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

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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 izolati (koyundan 97 ve broyler tavuklarından 349), polimeraz zincir reaksiyonu (PCR) kullanılarak Vtx-kodlayan genlerin (vtx1 ve vtx2) oluşmasını için test edilmiştir. Daha sonra, tüm geni kazanımlı VTEC izolatları, chuA, yjaA ve TSPE4.C2 olarak adlandırılan üç genetik sekans kullanılarak Clermont filotiplemeye dayanarak filogenetik olarak gruplandırılmıştır. Sonuç olarak, koyun kaynaklı izolatların %46,4’ünde (%45/97) vtx geni tanıyan E. coli saptanmış ve broyler için saptanan yüzdelere ise %8,3 (29/349) olmuştur. Genel olarak, filotipler, 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 koymus-tur. Buna ek olarak, sonuçlar ayrıca, koyun ve broyler tavuklarının VTEC izolatlarının yayılmasını B1 ve D filogenetik grupları arasında belirgin olarak farklı 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 suçlarının filogenetik grup atamasına göre önemli farklılıklar ortaya koymus-tur.

Anahtar kelimeler: Broyler tavukları, filogenetik gruplar, koyun, verotoksijenik Escherichia coli (VTEC)
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 (Faroqq 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 as 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 Urmi region, Iran (Saei and Ayremloou, 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) agar and incubated overnight at 37°C. These strains were then biochemically tested. 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 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 vtxF (5’-CAT TTA ATG TCG TGG CCA AGG-3’) and vtxR (5’-CAC CAG ACA ATG TAA CCG CTG-3’) were used for the amplification of vtx, which yielded a PCR product of 384 bp in size. The primer set vtxF (5’-ATC TTA ATG TCG TGG CCA AGG-3’) and vtxR (5’-GGC TCA TCG TAT ACA CAG GAG C-3’) were used for amplifying vtx, which allow the amplification of a DNA fragment at approximately 384 bp. E. coli ATCC43895 was used as positive control. Polymerase chain reaction and electrophoresis of products were described as performed previously (Saei and Ayremloou, 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 (A1, A2, B1, B2, C1, C2, D1, D2) of E. coli were determined using the BioNumerics software.
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 vtx1, 28 (37.8%) for vtx2, and 8 (10.8%) for both vtx1 and vtx2. Twenty-seven of sheep VTEC isolates were positive for vtx1, 26 contained vtx2, and 6 possessed both vtx1 and vtx2. The corresponding counts in broiler isolates were 11, 16, and 2, respectively.
The vtx genes in VTEC isolates from sheep and broiler chickens in relation to main phylogenetic groups are shown in Table 3.

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 |
| vtx1               | 8     | 17   | 1  | 1   | 4   | 1  | 3  | 3 |
| vtx2               | 3     | 8    | 1  | -   | 6   | 3  | 2  | 5 |
| vtxv, vtx2         | 2     | 4    | -  | -   | 1   | -  | -  | 1 |
| Total              | 13    | 29   | 2  | 1   | 11  | 4  | 5  | 9 |

* Verocytotoxin

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 broilers 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 B2v, 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 vtx1 was the predominant gene over vtx2, and vtxv-vtx2 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 vtx1.
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; Tahamant 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 *ibe*A 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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