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Research Article

Virulence Properties of *Escherichia coli* Isolated from Clinical Bovine Mastitis

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Abstract: The aim of this study was to detect the presence of the genes for some virulence factors in *Escherichia coli* isolates from clinical bovine mastitis. A total of 100 *E. coli* isolates, each isolated from individual cows in different herds between 2000 and 2005, were examined. Two multiplex polymerase chain reaction (PCR) assays were used to detect the presence of the genes encoding Shiga toxins 1 and 2 (*stx1* and *stx2*), intimin (*eaeA*), heat-stable enterotoxin a (*Sta*), and F5 (K99), F41, and F17 fimbriae. In the PCR assays, 9 isolates were positive for the genes of F17 fimbriae and only 1 isolate was positive for the *eaeA* gene. The results demonstrated that none of the potential virulence factors investigated was commonly observed in *E. coli* isolates from bovine clinical mastitis. Shiga toxin-producing *E. coli* (STEC), which is potentially pathogenic for humans, was not detected by PCR among the isolates from the cases of bovine mastitis.

Key Words: *Escherichia coli*, bovine mastitis, multiplex PCR, virulence factors

Klinik İnek Mastitislerinden İzole Edilen *Escherichia coli* Suşlarının Virülens Özellikleri

Özet: Bu çalışmada ineklerin klinik mastitis vakalarından izole edilen *E. coli* suşlarında bazı virülens faktör genlerinin araştırılması amaçlandı. Her biri farklı sürülere ait, 2000-2005 yılları arasında izole edilen toplam 100 *E. coli* suşu incelendi. Shiga toxin 1 ve 2 (*stx1* ve *stx2*), intimin (*eaeA*), heat-stable enterotoxin a (*Sta*), F5 (K99), F41 ve F17 fimbria'larını kodlayan genlerin tespiti amacıyla iki ayrı multipleks PCR uygulandı. PCR testleri sonucu 9 suş F17 fimbria geni ve sadece 1 suş *eaeA* geni pozitif bulundu. Sonuçlar klinik inek mastitislerinden izole edilen *E. coli* suşlarında araştırılan potansiyel virülens faktörlerinin hiç birinin yaygın olarak bulunmadığını gösterdi. İneklerde mastitis vakalarından izole edilen *E. coli* suşları arasında PCR yöntemi ile insanlarda hastalık oluşturma ihtimaline sahip Shiga toksin üreten *E. coli* (STEC) tespit edilmedi.

Anahtar Sözcükler: *Escherichia coli*, inek mastitis, multipleks PCR, virülens faktörleri

Introduction

Escherichia coli is considered an environmental pathogen and one of the most important causes of bovine clinical mastitis, which is mostly observed in the early lactation period and in high-producing cows with low somatic cell counts (1). It was reported that the incidence of *E. coli* mastitis has increased in some countries in recent years (2); nevertheless, there is no such evidence indicating the same trend in Turkey. *E. coli* is a normal inhabitant of the gastrointestinal tract of animals and humans and some particular strains of *E. coli* cause intestinal or a variety of extra-intestinal infections. Strains of *E. coli* are traditionally characterized by serological identification of somatic O, flagellar H, capsular K, and fimbrial F antigens (3,4). The expression of these different antigens on bacterial cell walls results in hundreds of serologically distinct *E. coli* serotypes. Differentiation of pathogenic strains from normal flora strains depends on the identification of virulence characteristics. *E. coli* strains can further be classified according to the presence of virulence factors such as enterotoxigenic *E. coli* (ETEC), attaching and effacing *E. coli* (AEEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and Shiga toxin-

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producing *E. coli* (STEC or VTEC) (5,6). Virulence factors associated with strains of *E. coli* include adhesins, toxins, cell wall, capsule production, and serum resistance (3). However, no unique virulence factor has been identified so far that is specific to *E. coli* isolates from bovine mastitis. *E. coli* isolates from bovine intramammary infections belong to a wide range of serogroups (7-9). DNA fingerprinting analyses of such strains have also demonstrated a high degree of variability (8-10). Therefore, it has been suggested that *E. coli* from mastitis are opportunistic bacteria originating from the cow's environment.

The pathogenesis of *E. coli* mastitis is different from that of other *E. coli* infections such as colibacillosis in calves. Studies show that adherence of *E. coli* to the epithelial surface does not play a role in the pathogenesis of bovine mastitis; bacteria multiply in the mammary secretion without attachment to the mammary epithelium (1,3). It is accepted that bacterial, environmental, management, and cow factors may affect the occurrence and severity of mastitis. Studies suggest that the severity of *E. coli* mastitis mainly depends on cow factors rather than the characteristics of *E. coli* (1,11).

Recent developments of molecular techniques facilitated the detection of virulence factors (5). Because the expression of the genes encoding some virulence factors can be affected by a variety of environmental conditions, the phenotypic determination of some virulence factors may be difficult or misleading. Although there are several studies on the molecular identification of virulence factors of *E. coli* from bovine mastitis in some countries (9,12-14), there is scant information in Turkey.

The objective of this study was to investigate some of the common virulence factors of *E. coli* from bovine mastitis by PCR.

Materials and Methods

E. coli isolates

A total of 100 *E. coli* isolates from milk samples submitted to Konya Veterinary Control and Research Institute diagnostic laboratory between 2000 and 2005 were examined. Each isolate was obtained from individual cows with clinical mastitis from different herds that were small and usually kept indoors. *E. coli* isolated only as a pure culture was examined in the study. The isolates were identified using standard microbiological techniques (4) and stored at -70 °C until further testing. The strains were also subcultured on Sorbitol MacConkey Agar (Oxoid Ltd., Hampshire, UK) and incubated at 37 °C overnight. Sorbitol-negative colonies were tested with an O157 latex agglutination kit (Oxoid) for the detection of O157:H7 *E. coli*.

The distribution of *E. coli* isolates according to years was as follows: 9 in 2000, 18 in 2001, 27 in 2002, 26 in 2003, 7 in 2004, and 13 in 2005.

PCR tests

Bacterial DNA for PCR analyses was obtained by resuspending colonies of bacteria grown on MacConkey agar plates in 500 µl of ultrapure water and boiling at 100 °C for 10 min to lyse the organisms and release the DNA.

A multiplex PCR that detects the genes encoding Shiga toxins (*stx1* and *stx2)*, intimin (*eaeA*), heat-stabile enterotoxin a (*Sta),* and fimbriae of F41 and K99 was performed using the primers described by Franck et al. (5) (Table 1). The PCR assay was carried out in a total volume of 50 µl of mixture containing PCR buffer (Promega, Madison, WI, USA), 1.5 mM of $MgCl_2$, 250 µM each of the deoxynucleoside triphosphates, 0.5 µM each of the virulence gene-specific primers, 1.25 U of Taq polymerase (Promega), and 5 µl of template DNA. The amplification conditions included 25 cycles of a denaturation step at 94 °C for 30 s, primer annealing at 50 °C for 45 s, and extension at 70 °C for 90 s. The extension time was ramped for an additional 3 s per cycle and a final extension step of 10 min at 70 °C was performed (5). The PCR products were analyzed by electrophoresis through 1.5% agarose gel, after which the gel was stained with ethidium bromide and photographed. *E. coli* strains of O101:H-: K99+, F41+ (Pendik Veterinary Control and Research Institute, İstanbul), which was K99-, F41-, and *Sta*-positive, and O157:H7 (Refik Saydam Hygiene Center, Ankara), which was *stx1*- and eaeA-positive, were used as positive controls in the mutiplex PCR.

For the detection of F17 fimbriae, a second multiplex PCR using 3 oligonucleotide primers (P7, P8, and P9) designed to be specific to the 2 subfamilies of the F17 adhesin genes as described by Bertin et al. (15) was used (Table 2). This PCR was carried out in a total volume of 50 µl of mixture containing PCR buffer (Promega), 1.5 mM of $MgCl_2$, 250 µM each of the deoxynucleoside triphosphates,

Table 1. Primers used in the multiplex PCR for *Stx1*, *Stx2*, Intimin, F41, F5 (K99), and *Sta* genes.

	Virulence factor	Primer sequence (5'-3')	Size of product (bp)
1.	*Stx1* (F)	TTC GCT CTG CAA TAG GTA	555
	Stx1 (R)	TTC CCC AGT TCA ATG TAA GAT	
2.	*Stx2* (F)	GTG CCT GTT ACT GGG TTT TTC TTC	118
	Stx2 (R)	AGG GGT CGA TAT CTC TGT CC	
3.	*Intimin* (F)	ATA TCC GTT TTA ATG GCT ATC T	425
	Intimin (R)	AAT CTT CTG CGT ACT GTG TTC A	
4.	F41 (F)	GCA TCA GCG GCA GTA TCT	380
	F41 (R)	GTC CCT AGC TCA GTA TTA TCA CCT	
5.	K99 (F)	TAT TAT CTT AGG TGG TAT GG	314
	K99 (R)	GGT ATC CTT TAG CAG CAG TAT TTC	
6.	*Sta* (F)	GCT AAT GTT GGC AAT TTT TAT TTC TGT A	190
	Sta (R)	AGG ATT ACA ACA AAG TTC ACA GCA GTA A	

Table 2. Primers used in the multiplex PCR for F17 fimbrial genes.

Primer	Primer sequence (5'-3')	Size of product (bp)
P7 (for subfamily I adhesins: F17a-G, F111)	CGG AGC TAA TAC TGC ATC AAC C	615
P8 (for subfamily II adhesins: F17b-G, F17c-G/*GafD*)	CGT GGG AAA TTA TCT ATC AAC G	615
P9 (for subfamilies I and II adhesin genes)	TGT TGA TAT TCC GTT AAC CGT AC	

0.6 µM of each primer, 1.25 U of Taq polymerase (Promega), and 5 µl of template DNA. The amplification parameters used were: following an initial denaturation at 94 °C for 5 min, 25 cycles of denaturation at 94 °C for 2 min, primer annealing at 55 °C for 1 min, and extension at 72 °C for 1 min (15). *E. coli* 11A (O?:K99, FY [F17 fimbria formerly called FY]) provided from Pendik Veterinary Control and Research Institute, İstanbul, was used as a positive control in this PCR.

Results

Microbiological culture of 100 *E. coli* isolates established that only 2 were sorbitol negative on Sorbitol MacConkey agar plates. These 2 isolates were subsequently shown to be negative for O157:H7 by both the latex agglutination test and PCR for the Shiga toxins (*stx1* and *stx2*) and *eaeA* genes. The PCR results established that 9 isolates were positive for the gene of F17 fimbriae, and 1 isolate was positive for the *eaeA* gene.

Discussion

Possible virulence factors of *E. coli* isolates from bovine mastitis have been previously published by various studies using molecular (12-14) and phenotypic (16-21) methods. These studies indicate that no specific virulence factor was found that is commonly produced by *E. coli* isolates from mastitis. In the present study, 9% of the strains were positive for the F17 fimbriae and only 1 strain was positive for the intimin gene. No genes for the other virulence factors, *Stx1, Stx2, Sta,* F41, and K99, were detected.

Fimbriae are important virulence factors of *E. coli*. F5 and F41 are common adhesins in *E. coli* isolated from diarrheic calves (6). The F17-related adhesins are prevalent in *E. coli* strains isolated from calves with diarrhea or septicemia and from lambs. The F17 family includes the F17a, F17b, F17c, and F111 fimbriae produced by bovine *E. coli* isolates (15). Although F5 and F41 were not detected among the mastitis isolates, the

presence of F17 fimbriae in mastitis isolates has been reported. Lipman et al. (14) found F17 fimbriae in 11 of 20 *E. coli* mastitis strains. Kaipainen et al. (13) reported the prevalence of the genes for F17 fimbriae in 9% of Finnish isolates and 1% of Israeli isolates from mastitis.

The importance of Shiga toxin-producing *E. coli* (STEC) has increased in recent years because of a food-borne infection caused by STEC O157:H7. *E. coli* O157:H7, designated as enterohemorrhagic *E. coli* (EHEC), is the predominant and most virulent serotype in a pathogenic subset of STEC (22). Cattle are considered the main reservoir of this serotype. The main virulence factors of STEC include at least 1 Shiga toxin (*Stx1* or *Stx2*), together with the outer membrane adhesion protein associated with attaching and effacing lesions, intimin. An objective of this study was also to investigate the prevalence of STEC in Turkish bovine mastitis isolates. None of the virulence factor genes associated with STEC have been detected in this study with the exception of only 1 isolate which was positive for the *eaeA* gene only. Wenz et al. (9) similarly detected the *eaeA* gene in only 1 of 123 mastitis isolates. No Shiga toxin in *E. coli* isolates from mastitis was reported in some studies (14,23). Barrow and Hill (16) found that only 1 strain of 237 *E. coli* from mastitis produced Shiga toxin. Stephan and Kühn (24) detected Shiga toxin 1 or 2 together with *ehly* gene in 4 of 145 samples. Nevertheless, in some other studies STEC genes have been found in higher rates. Bean et al. (12) detected *stx1* in 31% and *eaeA* in 3.5% of 80 mastitic isolates from pasture-fed cows. Lira et al. (25) found STEC in 12.0% of 182 *E. coli* isolates from bovine mastitis.

Enterotoxin production is also not a common property of *E. coli* isolates from mastitis. Barrow and Hill (16) detected enterotoxin in only 1 of 237 isolates. Sanchez-Carlo et al. (20) found only 1 heat-stable enterotoxin and 2 heat-labile enterotoxin positive isolates among 184 *E. coli* isolates from cows with acute mastitis. Lipman et al. (14) found no strains that are positive for enterotoxin genes (LT and ST1) among 20 isolates. Similarly, in this study no heat-stable enterotoxin gene was detected.

Taking into consideration that *E. coli* from mastitis originates from fecal flora or the cow's environment, virulence characteristics of the isolates from mastitis in this study may also reflect the general situation of bacteria in the environment.

In conclusion, in agreement with some previous studies conducted in other countries, no prevalent virulence factors were detected in *E. coli* isolates causing clinical bovine mastitis in Turkey.

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