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# traT and CNF2 genes of *Escherichia coli* isolated from milk of healthy cows and sheep

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**Abstract**

The objectives of the present study were to isolate *Escherichia coli* from milk of apparently healthy cows and sheep and to investigate the presence of traT and cytotoxic necrotising factor-2 (CNF2) virulence genes by multiplex polymerase chain reaction (PCR). Milk samples collected from a total of 1028 apparently healthy ruminants (737 cows and 291 sheep) in eastern Turkey were streaked onto 5% sheep-blood agar. *E. coli* was isolated and identified by biochemical tests in 5.9% (61/1028) of milk samples. Correct amplification with the molecular length of 232 bp was obtained from all *E. coli* isolates by the species-specific PCR. The isolation rates of the agent were calculated to be 7.6% (56/737) in cows and 1.7% (5/291) in sheep. The difference between these proportions was statistically significant ($p<0.001$). Multiplex PCR showed that traT and CNF2 genes were present in 62.3% and 6.6% of all isolates, respectively. Both genes were present in 16.4% of the isolates, with only 14.7% having neither gene.


*Keywords:* *E. coli*; traT; CNF2; Milk; Cow; Sheep

## 1. Introduction

Mastitis is the inflammation of mammary gland associated mostly with microbial infections. The eradication of mastitis in cows and sheep is difficult owing to its complex aetiology. It is of great economic significance particularly in the dairy industry worldwide, causing millions of dollars in losses due to reduced milk production, treatment costs, culling and death. An annual cost of approximately £170 million has been estimated in the dairy cow population of the UK due to clinical mastitis (Bradley, 2002). Losses associated with subclinical mastitis, which are difficult to estimate, should also be considered in order to have a better understanding of the economical impact of mastitis, which has been reported to be responsible for nearly 40% of total costs of common production diseases (Kossaibati and Esslemont, 1997). It must be underlined that mastitis is important for public health too, as the massive use of antibiotics in the treatment of food producing animals may contribute to the emergence of resistant bacteria entering the food chain (White and McDermott, 2001).

In recent years, the incidence and prevalence of contagious mastitis has been reduced due to strict control programmes, but environmental mastitis continues to cause serious problems in the dairy industry throughout the world (Bradley, 2002). *Escherichia coli* is the main causative agent of environmental mastitis and has been reported to be responsible for 35% of clinical cases in bovine mastitis compared to 10% caused by contagious pathogens in the UK (Bradley and Green, 2001). In Israel, coliform mastitis has been reported to account for more than 60% of clinical mastitis (Shpigel et al., 1998). The increase in the incidence of *E. coli* associated mastitis is thought to be due to routine use of dry-cow therapy and to post-milking teat dipping which is considered as an effective method to reduce mastitis caused by Gram positive microbes but is not equally efficient against Gram negative bacteria including *E. coli* (Erskine and Eberhart, 1991; Fang and Pyorala, 1996). In contrast to the UK and Israel, the prevalence of environmental mastitis associated with *E. coli* has been

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estimated to be less than 10% compared with more than 50% of contagious mastitis (caused by *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Streptococcus disgalactiae*) in dairy cattle and sheep populations of Turkey (Arda and Istanbulluoglu, 1979; Baysal and Kenar, 1989; Gulcu and Ongor, 2002; Turutoglu et al., 1995).

Clinical signs of mastitis caused by *E. coli* may vary from mild to very severe, occasionally fatal forms and various virulence factors of the agent may play role in this. Previous studies have mainly concentrated on the phenotypic expression of virulence factors and serum resistance has been suggested as the major virulence factor for *E. coli* mastitis (Barrow and Hill, 1989; Fang and Pyorala, 1996). The traT gene encodes an outer membrane protein which is believed to play a role in serum resistance (Pramoonjago et al., 1992). However, some studies failed to establish an association between the presence of traT and serum resistance (Nemeth et al., 1991; Pfaff-McDonough et al., 2000). This gene has been reported in mastitic milk in high proportions (Kaipainen et al., 2002; Nemeth et al., 1991). Some *E. coli* strains which are able to produce two types of cytotoxic necrotising factors (CNF1 and CNF2) are classified as necrotoxigenic *E. coli* (NTEC). Although the role of these toxins in the pathogenesis of *E. coli* is yet to be clarified, CNF2 has been reported to be present in strains isolated from diarrhoeic and septicaemic cases (Orden et al., 1999; Pohl et al., 1993). However, the fact that this toxin gene has also been found in *E. coli* present in the normal intestinal flora of healthy ruminants (Blanco et al., 1998; Burns et al., 1996) casts doubt on the role of NTEC in diarrhoea. The presence of CNF2-producing *E. coli* strains in mastitic milk has rarely been reported (Kaipainen et al., 2002; Pohl et al., 1993).

Recent advances in molecular biology, in particular the development of the polymerase chain reaction (PCR), have facilitated the investigation of virulence factors of pathogenic *E. coli* strains isolated from intestinal and extra intestinal infections including mastitis (Kaipainen et al., 2002; Orden et al., 1999). The objectives of the present study were to isolate *E. coli* from milk of apparently healthy cows and sheep and to investigate the presence of traT and CNF2 virulence genes by multiplex PCR.

## 2. Materials and methods

### 2.1. Material

Milk samples were collected from a total of 1028 apparently healthy ruminants (737 cows and 291 sheep), chosen randomly in Elazig province located in eastern Turkey between May and November 2002. No abnormalities were observed to suspect clinical mastitis in all the animals. The cows sampled here were Holstein breed and aged between 5 and 7 years, while the sheep were local breeds and aged between 3 and 5 years. All the animals were at the late stage of lactation. All necessary hygienic precautions were taken in order to avoid cross-contamination during sample collection. The samples were immediately transported to the laboratories and were processed for culture.

### 2.2. Culture and biochemical identification

Milk samples were inoculated on blood agar base (Merck, Darmstadt, Germany) supplemented with 5% defibrinated sheep blood. After the incubation of plates aerobically for 24 h at 37 °C, suspicious colonies were transferred onto eosin methylene blue (EMB) agar and MacConkey agar for further identification. Then, routine biochemical tests, i.e. catalase, oxidase, citrate, $H_2S$, methyl red, indole and Voges–Proskauer (VP) tests were carried out to identify the isolates. The colonies that were identified as *E. coli* were kept at −20 °C within glycerol nutrient broth.

### 2.3. DNA extraction and PCR

A few colonies from each isolate were transferred into an Eppendorf tube containing 300 μl distilled water, 300 μl of TNES buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.2% SDS) and Proteinase K (200 μg/ml). This suspension was incubated at 37 °C for 2 h. Following 10 min of boiling, the same amount of phenol (saturated with Tris–HCl) was added to the suspension. The suspension was shaken vigorously by hand for 5 min and then, centrifuged at 11,600*g* for 10 min. The upper phase was carefully transferred into another Eppendorf tube and sodium acetate (0.1 volume) and ethanol (2.5 volumes) were added to the suspension which was left at 20 °C overnight to precipitate the DNA. The pellet, obtained following the centrifugation at high speed for 10 min, was washed twice with 95% and 70% ethanol respectively, each step followed by 5 min centrifugation. Finally the pellet was dried and resuspended in 50 μl of distilled water.

The PCR was performed in a touchdown thermocycler (Hybaid, Middlesex, England) in a total reaction volume of 50 μl containing 5 μl of 10× PCR buffer (750 mM Tris–HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 5 μl 25 mM $MgCl_2$, 250 μM of each deoxynucleotide triphosphate, 1.25 U of *Taq* DNA Polymerase (MBI, Fermentas), 1 μM of each primer and 5 μl of template DNA. A pair of primers derived from 16S rRNA gene of *E. coli* (Riffon et al., 2001) was used to identify isolates. Two pairs of primers specific to traT and CNF2 genes (Kaipainen et al., 2002) were used in the multiplex PCR. Two amplification procedures were applied. In the first PCR using species specific primers,

amplification was obtained with 35 cycles following an initial denaturating step at 94 °C for 2 min. Each cycle involved denaturation at 94 °C for 45 s, annealing at 64 °C for 1 min and synthesis at 72 °C for 2 min. In the multiplex PCR, amplification was obtained with 25 cycles involving denaturation at 95 °C for 2 min, annealing at 55 °C for 1 min, and synthesis at 72 °C for 1 min.

The amplified products were detected by ethidium bromide (0.5 μg/ml) staining after electrophoresis at 80 V for 2 h in 1.5% agarose gels. PCR products with the molecular size of 232, 307 and 654 bp were considered indicative for identification as *E. coli*, traT positive *E. coli* and CNF2 positive *E. coli*, respectively.

### 2.4. Statistical analysis

A chi-squared ($\chi^2$) test was used to estimate differences between the isolation rates of *E. coli* in cows and sheep, whereby a probability of less than 0.05 was considered as statistically significant.

## 3. Results

*Escherichia coli* was isolated and identified by biochemical tests in 5.9% (61/1028) of milk samples collected from cows and sheep. All 61 isolates were catalase, indole and methyl red positive, oxidase, citrate, $H_2S$ and VP negative. They all produced distinctive metallic sheen on EMB agar and bright pink colonies on MacConkey agar. The isolation rates of the agent by species were estimated to be 7.6% (56/737) in cows and 1.7% (5/291) in sheep. The difference between these proportions was statistically significant ($p = 0.0006$). Correct amplification with the molecular length of 232 bp was obtained in the analysis of all the isolates by the species specific PCR which confirmed the results of biochemical tests.

In multiplex PCR, traT and CNF2 genes were determined to be present in 62.3% and 6.6% of all isolates, respectively. While 16.4% of the isolates were containing both genes, in only 14.7% neither gene was present (Fig. 1). The distribution of the virulence genes by animal species is shown in Table 1.

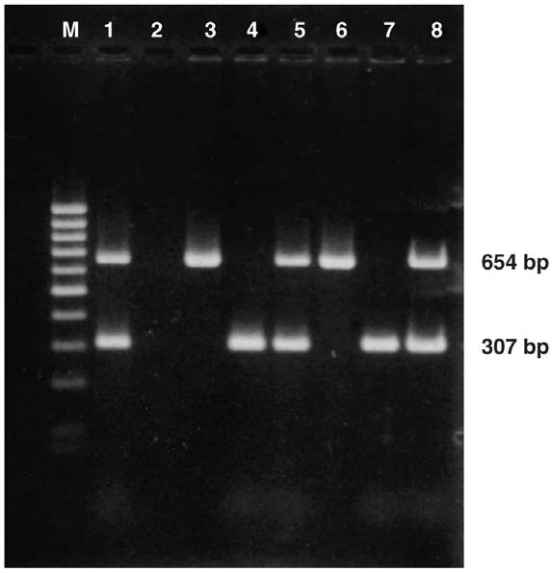


Fig. 1. An ethidium bromide-stained agarose gel of multiplex PCR products showing the virulence genes in *E. coli* strains isolated from milk samples of cows and sheep. M: molecular marker (DNA ladder, 100 bp); lanes 1, 5, 8: traT and CNF2 positive *E. coli*; lanes 3, 6: CNF2 positive *E. coli*; lanes 4, 7: traT positive *E. coli*; and lane 2: negative control (distilled water).

## 4. Discussion

Although the potential role of *E. coli* in clinical mastitis has been well documented, there is a paucity of information on the role of this agent in subclinical mastitis. This study investigated the presence of *E. coli*, the most prevalent bacterial species isolated from clinical mastitis in many countries including the UK, in milk of cows and sheep located in eastern Turkey and the overall isolation rate was determined to be approximately 6.0%. As there were no detectable abnormalities during the palpation of mammary gland of animals, it could be said that none of the animals sampled in this study had clinical mastitis. Although no attempts were made to determine somatic cell counts, bacterial culture results suggest that this proportion could be interpreted as the representative of prevalence of subclinical mastitis caused by *E. coli*. We also searched the presence of traT and CNF2 genes that were previously reported to be the most common virulence factors in mastitic milk samples (Kaipainen et al., 2002). Although there are a wide range of different virulence factors which may play role in the pathogenesis of *E. coli* in mastitis, inclusion of other genes was beyond the scope and budget of this study. Despite this, only a small proportion (14.7%) of *E. coli* isolates was determined to possess neither virulence factor analysed.

Table 1
Multiplex PCR results showing the distribution of virulence genes by animal species

| Species (No. of isolates) | traT gene (%) | CNF2 gene (%) | traT + CNF2 (%) | Negative (%) |
|---|---|---|---|---|
| Cows (56) | 35 (62.5) | 3 (5.3) | 10 (17.9) | 8 (14.3) |
| Sheep (5) | 3 (60) | 1 (20) | – | 1 (20) |
| Total (61) | 38 (62.3) | 4 (6.6) | 10 (16.4) | 9 (14.7) |

The isolation rate of *E. coli* was significantly higher in cows than in sheep. As in other parts of the world, bovine mastitis has been paid more attention in Turkey when compared with ovine mastitis due possibly to its higher incidence and therefore to its more severe economical consequences in cows. The proportion (7.6%) obtained in this study was slightly lower than those of 8% and 10% reported in previous studies carried out in different parts of the country (Arda and Istanbulluoglu, 1979; Turutoglu et al., 1995). However, it should be underlined that the latter figures were estimated from the examination of cows with clinical mastitis. In this respect, it could be said that *E. coli* accounts for similar proportions of clinical and subclinical mastitis in dairy cows in Turkey.

There is a dearth of information on the prevalence, distribution and causative agents of mastitis in sheep population of Turkey. In a few studies carried out in different regions, *E. coli* has been implemented as the causative agent in 6% to 10% of clinical mastitis in sheep (Baysal and Kenar, 1989; Gulcu and Ongor, 2002). These proportions were higher than that (1.7%) calculated in apparently healthy sheep in the present study. These figures suggest that *E. coli* in subclinical mastitis in sheep is less frequent compared with clinical mastitis in Turkey. In contrast, *E. coli* has been reported to be responsible for 20% of subclinical mastitis in sheep populations of southern Jordan (Al-Majali and Jawabreh, 2003). It is possible that some factors such as climate, sampling, management and nutrition differences might have contributed to this. In addition, the number of sheep examined here is rather small to draw a firm conclusion about the potential role of *E. coli* in subclinical ovine mastitis in Turkey.

The proportion of *E. coli* isolates with only traT gene was calculated to be 62%. When the isolates carrying both genes were also considered, the percentage rose up to 78.7% (48/61). This was significantly higher than the 37% and 41% reported in Finnish and Israeli cattle, respectively (Kaipainen et al., 2002). The detection of traT gene in a high proportion of isolates of mastitis origin may indicate a role for the gene in the pathogenesis of mastitis caused by *E. coli*, because this gene has been linked with serum resistance and more than 60% of strains isolated from mastitis have been reported to be serum resistant (Fang and Pyorala, 1996; Nemeth et al., 1991; Pramoonjago et al., 1992). However, there is some controversy regarding the association of traT and serum resistance (Nemeth et al., 1991; Pfaff-McDonough et al., 2000).

The CNF2 gene has been shown to be very common in faeces of ruminants with diarrhoea and with no health problems (Orden et al., 1999; Pohl et al., 1993). However, very few studies are available reporting the presence of CNF2 in isolates from mastitic cases. This gene was calculated to be present in 6.6% of *E. coli* strains isolated from milk samples in the current study. When the isolates carrying both genes were considered, this percentage was calculated as 23% (14/61). This figure was relatively higher than those of 14% and 3.5% reported in Finnish and Israeli cattle, respectively (Kaipainen et al., 2002). It is likely that the CNF2 synthesizing *E. coli* strains in milk were originated from faecal contamination.

Although it has widely been accepted that *E. coli* is an opportunistic intramammary pathogen with environmental origin, the latest reports of recurrent cases of mastitis associated with *E. coli* (Bradley, 2002; Dopfer et al., 1999) suggest that the agent is changing its behaviour and perhaps carrying contagious character which needs to be investigated in detail. Also, large scaled studies are necessary to improve our understanding of the potential role of *E. coli* and relevant virulence factors in the aetiology of both clinical and subclinical mastitis in cows and sheep and to provide quantitative data on the economical significance of this disease complex which may help develop effective control and eradication strategies.

## References


Al-Majali, A.M., Jawabreh, S., 2003. Period prevalence and etiology of subclinical mastitis in Awassi sheep in southern Jordan. Small Ruminant Research 47, 243–248.

Arda, M., Istanbulluoglu, E., 1979. Mastitislere neden olan aerob, anaerob ve mantarlarn izolasyonu, identifikasyonu, bunlara karşı etkili olan antibiyotik ve fungisitlerin saptanmas. Ankara Universitesi Veteriner Fakultesi Dergisi 26, 14–29.

Barrow, P.A., Hill, A.W., 1989. The virulence characteristics of strains of *Escherichia coli* isolated from cases of bovine mastitis in England and Wales. Veterinary Microbiology 20, 35–48.

Baysal, T., Kenar, B., 1989. Konya ve yöresindeki koyunlarda klinik ve subklinik mastitis olgularndan aerob etken izolasyonu ve identifikasyonu. Etlik Veteriner Mikrobiyoloji Dergisi 6, 55–66.

Blanco, M., Blanco, J.E., Mora, A., Blanco, J., 1998. Prevalence and characteristics of necrotoxigenic *Escherichia coli* CNF1+ and CNF2+ in healthy cattle. Research in Microbiology 149, 47–53.

Bradley, A., 2002. Bovine mastitis: an evolving disease. The Veterinary Journal 164, 116–128.

Bradley, A.J., Green, M.J., 2001. Aetiology of clinical mastitis in six Somerset dairy herds. The Veterinary Record 148, 683–686.

Burns, A.L., Ball, H.J., Finlay, D.A., 1996. CNF producing *Escherichia coli* isolated from cattle in Northern Ireland. Veterinary Microbiology 49, 235–241.

Dopfer, D., Barkema, H.W., Lam, T.J., Schukken, Y.H., Gaastra, W., 1999. Recurrent clinical mastitis caused by *Escherichia coli* in dairy cows. Journal of Dairy Science 82, 80–85.

Erskine, R.J., Eberhart, R.J., 1991. Post-milking teat dip use in dairy herds with high or low somatic cell counts. Journal of American Veterinary Medical Association 199, 1734–1736.

Fang, W., Pyorala, S., 1996. Mastitis-causing *Escherichia coli*: serum sensitivity and susceptibility to selected antibacterials in milk. Journal of Dairy Science 79, 76–82.

Gulcu, H.B., Ongor, H., 2002. Bacteriological examination of the udder samples collected from sheep and goats slaughtered at a local abattoir in Elazig. Veteriner Bilimleri Dergisi 18, 67–69.

Kaipainen, T., Pohjanvirta, T., Shpigel, N.Y., Shwimmer, A., Pyorala, S., Pelkonen, S., 2002. Virulence factors of *Escherichia coli* isolated from bovine clinical mastitis. Veterinary Microbiology 85, 37–46.

Kossaibati, M.A., Esslemont, R.J., 1997. The costs of production diseases in dairy herds in England. The Veterinary Journal 154, 41–51.

Nemeth, J., Muckle, C.A., Lo, R.Y., 1991. Serum resistance and the traT gene in bovine mastitis-causing *Escherichia coli*. Veterinary Microbiology 28, 343–351.

Orden, J.A., Ruiz-Santa-Quiteria, J.A., Cid, D., Garcia, S., de la Fuente, R., 1999. Prevalence and characteristics of necrotoxigenic *Escherichia coli* (NTEC) strains isolated from diarrhoeic dairy calves. Veterinary Microbiology 66, 265–273.

Pfaff-McDonough, S.J., Horne, S.M., Giddings, C.W., Ebert, J.O., Doetkott, C., Smith, M.H., Nolan, L.K., 2000. Complement resistance-related traits among *Escherichia coli* isolates from apparently healthy birds and birds with colibacillosis. Avian Diseases 44, 23–33.

Pohl, P., Oswald, E., Van Muylem, K., Jacquemin, E., Lintermans, P., Mainil, J., 1993. *Escherichia coli* producing CNF1 and CNF2 cytotoxins in animals with different disorders. Veterinary Research 24, 311–315.

Pramoonjago, P., Kaneko, M., Kinoshita, T., Ohtsubo, E., Takeda, J., Hong, K.S., Inagi, R., Inoue, K., 1992. Role of TraT protein, an anticomplementary protein produced in *Escherichia coli* by R100 factor, in serum resistance. Journal of Immunology 148, 827–836.

Riffon, R., Sayasith, K., Khalil, H., Dubreuil, P., Drolet, M., Lagace, J., 2001. Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. Journal of Clinical Microbiology 39, 2584–2589.

Shpigel, N.Y., Winkler, M., Ziv, G., Saran, A., 1998. Clinical, bacteriological and epidemiological aspects of clinical mastitis in Israeli dairy herds. Preventive Veterinary Medicine 35, 1–9.

Turutoglu, H., Atesoglu, A., Salihoglu, H., Ozturk, M., 1995. Marmara bolgesi sut ineklerinde mastitise neden olan aerobik etkenler. Pendik Veteriner Mikrobiyoloji Dergisi 26, 125–137.

White, D.G., McDermott, P.F., 2001. Emergence and transfer of antibiotic resistance. Journal of Dairy Science 84 (E. Suppl.), E151–E155.