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Molecular detection of nine clinically important non-O157 *Escherichia coli* serogroups from raw sheep meat in Chaharmahal-va-Bakhtiari province, Iran

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ABSTRACT

STEC isolates and also *stx*-negative *Escherichia coli* isolates from sheep meat from the Chaharmahal-va-Bakhtiari province, Iran were analyzed for nine clinically important non-O157 serotypes by PCR. A total of 90 *E. coli* isolates were tested. *Stx*-positive and *eae*-positive *E. coli* isolates did not belong to the nine most clinically relevant non-O157 STECs. Of the 80 non-STEC isolates, two belonged to the O103 and two belonged to the O128 groups. *Stx*-negative *E. coli* O103 and O128 strains isolated have potential in acquiring *stx* genes and continuing into the digestive system of consumers. Further studies are needed to analyze virulence characteristics of these *E. coli* strains to determine their potential role in causing disease in humans. For the sake of public health, it is important to monitor and investigate non-O157 diarrheagenic *E. coli* strains in meat in order to control and prevent them.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) can cause a range of clinical disease from relatively mild watery diarrhea to more serious complications of hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and can lead to the death of individuals with compromised immune systems (Gyles, 2007; Levine et al., 1987).

Ruminants are thought to be the main reservoir of STEC. Transmission of STEC can occur through consumption of contaminated foods, such as raw or undercooked meats, ingestion of contaminated water, or via direct contact with infected animals or people (Griffin & Tauxe, 1991; Gyles, 2007; Karmali, Gannon, & Sargeant, 2010; Shooter, Cooke, Rousseau, & Breaden, 1970).

Today, the detection of virulence-associated genes without the corresponding strain identification is considered a presumptive diagnosis, but is valid for the identification of carriage status (Etcheverría et al., 2010).

About 250 different O serogroups of *E. coli* have been shown to produce Shiga toxin, and over 100 of these have caused diseases in humans (Johnson, Thorpe, & Sears, 2006). Non-O157 STECs are responsible for the majority of STEC infections worldwide, representing a major public health concern (Johnson et al., 2006; Scallan et al., 2011). Although not all the STEC strains are pathogenic, some STECs can cause the most severe manifestations of STEC disease and can be as virulent as O157. The most common and dangerous non-O157 STECs are O26, O45, O91, O103, O111, O113, O121, O128, and O145 (Bettelheim, 2007; Brooks et al., 2001; Johnson et al., 2006).

It has been shown that patients infected with enterohemorrhagic *E. coli* (EHEC) carry both toxigenic and non-toxigenic variants. The significance of these findings should not be underrated because in the identification of EHEC infections only searching for *stx*-producing strains may give misleading or incorrect information and provide misleading answers to the epidemiological situation. In addition, *stx*-negative *E. coli* strains have potential in acquiring the *stx* gene at many stages along the farm to table food continuum and continuing into the digestive system of consumers, thus emerging as a potential source of new STEC strains. Livestock can harbor these non-toxigenic potential EHEC which acquire their *stx*-converting bacteriophages (Bettelheim, 2008; Beutin, 2006; Bielaszewska et al., 2007; Schmidt, Scheef, Huppertz, Frosch, & Karch, 1999; Wetzel & LeJeune, 2007; Zhang et al., 2000).

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Although prompt diagnosis of pathogenic *E. coli* contaminated food sources is important for effective prevention of foodborne illness, this may take several days to complete. Significant advantages of PCR assays over traditional serological tests for diagnosis of clinically important non-O157 serotypes are its speed, safety, high sensitivity and specificity (Lin et al., 2011). These advantages can result in a significant reduction in the time necessary for identification of clinically important non-O157 serotypes in contaminated food, reducing foodborne illness outbreaks.

Clinically important non-O157 serotypes are more common in ruminants and humans are potentially more exposed to these strains. As surveys in foods have rarely focused on the most clinically relevant *E. coli* serotypes, their occurrence is likely to be underrated (Auvray et al., 2007).

Although sheep meat consumption is higher than other red meats in Iran, few studies have been conducted to evaluate the occurrence of pathogenic *E. coli* in sheep meat. Most scientific papers in Iran are limited to the frequency of the O157 serotype in meat and meat products (Bonyadian, Zahraei Salehi, Momtaz, & Hasanpour, 2010; Kargar, Daneshvar, & Homayoon, 2011a; Kargar, Daneshvar, & Homayoun, 2011b; Rahimi, Kazemeini, & Salajegheh, 2012; Rahimi et al., 2012), and only recently 49 sheep meat STEC isolates obtained from four different locations were evaluated for detection of the most clinically relevant *E. coli* serotypes (Momtaz, Dehkordi, Ezadi, & Arab, 2013). To our knowledge, this study is the first to investigate and compare both non-STEC and STEC clinically important non-O157 serotypes in sheep meat in Iran.

2. Materials and methods

2.1. Lack of international standard method

Currently, there is no international standard method for the detection and isolation of the nine clinically important non-O157 serotypes, largely due to the broad range of phenotypic and genotypic characteristics that this group of pathogens displays. Although a number of enrichment protocols have been reported which allow for the isolation of some STEC serogroups, use of PCR as a prescreening tool in the protocols may be suboptimal as it may fail to detect O-positive samples that, despite their pathogenic potential, do not contain virulence genes. Different enrichment media were used for the isolation of *E. coli* O157 and non-O157 STEC, but Tryptone Soy Broth (TSB) is reported as the most frequently used and the most successful enrichment media (Vimont, Vernozy-Rozand, & Delignette-Muller, 2006). Many researchers have found that using selective agents (e.g. novobiocin) to improve the isolation of STEC serogroups, results in significantly reduced recovery of non-O157 STEC (Drysdale, MacRae, Strachan, Reid, & Ogden, 2004; Vimont, Delignette-Muller, & Vernozy-Rozand, 2007). USDA's Food Safety Inspection Service (FSIS) does not recommend using enrichment media containing supplements such as novobiocin for the detection of STEC (USDA-FSIS, 2013). Although previous studies recommended 37 °C (Doyle & Schoeni, 1984; Raghubeer & Matches, 1990) for the isolation of pathogenic serotypes, recent studies have reported an average optimal growth temperature around 41 °C (Gonthier, Gue'rin-Fauble'e, Tilly, & Delignette-Muller, 2001).

2.2. Sample collection, preparation and E. coli identification

Chaharmahal-va-Bakhtiari province lies in the southwestern part of the Iran. It has one of the highest levels of per capita consumption of red meat in the country.

Overall 270 raw sheep meat samples were collected. The samples were purchased from farm butchers from October 2010 to January 2011. The carcasses from which the meat samples were collected were clinically healthy and the meat samples showed normal physical characteristics. The meat was sampled by cutting meat slices (thickness 1–1.5 cm) from the external surfaces of three different locations (leg, flank and neck) of the post-washed carcass. The samples were transferred to the laboratory in sterile containers under aseptic conditions using an ice box.

Twenty five grams of each sample was placed in a Stomacher bag containing 225 ml of modified Tryptone Soy Broth (mTSB) (Merck, Germany). After stomacher homogenization for two min at normal speed, the samples were incubated for 24 h at 41 °C (Gonthier et al., 2001).

Fifty micro liter quantities of the suspension were plated on MacConkey agar (Merck) and Eosin–Methylene Blue (EMB) agar (Merck). The plates were incubated at 37 °C for 24 h for MacConkey agar and for 24 h for EMB agar. Colonies from each plate with typical *E. coli* morphology were selected and examined.

2.3. DNA isolation

Bacterial strains were grown overnight in Tryptone Soy agar (Merck) at 37 °C. Ten colonies were suspended in 100 μL of sterile distilled water. After boiling the suspension for 13 min; it was frozen and subsequently centrifuged at 32,800 ×*g* for 15 min to pellet the cell debris (Reischl et al., 2002). The supernatant was used as a template for amplification.

2.4. Polymerase chain reaction and electrophoresis

A total of 90 *E. coli* isolates, which were recovered from sheep meat were examined by PCR for nine clinically important non-O157 serotypes. Oligonucleotide primers and annealing temperatures used are listed in Table 1. The reaction mixture (25 μL) contained 0.5 μL of each primer at 10 mM (SinaClon Bioscience, Tehran, Iran), 2.5 μL of 10× PCR buffer, 1.5 μL of 50 mM MgCl2, 0.5 μL of 10 mM dNTP mix, 0.3 μL of 5 U/μL Taq DNA polymerase (SinaClon Bioscience, Tehran, Iran), and 1 μL of template DNA. Amplification reactions were carried out using a thermal cycler (Master Cycler Gradiant, Eppendurf, Germany). The PCR products were subjected to electrophoresis on 1.5% agarose gels.

2.5. Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed by the Kirby–Bauer disk diffusion method using Mueller–Hinton agar (Merck), according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2006). After incubating the inoculated plate aerobically at 37 °C for 18–24 h, the susceptibility of the *E. coli* isolates to each antimicrobial agent was measured and the results interpreted in accordance with the interpretive criteria provided by CLSI (2006).

2.6. Statistical analysis

The data were analyzed by using SPSS software (Version 17. SPSS Inc., USA).

3. Results

Although 10 STEC isolates, *stx*-positive and *eae*-positive *E. coli* isolates were identified they did not belong to the nine most clinically relevant non-O157 STECs. Of the 80 non-STEC isolates, two belonged to the O103 and two belonged to the O128 groups. These four non-STEC isolates were obtained in the autumn. All ten STEC isolates harbored only the *stx*1 gene and no isolate contained the *stx*2 gene. More details are shown in Table 2.

The antimicrobial profiles of the isolates showed extensive resistance to erythromycin and high sensitivity to ciprofloxacin, gentamicin, and nitrofurantoin (Fig. 1). None of the clinically important non-O157 isolates were resistant to ciprofloxacin and nitrofurantoin, but the resistance of stx-positive and eae-positive isolates to ciprofloxacin and nitrofurantoin was 5.9% and 17.6% respectively. All of the isolates were resistant to erythromycin. 58.8% of the stx-positive and eae-positive