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PREVALENCE AND SOME VIRULENCE GENES OF *ESCHERICHIA COLI* O157 ISOLATED FROM CHICKEN MEATS AND GIBLETS

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Abstract

***Escherichia coli* O157 related foodborne illnesses continue to be one of the most important global public health problems in the world. This study aims to determine *E. coli* O157 prevalence in 375 chicken meat parts and giblets. The samples were collected randomly from several supermarkets and butchers in Diyarbakir, a city in southeast Turkey. They were analyzed and confirmed using the immunomagnetic separation (IMS), Vitek® 2 microbial identification system and polymerase chain reaction (PCR) method. This study also aims to detect the presence of $fliC_{H7}$, *eaeA*, stx_1, stx_2 and *hlyA* genes by using PCR. The overall *E. coli* O157 prevalence in chicken meat parts and giblets was 1.3%. All of the *E. coli* O157 isolates carried $rfbE_{O157}$ and *eaeA* genes; but not any $fliC_{H7}$ and *hlyA* genes. The *E. coli* O157 isolates obtained from drumstick and breast meat carried either stx_1 or stx_2 genes, which were related to important virulence factors of the disease.**

Key words: *E. coli* O157, chicken meat, stx_1, stx_2, *eaeA*, *hlyA*

Verotoxin-producing *E. coli* (VTEC) or Shiga toxin–producing *E. coli* (STEC) can lead to sporadic cases and outbreaks that can cause several illnesses, such as hemolytic colitis (HC) and hemolytic uremic syndrome (HUS), following the onset of diarrhea. The strain of *E. coli* is also known as enterohemorrhagic *E. coli* (EHEC); and EHEC-related diseases are most frequently caused by the *E. coli* O157:H7 serotype. This bacterium can be transmitted to humans through contaminated food, water, direct contact with animals and human-to-human transmission, and can cause serious diseases (Chinen et al., 2009; Karmali et al., 2010; Baker et al., 2016). Shigatoxigenic *E. coli* serotype O157 has the highest prevalence in the world in recent years. For example, it has been reported that 6,658 of 13,524 STEC-sourced infections in Europe were caused by the O157 serotype of *E. coli* between 2007 and 2010 (Messens et al., 2015). In England and Wales, 335 outbreaks took place between 1983 and 2012, and 14,184 laboratory-confirmed STEC O157 cases were observed

between 1997 and 2012. Among 101 outbreaks identified between 1983 and 2012 (30.1%), 1,418 cases (corresponding to 45.9% of 3,107 cases) were reported to be related to contaminated foods (Adams et al., 2016). Between 2003 and 2012, 390 outbreaks of *E. coli* O157-caused diseases occurred in the United States, resulting in 4,928 cases, 1,272 hospitalizations and 33 deaths; 65% of these outbreaks were reported to be food-related (Heiman et al., 2015).

The pathogenicity of the *E. coli* O157:H7 is related to several virulence factors, such as intimin and hemolysin, which also contain Stx1 and Stx2 and are encoded by the stx_1 and stx_2 genes. These virulence factors can lead to HC and HUS. Intimin, which is encoded by the *eaeA* gene, plays a role in virulence factors responsible for the bacterium's adherence to the intestinal mucosa. A significant link has been identified between intimin and the occurrence of HC and HUS in humans (Paton and Paton, 1998). Hemolysin is encoded by the *hlyA* gene and lyses erythrocytes. When erythrocytes are lysed, the heme molecule (the iron source for growing bacteria) dissociates from the hemoglobin molecule (Nataro and Kaper, 1998; Sami and Roya, 2007). Several hemolysin groups have been defined in different pathological groups of *E. coli* species. The most common group is α-hemolysin, which is related to the *hlyA*, *hlyB*, *hlyC* and *hlyD* genes (Schmidt et al., 1995). The $rfbE_{O157}$ gene is used to distinguish the *E. coli* O157:H7 serotype from non-O157 serotype (Abong'o and Momba, 2008); and the presence of the $fliC_{H7}$ gene is used to determine flagellar antigen groups (Fields et al., 1997).

Chicken meat is the most common and leading product among meats produced by the poultry industry worldwide. Because it is a nutritious, widely consumed and inexpensive food, its production has increased gradually. However, microbial cross-contamination is inevitable during the process in which chicken meat reaches the consumer after production; and this increases the risks to public health. Although *E. coli* bacteria (particularly O157 serotype) are not common in the intestinal flora of poultry, they can be found in poultry, which shows that poultry meat may cause intestinal or extra-intestinal infections in humans (Allerberger, 2015; Manges, 2015). The aim of this study was to (i) detect the prevalence of *E. coli* O157 in chicken meat and giblets sold at retail level and (ii) investigate $fliC_{H7}$ and virulence-related genes in *E. coli* O157 strains isolated from the chicken samples along with the possible public health risks related to these genes.

Material and methods

Bacterial strains

In this study, *E. coli* O157:H7 ATCC 43894 and ATCC 43895 reference strains were used as positive control during microbiological and PCR analyses.

Study design and collection of chicken samples

In this study, 375 samples of chicken meat parts and giblets, including drumsticks (n = 75), breasts (n = 75), wings (n = 75), livers (n = 75) and gizzards (n = 75) were

collected randomly from 11 supermarkets and 15 butchers in Diyarbakir. All of the analyzed samples were collected from legally sold, unexpired products stored in refrigerated conditions. Then, the samples were brought to the laboratory in their original package and processed within two hours.

Pre-enrichment and immuno-magnetic separation

E. coli O157 was isolated as reported by Cagney et al. (2004). Twenty-five-gram samples were taken from meat parts (drumstick, breast and wing) and giblets (liver and gizzard) and then homogenized in 225 ml of sterile modified tryptone soy broth (mTSB) (Merck) with 20 mg/l Novobiocin in a stomacher bag for two minutes. Then, they were incubated at 41.5±0.5°C for 24 hours. After incubation, the immuno-magnetic separation (IMS) method was applied as recommended by the manufacturer (Dynabeads® anti–*E. coli* O157, ThermoFisher).

Isolation and confirmation of *E. coli* O157 and *E. coli* O157:H7

The 50 µl suspensions obtained from IMS were plated sorbitol MacConkey agar containing Cefixime (0.05mg/L) – Tellurite (2.5mg/L) (CT-SMAC) (LAB M,) and CHROM agar O157 (CHROM agar) and incubated at 37°C for 22–24 hours. Then the suspected colonies were transferred to tryptic soy agar with 6 g yeast extract (TSA-YE) and incubated at 37°C for 24 hours. The colonies were confirmed by using Vitek 2 identification Gram negative cards (bioMérieux). The isolates, which were identified as *E. coli* O157 or *E. coli* O157:H7 using O157 and H7 antisera, were subjected to latex agglutination test. The isolates obtained from this procedure were kept in the microbank tubes (Pro-Lab Diagnostic) at –80ºC until use for PCR.

DNA extraction

DNA was extracted from the *E. coli* O157 isolates using a GF-1 Bacterial DNA Extraction Kit (Vivantis), as recommended by the manufacturer.

Determination of $rfbE_{O157}$ and $fliC_{H7}$ genes by PCR

Each 25µl PCR reaction consisted of 2.5µl of 10X PCR buffer (Fermentas), 2.5µl of $MgCl_2$ (Fermentas), 2µl of deoxynucleoside triphosphate mix (dNTPs, Vivantis), 0.2µl of Taq DNA polymerase (Fermentas), 1.25µl of each $rfbE_{O157}$ or $fliC_{H7}$ primer (15 pmol/µl, Biomers) (Table 1), 5µl of DNA template and 7.8µl molecular grade water. PCR amplification conditions for $fliC_{H7}$ genes was performed with an initial denaturation of 94°C for two minutes, followed by 35 cycles, each consisting of 94°C for 20 s, 57°C for one minute and 72°C for one minute. The final extension cycle was performed for 10 minutes at 72°C (ABI Veriti Thermal Cycler) (Cagney et al., 2004). Amplification for $rbfE_{O157}$ was performed with initial denaturation of 94°C for five minutes, followed by 35 cycles of denaturation at 94°C for one minute, annealing at 53°C for one minute, extension at 72°C for one minute and a final extension cycle at 72°C for seven minutes (Maurer et al., 1999).

Table 1. Primer sequences and lengths of PCR amplification products

Target gene	Oligonucleotide sequence (5'–3')	Gene product	PCR amplicon (bp)	References
$rfbE_{O157}$	CGTGATGATGTTGAGTTG AGATTGGTTGGCATTACTG	LPS O157	420	Maurer et al. (1999)
$fliC_{H7}$	GCGCTGTCGAGTTCTATCGAGC CAACGGTGACTTATCGCCATTCC	H7	625	Fratamico and et al. (2000)
stx_1	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	Shigatoxin 1	180	Paton and Paton (1998)
stx_2	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	Shigatoxin 2	255	Paton and Paton (1998)
eaeA	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	Intimin	384	Paton and Paton (1998)
hlyA	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	Enterohemolysin	534	Paton and Paton (1998)

Multiplex PCR for the detection of some virulence factors

mPCR conditions were optimized using the previous recommendations with some modifications (Paton and Paton, 1998). Multiplex PCR was carried out with the primers described by Paton and Paton (1998) for *eaeA* (intimin), stx_1 (Shiga toxin 1), stx_2 (Shiga toxin 2) and *hlyA* (enterohemolysin) genes and listed in Table 1. The mPCR cycling conditions included an initial denaturation at 95°C for two minutes followed by 35 cycles, with one cycle consisting of one minute at 94°C, one minute at 62°C and one minute at 72°C with a final chain elongation at 72°C for seven minutes.

The amplified products in PCR were run in 1% agarose gel containing Safe-View™ (ABM, Canada), using a 100-volt for one hour. After running, the gel image was captured with the imaging system Quantum ST4 (Vilber Louma, Germany).

Statistical analysis

Statistical analysis was performed using the SPSS package version 11.5 (SPSS Chicago, IL) to carry out chi-square test and Fisher's exact test. $P<0.05$ was determined to be the threshold for statistical significance.

Results

In this study, *E. coli* O157 contamination was detected in five of the 375 chicken samples (Table 2) and *E. coli* O157:H7 was found in none of the samples. The Vitek 2 results of 20 isolates previously identified to have presumptive *E. coli* O157 using the IMS method showed that only five samples had *E. coli* O157, and the rest of the samples were found to have *E. coli*. The isolates identified to have *E. coli* O157 according to the Vitek 2 results were also found to be positive for the $rbfE_{O15}$ *gene.* Table 2 shows the distribution of the $fliC_{H7,}$ *eaeA*, *hlyA*, stx_1 and stx_2 virulence genes. None of the isolates were found to carry the $fliC_{H7}$ and *hlyA* genes.

Table 2. Prevalence of *E. coli* O157 isolated from chicken meat parts

Chicken sample	No (%) of positive samples with E. coli O157[x/y]	Presence of some virulence factors in the isolates					
		$rfbE_{O157}$	$fliC_{H7}$	*eaeA*	*hlyA*	stx_1	stx_2
Leg quarter	75/1 (1.3%)	1	-	1	-	1	-
Breast meat	75/1(1.3%)	1	-	1	-	-	1
Wing	75/2 (2.6%)	2	-	2	-	-	-
Liver	75/1 (1.3%)	1	-	1	-	-	-
Gizzard	75/- (0%)	-	-	-	-	-	-
Total	375/5 (1.3%)	5	-	5	-	1	1

x/y: Number of samples analyzed/number of positive samples.
–: Not detected.

Discussion

Although beef is accepted as the main reservoir for *E. coli* O157 (Sami and Roya, 2007; Chinen et al., 2009; Karmali et al., 2010), an increasing number of studies have shown that chicken meats and chicken meat products can also be the sources of this pathogen (Chinen et al., 2009; Alonso et al., 2012; Karadal et al., 2013; Momtaz and Jamshidi, 2013; Ahmed and Shimamoto, 2014; Akbar et al., 2014). In this study, *E. coli* O157 positivity was detected in 1.3% of the chicken meat and giblet samples. The difference in *E. coli* O157 prevalence was not statistically significant between drumstick, breast, wing, and liver samples ($P>0.05$). Studies of *E. coli* O157 prevalence in chicken meat parts have shown a prevalence of 9.3% in Slovakia (Pilipcinec et al., 1999), 0.4% in India (Wani et al., 2004) and 1.7% in the Netherlands (Schouten et al., 2005). In a study conducted in Turkey, only one broiler chicken liver sample was found to be contaminated with *E. coli* O157 among 1,000 samples (0.1%); four broiler chicken cecum samples were found to be contaminated with *E. coli* O157 among 1,000 samples (0.4%); and *E. coli* O157 contamination was not detected in any broiler chicken carcass samples (Kalin et al., 2012). In another study conducted in Turkey with processed chicken meat products, one sample was found to be *E. coli* O157 positive (1%) (Karadal et al., 2013). The differences among these study results may be related to the number and type of the sampling, the geographical region, seasonal and operational standards and the distribution of the *E. coli* O157 microorganism in the study regions.

The use of the IMS method to detect *E. coli* O157 in food, environmental and clinical samples increases the possibility to isolate this pathogen. However, the IMS method can cause false-positive results due to the competitive microorganism flora found in many food and feces samples (Fukushima and Seki, 2004; Ongor et al., 2007; Kim et al., 2014). In this study, Vitek 2 indicated five of the 20 suspicious *E. coli* O157 isolates to be *E. coli* O157 positive, and the rest of the samples to be contaminated with *E. coli*. In addition, each *E. coli* O157 positive isolate was confirmed by PCR to carry the $rfbE_{O157}$ gene. It is important to confirm the identification of the *E. coli* O157 using molecular methods or Vitek 2 microbial identification system in order to prevent false-positive results of the IMS method.

Stx1, Stx2, enterohemolysin and intimin, which are respectively encoded by *stx1*, *stx2*, *hlyA* and *eaeA* genes, are the virulence factors that cause *E. coli* O157-related infections in humans (Schmidt et al., 1995; Tarr et al., 2005). In this study, all *E. coli* O157 isolates were carrying the *eaeA* gene, but none of them were carrying the *hlyA* gene. Even if the pathogen only carries intimin, it can cause diarrhea in humans. A strong association has been reported between intimin and *E. coli* O157-related clinical cases (Paton and Paton, 1998). Shigatoxins are the key virulence factors for the development of STEC dependent diseases; and Stx2 is 1,000 times more cytotoxic than Stx1. Therefore, Stx2 has more significant effects on the occurrence of HUS in humans (Tarr et al., 2005; Mir et al., 2016). A study in Argentina determined the prevalence of STEC in chicken carcasses, giblets and chicken burgers to be 3.3%, 2.3% and 10.3%, respectively (Alonso et al., 2012). The same study also found that of the 79 isolates, 44 were carrying *stx2* and 14 were carrying both *stx1* and *stx2*. A study conducted in Iran reported that 31 (7.3%) of 422 chicken samples were contaminated with *E. coli* O157:H7, most of which were positive for the *stx1* gene (Momtaz and Jamshidi, 2013). A study in Egypt examined 160 chicken breast samples and 160 chicken drumstick samples for *E. coli* O157:H7 using culture and PCR methods. The researchers reported that four drumstick samples (2.5%) were contaminated with *E. coli* O157; of them, one was *stx1* positive, two were *stx2* positive and one was both *stx1* and *stx2* positive (Ahmed and Shimamoto, 2014). A study conducted in Turkey found that one of 100 (1%) processed chicken samples was contaminated with *E. coli* O157, and that the isolate obtained from this sample was carrying the *stx1* gene (Karadal et al., 2013). In the present study, the isolates obtained from one drumstick sample were carrying the *stx1* gene, obtained from one breast sample were carrying the *stx2* gene, and none of the *E. coli* O157 isolates were found to carry both *stx1* and *stx2*. In addition, the $fliC_{H7}$ gene was not detected in any of the isolates found to be *E. coli* O157 positive. Various hygienic conditions at the stages of slaughtering, processing, cutting and selling poultry may be the reason for the presence of *E. coli* O157 and the difference in detected genes among the samples.

In Turkey, STEC infections have taken their place among the "infectious diseases which need to be notified" in the notification system for communicable diseases, group D. Some studies have reported that some O157 and non-O157 strains of *E. coli* were isolated from tourists who visit Turkey; however, a limited number of notifications have also been reported from Turkey (Hascelik et al., 1991; Eurosurveillance, 1999; Smith-Palmer et al., 2005; Erdogan et al., 2011). In a recent study of Gulesen et al. (2016), this pathogen was detected in 28 (7.1%) diarrhea-related bloody fecal samples of 395 patients in Turkey who were suspected of having STEC. The same study also reported that of the samples, 18 were carrying the *stx2*, six were carrying the *stx1* and four were carrying both the *stx1* and *stx2*. However, the study gave no information about whether the bloody diarrhea was caused by foods or not. In another study conducted in Turkey, feces samples were collected from 367 patients with diarrhea in five different hospitals. Ten (2.7%) of these samples were found to be *E. coli* O157-positive, although none of the isolates were carrying the $fliC_{H7}$, stx_1, stx_2 or *hlyA* genes. In addition, the pulsed-field gel electrophoresis (PFGE) found no clonal relationship between the 10 samples of O157 obtained from human feces

samples and the five isolates of *E. coli* O157 obtained from chicken liver and cecum samples (Kalin et al., 2012). The above mentioned studies have indicated that it is hard to find a direct relation between STEC-related diseases and chicken meat samples in Turkey.

The present study showed that the *E. coli* O157 pathogen can exist in chicken drumstick, wing and liver samples, even though its prevalence was quite low. In these analyzed samples, the presence of several genes associated with HC and HUS onset especially in humans, indicates that chicken products have potential risks to public health. Equipment and staff involved in slaughtering, processing, cutting up and selling these poultry products may be the source of this contamination. It is possible to prevent foodborne diseases due to *E. coli* O157 by applying appropriate hygiene and sanitation methods in each step of chicken meat production and selling process.

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