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ORIGINAL ARTICLE

Molecular depiction and Antimicrobial Resistance Pattern of *Escherichia coli* O157:H7 isolated from different stages of Slaughter in Chicken abattoir

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ABSTRACT
From clinical and epidemiological perspectives, it is important to know the distribution and antibiotic resistance pattern of Escherichia coli O157:H7 in various stages of chicken slaughter in abattoir. Two-hundred and forty swab samples were taken from the external parts of chickens. Samples were cultured and those that were E. coli, were analyzed for presence of stx1, stx2, eaeA and ehly genes. Antimicrobial susceptibility was performed using disk diffusion method. Of 240 swab samples, 90 (37.5%) were positive for E. coli O157. Of 90 E. coli O157 isolates, 31 samples was serotype O157:H7 and 59 samples were O157:NM. The most commonly contaminated stages were after offal discharge. The distribution of stx1, stx2, eaeA and ehly virulence factors were 78.88%, 42.22%, 23.33% and 16.66%, respectively. Bacterial strains exhibited the highest levels of resistance to tetracycline (95.55%), gentamicin (94.44%), ampicillin (92.22%), enrofloxacin (87.77%), sulfamethoxazole (82.22%) and trimethoprim (76.66%). The presence of O157:H7 in chicken carcasses of various stages of slaughter in abattoir, emphasizes the importance of implementing the Hazard Analysis and Critical Control Point (HACCP) system, as well as the need for implementing, evaluating, and validating antimicrobial interventions to reduce the presence of potential pathogenic microorganisms.
Key words:Abattoir, Antimicrobial, Distribution, Escherichia coli, Resistance, Shiga toxigenic.

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INTRODUCTION

More than 100 serotypes of *E. coli* produce Shiga or Shiga-like toxins. The most commonly identified Shiga toxin-producing *E. coli* (STEC) all around the world is the *E. coli* O157:H7 (1). Infection with the O157:H7 serotype can result in a spectrum of outcomes, ranging from asymptomatic carriage to uncomplicated diarrhea, hemolytic uremic syndrome (HUS), Hemorrhagic Colitis (HC), bloody diarrhea, hemolytic anemia, thrombocytopenia, and acute renal failure [1]. Persons of all ages are susceptible. Very young children and the elderly are more likely to develop severe illness and HUS. In the U.S., it is estimated that 70,000 infections per year are caused by the *E. coli* O157:H7 [2]. The ability of bacterium in production of various shiga toxins (Stxs), the intimin (*eae*) protein and the plasmid-encoded protein known as hemolysin (*ehly*) often cited as a cause of mentioned diseases [1].

Meat, milk, unpasteurized dairy products, ground beef, apple cider, egg, chicken meat and foods with animal origin have been associated with severe outbreaks of *E. coli* O157:H7 [3]. Domestic and wild animals and also poultries are sources of the O157:H7 serotype [4]. Contamination of meat with fecal materials in the slaughtering process is the main transmission route of *E. coli* O157:H7 [5].

Diseases caused by *E. coli* often require antimicrobial therapy; however, antibiotic-resistant strains of this bacterium cause longer and more severe illnesses than their antibiotic-susceptible counterparts. Several studies have shown that antibiotic resistance in *E. coli* strains has increased over time [6,7]. In keeping with this, an epidemiological investigation in Iran revealed that the O157:H7 serotype of *E. coli* was the most commonly detected strains in foods with animal origins and that there was a high incidence of resistance (30-80%) to commonly used antibiotics [1,8].

Data about the prevalence of *E. coli* O157:H7 in various stages of chicken slaughter in abattoir are rare. Therefore, the present study was conducted to determine the prevalence of *E. coli* O157:H7/NM in various stages of chicken slaughter in abattoir and investigate the distribution of *stx1*, *stx2*, *eaeA* and *ehly* virulence genes and pattern of antibiotic resistance.

MATERIALS AND METHODS

Samples

From December 2013 to March 2014, a total of 240 swabs were collected from various stages of chicken slaughter in abattoir including after filling (n=60), after offal discharge (n=60), after chilling (n=44), after cooling (n=34) and before packaging (n=42). All samples were collected from the abattoir in various parts of Iran. Swab samples were taken from the surface of chickens. Swab samples were located on the sterile tubes contain diluted *Normal Saline (Merck, Germany).* All samples were immediately transported to the laboratory in cooled boxes. All techniques were applied to prevention of cross contamination within and between townships.

Bacterial isolation

The protocol suggested by Rahimi et al.. [3] was followed in order to study the presence of *E. coli* O157:H7 in collected samples.The microbiological examination was started within 6 h of sample collection. All samples were added to the nutrient broth (Merck, Germany) and the cultures incubated. Cultures were streaked onto MacConkey sorbitol agar (Merck, Germany) and the plates incubated overnight at 37°C. From each plate (one plate for each sample), 5 to 10 suspected *E. coli* colonies (sorbitol negative and positive) were selected and sub-cultured onto presumptive diagnostic medium and incubated overnight at 37°C. All sorbitol negative colonies were tested for the O157 antigen by latex agglutination (Oxoid) [9] and up to five agglutination positive colonies were taken for PCR analysis.

DNA extraction and PCR amplification

Bacterial strains were grown overnight in Luria-Bertani broth (Merck, Germany) at 37°C. Genomic DNA was extracted based on the instruction of the commercial genomic DNA extraction kit (Fermentase, Germany). The extracted DNA was quantified by spectrophotometric measurement at a wavelength of 260 nm according to the method described in previous investigation (10).

The protocol suggested by Rahimi et al. [3] was followed in order to study the distribution of various virulence factors and putative genes. All oligonucleotide primers were obtained from a commercial source (Cinna Gen, Iran). The H7 (*fliCh7*) gene of *E. coli* O157:H7 was detected based on the PCR technique [11]. List of primers used for detection of *fliCh7* gene is shown in Table 1 [12]. The PCR reaction was carried out in a final volume of 50 µl containing 1×Reaction Buffer (Fermentas, GmbH, Germany), $MgCl_2$ (Fermentas, GmbH, Germany), each of the four deoxynucleoside triphosphates (dNTPs) (Fermentas, GmbH, Germany), Taq DNA polymerase (Fermentas, GmbH, Germany), 0.50 µM of primers and 10 µl DNA. DNA amplification reactions were carried out using a DNA thermal cycler (Master Cycler Gradiant, Eppendr of, Germany) with the following program: one cycle of 2 min at 94°C, 35 cycles of denaturation at 94°C for 20 s, annealing at 54°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min.

Study the distribution of virulence factors

Presence of *stx1*, *stx2*, *eaeA* and *ehlyA* genes in the *E. coli* O157:H7/NM isolates were studied using the PCR method [11,12]. List of primers used for detection of *stx1*, *stx2*, *eaeA* and *ehlyA* virulence genes are shown in Table 1 [12]. The protocol of Rahimi et al. [6] has also been used. The PCR reaction was carried out in a final volume of 50 µL containing incomplete 1× Reaction Buffer [160 mM (NH4) 2SO4, 670 mMTris-HCl (pH 8.8); 0.1% Tween-20] (Fermentas, GmbH, Germany), 3.0 mM MgCl2 (Fermentas, GmbH, Germany), 400 µM each of the four deoxynucleoside triphosphates (dNTPs) (Fermentas, GmbH, Germany), 2.5 U Taq DNA polymerase (Fermentas, GmbH, Germany), 0.50 µM of all primers that were used. Then, 10µL of DNA was added to reaction mixture.

Gel electrophoresis

All the PCR products were electrophoreses on 1.5% agarose gels that were strained with ethidium bromide and examined under ultraviolet illumination.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of the *E. coli* O157:H7 isolates was performed using the Kirby–Bauer disc diffusion method and Mueller–Hinton agar (Merck, Germany) according to Clinical and Laboratory Standards Institute (CLSI) instructions described in the recent study (2). Inoculated plates were incubated aerobically at 37°C for 18–24 h, after which antimicrobial susceptibility in the *E. coli* isolates were tested. Tetracycline (30 µg/disk), ampicillin (10 u/disk), penicillin (10 u/disk), sulfamethoxazole (25 µg/disk), streptomycin (10 µg/disk), sulfonamide (100 µg/disk), chloramphenicol (30 µg/disk), gentamicin (10 µg/disk), trimethoprim (5 µg/disk), ciprofloxacin (5 µg/disk), enrofloxacin (5 µg/disk),

cephalothin (30 µg/disk), and nitrofurantoin (300 µg/disk) were tested. The results were interpreted in accordance with CLSI criteria described in the recent study (2). *E. coli*ATCC 25922 was used as quality control for antimicrobial susceptibility determination.

Statistical analysis

Differences in the prevalence of *E. coli* O157:H7in various stages of the slaughter in chicken abattoir were analyzed using a chi-square test in SPSS for Windows (release 18.0 standard version, SPSS Inc., Chicago, Illinois). The differences were considered statistically significant when $p < 0.05$.

RESULTS

All of the swab samples collected from various stages of chicken slaughter were examined using culture and PCR techniques. From 240 swab samples, 90 (37.5%) were positive for *E. coli* O157 (Table 2). The swab samples of after offal discharge stage were the most commonly contaminated (75%). Statistical analysis showed significant differences ($p < 0.05$) for the prevalence of *E. coli* O157:H7 between after offal discharge and before packaging stages. Out of 90 *E. coli* O157 isolates, 31 samples was serotype O157:H7 and 59 samples were O157:NM.

The distribution of *stx1*, *stx2*, *eaeA* and *ehly* virulence factors in *E. coli* O157 strains isolated from various stages of chicken slaughter in abattoir were 78.88%, 42.22%, 23.33% and 16.66%, respectively (Table 2). Significant differences ($p < 0.05$) were also seen between the distribution of *stx1* and other virulence factors.

Antimicrobial resistance pattern in the *E. coli* O157 strains isolated from the various stages of chicken slaughter in abattoir is shown in Table 3. *E. coli* O157 strains exhibited the highest level of resistance to tetracycline (95.55%), gentamicin (94.44%), ampicillin (92.22%), enrofloxacin (87.77%), sulfamethoxazole (82.22%) and trimethoprim (76.66%). There were statistically significant differences ($p < 0.05$) amongst the incidences of resistance to tetracycline and gentamicin with streptomycin, chloramphenicol, cephalothin and nitrofurantoin antibiotic agents. There were statistically significant differences ($p < 0.05$) amongst the incidences of antibiotic resistance and virulence factors in the *E. coli* O157 strains of various stages of chicken slaughter. Our work has identified high presence of *E. coli* O157:H7, STEC virulence factors and antibiotic resistance pattern against commonly used antibiotics in various stages of chicken slaughter in abattoir.

DISCUSSION

Our results showed that the most commonly contaminated stages in chicken slaughter was after offal discharge, followed by after filling and after cooling stages. Alonso et al. (13) reported that *E. coli* strains were detected in 6 to 28% of cloacal samples, 39 to 56% of unwashed eviscerated carcasses, and 4 to 58% of washed carcasses. Svobodová et al. (14) showed that *E. coli* counts dropped with each subsequent processing step just as Total Viable Counts (TVC) values. They reported that the mean values after plucking and after evisceration were 3.5 log ± 0.7 and 3.1 log ± 0.7, respectively; evisceration had no effect on *E. coli* counts ($p<0.317$) and a marked drop occurred after washing, where mean *E. coli* counts were 2.7 log ± 0.6 and then after chilling where they were around 1.8 log ± 0.8 ($p<0.001$). The results of Pannuch et al. [15] exhibited the high atypical *E. coli* contamination rate as 6 to 28% in cloacal swabs, 39 to 56% in unwashed eviscerated carcasses, and 4 to 58% in washed carcasses, In addition, Farooq et al. [16] investigated 212 fecal samples collected from chickens, ducks and pigeons. They demonstrated that 33 pathogenic *E. coli* strains (15.56%) were classified as atypical strains. Thus, avian species are evidenced to play a role as an important *E. coli* reservoir. Close contact of surface area of chickens with the contents of the intestinal tract in the offal discharge stage or their contact with the infected attachments like feathers are the main factors for the higher rates of chicken contamination in these stages. Therefore, our results showed that evisceration had strong effect on surface contamination and it is in contrast with the results of Svobodová et al. [14].

A number of studies have been carried out to determine the prevalence of *E. coli* O157:H7 in poultry. Rahimi et al. (3) showed that fourteen (4.7%) of 295 samples were positive for *E. coli* O157:H7 and the highest prevalence of bacterium was found in beef meat samples (8.2%), followed by water buffalo (5.3%), sheep (4.8%), camel (2.0%), and goat (1.7%). They reported that as the potential of contamination with *E. coli* O157:H7 can be considerable in slaughterhouses, the maintenance of slaughter hygiene and regular microbiological monitoring of carcasses are essential tools in minimizing the risk of direct and cross-contamination. In another study in Czech from a total of 987 samples, 22 strains (2.2%) were identified as *E. coli* O157 and only nine poultry meat samples were positive for all *stx1, stx2, eaeA*, and *ehxA* virulence genes [17]. Vernozy-Rozand et al.. [18] Analyzed 250 samples of meat and meat products in France, and found 4 chicken meat samples (1.6%) to be positive for non verotoxin producing-*E. coli* O157. The results of our investigation, in which 37.5% (90 positive out of 240 samples)

contamination rate of *E. coli* O157:H7 was found, generally agrees with the results obtained in previous studies (18). Akkaya et al. (1) reported that *E. coli* O157:H7 was isolated from 2 of the 190 samples (1.05%) collected from chickens in abattoir and both isolates were found to be capable of synthesizing toxins. Similar results have been reported by Momtaz and Jamshidi [1] while the results of Lee et al. [19] are in contrast with our investigation.

stx1, *stx2*, *eaeA* and *ehly* were detected in 78.88%, 42.22%, 23.33% and 16.66% of *E. coli* strains isolated from various stages of chicken slaughter, respectively. Results showed that *E. coli* O157:H7 strains of after offal discharge and after filling stages had the highest incidence of STEC virulence factors. BinduKiranmayi and Krishnaiah [20] reported that of the 27 samples positive for *E. coli* O157:H7, 12 (44.4%) were positive for *stx1*, 7 (26%) were positive for *stx2* and 5 (18.5%) were positive for both *stx1* and *stx2* which was similar to our results. Dipineto et al. (2006) [21] showed that the *E. coli* O157:H7 was detected in 26 of the 720 cloacal swabs of living layer hen samples (3.61%) and all of them were positive for *stx1*, *stx2*, *eaeA* and *ehly* genes. In previous study which was conducted in Maryland, among the 7,258 *E. coli* isolates recovered from retail meats collected in four states from 2002 to 2007, 17 (0.23%) tested positive for the presence of a *stx* genes and were considered STEC [22].

Due to the high indiscriminate use of antibiotics especially in veterinary medicine, antibiotic resistance against most effective antibiotics has been occurred. Several programs like the Danish Integrated Antimicrobial Resistance Monitoring and Research Programmed (DANMAP) on Denmark and the Japanese Veterinary Antimicrobial Resistance Monitoring (JVARM) on Japan (23) have been described the resistance to various antibiotic drugs in veterinary medicine. In the present study, the levels of resistance to some antimicrobial agents (tetracycline, gentamicin, ampicillin, enrofloxacin, sulfamethoxazole and trimethoprim) were high (70-95%). Totally, 33.33% and 22.22% of *E. coli* O157:H7 strains of our investigation were resistance to chloramphenicole and nitrofurantoin, respectively.

Chloramphenicole and nitrofurantoin are two forbidden antibiotics. The high presences of resistance to these two antibiotics showed the irregular and unauthorized use of these drugs in veterinary treatment in Iran. Veterinarians in many fields of veterinary such as large animal internal medicine, poultry and even aquaculture, use these antibiotics as a basic one. Therefore, In a very short period of time, antibiotic resistance will appear. In the same study in Thailand, the *E. coli* isolates have100% resistance to tetracycline, ampicillin and erythromycin while resistance to cephalothin and sulfonamide+trimethoprim was 73.3% and 26.7%, respectively (24). Another similar investigation showed 82.4% resistance of *E. coli* isolated from broiler chickens to tetracycline (25). Li et al. (2011) (26) showed that 4 out of 70 (5.7%) minced beef meat, 2 out of 50 (4.0%) minced mutton, 1 out of 50 (2.0%) pork meat, 2 out of 50 (4.0%) minced chicken meat, and 1 out of 50 (2.0%) raw chicken meat were positive for *E. coli* STEC strains and from these positive samples, twenty (100%) were resistant to sulfamethoxazole-trimethoprim, 16 (80.0%) to ciprofloxacin, 12 (60.0%) to tetracycline, 9 (45.0%) to ampicillin, followed by 6 (30.0%) to gentamycin, 6 (30.0%) to streptomycin, 4 (20.0%) to chloramphenicol, and 3 (15.0%) to cephalothin.

Prescription of tetracycline is not recommended in cases with *E. coli* infections. To our knowledge, ciprofloxacin and enrofloxacin are the most common antibiotics for *E. coli* infections, but our results showed 57.77% and 87.77% resistance to these drugs, respectively, and it is similar to previous studies (82% resistance to ciprofloxacin) (27). Another study showed that in some countries *c*. 300 000 kg of antibiotics are used yearly on veterinary prescription in animals, of which 10% is used in poultry (28). It seems that the high and irregular prescriptions of antibiotics are the major reasons for emergence of antibiotic resistance. Study in Kenya indicated that tetracycline (75.9%) and cotrimoxazole (72.4%) had the highest antibiotic resistance in *E. coli* isolated from broiler slaughtered chicken (29). Safarpoor Dehkordi et al. (30) reported that the prevalence of antibiotic resistance in STEC strains of food products against tetracycline, penicillin, ampicillin and streptomycin were 84%, 46%, 38% and 36%, respectively.

These differences in the levels of antibiotic resistance in various countries showed that the resistance properties of STEC strains are closely depend on geographic regions. In the other hand, the antibiotic resistances in STEC sero-groups isolated from chicken meat samples are closely depend on antibiotic prescriptions. Prescription of antibiotics in various countries is related to common and conventional antibiotics which are available.

The reason for the presence of *E. coli* O157:H7 on chicken carcasses, may result from cross-contamination during slaughter, and/or processing or during transportation. Cross contamination of the carcasses at the chicken abattoir can be at various stages such as, during evisceration, scalding, plucking, and/or cutting processes. In addition, the presence of *E. coli* O157:H7 on chicken carcasses suggests that chickens may be natural carriers of the microorganism, or contamination may be coming from other sources such as transportations, and/or water used during slaughtering for various purposes. The high proportion of contaminated carcasses during chicken packaging and processing represents a risk for the consumers and a challenge to improve procedures for those working in the sanitary control service.The above data

highlight large differences in the prevalence of *E. coli* O157:H7in the different studies, as well as differences in virulence genes and antibiotic resistance properties in the food samples. This could be related to differences in the type of sample (meat, milk, vegetable, beef, poultry and …) tested, number of samples, method of sampling, experimental methodology, geographical area, antibiotic prescription preference among clinicians, antibiotic availability, and climate differences in the areas where the samples were collected, which would have differed between each study.

Table 1.Oligonucleotide primers used for detection of *Escherichia coli* O157:H7 and putative virulence factors based on the study of Rahimi et al. (2012) (6).

Genes	Primer sequences (5'–3')	Fragment size (bp)
stx1	F-TGTAACTGGAAAGGTGGAGTATACA R-GCTATTCTGAGTCAACGAAAAATAAC	210
stx2	F-GTTTTTCTTCGGTATCCTATTCC R-GATGCATCTCTGGTCATTGTATTAC	482
ehly	F-ATTACCATCCACACAGACGGT R-ACAGCGTGGTTGGATCAACCT	397
eaeA	F-ACGATGTGGTTTATTCTGGA R-CTTCACGTCACCATACATAT	166
fliCh7	F-GCGCTGTCGAGTTCTATCGAGC R-CAACGGTGACTTTATCGCCATTCC	625

Table 2. Distribution of *Escherichia coli* O157:H7 and STEC virulence factors in various stages of chicken slaughter in abattoir.

Types of samples	No. of examined samples	*E. coli* O157 (%)	Distribution of virulence factors (%)			
			Stx1	*Stx2*	*eaeA*	*ehly*
After filling	60	20 (33.33)	15	10	7	4
After offal discharge	60	45 (75)	39	20	11	8
After chilling	44	10 (22.72)	7	3	1	1
After cooling	34	9 (26.47)	6	3	1	1
Before packaging	42	6 (14.28)	4	2	1	1
Total	240	90 (37.5)	71 (78.88)	38 (42.22)	21 (23.33)	15 (16.66)

Table 3.Antimicrobial resistance pattern in *Escherichia coli* O157:H7 isolated from various stages of chicken slaughter in abattoir.

Types of samples	*E. coli* O157:H7	Pattern of antibiotic resistance (%)											
		TE30*	**S10**	**C30**	**SXT**	**GM10**	**NFX5**	**CF30**	**CIP5**	**TMP5**	**F/M300**	**AM10**	**P10**
After filling	20	20	8	6	17	20	18	6	13	16	4	19	15
After offal discharge	45	44	18	17	39	43	41	16	30	37	13	44	34
After chilling	10	9	2	3	7	9	8	2	4	6	1	9	6
After cooling	9	7	3	3	7	8	7	3	3	6	1	7	6
Before packaging	6	6	2	1	4	5	5	1	2	4	1	4	3
Total	90	86 (95.55)	33 (36.66)	30 (33.33)	74 (82.22)	85 (94.44)	79 (87.77)	28 (31.11)	52 (57.77)	69 (76.66)	20 (22.22)	83 (92.22)	64 (71.11)

*TE30= tetracycline (30 µg/disk); S10= streptomycin (10 µg/disk); C30= chloramphenicol (30 µg/disk); SXT= sulfamethoxazole (25 µg/disk); GM10= gentamicin (10 µg/disk); NFX5= enrofloxacin (5 µg/disk); CF30= cephalothin (30 µg/disk); CIP5= ciprofloxacin (5 µg/disk); TMP5= trimethoprim (5 µg/disk); F/M300= nitrofurantoin (300 µg/disk); AM10= ampicillin (10 u/disk); P10= penicillin (10 u/disk).

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