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Original article

ANTIMICROBIAL RESISTANCE PATTERNS AND PHYLOGENETIC ANALYSIS OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* STRAINS FROM GOATS USING BOTH CLERMONT PHYLOGENETIC SCHEMES

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Summary

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Foodborne transmission of Shiga toxin-producing *Escherichia coli* (STEC) poses a threat to public health. The Clermont typing schemes (previous and revised) have been used widely to phylotype *E. coli*. The present study was conducted to compare the relationship of the Clermont phylogenetic schemes in STEC strains isolated from goats and antibiotic resistance patterns in the southeast of Iran. Overall 52 strains carrying the *stx* gene were used for subsequent analysis. All strains were determined by analysing the genomic DNA with a PCR-based method using the two Clermont *et al.* (2000, 2013) schemes. Extended spectrum beta-lactamase (ESBL) producing strains were confirmed by the double disk-diffusion method. STEC strains were also tested for susceptibility to 20 antimicrobials agents. In the original Clermont method, the prevalent phylogroups were B1 (69.2%) and A (28.8%). The significant phylogenetic groups of strains according to the revised Clermont method were B1 (82.7%), A (13.5%) and unknown (3.8%). However, STEC strains underwent changes as noted from A to B1 (17.3%), B1 to unknown (3.8%), B1 to A (1.9%) and D to B1 (1.9%) groupings. Of the 52 *stx*-positive strains, two ESBL producing strains were detected. Susceptibility data showed that the most frequent resistance phenotype was related to cefazolin (90.4%), streptomycin (88.5%), ampicillin (86.5%) and oxytetracycline (82.7%) respectively. Although the overall frequency of the reassigned phylotypes was not significant, most changes occurred within the A phylotype. Therefore, implementation of the new method on isolates belonging to the A phylotype in the old method seems to be necessary to obtain accurate results.

Key words: drug resistance, extended-spectrum beta-lactamase, goat, phylogeny, STEC

INTRODUCTION

The Shiga toxin-producing *Escherichia coli* (STEC) strains represent a threat to public health (Ferreira *et al*., 2014). Since goat meat is consumed in many countries especially in the Middle-East, the identification and characterisation of STEC strains are of significant economic importance (Ghanbarpour *et al*., 2017). The STEC strains are characterised by their ability to produce Shiga toxin genes, which can be divided into two groups: *stx1* and *stx2*. Accessory virulence factors such as intimin and enterohaemolysin which are encoded by *eae* and *ehxA* genes, respectively play an important role in STEC pathogenicity (Fu *et al*., 2017). Nowadays, it is evident that resistant bacteria (saprophytes and pathogens) found in small ruminants could be disseminated into the food chain and therefore may serve as reservoirs and transfer of resistant bacteria to humans (Landers *et al*., 2012).

For the first time, Herzer *et al*. (1990) used multilocus enzyme electrophoresis (MLEE) to classify *E. coli* in the ECOR collection into phylogenetic groups (A, B1, B2 and D). In 2000, Clermont *et al*. described a simple triplex-PCR method to detect *chuA* and *yjaA* genes and DNA fragment TSPE4.C2. Regarding the presence/absence of amplicons, *E. coli* strains are assigned into four groups: A, B1, B2, and D. In 2013, Clermont *et al*. added an *arpA* gene target to those three candidate markers and revised the phylogenetic typing scheme to classify an *E. coli* isolate into one of the phylogroups A, B1, B2, C, D, E, F, and clade I.

Since most previous studies have used the original method for phylotyping, in the present study, both methods in relation to STEC resistance patterns were compared in order to determine if the data available from the original scheme were applicable in the revised method. The present study was conducted to compare the relationship of the Clermont phylogenetic schemes (previous and revised) in STEC strains isolated from goats and antibiotic resistance patterns in the southeast of Iran.

MATERIALS AND METHODS

Sampling and E. coli isolation

Overall 52 STEC strains were confirmed in goats from Kerman province (south-eastern Iran). Sterile swabs were used for sample collecting from faeces of goats. Samples were transported in Amies medium (Difco, USA) to the laboratory for immediate processing. Each swab samples plated on sorbitol Mac Conkey agar (SMAC) (Merck, Germany) and incubated at 37 °C for 24 h. *E. coli* colonies were picked from SMAC and confirmed as *E. coli* by standard biochemical and bacteriological tests. The strains were used for subsequent PCR analysis for the detection of *stx1*, *stx2*, *eaeA*, and *ehxA* genes (see the next section).

DNA extraction and confirmatory PCR for STEC strains

Crude DNA was extracted from STEC and reference strains by the boiling method as described previously (Dashti *et al*., 2009). STEC strains were confirmed by a multiplex-PCR assay detecting the *stx1*, *stx2*, and *eaeA* genes as described by Paton & Paton (2002). Screening for the *ehxA* gene was done by conventional PCR with the primers and amplification conditions as described by Schmidt *et al*. (1995) (Table 1).

Table 1. Primer sequences used for PCR analysis

Gene		Primer sequence (5′-3′)	Size (bp)	References
stx1	STEC	F: ATAAATCGCCATTCGTTGACTAC R: AGAACGCCCACTGAGATCATC	180	Paton & Paton, 1998
stx2		F: GGCACTGTCTCTCTGAAACTGCTCC R: TCGCCAGTTATCTGACATTCTG	255	
eaeA		F: GACCCGGCACAAGCATAAGC R: CCACCTGCAGCAACAAGAGG	384	
ehxA		F: GGTGCAGCAGAAAAAGTTGTAG R: TCTCGCCTGATAGTGTTTGGTA	1551	Schmidt *et al.,* 1995
chuA	Phylo-group; Triplex	F: GACGAACCAACGGTCAGGAT R: TGCCGCCAGTACCAAAGACA	279	Clermont *et al.,* 2000
yjaA		F: TGAAGTGTCAGGAGACGCTG R: ATGGAGAATGCGTTCCTCAAC	211	
TspE4.C2		F: GAGTAATGTCGGGGCATTCA R: CGCGCCAACAAAGTATTACG	152	
chuA	Phylo-group; Quad-ruplex	F: ATGGTACCGGACGAACCAAC R: TGCCGCCAGTACCAAAGACA	288	Clermont *et al.,* 2013
yjaA		F: CAAACGTGAAGTGTCAGGAG R: AATGCGTTCCTCAACCTGTG	211	
arpA		F: AACGCTATTCGCCAGCTTGC R: TCTCCCCATACCGTACGCTA	400	
TspE4.C2		F: CACTATTCGTAAGGTCATCC R: AGTTTATCGCTGCGGGTCGC	152	
trpA	Group C	F: AGTTTTATGCCCAGTGCGAG R: TCTGCGCCGGTCACGCCC	219	Clermont *et al.,* 2013
arpA	Group E	F: GATTCCATCTTGTCAAAATATGCC R: GAAAAGAAAAAGAATTCCCAAGAG	301	Clermont *et al.,* 2013
trpA	Internal control	F: CGGCGATAAAGACATCTTCAC R: GCAACGCGGCCTGGCGGAAG	489	Clermont *et al.,* 2013

Phylogenetic typing

The STEC strains were assigned to one of the four described *E. coli* phylogenetic groups (A, B1, B2, or D) using a triplex-PCR based phylotyping scheme (Clermont *et al*., 2000) and classified into one of the eight phylogroups (A, B1, B2, C, D, E, F, or clade I) using a revised phylotyping protocol (Clermont *et al*., 2013). The PCR assay targeting the phylogroup genes and primer pairs used are reported in Table 1.

Antibiotic susceptibility testing

All STEC strains were subjected to antimicrobial susceptibility tests using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2016). The antibiotic disks used in the experiment were 10 µg ampicillin (AM), 10/10 µg ampicillin-sulbactam (SAM), 30 µg cefazolin (CZ), 30 µg ceftriaxone (CRO), 30 µg cefuroxime (CXM), 30 µg cefotaxime (CTX), 30 µg ceftazidime (CAZ), 10 µg

gentamicin (GM), 30 µg amikacin (AK), 30 µg kanamycin (K), 10 µg streptomycin (S), 30 µg tetracycline (T), 30 µg doxycycline (D), 30 µg oxytetracycline (TE), 5 µg ciprofloxacin (CP), 30 µg nalidixic acid (NA), 30 µg enrofloxacin (NFX), 1.25/ 23.75 µg trimethoprim-sulfamethoxazole (SXT), 30 µg chloramphenicol (C), 30 µg florfenicol (FF) (PadtanTeb. Co., Iran). The *E. coli* ATCC 25922 strain was used for quality control in this experiment.

Extended-spectrum beta-lactamase-producing strains

ESBL production was confirmed using a double-disc synergy test as a standard disc-diffusion assay recommended by the Clinical Laboratory Standards Institute (CLSI, 2012) guidelines. Discs containing cefotaxime (30 µg) and ceftazidime (30 µg) were placed at a distance of 30 mm (opposite sides) around discs containing cefotaxime/clavulanic acid (30/10 mg) and ceftazidime/clavulanic acid (30/10 mg). A positive test result was defined as a ≥5 mm increase in zone diameter compared to a disk without clavulanic acid. Quality controls were conducted using *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 strains.

RESULTS

Phylogenetic analysis

All STEC strains were analysed using both Clermont methods. Most strains were classified as B1 and A groups by the old typing scheme and similar results were observed using the revised phylogenetic typing schemes. The old Clermont method revealed that from 52 STEC strains, 28.8% (15/52 strains) belonged to A, 69.2% (36/52) belonged to B1, and 1.9% (1/52) belonged to D phylogenetic groups. Phylotyping according to the revised Clermont method indicated that the strains could be categorised into three groups: A (7/52; 13.5%), B1 (43/52; 82.7%) and unknown (2/52; 3.8%). However, STEC strains that underwent changes were noted as A to B1 (17.3%), B1 to unknown (3.8%), B1 to A (1.9%) and D to B1 (1.9%) groupings. Here, the STEC strains underwent not significant reassignment among strain reclassification from the A to the B1 phylogenetic group.

Table 2. STEC strains genes in relation to Clermont phylogenetic methods

Clermont scheme 2000						Clermont scheme 2013					
No	phylotype	genes				No	phylotype	genes			
		stx1	*stx2*	*eae*	*ehxA*			*stx1*	*stx2*	*eae*	*ehxA*
15	B1	+	–	–	–	20	B1	+	–	–	–
10	A	+	–	–	–	10	B1	+	–	–	+
10	B1	+	–	–	+	8	B1	+	+	–	+
6	B1	+	+	–	+	6	A	+	–	–	–
4	A	+	+	–	+	3	B1	+	+	–	–
2	B1	+	–	+	+	2	B1	+	–	+	+
2	B1	+	+	–	–	1	A	+	+	–	+
1	A	+	+	-	–	1	unknown	+	+	–	+
1	B1	+	–	+	–	1	unknown	+	–	+	–
1	D	+	–	–	–						
Total		52	13	3	22	Total		52	13	3	22

STEC strains

Among the strains examined, all harboured the *stx1* gene, 13 strains (25%) carried the *stx2* gene, and 3 strains (5.8%) possessed the *eae* gene. Twenty-two strains (42.3%) were positive for the *ehxA* gene. Table 2 shows the distribution of STEC strains by the old and revised phylogenetic grouping schemes.

Antimicrobial susceptibility

The STEC strains were surveyed for susceptibility to 20 antibiotic agents. The antibiotic susceptibility profile showed that all of the strains examined were resistant to one or more antimicrobials. They were resistant most commonly to cefazolin (90.4%), streptomycin (88.5%), ampicillin (86.5%) and tetracycline (82.7%). Different rates of antibiotic resistance were recorded against amikacin (80.7%), ceftriaxone (67.3%), florfenicol (57.7%), kanamycin (51.9%), ciprofloxacin (23.1%), cefotaxime (23.1%), ampicillin/sulbactam (21.1%), cefuroxime (21.1%), doxycycline (15.4%), gentamicin (9.6%), ceftazidime (7.7%), tetracycline, nalidixic acid and enrofloxacin (each 5.8%). However, only one strain was resistant to trimethoprim-sulfamethoxazole (1.9%) and none of the strains were resistant to chloramphenicol. There were significant differences in the resistance rates to cefazolin, streptomycin, ampicillin, tetracycline, amikacin, ceftriaxone, florfenicol, and kanamycin in the STEC strains.

Antimicrobial susceptibility patterns

Forty-seven antibiotic resistance patterns were detected. Table 3 indicates the antibiotic resistance patterns of the STEC strains in relation to their phylogenetic background. The phylotyping of antibiotic resistant patterns indicated that these strains were segregated mostly in the B1 and A groups.

Detection of ESBLs production

The double disk diffusion test of β-lactams and β-lactam/inhibitor combinations indicated that 2/52 (3.8%) STEC strains were ESBL producers. The ESBL production of STEC strains in relation to original and revised phylogenetic backgrounds is shown in Table 3.

DISCUSSION

Consumption of food contaminated with STEC strains and producing stx toxins in the intestinal tract can cause serious infection in humans, including haemolytic-uremic syndrome and neurological damage (Mcgannon *et al*., 2010; Hoang Minh *et al*., 2015). These bacteria could also serve as reservoirs for the spread of antimicrobial resistance to humans (Iwu *et al*., 2017). The focus of the present study was to evaluate the impact of the previous and revised Clermont methods (2000 and 2013) on the phylogenetic typing of STEC strains and antibiotic resistance patterns of goat in Iran. Several studies have reported that with the old Clermont method, diarrhoeagenic *E. coli* pathotypes belonged to the A, B1, and D phylogenetic groups, while most non-pathogenic commensal strains belonged to groups A and B1 (Carlos *et al*., 2010; Mokracka *et al*., 2011). The results of the current study show that most groups of STEC strains are in the B1 and A groups rather than in the D group. A study on strains of *E. coli* isolated from the faeces of cows, goats, and sheep showed that group B1 was prevalent among these hosts. However, most *E. coli* strains belonged to this group were found in hosts that are able to survive in the environment, and the diet of these hosts consisted

Table 3 Antibiotic resistance patterns and ESBL of STEC strains in relation to Clermont phylogenetic methods

Antibiotic resistance patterns	Clermont (2000)			Clermont (2013)		
	phylotype			phylotype		
	A	B1	D	A	B1	unknown
AK, T	1	–	–	1	–	–
CZ, K, T	1	–	–	1	–	–
CZ, AK, ST	1	1	–	1	1	–
AM, CZ, K, S, T	1	–	–	–	1	–
AM, CZ, CRO, T	–	1	–	–	1	–
AM, CRO, K, S, T	1	–	–	–	1	–
AM, CZ, AK, S, FF	–	2	–	–	2	–
AM, CRO, CXM, ST	1	–	–	–	1	–
CZ, AK, S, NA, NFX*	–	1	–	–	1	–
AM, CRO, AK, K, S, T	1	–	–	1	–	–
AM, CZ, CRO, AK, S, T	–	1	–	–	1	–
AM, CZ, AK, K, S, T, FF	1	1	–	–	2	–
AM, CZ, CRO, AK, S, FF	1	–	1	–	2	–
AM, CZ, CRO, AK, S, CP	–	1	–	–	1	–
AM, CZ, CRO, AK, K, S, T	–	1	–	–	1	–
AM, CZ, CRO, AK, S, T, FF	1	1	–	–	2	–
SAM, CZ, CTX, AK, K, S, T	1	–	–	–	1	–
CZ, CRO, CXM, AK, S, T, FF	–	1	–	–	1	–
AM, CZ, CRO, CTX, S, T, FF	–	1	–	–	1	–
AM, CZ, CRO, CTX, S, T, CP	–	1	–	–	1	–
AM, CZ, CRO, CTX, TE, T, FF	–	1	–	–	1	–
AM, CZ, CRO, AK, S, D, T, FF	–	1	–	–	1	–
AM, CZ, CRO, AK, K, S, T, CP	–	1	–	–	1	–
AM, SAM, CZ, CRO, K, S, T, CP	–	1	–	–	1	–
AM, CZ, CXM, CTX, S, T, NFX	–	1	–	–	1	–
AM, CZ, CRO, AK, K, S, D, T, FF	–	1	–	–	1	–
AM, SAM, CZ, CRO, AK, K, S, T	–	1	–	–	1	–
AM, CAZ, AK, K, T, CP, NFX, FF	–	1	–	–	1	–

*ESBL strains positive.

Table 3 (cont'd). Antibiotic resistance patterns and ESBL of STEC strains in relation to Clermont phylogenetic methods

Antibiotic resistance patterns	Clermont (2000)			Clermont (2013)		
	phylotype			phylotype		
	A	B1	D	A	B1	unknown
AM, CZ, CTX, AK, K, S, T, CP, FF	–	1	–	–	1	–
AM, CZ, CRO, AK, K, S, T, CP, FF	–	1	–	–	1	–
AM, CZ, CRO, CTX, CAZ, AK, S, T	–	1	–	–	1	–
AM, CZ, CRO, AK, S, T, CP, NA, FF	–	1	–	–	1	–
AM, SAM, CZ, CRO, AK, K, S, T, FF	–	1	–	–	1	–
AM, CZ, CRO, AK, S, T, CP, SXT, FF	–	1	–	1	–	–
AM, CZ, CRO, CXM, AK, K, S, T, FF	–	1	–	–	1	–
AM, CZ, CXM, AK, K, S, TE, D, T, FF	–	1	–	–	–	1
AM, CZ, CRO, CXM, GM, AK, K, S, T	–	1	–	–	–	1
AM, CZ, CRO, CTX, AK, K, S, D, T, FF	1	–	–	–	1	–
AM, SAM, CZ, CXM, GM, AK, S, T, FF	–	1	–	–	1	–
AM, SAM, CZ, CRO, CTX, AK, S, D, T, FF	–	1	–	–	1	–
AM, SAM, CZ, CXM, CAZ, AK, K, S, T, FF	–	1	–	–	1	–
AM, CZ, CRO, CTX, GM, AK, K, S, D, T, FF	–	1	–	–	1	–
AM, CZ, CRO, GM, AK, K, S, T, CP, NA, FF	1	–	–	1	–	–
AM, SAM, CZ, CRO, GM, AK, K, D, T, CP, FF	1	–	–	1	–	–
AM, SAM, CZ, CRO, CXM, CTX, AK, K, S, T, FF*	1	–	–	–	1	–
AM, SAM, CZ, CRO, CXM, AK, K, S, TE, D, T, CP	–	1	–	–	1	–
AM, SAM, CZ, CRO, CXM, CTX, CAZ, AK, K, S, T, FF	–	1	–	–	1	–
Total	15	36	1	7	43	2

*ESBL strains positive; Ampicillin (AM), ampicillin-sulbactam (SAM), cefazolin (CZ), ceftriaxone (CRO), cefuroxime (CXM), cefotaxime (CTX), ceftazidime (CAZ), gentamycin (GM), amikacin (AK), kanamycin (K), streptomycin (S), tetracycline (T), doxycycline (D), oxytetracycline (TE), ciprofloxacin (CP), nalidixic acid (NA), enrofloxacin (NFX), trimethoprim-sulfamethoxazole (SXT), chloramphenicol (C), florfenicol (FF).

mostly of unprocessed, natural foods for ruminants (grass and straw), whereas B1 isolates became much less commonly observed in partially processed foods served to other hosts, such as dairy cattle in the barn (Carlos *et al*., 2010; Bok *et al*., 2015). A previous study by Lecointre *et al*. (1998) verified that groups A and B1 were sister groups. Logue *et al*. (2017) described results similar to the present findings, showing that A and B1 isolates appeared to cluster together with equal distribution among avian faecal *E. coli* and avian pathogenic *E. coli*. The current results showed that about 75% of STEC strains detected in the revised typing scheme retained their old phylogenetic group. However, about 25% of the strains moved into new groups not recognised in the old typing (i.e. A, B1, and unknown). This was particularly observed among strains that were reclassified from A to B1 or from B1 to unknown (old to new). In the current study, most of the B1 strains appeared among STEC. The level of gene carriage was relatively high with most having the *stx1* gene, although some had combination genes (*stx1*, *stx2,* and *ehxA*) (Table 2). These results also correlated with a study on STEC strains from ruminants in which the majority of strains carried the *stx1c* and/or *stx2d*, *ehxA*, and *saa* genes and belonged to the B1 phylogroup (Ishii *et al*., 2007).

In general, the current results showed a high prevalence of resistance rates to at least one agent among 95% of STEC strains. The results are in agreement with previous reports that have indicated much higher rates of resistance among *E. coli* isolates from livestock animals (Knezevic & Petrovic, 2008; Ogunleye *et al*., 2013). The multidrug resistance was significantly higher among these strains, indicating resistance to 19 out of 20 tested antimicrobial agents. The existence of 47 antibiotic resistance patterns showed high variability among STEC strains of caprine origin. A study in Poland indicated a high prevalence of resistance among *E. coli* isolated from dairy cows (82.3%) and beef cattle (58.5%), but multidrug resistance was lower at 17% and 8.5% among dairy and beef cattle, respectively (Bok *et al*., 2015). ESBL producing *E. coli* carrying *AmpC* gene have been isolated from food animals in many European countries. The percentages of ESBL producing *E. coli* in food-producing animals, varied from 0.2% to 40% in European countries, (Liebana *et al*., 2012) to 25.3% in India (Mandakini *et al*., 2015). In a study conducted in south east of Iran the prevalence of ESBL producing *E. coli* in nosocomial and community-acquired isolates was 31% and 21% respectively (Dehghan *et al*., 2017). The current results showed that the B1 phylogenetic group included a greater number of antibiotic-resistant strains than isolates belonging to the other groups (A, D, and unknown). Most of the ESBL-producing *E. coli* isolates were classified into the A and B1 phylogroups, perhaps due to the greater antibiotic exposure of the strains in these groups in the faecal flora (Birgy *et al*., 2012). Bukh *et al*. (2009) reported that isolates belonging to the B1 phylogroup were less resistant to multiple antibiotics than those in groups A and D. The present results disagree with this observation, because the higher frequency of the phylogenetic group B1 in STEC strains from goat might be a consequence of selection pressure by the use of different antibiotic categories.

CONCLUSION

The present study indicates that in both Clermont typing schemes, B1 and A phy-

logenetic groups represented the majority of strains involved in STEC. It is noteworthy that the highest prevalence of antimicrobial resistance was observed in STEC strains from goat, which underlines the importance of this animal species as a reservoir. The antimicrobial drug-resistant strains were associated with phylogenetic distribution toward groups B1 and A. The comparison of phylogenetic typing schemes indicated that most of the strains remained where they were originally assigned (previous scheme). In total, these results are of importance in phylogenetic typing schemes and in the epidemiological surveillance of STEC strains.

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