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Research Article

Evaluation of Virulence Factors and Phylogrouping of *Escherichia coli* Strains Isolated from Acute Bovine Mastitis in Turkey

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Abstract

The objective of this study was to determine the phylogenetic distribution of commonly studied virulence factors of 155 E. coli isolated from acute bovine mastitis. In the study, A, B1, B2, C, D, E, F phylogroups, and Clade I were defined. B1 and C were found to be the most prevalent phylogroups covering commensal strains with a ratio of 45.2% and 37.4%, respectively. The other phylogroups determined in the study were F, D, A, and E with a range of 0.6%-8.9%. Commonly known virulence genes; *clpg, F17, afa, iucC, iuc*D, *cnf1, cnf2, kps,* and *tra*T were selected to determine the virulence factors. The only virulence factor gene existing within the strains was *tra*T (66.4%). However, any relation between phylogroups and virulence factors was not determined. Phylogroups C, E and F were found to be new phylogroups for the first time in acute bovine mastitis cases.

Keywords: *Escherichia coli, Bovine mastitis, Phylogroup, Virulence factor*

Akut Sığır Mastitislerinden İzole Edilen *Escherichia coli* Suslarının Filogruplandırılması ve Virulens Faktör Değerlendirmesi

Özet

Çalışmanın amacı akut sığır mastitinden izole edilen 155 E. coli izolatında, yaygın olarak çalışılan virulens faktörlerinin filogenetik dağılımını belirlemekti. İzolatların filogruplandırması yapıldığında, A, B1, B2, C, D, E, F grupları ve Sınıf I tespit edildi. Kommensal suşları oluşturan, B1 ve C sırasıyla, %45.2 ve %37.4 oranla en yaygın filogruplar olarak bulundu. Bu çalışmada, diğer filogruplar, %0.6-%8.9 aralığındaki oranda F, D, A, and E olarak tespit edildi. Virulens faktörlerini belirlemek için en çok bilinen virulens genleri; *clpg, F17, afa, iucC, iuc*D, *cnf1, cnf2, kps* ve *tra*T seçildi. *tra*T, suşların büyük çoğunluğunda (%66.4) tespit edilen virulens genidir. Filogruplar ve virulens faktörleri arasında herhangi bir ilişki belirlenmedi. Akut sığır mastitis olgularında, C, E. F filogrupları ilk kez tespit edildi.

Anahtar sözcükler: *Escherichia coli, Sığır mastitis, Filogrup, Virulens faktörü*

INTRODUCTION

Escherichia coli is the most common pathogen of acute bovine mastitis worldwide [1-3]. Clinical signs of *E. coli* mastitis differ from severe or even fatal forms to mild mastitis. Association between the virulence factors of the isolate and the clinical severity of mastitis has not been clarified, yet [3].

Escherichia coli have a clonal genetic structure with a low level of recombination which leads *E. coli* strains to be grouped. These groups also have the same phenotypic and genotypic characteristics, ecological niche and ability to cause disease which helps researchers to understand the epidemiology of *E. coli*. According to rapidly expanding multi locus sequence data for *E. coli* isolated from different hosts and habitats, at first mainly four phylogenetic groups A, B1, B2 and D were discovered [4,5]. Most of the commensal and diarrheagenic strains belong to group A and B1 whereas extra-intestinal *E. coli* strains belong to group B2 and D [6,7]. Bovine *E. coli* mastitis isolates had been shown to belong to phylogroup A, mostly constituted commensal (non-pathogenic) strains [8-10]. For determining the new phylogroups in 2013, Clermont *et al*.[11] improved

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their former method [4] and developed a new quadruplex-PCR by using additional *arp*A gene target to *chu*A, *yja*A, TspE4.C2 target genes, and also *trp*A target genes, and enabled to differentiate A, B1, B2, C, D, E, F groups, and Clade I.

Several virulence factors such as adhesion, invasion, capsule production, ability to resist serum complement, siderophores were determined from pathogenic *E. coli* isolated in case of diarrhea, septicemia and meningitis, infection of urinary tract, and mastitis. Virulence factors of *E. coli* have different functions which formed against to host defense system to colonize, multiply and survive in the udder [12]. Several adhesins were detected in *E. coli* isolated from bovine mastitis. The family of F17 fimbriae and *afa* adhesin facilitate the adherence of pathogenic *E. coli* to various host tissues and mammary gland as well. The family of F17 (F17A) fimbriae comprising F17a-A, F17b-A, F17c-A and F17d-A also fimbrial adhesin *afa* facilitate the adherence of *E. coli* to various host tissues were frequently detected in *E. coli* from bovine mastitis [13-15]. CS13A adhesin encoding *clp*G gene has been detected in *E. coli* isolates from bovine mastitis and bovine with septicemia or diarrhea [16]. Siderophores are the important components for the virulence of *E. coli* strains. The presence of virulence genes (*iuc*ABCD) encoding aerobactin iron uptake system was reported significantly higher in *E. coli* isolates from bovine mastitis than fecal isolates [17]. The *tra*T gene encoding an outer membrane protein plays an important role in serum resistance was reported in mastitic milk in high proportions [18]. Capsular polysaccharide gene reported in coliform mastitis is known to be related to the resistance to serum bactericidal activity and opsonization [19]. Cytotoxic necrotizing factors (CNF) are putative virulence factors of pathogenic *E. coli* strains. CNF1 and CNF2 producing isolates termed as necrotoxigenic *E. coli* (NTEC) are associated with enteritis and intestinal infections, mastitis, pneumonia and metritis [20,21].

The purpose of this study was to determine the phylogenetic groups and the most common virulence genes (*tra*T, *F17*, *afa*, *iuc*C, *iuc*D, *cnf*1, *cnf*2, *clpg*, *kps*) of *E. coli* strains isolated from acute bovine mastitis.

MATERIAL and METHODS

Escherichia coli Strains

A total of 155 *E. coli* strains was isolated from acute bovine mastitis and identified according to conventional biochemical tests [22]. *Escherichia coli* positive control strains for phylogrouping were obtained from culture collection of Ankara University Faculty of Veterinary Medicine Department of Microbiology. *Salmonella* Typhimurium 2696 for *tra*T, *E. coli* AVMC95-03 for *F17*; *E. coli* AVMC95-07 for *cnf*1; *E. coli* S5 for cnf2; *E. coli* 31A for *clpg*; *E. coli* AVMC95-05 for *afa*; *E. coli* U9-41 for *kps*; *E. coli* AVMC95-18 for *iuc*D and *iuc*C were used as positive control strains for PCR.

Determination of Phylogroups and Virulence Genes

DNA extraction from *E. coli* strains was performed by DNA isolation kit (QIAamp DNA Mini Kit; Qiagen, Cat no: 51104) according to the manufacturer's instructions. Isolated DNAs were served as template for determining phylogroups and virulence genes of *E. coli*. PCR for determining the phylogroups of *E. coli* was performed as previously described by Clermont *et al*.[11].

The genes of *iuc*D, and *cnf*1 were examined by a multiplex PCR as described by Yamamoto *et al*.[23]. The *iuc*C gene was analyzed by PCR as described by Bingen *et al*.[24]. The presence of *F17* and *clp*G genes were analyzed by PCR as stated by Bertin *et al*.[14] and Bertin *et al*.[16], respectively. C*nf*2 gene was determined by the procedure described by Kaipainen *et al*.[12]. The PCR procedures described by Yamamoto *et al*.[23] and Johnson *et al*.[25], previously were used to determine *afa* and kps, respectively. The PCR products were analyzed in 1.5% agarose gel containing ethidium bromide. The oligonucleotide primers used for amplification of the genes and expected size of products were presented in *Table 1*.

RESULTS

Of 155 *E. coli* isolates examined by Quadruplex-PCR [11] from bovine mastitis, 71 (45.2%) were found to belong to group B and 58 (37.4%) were found to belong to group C. Nine (5.7%) *E. coli* isolates were determined in group A, 2 (1.2%), 14 (8.9%), and 1 (0.6%) *E. coli* isolates were found to belong to groups D, E, and F, respectively *(Table 2)*.

Among the isolates, 148 out of 155 possess either one or more virulence factors examined in the study. The virulence genes associated with adhesion *(clpg, F17, afa*),

Table 1. *Oligonucleotide primers used to determine the virulence genes*

Gene	Primer Sequence	PCR Product Size (bp)	References
clpG	GGGCGCTCTCTCCTTCAAC CGCCCTAATTGCTGGCGAC	402	Bertin *et al*.[18]
cnf1	AAGATGGAGTTTCCTATGCAGGAG CATTCAGAGTCCTGCCCTCATTATT	498	Yamamoto *et* al.[23]
cnf2	ACTGAAGAAGAAGCTGGGAATA ATAAGTTGAGCCGAGCGAGG	654	Kaipainen *et al*.[12]
F17	GCAGAAAATTCAATTTATCCTTGG CTGATAAGCGATGGTGTAATTAAC	537	Bertin *et al*.[14]
*iuc*D	TACCGGATTGTCATATGCAGACCG AATATCTTCCTCCAGTCCGGAGAAG	602	Yamamoto *et al*.[23]
*iuc*C	AAACCTGGCTTACGCAACTGT ACCCGTCTGCAAATCATGGAT	269	Bingen *et al*.[24]
*tra*T	GATGGCTGAACCGTGGTTATG CACACGGGTCTGGTATTTATGC	307	Kaipainen *et al*.[12]
kps	GCGCATTTGCTGATACTGTTG CATCAGACGATAAGCATGAGCA	272	Johnson *et al*.[25]
afa	GCTGGGCAGCAAACTATAACTCTC CATCAAGCTGTTTGTTCGTCGCCG	750	Yamamoto *et al*.[23]

Table 2. *Phylogroup distribution of E. coli strains (number 'N' and percentage '%')*

Parameter	Phylogroups					
	A	B1	C	D	E	F
N of isolates and (%)	9 (5.73)	71 (45.2)	58 (37.4)	2 (1.27)	14 (8.91)	1 (0.63)

Table 3. *Frequency of virulence genes among each phylogroups of E. coli*

Gene	Number Positive Isolates (n=155) and Percentage (%)	Number of Virulence Genes Among Each Phylogroups					
		A	B1	C	D	E	F
*tra*T	103 (66.4)	4	52	36	1	9	1
*cnf*2	4 (2.5)	0	3	0	0	1	0
afa	15 (9.6)	1	8	4	0	1	1
*iuc*D	9 (5.8)	0	4	5	0	0	0
*iuc*C	9 (5.8)	1	3	5	0	0	0
clpg	1 (0.6)	0	0	0	0	0	1
kps	2 (1.2)	0	0	0	1	1	0
F17	5 (3.2)	0	2	2	0	1	0
cnf1	0 (0)	0	0	0	0	0	0

Table 4. *Patterns of virulence factors within each phylogroups*

Group	Number of Isolates	Patterns of Virulence Genes
B1	1	*tra*T, *iuc*C, *iuc*D, *afa*,
B1	1	*tra*T, *iuc*C, *afa*
B1	1	*tra*T, *iuc*C, *iuc*D
B1	5	*tra*T, *afa*
B1	2	*tra*T, *F17*
B1	2	*tra*T, *cnf2*
C	2	*tra*T, *iuc*C, *iuc*D, F17
C	3	*tra*T, *iuc*C, *iuc*D
C	4	*tra*T, *afa*
C	1	*tra*T, *iuc*D
E	1	*tra*T, cnf2, *afa*
E	1	*tra*T, kps
E	1	*tra*T, F17
A	1	*tra*T, *afa*, *iuc*C
F	1	*tra*T, *afa*, *clpg*

aerobactin production *(iucC, iucD)*, cytotoxic necrotizing factors *(cnf1, cnf2)*, capsule production *(kps)*, and serum resistance *(traT)* were found to be with a range of 0.6%-66.4%. None of the isolates were had *cnf*1 gene. The most prevalent virulence gene from acute bovine mastitis was *tra*T, coding serum resistance with a percentage of 66.4. The genes associated with adhesion were ranging from 0.6% to 9.6%. Each aerobactine production genes were determined to be 5.8%. The remaining virulence genes, *cnf2* and *kps* were determined as 2.5% and 1.2%, respectively. The distribution of virulence genes among the phylogroups were summarized in *Table 3* in detail. The combination of virulence factors with phylogroups determined in the study was shown in *Table 4*.

DISCUSSION

The most prevalent phylogroups determined in this study were B1 and C according to Clermont *et al*.[11]'s phylogrouping. Following dominant phylogroups from acute bovine mastitis in this study were determined as E and A phylogroups. The least two phylogroups determined from acute bovine mastitis were found to be D and F. Most of the commensal and intestinal strains have been reported as group A and B1, extra-intestinal *E. coli* strains belonged to group B2 and D in other studies [6,7]. In the previous studies, bovine mastitis isolates have been generally belonged to A and B1 groups [8,9,26-28]. However, phylogroup C was defined to be closely related to, but distinct from B1 [29,30]. Phylogroup E was designated as comprising the unassigned strains of O157:H7 which is enterohemorrhagic classified under intestinal pathogenic *E. coli* [11]. According to the results of this study, most of *E. coli* isolates from bovine acute mastitis were determined as commensal and intestinal origin in compatible with the previous studies [17,26,30]. Newly designated phylogroup F has been termed as a sister group to phylogroup B2 [30]. Hence, two isolates in pylogroup D and 1 isolate in phylogroup F were found to be extra-intesinal *E. coli* in this study. Due to being the first time for reporting phylogroups C, E, F from acute bovine mastitis, we could not compare the newly designated phylogroups of mastitic bovine *E. coli*.

Several panels of virulence factors have been studied in the previous studies [3,12,18,28,31]. The most common studied virulence genes representing pathogenic potential were

choosen in the study. Among the virulence genes examined, except *tra*T, the remaining virulence factor genes; *cnf*2, *cnf*1, *iuc*D, *iuc*C, *afa*, F17, clpg, *kps*, were found to be uncommon among the *E. coli* isolates.

Serum resistance is the most studied and commonly reported virulence factor related to bovine mastitis with a range of 16.7% and 99.5% [12,18,28,31-33]. In our study, 66.4% of *E. coli* isolates were determined to have *tra*T gene which causes serum resistance. There are two controversial views about *tra*T gene. One of them assumes that the presence *tra*T gene in a high proportion of mastitis isolates may indicate a role of *tra*T in the pathogenesis of mastitis caused by *E. coli* and other mastitis pathogen species as well [34]. The second one assumes that serum resistance has not been attributed only to mastitis, also environmental strains may carry *tra*T gene [11]. In this respect, the first assumption is more logical that *tra*T is necessary for virulence in the mammary gland.

The prevalence of *cnf*2 found in *E. coli* strains isolated from bovine mastitis was with a range of 3%-17% [3,12,27,35]. In this study, *cnf*1 gene was not detected in *E. coli* isolates from bovine mastitis whereas 2.5% of the isolates were found to be *cnf*2 positive. Similar result was previously described by Kaipainen *et al*.[12] that *cnf*1 was far less prevalent than *cnf*2 in bovine *E. coli* strains. Although *cnf*2 and *cnf*1 genes were investigated in mastitic bovine *E. coli* isolates, the role of *cnf* toxins in the pathogenesis of bovine mastitis is not clarified, but supposed be associated with urinary tract infections and meningitis.

Siderophores are one of the most commonly studied virulence factors [12,36]. Each *iuc*D and *iuc*C genes, responsible for aerobactin production were found to be 5.80% positive. In previous studies, ratio of the presence of aerobactin genes were determined to be approximately 20% [12,17,27,36]. Taking into account of iron acquisition in the pathogenesis of systemic infections, siderophores are well-defined virulence factors in Gram(-) bacteria acquiring iron and enhances the pathogenicity of the microorganism [37]. Aerobactins enables *E. coli* to acquire iron from lactoferrin and iron binding protein in milk, subsequently multiply and cause mastitis. In this study, aerobactin rate was expected to be high within the *E. coli* isolates due to importance acquisition of iron from lactoferrin. However, our findings overlapping a previous study performed by Linggood *et al*.[38] showed that aerobactin is probably not involved in the virulence of *E. coli* isolated from bovine mastitis. We also examined whether the genes *(F17*, *clpg*, and *afa)* encoding adhesion proteins were necessary for the pathogenesis of *E. coli* isolated from acute bovine mastitis or not. *F17* and *clpg* genes were reported to be relevant in *E. coli* isolates from bovine mastitis [14,16,39]. The prevalence of *F17* related gene in other countries from bovine mastitis were reported to be 1% [12] to 20.4% [27]. The prevalence of *clpg* gene from bovine mastitis was determined between 0.78%-29.62% in the previous studies [3,27,28]. In the study, virulence genes, *afa*, *clpg, F17* encoding adhesions were found with a range of 0.6%-9.6%. The low prevalence of these genes encoding adhesion proteins from acute bovine mastitis was interpreted as attachment of bacteria to mammary epithelium may have a less importance in the pathogenesis of acute bovine mastitis [40]. Unless adherence to epithelial tissue of mammary gland, *E. coli* can adjust metabolically to mammary secretion. The last virulence gene, *kps*, examined in the study was determined in two *E. coli* isolates. In compatible with our result, Fernandes *et al*.[28] reported only one isolate possessing *kps* gene.

The agent features of *E. coli* comprising ability to utilize lactose as an energy source and survive in the mammary gland though the oxygen tension is very low, makes itself possible to cause mastitis. Peracute infections due to *E. coli* will not be explained with the role of expected virulence factors such as adhesion proteins, siderophores, cytotoxic necrozan factors, in the pathogenesis of infection. The possible candidate virulence factor for the pathogenesis of bovine mastitis is endotoxin which does not directly effect the secretory cell but distrups the blood flow [41] and cause decreased milk production by systemic and local effects of itself [42].

As a conclusion, the results obtained in this study indicate that neither a specific set of virulence factors nor any phylogroup-virulence factor association was determined in compatible with the former studies [3,12,13,27]. Phylogroups C, E, F were found to be as new phylogroups for the first time in acute bovine mastitis cases.

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