains isolated from mastitic milk samples from dairy cattle in Turkey bearing in mind that *E. coli* O157:H7 strains are important food pathogens in humans.

The presence of *E. coli* O157:H7 strains in mastitic bovine milk can cause public health problems. We suggest that there is a need for improving the hygienic conditions of the farms. More legislative measurements have to be taken to prevent people from selling non-pasteurized milk. Further researches should be designed to investigate a genetic relationship between animal and human isolates and the importance of this agent in mastitis because of its zoonotic characteristics. This could reduce transmission of *E. coli* O157:H7 to humans.

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