Identification, characterization and molecular epidemiology of *Escherichia coli* isolated from lamb and goat kids with diarrhoea

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Abstract

Neonatal diarrhoea is a serious health problem on commercial farms. Enterovirulent *Escherichia coli* is a significant aetiological agent of neonatal diarrhoea. In this work, identification and classification of *E. coli* isolates obtained from lambs and goat kids with diarrhoea were studied along with antibiotic resistance and clonal relationships of enterovirulent strains. A total of 107 *E. coli* strains isolated from animals on 43 farms were investigated. Specific virulence genes were determined by multiplex and uniplex polymerase chain reaction. Testing of antibiotic susceptibility was carried out by the Vitek II compact system. The relationship of *E. coli* isolates was determined by enterobacterial repetitive intergenic consensus polymerase chain reaction. A total of 39 (36.4%) enterovirulent *E. coli* strains were identified and of this 19 (48.7%) were shiga toxigenic, 12 (30.8%) enterotoxigenic and 8 (20.5%) enteropathogenic. Three isolates (7.7%) were found to be positive for extended spectrum beta lactamase; 10 (25.6%) isolates showed multi-drug resistance to antimicrobials. A total of 28 types were detected by enterobacterial repetitive intergenic consensus polymerase chain reaction. Twenty strains had distinct types while 5 types were common for 2 strains and 3 types were common for 3 strains. This is the first current determination of types, clonality and antibiotic resistance of enterovirulent *E. coli* isolated from small ruminants with diarrhoea. The results of this study showed that the rates of shiga toxigenic, enterotoxigenic and enteropathogenic isolates of *E. coli* are high in the western part of Turkey. Although these isolates were not clonal, presence of multidrug resistant isolates may cause public health problems.

E. coli, virulence genes, antimicrobial susceptibility, ERIC PCR

Among bacterial pathogens worldwide, enterovirulent *Escherichia coli* ranks among the most common causative agents of bacterial diarrhoea in several animal species as well as in humans. The strains of *E. coli* can be divided into five groups or pathotypes. Types of diarrhoeagenic *E. coli* include enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC) and enteroaggregative (EAEC) enterohaemorrhagic *E. coli* (EHEC) (Nataro and Kaper 1998). The Shiga toxin-producing group of *E. coli* strains is capable of producing toxins very similar to the one produced by *Shigella dysenteriae* type 1. Therefore, these bacteria are often called Shiga toxin-producing *E. coli* (STEC). Active Shiga toxins may be detected using Vero cell toxicity test (Beutin et al. 2007). This is why these bacteria are also called verotoxin or verocytotoxin-producing *E. coli* (VTEC). The Shiga toxins produced by *E. coli* may cause anything from uncomplicated diarrhoea to haemorrhagic colitis, which can progress into haemolytic uremic syndrome (HUS). Thus, the bacterium is often called also EHEC. Animals are the main reservoir for STEC (Nataro and Kaper 1998).

Strains of pathogenic *E. coli*, which have acquired virulence genes, have the ability to cause diarrhoeal disease. The shigatoxigenic (STEC or VTEC) strains produce shiga toxins 1 and/or 2 (*stx*1, *stx*2) while EHEC produce *stx*1 and/or *stx*2 and intimin (*eae*A). The ETEC strains produce heat labile (LT) and/or heat stable (ST) enterotoxins, while EPEC strains may have the EPEC adherence plasmid (EAF) that carries the bundle forming pilus (*bfp*). The EIEC strains have the ability to invade the colon epithelial cells due to presence

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of a plasmid that carry the *ial* gene. The EAEC strains are characterized by presence of aggregative adherence factors (AAFs). The expression of these factors is regulated by transcription activation factor encoded by the *aaf*II gene. Molecular methods remain the most suitable techniques for differentiating diarrhoeagenic strains of *E. coli* from non-pathogenic ones (Nataro and Kaper 1998).

The aim of this work was to classify pathogenic *E. coli* strains, their antimicrobial resistance profiles and clonal relations of these strains isolated from lambs and goat kids with diarrhoea.

Materials and Methods

Sample collection on farms

A total of 107 *E. coli* isolates obtained from 79 lambs and 28 goat kids on 43 farms were investigated. Samples were mainly collected between 2007 and 2009 from sheep and goat farms located in Izmir located in the western part of Turkey. Data on sex and breed of animals were not available. Both species were mostly reared under semi extensive husbandry for dairy and/or meat production. Two or three samples were taken from every farm. Animals receiving antimicrobial treatment and animals older than 45 days were excluded from the study. Swabs were taken from the rectum of live animals and placed immediately in 1 ml of 0.1 M sterile phosphate buffered saline (PBS) and diluted 1:10 in PBS.

Isolation and identification of *E. coli*

Faeces samples were inoculated onto McConkey agar. Agar plates were incubated at 37 °C and bacterial growth was evaluated after 24 and 48 h. Gram negative microorganisms were selected using MacConkey agar and identified with biochemical tests (Quinn et al. 1994). The confirmation of the *E. coli* identifications was done with the Vitek II system (Biomerieux, France).

Testing of antimicrobial susceptibility

The activities of 15 antibiotics (ampicillin, amoxicillin-clavulanic acid, piperacillin, gentamicin, tobramicin, amikacin, imipenem, tetracycline, cefpodoxime, ceftiofur, enrofloxacin, marbofloxacine, chloramphenicol, trimethoprim-sulfamethoxazole and nitrofurantoin) and determination of the presence of extended spectrum beta lactamase (ESBL) were carried out with the Vitek II compact system using the Vitek II AST-GN38 cards as described previously (Ling et al. 2001). The *E. coli* reference strain (ATCC 25922) was used as quality control. Interpretation of results was performed according to National Committee for Clinical Laboratory Standards (CLSI 2007).

Virulence genes and clonality of *E. coli* strains

Genomic DNA from individual pure cultures of *E. coli* isolates was extracted by InstaGene matrix (Bio-Rad Laboratories, Canada) according to the manufacturer's instructions. Genes of virulence *stx*1 and/or *stx*2, *st* and/or *lt*, *eae* and/or *bfp, ial* and *aaf*II specific for strains EHEC, ETEC, EPEC, EIAC and EAEC, respectively, were used in this study. Multiplex PCR was performed for the detection of *stx*1, *stx*2 and *eae* genes; uniplex PCR was used for detection of *lt*, *st*, *bfp, ial* and *aaf*II genes. PCR conditions are given in Table 1. The primers used in

Table 1. PCR conditions for the detection of virulence genes and clonality of pathogen *E. coli* strains.

	uPCR[1]			mPCR[2]			ERIC PCR		
	Temperature (°C)	Duration (min)	Cycles	Temperature (°C)	Duration (min)	Cycles	Temperature (°C)	Duration (min)	Cycles
Initial denaturation	94	5	1	94	5	1	94	5	1
Denaturation	94	1	35	94	1	35	94	1	35
Annealing	50	1	35	51	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 *bfp*, *ial*, and *aaf*II were tested by uPCR, [2]Presence of genes *stx*1, *stx*2, *eae* and *lt, st* were tested by mCR
ERIC - Enterobacterial Repetitive Intergenic Consensus

PCR for *stx*1, *stx*2, *lt*, *st*, *ial* and *eae* genes were those as reported by Lopez-Saucedo et al. (2003), for *bfp*A (Stacy-Phipps et al. 1995), for *aaf*II (Vidal et al. 2005) and for enterobacterial repetitive intergenic consensus (ERIC) (Versalovic et al. 1991). Reference strains of *E. coli* ATCC 35150 (EHEC; *stx*1, *stx*2, *eae* positive), ATCC 35401 (ETEC; *lt*, *st* positive), and ATCC 43893 (EIEC; *ial* positive) were used as positive control strains and ATCC 25922 was used as negative control strain. The strains EAEC (*aaf*II positive) and standard EPEC (*bfp*A positive) could not be obtained due to the restrictions on export. Uniplex PCR was used to detect the clonality by enterobacterial repetitive intergenic consensus (ERIC) PCR. For ERIC PCR, genetic relationships were calculated and a dendogram was designed by the Bio-1D++ program (Vilber Lourmat, Germany), relying on the existence of bands. In this study, a *bfp*A positive PCR amplicon was sequenced by a private company (Macrogen, Korea). The amplification products were analysed by electrophoresis on 1.5% agarose gel at 100 V for 30 min in Tris-acetate-EDTA buffer and revealed in ethidium bromide (20 µg/ml).

Results

Among 107 *E. coli* strains isolated, 39 (36.4%) were found to carry at least one of the virulence genes tested by PCR (Table 2, Plate I, Fig. 1). Isolates of *E. coli* were mostly resistant to tetracycyline (40%). Three isolates were found to be ESBL positive. A total of 22 isolates (56.4%) were found to be susceptible to all antimicrobials, whereas 10 (25.6%) of them showed multi antimicrobial resistance (resistance against three or more antimicrobials). Seven, 3, 2, 3 and 2 isolates were resistant to 1, 3, 4, 5 and 11 antimicrobials, respectively.

Table 2. Classification of enterovirulent *E. coli* strains isolated from lamb and goat kids with diarrhoea by multiplex and uniplex PCR.

Category	Virulence genes	Number of virulence genes (%)	Number of enterovirulent isolates (%)
STEC	*stx*1	1 (2.6)	
	*stx*2	13 (33.2)	15 (38.4%)
	*stx*1+ *stx*2	1 (2.8)	
EHEC	*stx*2+ *eae*A	4 (10.2)	4 (10.2%)
ETEC	*st*	1 (2.6)	12 (30.8)
	lt	11 (28.2)	
EPEC	*eae*A	7 (17.8)	8 (20.6)
	bfp	1 (2.6)	
EIEC	*ial*	0	0
EAEC	*aaf*II	0	0
Total (%)		39 (100)	

STEC - shigatoxigenic EHEC - enterohaemorrhagic, ETEC - enterotoxigenic, EPEC - enteropathogenic, EIEC – enteroinvasive, EAEC - enteroaggregative strains

Information on ERIC types, category, and nine antimicrobial resistance profiles of enterovirulent isolates are given in Table 3. All isolates tested by ERIC PCR had amplicons ranging from approximately 150 to 1500 bp. According to the dendogram (Plate I, Fig. 2), two groups with 30% genetic proximity were determined. Among 39 strains tested, a total of 28 types were detected. Twenty strains had distinct types while 5 types were common for 2 strains and 3 types were common for 3 strains. Clonal spread of a common strain was not detected among the animals tested in the present study.

Discussion

E. coli is an important bacterial agent of neonatal diarrhoea that causes great economic losses in the farming industry. *E. coli* has been reported as the causative agent of diarrhoea

Table 3. Enterobacterial repetitive intergenic consensus types, category, and antibiotic resistance profiles of enterovirulent *Escherichia coli* isolated from lamb and goat kids with diarrhoea.

Number of isolates	ERIC type	Category	Antibiotic resistance profile
1	1	STEC	TET
2	6	STEC	-
3	8	STEC	TET
4	7	STEC	-
5	10	STEC	TET
6	12	ETEC	-
7	14	STEC	-
8	3	EHEC	TET, PIP, SXT, AMP
9	20	EHEC	TET
10	14	STEC	-
11	11	EHEC	-
12	4	STEC	TET, PIP, SXT, AMP, GEN
13	17	EPEC	-
14	21	STEC	-
15	21	STEC	-
16	22	STEC	-
17	5	EPEC	TET, PIP, SXT
18	17	EPEC	-
19	17	EPEC	-
20	15	STEC	TET, PIP, SXT, AMP, CHL
21	2	ETEC	TET, PIP, SXT, AMP, GEN, AMC, CEFP, CFT, TO, ENR, MAR ESBL +
22	13	ETEC	-
23	2	ETEC	TET, PIP, SXT, AMP, GEN, AMC, CEFP, CFT, TO, ENR, MAR ESBL +
24	24	ETEC	TET
25	18	ETEC	TET
26	19	ETEC	TET
27	25	ETEC	-
28	16	ETEC	TET, SXT, CHL, ENR, MAR
29	23	ETEC	-
30	9	EHEC	TET, SXT, AMP
31	9	STEC	TET, SXT, AMP
32	23	ETEC	-
33	23	ETEC	-
34	26	EPEC	-
35	26	EPEC	-
36	26	EPEC	-
37	27	STEC	-
38	27	STEC	-
39	28	EPEC	PIP, AMP, AMC, CEFP ESBL +

AMC - amoxicillin clavulanic acid, AMP - ampicillin, CEFP - cefpodoxime, CFT - ceftiofur, CHL - chloramphenicol, ENR - enrofloxacin, GEN - gentamicin, MAR - marbofloxacine PIP - piperacillin, SXT - trimethoprim sulfamethoxazole, TET - tetracycyline, TO - tobramicin, ESBL - extended spectrum beta lactamase, enterobacterial repetitive intergenic consensus - ERIC, EHEC - enterohaemorrhagic, STEC – shigatoxigenic

in small ruminants from Turkey (Gokce et al. 2010) and other states (Wani et al. 2004), however, research on classification of enterovirulent *E. coli* and its virulence genes is limited. Recent studies have reported (Wani et al. 2003; Bhat et al. 2008; Bandyopadhyay et al. 2011), the existence of at least one virulence gene in 41%, 36.7%, and 32.6% of *E. coli* isolates, respectively. In our study we found at least one virulence gene for EHEC or STEC, EPEC, ETEC, EIEC, and EAEC among 36.4% of the isolates.

The prevalence of STEC in our study was 17.8% of 107 *E. coli* isolates tested. This rate was higher than the findings reported by Wani et al. (2003) from India (6%), and was the same (17.8%) to the findings of Bhat et al. (2008). However, the latest report from India showed that the STEC rate has increased to 32% (Bandyopadhyay et al. 2011). This may be an indication that if necessary precautions are not taken, pathogenic *E. coli* ratio may increase also in our country. In our study, the *stx*2 gene was more prevalent than the *stx*1 gene. This result contrasts with reports which showed that STEC strains isolated from small ruminants harbour the *stx*1 gene more frequently (Bhat et al. 2008; Wani et al. 2009) but agrees with Bandyopadhyay et al. (2011), who also found a predominance of the *stx*2 gene in STEC strains isolated from small ruminant with diarrhoea in India. Jenkins et al. (2002) reported that among healthy cattle, prevalence of VTEC strains may vary depending on the season. They found that except for winter, *stx*2 rates were higher among cattle. This is significant because *stx*2 is a more important toxin for humans as the cause of the haemolytic uremic syndrome (Nataro and Kaper 1998).

Among newborn small ruminants, ETEC strains are the most important pathogen that causes diarrhoea. In our study, about 11.2% of 107 *E. coli* strains isolated from faeces of diarrhoeic lambs were harbouring specific gene(s) for ETEC. The latest study from India showed that 9% of the faecal isolates from lambs were ETEC. In India, among 22 ETEC positive isolates, five (23%) possessed *lt* genes, and another five possessed *st* genes (Bandyopadhyay et al. 2011). In our study, recovery of *lt* was in high frequency (28.2%) but *st* was in low frequency (2.6%). The differences between the ratio of *st* and *lt* in these studies may be due to geographical locations.

Diarrhoea associated with *E. coli* infections is often treated with antibiotics; however, therapy may be unsuccessful due to resistant strains in animals (Cid et al. 1996; Kolar et al. 2008). In our study, 10 (25.6%) isolates were found to be multi-drug resistant. The resistance level of Spanish isolates (Medina et al. 2011) was also high with 74.8% isolates resistant to at least 2 antimicrobials, 59.7% resistant to at least 4 antimicrobials, 34.9% resistant to at least 6 antimicrobials and 14% resistant to at least 8 antimicrobials. While tetracycline resistance rates were found to be 40.2% in our study, in previous studies (Blanco et al. 1996; Cid et al. 1996) the resistance was high (> 70%). High resistance rates to tetracycline may be due to extensive use of tetracycline in veterinary treatment.

In recent years, the dissemination of *E. coli* strains harbouring ESBL has caused great deal of concern. Although ESBL producing *E. coli* have been more clearly defined in the humans, they remain unclear in small ruminants. Hartmann et al. (2012) detected ESBL producing *E. coli* in livestock (5% of the animals tested) in France. In Europe, the isolation rates of such strains still remain low and have been only detected sporadically in cattle. Snow et al. (2011) detected ESBL producing *E. coli* on six farms in UK. To our best knowledge this is the first report of ESBL producing *E. coli* in lambs with diarrhoea in Turkey. Further epidemiological studies are necessary to investigate the status of the ESBL presence in the veterinary field.

The results of the present study showed that the isolation rates of STEC, ETEC, EPEC and EHEC strains were high. Although these isolates were not clonal, presence of multidrug resistant isolates may cause future public health problems.

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Plate I
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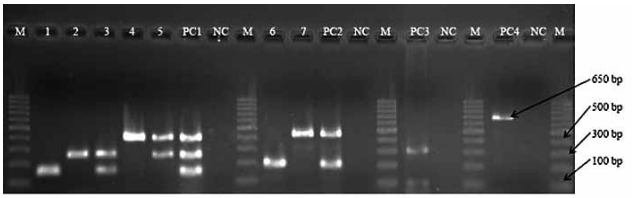

Fig. 1. Multiplex and uniplex PCR profiles detected among 39 isolates of *E. coli* with virulence gene.

1 - *stx*1 positive (1 strain; 150 bp), 2 - *stx*2 positive (13 strains; 255 bp), 3 - *stx*1 and *stx*2 positive (1 strain; 150 bp, 255 bp), 4 - *eae*A positive (7 strains; 384 bp) 5 - *stx*2 and *eae*A positive (4 strains; 255 bp, 384 bp) 6 - *st* positive (1 strain; 190 bp) 7 - *lt* positive (11 strains; 450 bp), M - Marker (100 bp DNA ladder) NC - negative control (ATCC 25922) PC - positive control, PC1 - EHEC (ATCC 35150; 150 bp, 255 bp and 384 bp), PC2 - ETEC (ATCC 35401; 190 bp and 450 bp), PC3 - *bfp* positive field isolate (1 strain; 300 bp), PC4 - EIEC (ATCC 43893; 650 bp)

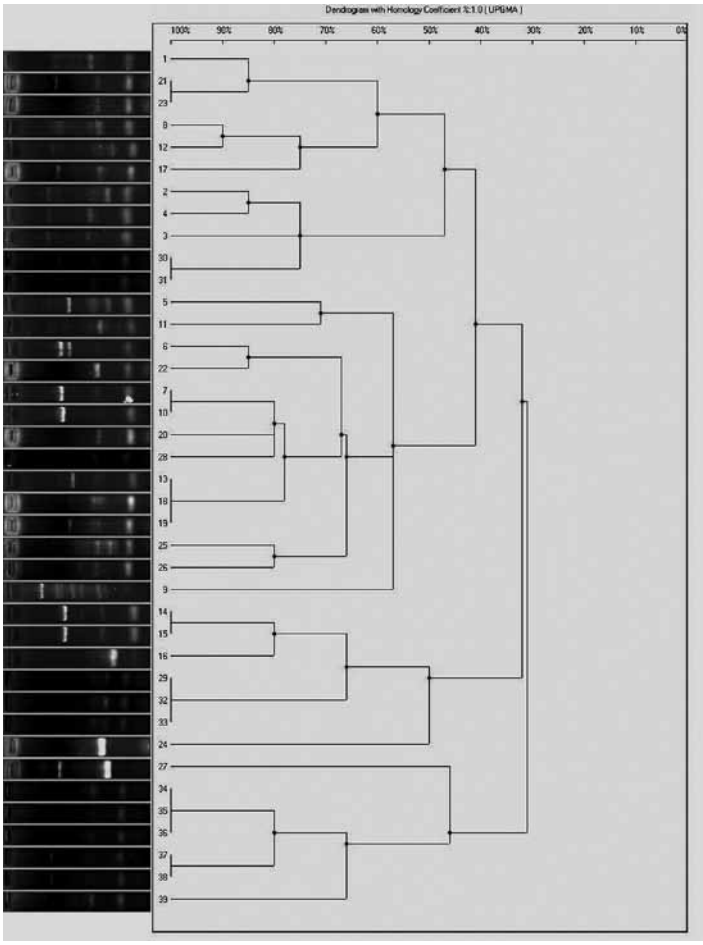

Fig. 2. Dendogram of enterovirulent *E. coli* strains isolated from lamb and goat kids with diarrhoea. Results from ERIC PCR showed presence of 28 types among 39 isolates tested. Although these isolates were not clonal, twenty strains had distinct types while 5 types were common for 2 strains and 3 types were common for 3 strains