

Original Research Article

Determination of Virulence Genes and Genetic Similarities of Mastitic Milk Originated *Escherichia coli* Isolates

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Abstract: The primary causes of mastitis are bacteria, such as *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus spp.* However, mastitis still continues to be a major problem in dairy animals due to economic losses to dairy farms. Turkey is ranked 27th in world and 3rd in European countries in terms of the assets of cattle and livestock potential. For this reason, mastitis is one of the important diseases for our country. Identification was made by conventional methods and confirmed using PCR. The virulence genes of *eae*, *traT*, *stx1*, *stx2* and *aer* were investigated individually by PCR. Twenty-one *E. coli* were isolated and identified from milk samples. Two and 3 of them were found to be positive for the *eae* and *traT* genes, respectively. However, no isolate was found positive for *stx1*, *stx2* and *aer* genes. In conclusion, it can be thought that mastitis isolates of *E. coli* pose risks to human health. Therefore, protective and hygienic measures should be taken in dairy farms for the contamination of *E. coli*.

Keywords: *aer*, *eae*, *E. coli*, Mastitis, PCR, *stx1*, *stx2*, *traT*.

INTRODUCTION

Mastitis is the most prevalent and costly disease of dairy cattles (Savaşan and Kaya, 2004; Ferguson *et al.*, 2007). Mastitis reduces milk yield, increases health cost and makes milk less suitable for both consumption and processing. Among the pathogens that cause mastitis predominantly *Staphylococcus spp.*, *Streptococcus spp.*, and *Escherichia coli* (Savaşan and Kaya, 2004; Bradley, 2002). *E. coli* was first described by Theodor Escherich in 1885. This bacterial precedence is known as *Bacterium coli commune* and is later referred to as *Escherichia coli*. *E. coli* has been considered as a non-pathogenic facultative microorganism found in humans in normal intestinal flora and in warm-blooded animals and birds for years (Nataro and Kaper, 1998). *E. coli* was first isolated from milk with mastitis in 1896 and reported that it was the second most common cause of cattle mastitis in the 1960s (Sumathi *et al.*, 2008). Studies have shown that, it is one of the pathogenic agents that cause diseases such as enteritis, urogenital infections, wound infections, mastitis, septicemia and meningitis. (Wasteson, 2002; Guler and Gunduz, 2007).

The association between the host organism and pathogenic *E. coli* strains depends on the presence of bacterial virulence factors (China and Goffaux, 1999; İzgür, 1999; Kuhnert *et al.*, 2000). Virulence factors play a role in martial infestation, fighting against the site defense system, and colonization. Genes encoding virulence factors could be found in the genome of the bacteria or in the plasmids. Primarily in the case of colonization of mucosal surfaces, extraintestinal infections, resistance to complementary bactericidal effect, passage of epithelial cell layer, ability to survive phagocytosis and survival is significant (Harel *et al.*, 1993). The main virulence factors of *E. coli* strains include a variety of factors such as adhesins, toxins, proteins released into the host cells, polysaccharide capsules, resistance to complement killing and aerobic siderophores (China and Goffaux, 1999; Harel *et al.*, 1993).

The aims of this study were to determine the virulence genes and to identify genetic similarities of *E. coli* isolates from mastitic milk.

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MATERIALS AND METHODS

Collection of Specimens and Bacterial Isolation

For the research, different cattle establishments as large scale (50 and over cows) enterprises and family establishments located in/around Aydın and İzmir provinces were visited. The milk samples were taken from at least one cow that had been in a period of many lactation. For the isolation of *E. coli*, milk samples were directly transferred to Blood Agar, MacConkey Agar and Eosin Methylene Blue Agar and incubated at 37°C for 18-24 h. The macroscopic and microscopic morphologies of the colonies were examined and biochemical tests specific for *E. coli* were performed (Scheutz and Strockbine, 2005).

Genotypic identification and determination of virulence genes

Following isolation and biochemical identification, the DNA's were extracted for examining the virulence genes of *E. coli*.

For the DNA extraction aim, boiling method was performed. Suspected colonies were inoculated to nutrient agar and incubated at 37°C for 12 h. The pure colonies were collected and suspended in 500 microliters of deionized water in DNase-RNase free eppendorf tubes. The suspensions were boiled at 100°C

for 10 min. After centrifugation for 5 min at 10,000xg, the supernatants were taken and stored for using as target DNA in PCR.

PCR were performed for confirmation of identification and examining the virulence genes. The identification of *E. coli* was confirmed as described by Abd El-Razik *et al.* (2010). After PCR, the band of 662 bp was considered positive for *E. coli*.

PCR was carried out to determine intimin (*eae*), Aerobactin (*aer*), outer membrane protein (*traT*), and shiga toxin (*stx1* and *stx2*) virulence genes of the *E. coli* isolates. *eae* specific PCR was performed as reported by Güler and Gündüz (2007), and the band presence of 425 bp was considered as positive. The PCR for *aer* was performed as reported by Oliveira *et al.* (2011), and the band presence of 602 bp was considered as positive. In order to determine the presence of the *traT* gene in isolates, the method described by Kaipainen *et al.* (2002) was carried out. The band of 307 bp was considered as positive. For *stx1* and *stx2*, PCR assay was carried out as described by Fitzmaurice (2003). The bands of 180 and 255 were evaluated as positive for *stx1* and *stx2*, respectively. The oligonucleotide primes used for PCR were shown in Table-1.

Table-1. Oligonucleotide primers used for PCR.

Target DNA	Oligonucleotide primers	Expected bands (bp)
<i>eae</i>	F5'- ATATCCGTTTTAATGGCTATCT-3' R5'- AATCTTCTGCGTACTGTGTTCA-3'	425
<i>aer</i>	F5'-TACCGGATTGTCATATGCAGACCGT-3' R5'- AATATCTTCCTCCAGTCCGGAGAAG-3'	602
<i>traT</i>	F5'-GATGGCTGAACCGTGGTTATG-3' R5'- CACACGGGTCTGGTATTTATGC-3'	307
<i>stx1</i>	F5'-ATAAATCGCCATTCGTTGACTAC-3 R5'-AGAACGCCCACTGAGATCATC-3	180
<i>stx2</i>	F5'-GGCACTGTCTGAACTGCTCC-3 R5'-TCGCCAGTTATCTGACATTCTG-3	255
ERIC2	5'-AAGTAAGTGACTGGGGTGAGCG-3	variable
<i>E.coli</i>	F5'-GCTTGACACTGAACATTGAG-3' R5'-GCACTTATCTCTTCCGCATT-3'	662

Genotyping of isolates

The isolates were genotyped using the ERIC-PCR reaction as described by Versalovic *et al.* (1991). Similarity coefficients for pairs of tracks were calculated using the unweighted pair group method with arithmetic averages (UPGMA).

RESULTS

Identification of isolates

A total of 21 isolates were identified as *E. coli* phenotypically and genotypically.

Determination of virulence genes

For determination of virulence genes, PCR was performed to detect *aer*, *traT*, *eae*, *stx1* and *stx2* genes. one (8.3%) and two (16.6%) of the 21 isolates of *E. coli* were found to be positive for *eae* and *traT* genes, respectively. None of the isolates carried *aer*, *stx1* and *stx2* gene.

Genotyping of isolates

RAPD-PCR patterns were grouped by the unweighted pair group method using arithmetic averages (UPGMA). Phylogenetic analysis of RAPD-PCR patterns obtained for *E. coli* revealed 21 unique

types (Fig- 1). The isolates were found as a similarity of 35% to 90%.

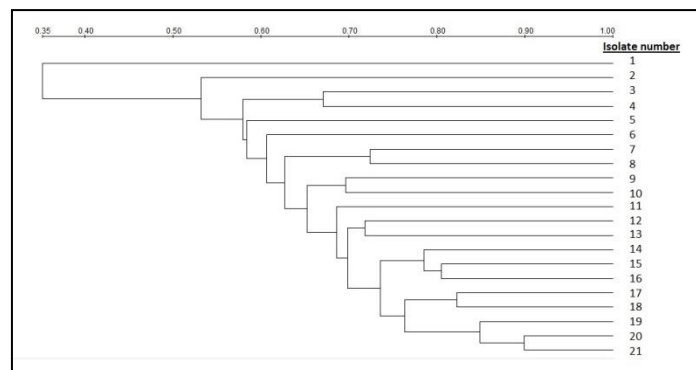


Fig-1. Phylogenetic analyses dendrogram of *E.coli* isolates.

DISCUSSION

Bovine mastitis is the most costly disease to the dairy industry worldwide as well as in Turkey. *E. coli* is a frequent cause of bovine mastitis. The rate of clinical mastitis occurring in cattle due to *E. coli* varies between countries. Recently, dairy managers have become more aware of *E. coli* mastitis infections and have made additional efforts to remove this pathogen from their herds. Such efforts have been hampered by the inability of dairy managers to detect all infections consistently due to their inability to detect multiple and perhaps some unknown reservoirs. In an Israeli study, 60.2% of 1124 clinical mastitis cases were characterized by coliform agents (2002). Schukken *et al.*, (1989) reported that they isolated 16.2% of *E. coli* from 1140 clinical mastitis cases in the Netherlands. In another study conducted in the Netherlands, 16.9% of mastitis cases were found to be caused by *E. coli* (Miltenburg *et al.*, 1996). It has been reported by Shipigel *et al.* (1998) that in Finland, the incidence of *E. coli*-induced mastitis is less than 20%. Bradley (2007) reported in 1998 that 14.4% of *E. coli* caused mastitis in the UK. Bradley and Green (2001) found that *E. coli*-induced mastitis was found in 34.7% of 6 dairy cows. Correa and Marin (2002) found 182 (8%) *E. coli* isolates from 2144 clinical and subclinical mastitis-infected dairy cows in a study conducted in Brazil. Unnerstad *et al.*, (2009) found that *E. coli* from 15.9% of mastitis cases examined in Sweden. Barrow and Hill (1998) reported that 5% of 237 *E. coli* strains isolated from bovine mastitis produce hemolysis in sheep blood agar. Hogan and Smith (2003) found that 2.6% of 76 *E. coli* strains causing mastitis lead to hemolysis in cattle and 3.9% in sheep erythrocytes. In a study conducted by Nemeth *et al.*, (1991), 53% of the 47 *E. coli* strains isolated from bovine mastitis by 64% of 95 *E. coli* strains isolated from bovine mastitis accounted for 47% of the 36 faecal-origin enterotoxigenic *E. coli* strains and 43% of fecal originated verotoxigenic *E. coli* strains were serum-resistant.

As an environmental mastitis pathogen, *E. coli* cause persistent and repeated intramammary infections

with a variable bacterial virulence factors. Therefore, determination of the virulence factors is important to identify the characters of infection. Lipopolysaccharide has been investigated for the *E. coli* strains causing mastitis (Nemeth and Muckle, 1991; Taylor, 1983), which is associated with K1 capsular antigen and exogenous bone proteins such as *TraT* and IsoPs proteins. Sanchez-Carlo *et al.* (1984) found that all strains of *E. coli* strains isolated from acute mastitis cows (100%) were serum-resistant in the test using cow, calf, human and guinea pig serum. In a study investigating serum resistance and *traT* gene in *E. coli* isolates from bovine mastitis, 43 of *E. coli* strains of 95 mastitis originated from the study, 36 faecal 31% of the original ETEC strains and 44% of the 43 VTEC strains were identified as *traT* genes (Nemeth and Muckle, 1991). Kaipainen *et al.*, (2002) have detected the *traT* gene in 37% of 160 *E. coli* strains originated from mastitis isolated from Finland and 41% of 113 *E. coli* strains isolated from Israel isolate from Israel. Lehtolainen (2004) determined the *traT* gene in 38% of *E. coli* strains originated from the mastitis investigated. In our study, 2 (16.6%) strains were found to be positive for *traT* gene.

Several studies have been carried out on the role of aerobactin in bovine mastitis. Nemeth *et al.*, (1991) found aerobactin presence in 20% of *E. coli* strains isolated from mastitis cases. Kaipainen *et al.*, (2002) are broad-spectrum (*aer*) isolates encoding 11.3% of *E. coli* strains originating in Finland originating in mastitis and 4.4% of Israeli origin *E. coli* strains. Lehtolainen (2004) found 8.4% of *E. coli* strains isolated from mastitis cows positive for aerobactin. In our study, no *aer* gene was detected in any of the strains examined. Cengiz *et al.*, (2014) did not detect any *aer* gene in 56 *E. coli* strains in parallel with our results.

In a study by Güler and Gündüz (2007), investigating potential virulence factors in *E. coli* strains isolated from clinical cow mastitis, only 1 (1%) of 100 *E. coli* strains were identified as *eae*. Wenz *et al.*, (2006) 123 have identified the *eae* gene in only 1 of

123 mastitis isolates. Bean *et al.*, (2004) found that 31% of the 80 milk samples examined had *stx1* and 3.75% had the presence of *eae*. In our study, 8.3% of the isolates found to be positive for *eae*. Cengiz *et al.*, (2014) found *eae* gene in 8 of 56 *E. coli* strains. This result is in parallel to our results.

Shigatoxin producing *E. coli* strains (STEC) are responsible for food-borne outbreaks, and cattle and other ruminants are the most important reservoirs of STEC. The shigatoxin is accepted as the additional virulence factor such as intimin, which provides attaching of STEC to the intestinal mucosa. Additionally, the STEC strains carry *eae* gene for intimin. In the current study, *stx1* and *stx2* genes were evaluated. In the previous studies, researchers also stated variations in the presence rates for these genes (Kobori *et al.*, 2004; Fremaux *et al.*, 2006; Rangel and Marin, 2009; Momtaz *et al.*, 2012). Although, Momtaz *et al.*, (2012) and Kobori *et al.*, (2004) stated a higher presence rate, other researchers (Fernandes *et al.*, 2011; Doğan *et al.*, 2006; Bean *et al.*, 2004) could not detect the gene in *E. coli* strains. In the presented study, *stx* genes were not found in any of isolates. The significant difference between the reports was associated with the origin of the strains (Fremaux *et al.*, 2006).

RAPD helps to understand the epidemiology, ecology, tracking outbreaks, and spreading of the microorganisms (Kılıç *et al.*, 2009; Johnson *et al.*, 2006; Suardana *et al.*, 2013). It allows phylogenetic tree to visualize and quantify the relationship between strains of bacterial species (Morshed and Peighambari, 2010; Norazah *et al.*, 2009). In the presented study, strains were divided into 21 genotypes which had a similarity of 30% to 90% using RAPD. This result showed that, *E. coli* strains which caused intramammary infections might be originating from various sources.

In conclusion, mastitis isolates of *E. coli* might be considered to pose a risk for human beings. For this reason, the protective and hygienic precautions should be taken in dairy farms for contamination of *E. coli*.

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