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Phylotyping of Antibiotic Resistant, Shiga Toxin-Producing and Atypical Enteropathogenic *Escherichia Coli* Strains Isolated from Ovine and Caprine Carcasses in Iran

Mohammad Reza Aflatoonian*, Hesam Alizade, Maziar Jajarmi, Reza Ghanbarpour, Mehrdad Shamsaddini Bafti, Asma Askari, Nasrin Adib, Mehrdad Khatami

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Abstract

The present study was carried out in order to survey phylogroups and molecular characterization of antibiotic resistance, shiga toxin-producing *Escherichia coli* (STEC) and atypical enteropathogenic *E. coli* (aEPEC) strains isolated from ovine and caprine carcasses in Iran. The *E. coli* isolates were collected from carcasses of sheep (53 isolates) and goat (52 isolates). The *E. coli* isolates were examined for STEC, aEPEC and nine antibiotic resistance genes. The Clermont et al. (2013) method was utilized to determine the phylogroup of the isolates. The most frequent phylogroup was A (44.8%) followed by B1 (40%). Sixteen isolates (15.23%) harbored *stx1* and/or *stx2* were classified as STEC. Three isolates (2.9%) were assigned to aEPEC. Out of the isolates, 7.7% isolates possessed the *qnrB* gene, and no bla_{TEM}, bla_{SHV}, bla_{OXA-1}, $bla_{CTX-M-15}$, *IMP* and *VIM* genes were detected. The moderately high prevalence of STEC was observed in ovine and caprine samples in Kerman, Iran. Practical applications : Since ovine and caprine are considered to be the lifeline agro-economy in many tropical countries, especially Iran, identification and characterization of diarrheagenic agents and antibiotic resistance are of significant economic importance. The presence of STEC and EPEC strains with antimicrobial resistance in ovine and caprine carcasses could be considered a threat to public health for being highly pathogenic for humans.

Keywords: *Escherichia coli*, Antibiotic resistance, Virulence genes, Ovine, Caprine

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) strains are associated with food and waterborne illness and represent a threat to public health (Ferreira, Filho, Pinto, Dias, & Moreira, 2014; Rajkhowa & Sarma, 2014). Enterohemorrhagic *E. coli* (EHEC) is a subset of STEC pathotype that has been described as the main pathogen responsible for causing serious and systemic diseases, e.g., hemolytic uremic

Mohammad Reza Aflatoonian*
Research Center for Tropical and Infectious Diseases, Kerman University of Medical Sciences, Kerman, Iran.
NanoBioElectrochemistry Research Center, Bam University of Medical Sciences, Bam, Iran.

Hesam Alizade
Research Center for Tropical and Infectious Diseases, Kerman University of Medical Sciences, Kerman, Iran.
Infectious and Tropical Disease Research Center, Hormozgan Health Institute, Hormozgan University of Medical Sciences, Bandar Abbas, Iran.

Maziar Jajarmi, Asma Askari, Nasrin Adib
Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran

Reza Ghanbarpour
Molecular Microbiology Research Group, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran

Mehrdad Shamsaddini Bafti
Anaerobic Bacterial Vaccines Research & Production and Molecular Microbiology Department, Kerman Branch, Razi Vaccine & Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Kerman, Iran

Mehrdad Khatami
NanoBioElectrochemistry Research Center, Bam University of Medical Sciences, Bam, Iran

***Email:**mraflatoonian@gmail.com

syndrome and hemorrhagic colitis, mostly among infants in developing countries (Pilipcinec, Tkacikova, Naas, Cabadaj, & Mikula, 1999; Askari Badouei, Morabito, Najafifar, & Mazandarani, 2016). Consumption of STEC-contaminated foodstuff of domestic ruminants, especially cattle and sheep, is one of the major reasons for STEC infection in humans (Ghanbarpour & Daneshdoost, 2012). All EHEC and enteropathogenic *E. coli* (EPEC) strains express *eaeA* gene, which encodes intimin. Intimin mediates attachment to epithelial cells and leads to attaching and effacing lesions in the gut mucosa. The absence of the shiga toxin (*stx*) genes that are found in EHEC can be differentiated between EPEC and EHEC (Alizade, Ghanbarpour, & Aflatoonian, 2014; Slinger, Lau, Slinger, Moldovan, & Chan, 2017). Animals and raw meat are considered as important reservoirs of atypical EPEC (aEPEC) in humans (Xu et al., 2016).

A new phylogenetic analysis has been done for categorizing of the *E. coli* species: A, B1, B2, C, D, E, F and clade I (Clermont, Christenson, Denamur, & Gordon, 2013). Pathogen isolates included verotoxin-producing *E. coli* mostly often found in groups B2 and D, while prevalence of antibacterial resistance was predominantly found in non-B2 phylogenetic group *E. coli* strains (Clermont, Bonacorsi, Bingen, & Bonacorsi, 2000; Wang et al., 2009; Alizade et al., 2014a).

Resistance to antimicrobial agents is a major public health concern and antibiotic usage in livestock production is thought to be a contributing factor. Antibiotics currently used in veterinary and human medicines are similar. Therefore, consumption of these antibiotics is expected to go up due to increased demand, particularly in emerging economies (Critically Important Antimicrobials for Human Medicine, 2011; Nguyen & Sperandio, 2012). Multi-drug resistance has been commonly observed in most extended spectrum beta-lactamase (ESBL)-producers in gram negative bacteria, exclusively in *E. coli*. More alarmingly, there has been co-resistance to all main classes of available antimicrobials such as aminoglycosides, fluoroquinolones and tetracycline (Ali et al., 2016). Resistance to quinolones has increased in *E. coli* isolates obtained from animals and humans during the last two decades. The main mechanism of resistance to quinolones is coded in the chromosome and plasmid-mediated quinolone resistance or quinolone-resistance protein (later named *qnrA*) (Aguilar-Montes de Oca, Talavera-Rojas, Soriano-Vargas, Barba-León, & Vazquez-Navarrete, 2015). Carbapenem resistance has become an urgent public health concern largely due to the fact that carbapenems are reserved as the last treatment option for multi-drug resistant infections in humans. Carbapenems are not approved for use in animal production throughout the world. Therefore, little is known about the prevalence of carbapenem-resistant *Enterobacteriaceae* in livestock populations and their associated environments (Webb et al., 2016).

The purposes of the present work were to (i) identify the phylogenetic groups of *E. coli* isolates based on the new Clermont method (ii) investigate the presence of shiga toxin-producing *E. coli* and atypical enteropathogenic *E. coli* and finally, (iii) determine the magnitude of *E. coli* resistance to β-lactam, carbapenem and quinolone from carcasses of ovine and caprine in southeastern Iran.

Materials and Methods

Sampling and identification of E. coli

Overall 105 *E. coli* isolates were obtained from carcasses of sheep (53 isolates) and goat (52 isolates) from slaughterhouse and butchers shops in Kerman province, Iran, in the summer of 2016. Specimens were collected using sterile swabs from external surfaces of each carcass. All swab samples were placed directly in tubes containing Amies transport medium (Becton Dickinson, BBL, and USA) and sent out to the laboratory for immediate processing. Each swab samples was streaked on Mac Conkey agar plates (Biolife Laboratories, Milan, Italy) and incubated at 37 °C for overnight. Lactose-fermenting colonies were selected for Gram staining and were confirmed to be *E. coli* by using biochemical and bacteriological standard tests. The confirmed *E. coli* isolates were stored for further analysis at -70°C in Luria-Bertani broth (Invitrogen, Paisley, Scotland) with 30% sterile glycerol.

DNA extraction

Freshly grown overnight cultures of *E. coli* isolates underwent streak culture on Luria Bertani agar (Bio-Rad, Marnes-la-Coquette, France). After incubation at 37°C for overnight, DNA was extracted using the boiling method.

Phylotyping assay

The quadruplex PCR method described by Clermont, Christenson, Denamur, and Gordon (2013) was used to assign the *E. coli* isolates. The presence/absence of the four PCR products *arpA*, *chuA*, *yjaA*, and TspE4.C2, an *E. coli* strain could be classified into one of the main phylogroups, A, B1, B2, C, D, E, F and clade I.

Detection of STEC and aEPEC

The isolates were subjected to a multiplex-PCR assay to detect the major virulence genes of EHEC and aEPEC (e.g., *stx1*, *stx2* and *eaeA*) as described previously (China, Pirson, & Mainil, 1996).

Detection of antibiotic resistance

All isolates were screened by multiplex PCR for *qnr* genes (*qnrA*, *qnrB* and *qnrS*) (Cattoir, Poirel, Rotimi, Soussy, & Nordmann, 2007). The carbapenemase encoding genes *IMP* and *VIM* were amplified as described previously by Garza-Ramos et al. (2008). The PCR assays for the different ESBL genes including bla_{TEM}, bla_{SHV}, bla_{OXA-1} and $bla_{CTX-M-15}$ were employed as previously described (Colom et al., 2003; Messai, Benhassine, Naim, Paul, & Bakour, 2006; Sharma, Sharma, & Ray, 2010). Specific primer sequences and the predicted size of the amplified products are described in Table 1.

Statistical analysis

Statistical analysis was done using SPSS, version 19.0 (SPSS, Inc., USA) for determining the relationship among incidences of antibiotics resistance genes, STEC genes and phylogenetic groups of *E. coli* isolated from carcasses of sheep and goat. *P*-value<0.05 was considered as statistically significant.

Results

According to data, the most frequent phylogenetic group was A (44.8%; 47/105) followed by B1 (40%; 42/105), unknown (7.6%; 8/105), E (6.6%; 7/105) and D (0.9%; 1/105). PCR assays for phylotyping of isolates indicated that groups A (54.7%; 29/53) and B1 (24.5%; 13/53) were predominant among sheep carcasses, whereas isolates from groups B1 (55.8%; 29/52) and A (34.6%; 18/52) were found in goat carcasses. Details of phylo-groups in *E. coli* isolates isolated from sheep and goat carcasses are shown in Table 2.

Among 105 *E. coli* isolates, 13 isolates (~13%) carried just *stx1*, two isolates (~2%) had only *eaeA*, and the remaining three isolates (~3%) possessed both *stx1* and *stx2* genes. One (~1%) isolate was positive for *stx1* and *eaeA* genes. Therefore, *stx1* gene was more prevalent than *eaeA* genes (13% vs. 2%), with significant difference ($p = 0.022$) (Table 3).

Phylogenetic analysis revealed that the most prevalent virulence genes belonged to the phylo-group B1 (*p-value* = 0. 000). It is worth noting that isolates that possessed *stx1* and *stx2* genes (3%) and *stx1* and *eaeA* genes (1%) from goat carcasses were assigned to the B1 and A phylo-groups, respectively. Out of the 52 isolates from goat carcasses, three were positive for *stx1* genes (from B1 phylo-group) and only one isolate possessed *eaeA* gene (from B1 phylo-group). Among 53 sheep carcass isolates, 10 isolates (18.9%) were positive for *stx1* gene and belonged to A (six isolates) and B1 (four) phylo-groups. One isolate was positive for *eaeA* gene, which belonged to the B1 phylo-group. None of the isolates possessed *stx2* gene (Table 3).

Totally, only eight out of the 52 *E. coli* isolates obtained from goat carcasses harbored at least one of the *qnr* genes chosen for analysis. The presence of *qnrS* in 5.8% (3/52) of the *E. coli* isolates examined from goat carcasses was most prevalent in the singlet, which belonged to the B1 phylo-group. Only two isolates were positive for *qnrB* and one isolate for *qnrA* gene (both from A phylo-group). Two of the *qnrA* positive isolates possessed *qnrB* (both from A phylo-group) (Table 3). None of the *E. coli* isolates were positive for bla_{TEM}, bla_{SHV}, bla_{OXA}, $bla_{CTX-M-15}$, *IMP* and *VIM* genes.

Discussion

In this study, we performed an analysis of the phylotyping and molecular epidemiology of STEC, aEPEC and antibiotic resistance in Iran, using a collection of 105 *E. coli* isolates recovered from carcasses of sheep and goat. The prevalence of STEC in sheep and goat meat samples was 18.9% and 13.4%, respectively (*p-value*=0.457). STEC is a group of pathogenic *E. coli* strains that can cause severe enteric and systemic disease in humans (Multi-country outbreak of STEC infection associated with HUS, 2016). Bai et al. (2016) in China found that 7.4% of the investigated *E. coli* isolates from raw meat samples were positive for STEC. Another study in Iran revealed that 11.9% of the 452 examined bovine *E. coli* isolates carried the EHEC-*hlyA* gene (Askari Badouei, Morabito, Najafifar, & Mazandarani, 2016). In the most recent estimates, STEC isolates were reported in 40.34% of *E. coli* isolates from diarrheic lambs in southeast of Iran. Moreover, *stx1*/*stx2* with a frequency of 9.3% was found to be the predominant gene profile (Ghanbarpour et al., 2017). Regarding the results, three goat samples were positive for both shiga toxin coding genes. The presence of the *stx1* and *stx2* genes in the STEC strains could indicate that these strains are associated with enhanced virulence and increased severity of clinical infections in humans (Friedrich et al., 2002). Literature review showed that goat milk and farm environment could be the natural reservoirs for particular STEC isolates that mainly harbor stx1/stx2 gene profile. Although the prevalence is lower than in cattle, goat milk and cheese have been seen as vehicles of foodborne disease outbreaks (Alvarez-Suarez et al., 2016).

aEPEC was more frequent in sheep than in goat meat samples (~4% versus ~2%) but it was not significant (*p-value*=0.419). The prevalence rate of EPEC (2.9%) in the current study was significantly lower than in previous studies (Türkyılmaz, Eskiizmirliler, Tunaligil, & Bozdogan, 2013; Ghanbarpour et al., 2017). *E. coli* strains, which harbor *eae* gene, are considered more virulent for humans than *eae*-negative strains. However, most pathogenic *E. coli* strains, isolated from the feces of sheep, goat and cattle are not positive for the *eae* gene (Cornick, Booher, Casey, & Moon, 2000; Osman, Mustafa, Elhariri, & Abd Elhamed, 2013).

Among the *qnr* genes, the *qnrS* gene was significantly present in the current study, which was comparable with the previous result reported by Liu et al. (2012). Quinolones encoding genes were not detected in our collection of sheep *E. coli* isolates. Plasmid-mediated quinolone resistance was detected in *Enterobacteriaceae* human isolates but is likely to be rare in isolates of animal food (Robicsek, Jacoby, & Hooper, 2006; Kirchner, Wearing, & Teale, 2011). However, another study in China reported that plasmid-mediated quinolone resistance is frequently found in the isolates of food-producing animals (Ma et al., 2009). The bla_{TEM}, bla_{SHV}, bla_{OXA}, $bla_{CTX\text{-}M\text{-}15}$ genes were not detected in this study. Nevertheless, a study in Switzerland reported moderately high prevalence of ESBL producers and high genetic diversity among *Enterobacteriaceae* in food producing animals (Geser, Stephan, & Hächler, 2012).

To the best of our knowledge, the present study is one of the first to use the quadruplex PCR assay (Clermont, Christenson, Denamur, & Gordon, 2013) allowing the identification of the minor phylogenetic groups C, E and F and clade I in carcasses of caprine and ovine. On the basis of biochemical profiling, *Escherichia* clades are phenotypically indistinguishable from *E. coli* but the sequence types are highly divergent. Clade I strains are most closely related to *E. coli* sensu stricto (Walk et al., 2009; Massot et al., 2016). The high frequency of the phylogroups A and B1 *E. coli* strains is consistent with recent data on human commensal strains in developing countries (Massot et al., 2016). In this study, about 45% and 40% of *E. coli* isolates belonged to A and B1 phylogenetic groups, respectively. In a few groups, the D strain was found (~1%) whereas almost 7.6% of strains were unknown and more in number than group E strains (6.6%). Majority of the STEC strains fell into the phylogenetic group B1, while other strains can be found in phylogenetic groups A and E (Ishii, Meyer, & Sadowsky, 2007). Ghanbarpour and Kiani (2013) reported that STEC positive *E. coli* isolates from faeces of healthy fat tailed sheep belong to B1 and A phylogenetic groups, which is similar to the results of the current study. Antibiotic resistance of *E. coli* is strongly related to the phylogenetic grouping (Alizade et al., 2015). In our study, the majority of antibiotic resistance genes positive isolates belonged to the reportedly less virulent group A, followed by group B1, which is similar to the results of the Klimiene et al. (2017).

Conclusion

Results of the present study indicate that *E. coli* isolates, which originated from the carcasses of sheep and goat, belong to the different phylo-groups and contain shiga toxin, intimin and quinolone coding genes. The relatively high prevalence of STEC found in these animal species represents a reservoir of STEC infection for humans in this region. Presence of the *eaeA* gene in a few isolates showed that these isolates could be more virulent in pathogenicity. It can be concluded that the prevalence of *qnr* genes in this study is very low. Furthermore, no ESBL producers were found in the examined isolates. However, monitoring and longitudinal studies on antibiotic resistance in bacteria of food-producing animals are essential in the future.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Specific primers used in this study

Gene	Primer sequence (5′-3′)	Annealing temp (°C)	Product size (bp)	Ref
stx1	AGAGCGATGTTACGGTTTG TTGCCCCCAGAGTGGATG	53	388	(18)
stx2	TGGGTTTTTCTTCGGTATC GACATTCTGGTTGACTCTCTT	53	807	(18)
eaeA	AGGCTTCGTCACAGTTG CCATCGTCACCAGAGGA	53	570	(18)
qnrA	AGAGGATTTCTCACGCCAGG TGCCAGGCACAGATCTTGAC	54	580	(19)
qnrB	GGMATHGAAATTCGCCACTG TTTGCYGYYCGCCAGTCGAA	54	264	(19)
qnrS	GCAAGTTCATTGAACAGGGT TCTAAACCGTCGAGTTCGGCG	54	428	(19)
bla_{TEM}	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATC	50	1080	(23)
bla_{SHV}	GGGTAATTCTTATTTGTCGC TTAGCGTTGCCAGTGCTC	50	928	(23)
bla_{OXA-1}	TCAACTTTCAAGATCGCAG GTGTGTTTAGAATGGTGA	48	609	(21)
$bla_{CTX-M-15}$	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT	60	550	(22)
IMP	GGAATAGAGTGGCTTAATTC GCCAAGCTTCTATATTTGCG	58	275	(20)
VIM	GTGTTTGGTCGCATATCGC CGCAGCACCAGGATAGAAG	58	380	(20)
chuA	ATGGTACCGGACGAACCAAC TGCCGCCAGTACCAAAGACA	59	288	(9)
yjaA	CAAACGTGAAGTGTCAGGAG AATGCGTTCCTCAACCTGTG	59	211	(9)
arpA	AACGCTATTCGCCAGCTTGC TCTCCCCATACCGTACGCTA	59	400	(9)
TspE4.C2	CACTATTCGTAAGGTCATCC AGTTTATCGCTGCGGGTCGC	59	152	(9)
trpA (Group C)	AGTTTTATGCCCAGTGCGAG TCTGCGCCGGTCACGCCC	59	219	(9)
arpA (Group E)	GATTCCATCTTGTCAAAATATGCC GAAAAGAAAAAGAATTCCCAAGAG	57	301	(9)

Table 2. Distribution of sheep and goat *E. coli* isolates in phylogenetic groups

Phylo-groups	Sheep no. (%)	Goat no. (%)	Total
A	29 (54.7)	18 (34.6)	47 (44.8)
B1	13 (24.5)	29 (55.8)	42 (40)
B2	-	-	-
C	-	-	-
D	-	1 (1.9)	1 (0.9)
E	3 (5.6)	4 (7.7)	7 (6.6)
F	-	-	-
Clade I	-	-	-
unknown	8 (15.1)	-	8 (7.6)
Total	53	52	105

Table 3. Details of positive *E. coli* isolates for selective of virulence and antibiotic genes according to phylogenetic background in sheep and goat samples

no	Isolates from sheep samples							no	Isolates from goat samples						
	stx1	*stx2*	*eaeA*	*qnrA*	*qnrB*	*qnrS*	phylo		*stx1*	*stx2*	*eaeA*	*qnrA*	*qnrB*	*qnrS*	Phylo
6	+	-	-	-	-	-	A	2	-	-	-	-	+	-	A
4	+	-	-	-	-	-	B1	2	-	-	-	-	-	+	B1
1	-	-	+	-	-	-	B1	1	-	-	+	-	-	-	B1
-	-	-	-	-	-	-	-	2	+	+	-	-	-	-	B1
-	-	-	-	-	-	-	-	2	-	-	-	+	+	-	A
-	-	-	-	-	-	-	-	1	-	-	-	+	-	-	A
-	-	-	-	-	-	-	-	1	+	+	-	-	-	+	B1
-	-	-	-	-	-	-	-	3	+	-	-	-	-	-	B1
-	-	-	-	-	-	-	-	1	+	-	+	-	-	-	A
Total	10	0	1	0	0	0			7	3	2	3	4	3	