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Phylogenetic Grouping of Verotoxigenic *Escherichia coli* (VTEC) Obtained from Sheep and Broiler Chicken in Northwestern Iran

Kuzeybatı İran'da Koyun ve Broyler Tavuğundan Elde Edilen Verotoksijenik Escherichia coli (VTEC) Filogenetik Grubu

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Abstract

Verotoxigenic *Escherichia coli* (VTEC) are major foodborne pathogens with an increasing public health concern. The purpose of this study was to investigate the occurrence and the phylogenetic groups of VTEC isolates from the feces of healthy sheep and broiler chickens at a slaughterhouse in Urmia region, Northwestern Iran. A total of 446 *E. coli* isolates (97 from sheep and 349 from broiler chickens) were assessed for the occurrence of the Vtx-encoding genes (vtx1 and vtx2) using polymerase chain reaction. Then, all the recovered VTEC isolates were phylogenetically grouped based on the Clermont phylotyping method using three genetic sequences, the so-called chuA, yjaA, and TSPE4.C2. The vtx gene-carrying *E. coli* was identified in 46.4% (45/97) of sheep-originated isolates and in 8.3% (29/349) of broiler chicken-originated isolates. In general, phylotyping revealed that 74 VTEC isolates segregated in the phylogenetic groups A (32.4%; designated as VTEC-A), B1 (44.6%; VTEC-B1), B2 (9.5%; VTEC-B2), and D (13.5%; VTEC-D). The results also showed that the dissemination of VTEC isolates of sheep and broiler chicken origin varied noticeably in their assignment to B1 and D phylogenetic groups ($p<0.01$). In addition, the virulent phylogenetic groups (B2 and D) were significantly more common in broiler chickens than in sheep ($p<0.01$). In conclusion, healthy sheep and broiler chickens could be a reservoir for VTEC belonging to virulent phylogenetic groups, thus representing a potential risk factor for public health. This study also demonstrated significant differences with respect to the phylogenetic group assignment of the VTEC strains between sheep and broiler chickens.

Keywords: Broiler chickens, phylogenetic groups, sheep, verotoxigenic *Escherichia coli* (VTEC)

Öz

Verotoksijenik *Escherichia coli* (VTEC), artan bir halk sağlığı sorunu olan başlıca gıda kaynaklı patojenlerdir. Çalışmanın amacı, Kuzeybatı İran'ın Urmia bölgesinde kesilen sağlıklı koyun ve broyler tavuklarının dışkılarından VTEC izolatlarının oluşum ve filogenetik gruplarını araştırmaktır. Bu çalışmada, 446 *E. coli* izolatı (koyundan 97 ve broyler tavuktarından 349), polimeraz zincir reaksiyonu (PCR) kullanılarak Vtx-kodlayan genlerin (vtx1 ve vtx2) oluşumu için test edilmiştir. Daha sonra, tüm geri kazanılmış VTEC izolatları, chuA, yjaA ve TSPE4.C2 olarak adlandırılan üç genetik sekans kullanılarak Clermont filotiplemeye dayanan filogenetik olarak gruplandırılmıştır. Sonuç olarak, koyun kaynaklı izolatların %46,4'ünde (45/97) vtx geni taşıyan *E. coli* saptanmış ve broylerler için saptanan yüzdeler ise %8,3 (29/349) olmuştur. Genel olarak, filotipleme, 74 VTEC izolatının, filogenetik olarak grup A (%32,4, VTEC-A olarak belirlenmiş), B1 (%44,6; VTEC-B1), B2 (%9,5; VTEC-B2) ve D (%13,5; VTEC-D) olarak ayrıldığını ortaya koymuştur. Buna ek olarak, sonuçlar ayrıca, koyun ve broyler tavuklarının VTEC izolatlarının yayılmasının B1 ve D filogenetik grupları arasında belirgin olarak farklılık gösterdiğini göstermiştir ($p<0,01$). Ayrıca, virütik filogenetik gruplar (B2 ve D), broyler tavuklarında koyunlara göre önemli ölçüde daha yaygın olarak saptanmıştır ($p<0,01$). Sonuç olarak, sağlıklı koyun ve broyler tavukları, virütik filogenetik gruplara ait VTEC için halk sağlığına potansiyel bir risk faktörü olan bir rezervuar olabilir. Çalışma ayrıca koyun ve broyler tavukları arasında VTEC suşlarının filogenetik grup atamasına göre önemli farklılıklar ortaya koymuştur.

Anahtar kelimeler: Broyler tavukları, filogenetik gruplar, koyun, verotoksijenik *Escherichia coli* (VTEC)

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Introduction

Escherichia coli is a bacterium generally found in the gut of warm-blooded animals (Kaper et al., 2004). Although most strains of this micro-organism are considered to be harmless symbionts of digestive tract, some strains cause human diseases. Verotoxigenic *Escherichia coli* (VTEC; also called Shiga toxin-producing *E. coli* or STEC) has emerged as an important zoonotic food-borne pathogen (Gyles, 2007) which can cause hemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) in human (Girardeau et al., 2005). These strains are defined by making of one or more cytotoxins, called verocytotoxin 1 (VT1) and verocytotoxin 2 (VT2), usually encoded by bacteriophages. However, strains of this pathotype appear to circulate as a part of the gut flora with ruminants such as cattle, sheep and goats serving as the major animal reservoirs (Horcajo et al., 2010; Oporto et al., 2008). Healthy birds have also been reported to carry VTEC (Farooq et al., 2009).

Escherichia coli strains can be categorized into four main phylogenetic groups A, B1, B2 and D, by assessing the presence or absence of three genetic sequences called *chuA* (existing in B2 and D phylogroups, absent from B1 and A), *yjaA* (existing in B2, absent from D) and TSPE4.C2 (existing in B1, absent from group A) (Clermont et al., 2000). These phylogenetic groups apparently differ in their ecological niches, history of life, tendency to cause disease (Gordon et al., 2008) and some characteristics such as their virulence genotype and genome size (Bergthorsson and Ochman, 1998; Girardeau et al., 2005). Commensal *E. coli* belongs generally to A and B1 phylogroups and rarely possess virulence genes (Dixit et al., 2004), whereas B2 and D strains are typically related to disease and carry a broad spectrum of virulence-factor genes (Nowrouzian et al., 2005). Phylotyping analyses have also revealed that the majority of the VTEC strains comprise phylogenetic group B1, representing that they most probably do not cause severe diseases in human (Girardeau et al., 2005; Ishii et al., 2007). In general, humans are infected with VTEC strains mostly through the ingestion of contaminated food or water or direct contact with animals, therefore identifying the sources of infection is an effective way towards decreasing the prevalence of this pathogen and thus reduce the risk of humans infection. Phylotyping of *E. coli* strains has previously been underscored as a valuable tool for bacterial source tracking (BST) and for surveillance programs in slaughterhouses (Carlos et al., 2010; Martins et al., 2013). Although calves have been considered to be a reservoir of VTEC in Urmia region, Iran (Saei and Ayremlou, 2012), there are limited information about the prevalence of VTEC in other food producing animals. The aims of the current study were to investigate the presence of VTEC and to determine their phylogenetic groups in feces from healthy sheep and broiler chickens at slaughter in Urmia, northwest of Iran.

Materials and Methods

Sample collection and *E. coli* isolates

A total of 446 fecal samples of apparently healthy sheep (n=97) and broiler chickens (n=349) were obtained during slaughter in Urmia region, Northwestern, Iran. All procedures in this study were in accordance with the ethical standards of the Animal Ethics Committee of Faculty of Veterinary Medicine, Urmia University (AECVU) and supervised by authority of Urmia University Research Council (UURC). The swab samples were placed directly in tubes containing Stuart transport medium (CM0111-Oxoid, Basingstoke, United Kingdom), and submitted to the laboratory for immediate processing. Each sample was streaked onto MacConkey agar (105465-Merck, Darmstadt, Germany) plates and incubated overnight at 37°C. Typical lactose-positive (pink *E. coli* colonies) colonies were further streaked on Eosin Methylene Blue (101347-EMB, Merck, Darmstadt, Germany) agar. From each plate, a single colony of typical morphology was selected and subcultured onto 5% sheep blood agar (110886-Merck, Darmstadt, Germany) for purity and biochemical tests. Furthermore, species-specific PCR was done as described previously (Riffon et al., 2001) using primers Eco 2083 (GCT TGA CAC TGA ACATTG AG) and Eco 2745 (GCA CTT ATC TCT TCC GCA TT). The confirmed *E. coli* isolates were kept in glycerol broth at -20°C for subsequent analysis.

Detection of *vtx* genes by PCR

The presence of the *vtx* genes in the *E. coli* isolates was examined by PCR using primers described earlier (Osek, 2003). The primer set vtx_1F (5′-CAG TTA ATG TCG TGG CGA AGG-3′) and vtx_1R (5′-CAC CAG ACA ATG TAA CCG CTG-3′) were used for the amplification of vtx_1, which yielded a PCR product of 384 bp in size. The primer set vtx_2F (5′-ATC CTA TTC CCG GGA GTT TAC G-3′) and vtx_2R (5′-GCG TCA TCG TAT ACA CAG GAG C-3′) were used for amplifying vtx_2, which allow the amplification of a DNA fragment at approximately 584-bp. *E. coli* ATCC43895 was used as positive control. Polymerase chain reaction and electrophoresis of products were performed as described previously (Saei and Ayremlou, 2012).

Phylogenetic group determination by triplex PCR

Three primer pairs used for the amplification of three genetic sequences called *chuA*, *yjaA* and TSPE4.C2 are presented in Table 1. Amplifications were done in a CORBETT thermocycler (Model CP2-003, Australia) with the following temperature profile: 1 cycle of 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and finally 1 cycle of 72°C for 7 min. The positive control used for the determination of three phylogenetic group markers ($chuA^+$, $yjaA^+$ and $Tspe4C2^+$) was *E. coli* reference strain ECOR62. Amplicons were electrophoresed on 1.5% (w/v) agarose gel containing ethidium bromide and visualized by the UV transilluminator. The sizes of PCR products were determined by comparing with GeneRuler 100 bp DNA ladder plus (Thermo Scientific, Germany). The main phylogenetic groups (A, B1, B2 and D) and subgroups (A_0, A_1, B_1, $B2_2$,

Table 1. Sequences of oligonucleotide primers used in this study

Gene	Sequence	Amplicon size	Reference
chuA	5'-GAC GAA CCA ACG GTC AGG AT-3'	279 bp	(Clermont et al., 2000)
	5'-TGC CGC CAG TAC CAA AGA CA-3'		
yjaA	5'-TGA AGT GTC AGG AGA CGC TG-3'	211 bp	(Clermont et al., 2000)
	5'-ATG GAG AAT GCG TTC CTC AAC-3'		
TSPE4.C2	5'-GAG TAA TGT CGG GGC ATT CA-3'	152 bp	(Clermont et al., 2000)
	5'-CGC GCC AAC AAA GTA TTA CG-3'		

$B2_3$, D_1, and D_2) of VTEC strains were determined according to the combinations of *chuA*, *yjaA*, and Tspe4.C2 markers as described earlier by Clermont et al. (2000) and Escobar-Paramo et al. (2004), respectively.

Statistical analysis

The Mann-Whitney U test was done using Statistical Package for the Social Sciences Software V22.0 (SPSS Statistics for Windows, IBM Corp.; Armonk, New York, USA) to compare the phylogenetic groups between the studied hosts. For each comparison, a p value less than 0.01 was considered significant.

Results

A total of 446 *E. coli* isolates comprising 97 from sheep and 349 from broiler chickens were obtained from same number of fecal samples by conventional culture as well as species-specific PCR techniques. Confirmed *E. coli* isolates were tested for the presence of vtx_1 and vtx_2 genes. VTEC isolates were recovered from 46.4% (45/97) in healthy sheep, and 8.3% (29/349) in broiler chickens.

The PCR results for phylotyping revealed that VTEC strains segregated mainly in phylogenetic group B1 (33 of 74 [44.6%]), designated as VTEC-B1. Of the remaining strains, 24 (32.4%), 10 (13.5%) and 7 (9.5%) segregated in main phylogenetic groups A (VTEC-A), D (VTEC-D), and B2 (VTEC-B2), respectively. The different banding patterns obtained by Clermont triplex PCR method for the phylogenetic groups are shown in Figure 1.

Of 45 sheep VTEC isolates, 29 isolates (64.5%) were phylogenetic group B1, 13 (28.9%) phylogenetic group A, 2 (4.4%) phylogenetic group B2, and 1 (2.2%) phylogenetic group D. Among the isolates recovered from broiler chickens, 11 isolates (38%) were allocated into phylogenetic group A, 9 (31%) phylogenetic group D, 5 (17.2%) phylogenetic group B2, and 4 (13.8%) phylogenetic group B1. The analysis of phylotyping results among the studied hosts is detailed in Table 2. As shown, with the exception of B2 phylosubgroups ($B2_2$, $B2_3$), all other phylogenetic subgroups A (A_0, A_1), B1, D (D_1, D_2) were identified in VTEC isolates from both sheep and broiler chickens.

There was no significant difference in carriage of phylogenetic groups A and B2 between sheep and broiler chickens, whereas

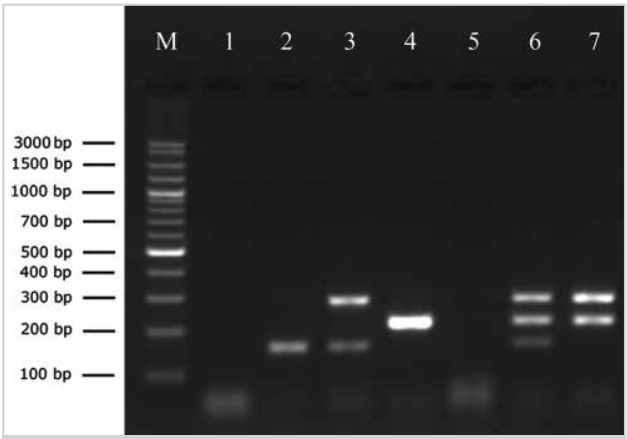

Figure 1. Triplex PCR patterns of representative *E. coli* phylogenetic groups. Lane M: GeneRuler™ 100 bp plus DNA ladder; Lane 1: negative control; Lane 2: group B1; Lane 3: group D; Lanes 4 and 5: group A; Lanes 6 and 7: group B2

Table 2. Phylogenetic groups and subgroups of VTEC strains isolated from sheep and broiler chickens

		Phylogenetic groups and subgroups of VTEC*						
		VTEC-A		VTEC-B1	VTEC-B2		VTEC-D	
Host	n	A_0	A_1	B_1	$B2_2$	$B2_3$	D_1	D_2
Sheep	45	5	8	29	2	-	1	-
Broiler chickens	29	2	9	4	-	5	5	4
Total	74	7	17	33	2	5	6	4

* Verotoxigenic *Escherichia coli*

B1 significantly associated with sheep and D with broiler chickens ($p<0.01$). Concerning commensal (A and B1) and virulent (B2 and D) phylogenetic groups, statistical analysis also showed that commensal strains and virulent strains significantly associated with sheep and broiler chickens, respectively ($p<0.01$).

Out of 74 VTEC isolates, 38 isolates (51.4%) were positive for vtx_1, 28 (37.8%) for vtx_2, and 8 (10.8%) for both vtx_1 and vtx_2. Twenty-seven of sheep VTEC isolates were positive for vtx_1, 26 contained vtx_2 and 6 possessed both *vtx1* and *vtx2*. The corresponding counts in broiler isolates were 11, 16, and 2, respec-

Table 3. The *vtx* genes in VTEC isolates from sheep and broiler chickens in relation to main phylogenetic groups

Phylogenetic group	Sheep				Broiler chickens			
vtx* genes	A	B1	B2	D	A	B1	B2	D
vtx_1	8	17	1	1	4	1	3	3
vtx_2	3	8	1	-	6	3	2	5
vtx_1, vtx_2	2	4	-	-	1	-	-	1
Total	13	29	2	1	11	4	5	9

* Verocytotoxin

tively. The *vtx* genes in sheep and broilers VTEC isolates in relation to main phylogenetic groups are shown in Table 3.

Discussion

According to Wasteson (2001), Verotoxigenic *Escherichia coli* (VTEC) is the only *E. coli* pathogenicity group of major interest from zoonotic standpoint. In the current study, VTEC were isolated more frequently (45/97; 46.4%) in feces from sheep. This is in agreement with previous studies and confirms the importance of sheep as VTEC reservoir (Oporto et al., 2008). The frequency detected in the present study was, however, higher than the 29.9% and 7.9% reported in Switzerland and Brazil, respectively (Maluta et al., 2014; Zweifel et al., 2004). Another study on collection of *E. coli* isolates from healthy fat-tailed sheep in Iran showed that 13% of isolates belonged to VTEC pathotype (Ghanbarpour and Kiani, 2013). Differences in farm-level factors such as feed composition and sanitation of drinking water may explain these discrepancies. A study of dairy cattle farms demonstrated that herd management factors related to cattle feeding practices were associated with fecal shedding of VTEC (Cho et al., 2013). In the current study, VTEC prevalence rate (29/349; 8.3%) in fecal samples of healthy broiler chickens was also higher than those reported in Kerman, southeastern of Iran (Ghanbarpour et al., 2011; Salehi, 2014). High incidence of VTEC observed in broilers may at least in part be due to geographical effects, hygienic measures and higher stocking density of birds in intensive chicken farming.

Despite the description of a new quadruplex PCR method to assign *E. coli* isolates to eight phylo-groups (A, B1, B2, C, D, E, F and clade I), Clermont genotyping triplex PCR is a cost effective and reasonably accurate method for detecting putative *E. coli* isolates from a variety of sample types (Higgins et al., 2007). Consistent with previous study (Girardeau et al., 2005), phylogenetic analysis revealed that VTEC isolates, irrespective of sheep or broilers origin, segregated mainly in phylogenetic groups A (24/74; 32.4%) and B1 (33/74; 44.6%). Selection through antibiotic pressure may explain this phenomenon, as most of antibiotic resistant *E. coli* strains have been shown to belong to the phylogenetic groups A and B1 (Obeng et al., 2012). Other speculation could be the ability of these phylogenetic groups to survive and persist in feces, manure, and soil in the environment. It is also hypothesized that bacteriophages carrying *vtx* genes probably could transduce with significant frequency to A and B1 phylogenetic group strains (Garcia-Aljaro et al., 2009).

Seven out of 74 (9.5%) VTEC strains analyzed in the study belonged to phylogenetic group B2, which is predominant among extraintestinal strains. In contrast to this result, none of the fecal isolates from domestic animals in South Korea and healthy fat-tailed sheep in southeastern of Iran belonged to B2 group (Ghanbarpour and Kiani, 2013; Unno et al., 2009). We supposed that they originated from food handlers or water contaminated with fecal material of humans. Carlos et al. (2010) stated that isolates belonging to the B2 group, particularly subgroup $B2_3$, represent an indicator for pollution by human feces.

According to statistical analysis, there were significant differences with respect to the phylogenetic group assignment of VTEC strains obtained from sheep and broilers. Carlos et al. (2010) also described a different dissemination of phylogenetic groups among *E. coli* strains isolated from humans, chickens, cows, goats, pigs and sheep, where high percentage of strains from the chicken samples were dominated by group A, whereas group B1 was predominant among *E. coli* strains from sheep. This non-random distribution of phylogenetic groups in the hosts may be due to ecological differences (e.g. in their behaviour, diet, antibiotic usage etc.) coupled with physiological differences (e.g. host genetic factors, gut characteristics, etc). A well-known example of the influence food ingestion may have is the prevalence of phylogenetic group A and B1 among omnivorous and herbivorous mammals, respectively (Carlos et al., 2010). Clermont et al. (2011) also concluded that gain (or loss) of few genes, e.g. adhesion-encoding genes, could contribute to the host specificity of non-B2 strains of different origin. Further studies of virulence factors which enable a phylogenetic group to colonize the gastrointestinal tract of different animal species are therefore needed to be evaluated.

Escherichia coli ST69, ST393, ST405 clones belonging to phylogenetic group D are increasingly reported as multidrug resistant strains causing extraintestinal infections (Novais et al., 2013). We found that the 10 VTEC strains studied belonged to the phylogenetic group D and significant differences on its association with hosts were also detected: only one VTEC isolate of phylogenetic groups D (2.2%) in sheep against 9 (31%) of broiler origin. Therefore, avian species appear to be a relevant reservoir of virulent phylogenetic group D. More expanded studies are needed to be undertaken in order to confirm this hypothesis.

In this study, we found that vtx_1 was the predominant gene over vtx_2 and vtx_1-vtx_2 in VTEC isolates in sheep. This distribution is in consistent with those previously described in two studies in sheep carried out in Spain (Blanco et al., 2003; Rey et al., 2003). However, in contrast with the results reported here, several studies have shown that most sheep VTEC carry vtx_1

and vtx_2 (Oporto et al., 2008; Vettorato et al., 2009). As results, vtx_2 was frequently found in broiler isolates. The same trend has been observed in chicken products in Argentina (Alonso et al., 2012). This is important because vtx_2-producing strains is more associated with severe disease in humans than vtx_1-producing strains (Paton and Paton, 2002).

Detection of *vtx* gene-carrying *E. coli* belonging to virulent phylogenetic groups (B2 and D), especially in broilers, represents a public health concern through fecal contamination of carcasses during slaughter operation at the processing facility. In Iran, studies have recently demonstrated that broiler and sheep carcasses could be considered as an important source of pathogenic *E. coli* (Bagheri et al., 2014; Tahamtan et al., 2010). On the other hand, high ratios of B2 and D isolates have been obtained from human clinical samples (Navidinia et al., 2013; Ramazanzadeh et al., 2013). However, further studies regarding phylogenetic background using other phylogenetic methods such as multilocus sequence typing (MLST), along with detection of serovars, *vtx* subtypes, and virulence genes are needed for predicting potential health hazards related to *E. coli* isolates from animals. In this regard, researches have pointed out the zoonotic potential of certain clonal groups such as avian pathogenic *E. coli* (APEC) O45:K1:H7-B2-ST95 (Mora et al., 2013) and O25b:K1:H4-B2-ST131 *ibeA* strains (Mora et al., 2010).

In conclusion, this study indicates that healthy sheep and broilers in Urmia region, Iran, could be considered as a source of VTEC strains. In addition, it demonstrates a different circulation of the *E. coli* phylogenetic groups in the analyzed host. Regarding the presence of *stx* gene-carrying *E. coli* belonging to virulent phylogenetic groups in fecal samples of healthy animals, sufficient discrimination among VTEC strains to assess their public health significance is therefore recommended.

Ethics Committee Approval: Ethics committee approval was received for this study from the Animal Ethics Committee of Faculty of Veterinary Medicine, Urmia University (AECVU) and supervised by authority of Urmia University Research Council (UURC).

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