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The Philippine Journal of Veterinary Medicine

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ORIGINAL ARTICLE

VIRULENCE FACTOR PROFILE AND ANTIBIOTIC RESISTANCE OF *Escherichia coli* O157 STRAINS ISOLATED FROM ANIMAL RAW MEAT

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ABSTRACT

The current research was done to study the prevalence of antibiotic resistance and distribution of virulence factors amongst the *Escherichia coli* O157 strains isolated from 180 animal raw meat samples (60 chicken, 30 camel, 40 bovine, 30 turkey, and 20 ovine). *E. coli* O157 strains harbored the highest prevalence of resistance against tetracycline (94.4%), trimethoprim (61.1%), co-trimoxazole (55.5%), and gentamicin (55.5%) antibiotics. The most commonly detected virulence factors amongst the *E. coli* O157 isolates were *fimH* (88.8%), *papC* (61.6%), *cnf1* (61.6%), *afa/draBC* (61.6%), *papGII* (55.5%), *csgA* (55.5%), and *cvaC* (55.5%). Findings exhibited that all bovine, ovine, caprine, and camel meat samples can be reservoirs of the virulent and antibiotic resistant *E. coli* O157 strains. Simultaneous presence of some virulence factors together were found in several *E. coli* O157 strains which showed their high pathogenicity. Retail ruminant meat may be reservoirs of virulent and resistant *E. coli* O157 strains.

Keywords*: antibiotic resistance pattern, Escherichia coli O157, raw meat, virulence factors*

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INTRODUCTION

Escherichia coli (*E. coli*) is a bacterial species found in the environment, foods, and intestines of people and animals. *E. coli* are a large and diverse group of bacteria. Although most strains of *E. coli* are harmless, others can give rise to disease. Some kinds of *E. coli* can cause diarrhea, while others urinary tract infections, respiratory illness and pneumonia, and other illnesses (Manning *et al.,* 2008).

Strains of *E. coli* that express Shiga and Shiga-like toxins gained this ability due to infection with a prophage containing the structural coding for the toxin. Nonproducing strains may become infected and produce Shiga-like toxins after incubation with Shiga toxin positive strains. The prophage responsible appears to have infected the strain's ancestors fairly recently as viral particles have been observed to replicate in the host if it is stressed in some way (e.g. antibiotics) (O'Brien *et al.,* 1984; Strockbine *et al.,* 1986).

Escherichia coli O157 is a serotype of the bacterial species *Escherichia coli* and is one of the Shiga toxin–producing types of *E. coli*. It is a cause of disease, typically foodborne illness, through consumption of contaminated and raw food, including raw milk and undercooked ground beef (Gally and Stevens, 2017; Karch et al., 2005).

Symptoms of the diseases brought about by *E. coli O157* include abdominal cramps and diarrhea that may in some cases progress to bloody diarrhea (haemorrhagic colitis). Fever and vomiting may also occur. Most patients recover within 10 days but in a small proportion of patients (particularly young children and the elderly), the infection may lead to a life-threatening disease such as haemolytic uraemic syndrome (HUS). HUS is characterized by acute renal failure, haemolytic anaemia, and thrombocytopenia (low blood platelets) (Kaper *et al.,* 2004).

It is estimated that up to 10% of patients with *E. coli O157* infection may develop HUS with a case-fatality rate ranging from three to five percent. Overall, HUS is the most common cause of acute renal failure in young children. It can cause neurological complications (such as seizure, stroke, and coma) in 25% of HUS patients and chronic renal sequelae, usually mild, in around

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50% of survivors (Paiba *et al.,* 2002).

The reservoir of *E. coli* O157 appears to be mainly cattle. In addition, other ruminants such as sheep, goats, and deer are considered significant reservoirs while other mammals (such as pigs, horses, rabbits, dogs, and cats) and birds (such as chickens and turkeys) have been found infected (Goncuoglu *et al.,* 2010).

E. coli O157 strains also possess multiple resistance against different types of antibiotics and high prevalence of certain virulence factors. In this study, molecular characterization of antibiotic resistance and virulence factors of the *E. coli* O157 isolated from raw meat samples were investigated.

MATERIALS AND METHODS

Bacterial strains

In this cross-sectional study, there are a total of 18 *E. coli* O157 isolates. Bacteria were isolated from different types of retail meat samples including bovine (n= 40), ovine (n= 20), camel (n= 30), chicken (n= 60), and turkey (n= 30) meat samples. Samples were randomly collected from different parts of the Isfahan province, Iran. A total of 20 grams of femur muscle were collected from each animal. *E. coli* isolates were approved using the culture and biochemical tests.

Meat samples were homogenized in 3 mL of sterile phosphate-buffered saline (PBS) (pH 7.2) by using a mortar and pestle. Three milliliters of each homogenized sample was blended with 225 mL of nutrient broth (Merck, Germany) for two minutes at normal speed using a Stomacher lab blender and incubated at 37 °C for 24 hours. A one milliliter sample of the nutrient broth culture was mixed with 9 mL of MacConkey broth (Merck, Germany) and further incubated at 37 °C for 24 hours. One loop of each tube was streaked on MacConkey agar (Merck, Germany). One colony from each plate with typical *E. coli* morphology was selected and examined by biochemical tests, including hydrogen sulfide, citrate, urease, and indole (Momtaz *et al.,* 2013).

Identification of *E. coli* O157 isolates were done using the PCR method. Genomic DNA was extracted from bacterial colonies using *DNA extraction kit* (*DNP™,* SinaClonBioScience, Tehran, Iran) according to the manufacturer's instructions. Two primer pairs were used for PCR amplification (Possé *et al.,* 2007): O17F: 5'- CGG ACA TCC ATG TGA TAT GG-3' and O157R: 5'-TTG CCT ATG TAC AGC TAA TCC-3' and fliCH7 F: 5'- GCGCTGTCGAGTTCTATCGAGC and fliCH7 R: 5'- AACGGTGACTTTATCGCCATTCC (SinaClonBioScience, Tehran, Iran).

Antibiotic susceptibility testing

Antimicrobial susceptibility profiles were performed by disc diffusion method in Mueller-Hinton agar with 18 different antibiotics. Selected disks for the study were: azithromycin (15 µg), trimethoprim (25 µg), rifampin (5 µg), meropenem (10 µg), nitrofurantoin (300 µg), chloramphenicol (30 µg), amikacin (30 µg), imipenem (30 µg), levofloxacin (5 µg), ciprofloxacin (5 µg), trimethoprim (5 µg), erythromycin (15 µg), tetracycline (30 µg), ceftazidime (30 µg), cephalotin (30 µg), tobramycin (10 µg), amikacin (30 µg), gentamicin (10 µg), and streptomycin (10 µg) (produced by PadTan-Teb, Iran), according to the instruction of Clinical and Laboratory Standards Institute (CLSI, 2017). *E .coli* ATCC 25922 was used as quality control organism in antimicrobial susceptibility determination. Antibiotic discs were selected from the most common antibiotics used to treat infectious diseases in poultry.

Detection of virulence factors

Table 1 represents the list of primers and PCR conditions used for amplification of virulence factors in the *E. coli* isolates. The PCR amplifications were performed using three different protocols. Detection of *afa/ draBC*, *cnf1*, *csgA cvaC*, *iutA,* and *fyuA* genes was performed using the PCR in a total volume of 25 µl, including 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 200 µM dNTPs each (Fermentas, St. Leon-Rot, Germany), 2.5 µl PCR buffer 10X, 50 pmoL of each primer, 1.25 U of Taq DNA polymerase (Fermentas, St. Leon-Rot, Germany), and 2.5 µl (40-260 ng/µl) of the extracted DNA template of the *E. coli* O157 isolates. Detection of *cnf2, kpsMT* II, *PAI,* and *papC* genes was performed using the PCR in a total volume of 25 µl, including 2 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 150 µM dNTPs each, 2.5 µl PCR buffer 10X, 75 pmoL of each primer, 1.5 U of Taq DNA polymerase, and 3 µl of the extracted DNA template of the *E. coli* O157 isolates.

Finally, detection of *fimH*, *ibeA*, *PapGII-III*, *sfa focDE,* and *traT* genes was performed using the PCR in a total volume of25 µl, including 2 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 200 µM dNTPs each, 2.5 µl PCR buffer 10X, 50 pmoL of each primer, 1.5 U of Taq DNA polymerase, and 5 µl of the extracted DNA template of the *E. coli* O157 isolates.

The DNA thermocycler (Eppendorf Mastercycler 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) was used in all PCR reactions for DNA amplifications.

Amplified samples were analyzed by electrophoresis (120 V/208 mA) in 1.5% agarose gel. The gel was stained with 0.1% DNA Safe Stain (0.4 µg/ml) (SinaClonBioScience, Tehran, Iran). The UVI doc gel documentation systems (UVItech, UK) was applied in the analysis of images (Fagan *et al.,* 1999).

Statistical analysis

Statistical analysis was done using the SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA). Chi-square test and Fisher's exact two-tailed test were used to assess any significant relationship between prevalence of *E. coli* strains, distribution of virulence factors, and antibiotic resistance properties. *P* value <0.05 was considered as statistically significant level.

Table 1. Primer sequences PCR conditions used for amplification of virulence factors in the *E. coli* O157 strains isolated from raw meat samples (Tavakol *et al.,* 2018).

Target gene	Primer Sequence (5'-3')	Size of product (bp)
afa/draBC	GCTGGGCAGCAAACTGATAACTCTC CATCAAGCTGTTTGTTCGTCCGCCG	750
cnf1	AAGATGGAGTTTCCTATGCAGGAG CATTCAGAGTCCTGCCCTCATTATT	498
csgA	ACTCTGACTTGACTATTACC AGATGCAGTCTGGTCAAC	200
cvaC	CACACACAAACGGGAGCTGTT CTTCCCGCAGCATAGTTCCAT	680
iutA	GGCTGGACATCATGGGAACTGG CGTCGGGAACGGGTAGAATCG	300
fyuA	TGATTAACCCCGCGACGGGAA CGCAGTAGGCACGATGTTGTA	880
cnf2	AATCTAATTAAAGAGAAC CATGCTTTGTATATCTA	543
***kpsMT* II**	GCGCATTTGCTGATACTGTTG CATCCAGACGATAAGCATGAGCA	272
PAI	GGACATCCTGTTACAGCGCGCA TCGCCACCAATCACAGCCGAAC	930
papC	GACGGCTGTACTGCAGGGTGTGGCG ATATCCTTTCTGCAGGGATGCAATA	328
fimH	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA	508
ibeA	AGGCAGGTGTGCGCCGCGTAC TGGTGCTCCGGCAAACCATGC	170
***PapG* II-III**	CTGTAATTACGGAAGTGATTTCTG ACTATCCGGCTCCGGATAAACCAT	1070
s*fa/focDE*	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	410
traT	GGTGTGGTGCGATGAGCACAG CACGGTTCAGCCATCCCTGAG	290

RESULTS

A total of 18 *E. coli* O157 isolates were tested for antibiotic resistance patterns and distribution of virulence factors. Table 2 represents the antibiotic resistance patterns of *E. coli* O157 strains isolated from different types of animal samples. Multiple resistance to antibiotics was observed in all isolates. The highest prevalence of resistance was against: tetracycline (94.4%), trimethoprim (61.1%), co-trimoxazole (55.5%), and gentamicin (55.5%).

On the other hand, *E. coli O157* strains obtained the lowest prevalence of resistance against tobramycin (16.6%), meropenem (16.6%), imipenem (11.1%), and azithromycin (16.6%) antibiotic agents. Statistically significant differences were observed between types of samples and prevalence of antibiotic resistance ($P<0.05$).

Table 2. Antibiotic resistance pattern of *E. coli* O157 strains isolated from raw meat samples.

No. Isolates	Source of Isolates	azithromycin	nitrofurantoin	chloramphenicol	meropenem	imipenem	lovofloxacin	ciprofloxacin	trimetoprim	tetracycline	ceftazidime	cephalotin	co-trimoxazole	tobramycin	amikacin	gentamicin	streptomycin	erythromycin	rifampin
1	Chicken meat	+	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-	+	-
2	Camel meat	-	-	+	-	-	-	+	-	+	+	+	+	-	+	+	+	-	+
3	Chicken meat	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
4	Bovine meat	-	-	+	-	+	+	+	+	+	-	+	-	+	+	-	+	+	-
5	Turkey meat	+	-	-	-	-	+	-	+	-	+	-	-	-	-	+	+	-	-
6	Bovine meat	-	+	+	+	-	-	+	+	+	-	+	+	-	+	+	-	+	-
7	Bovine meat	-	-	+	-	-	-	-	-	+	+	-	+	+	-	+	-	-	+
8	Chicken meat	-	+	-	+	-	-	-	+	+	-	-	+	-	-	+	-	-	-
9	Camel meat	-	-	-	-	-	+	+	+	+	+	-	-	-	+	-	-	+	-
10	Ovine meat	-	-	-	-	-	-	-	+	+	-	+	+	-	-	+	-	-	-
11	Camel meat	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	+	-	-
12	Chicken meat	-	-	-	-	-	+	-	+	+	+	-	-	-	+	+	+	+	-
13	Turkey meat	-	-	-	+	+	-	-	+	+	+	-	+	-	-	-	-	-	+
14	Chicken meat	+	-	-	-	-	-	-	+	+	-	+	-	+	-	+	-	-	-
15	Chicken meat	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-
16	Ovine meat	-	+	+	-	-	-	+	+	+	-	-	+	-	+	+	-	-	-
17	Bovine meat	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+
18	Turkey meat	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-
Total		3	4	5	3	2	4	6	11	17	7	5	10	3	6	10	5	7	4

Zone of inhibition diameters (mm) for antibiotics resistance is: azithromycin= ≤13; nitrofurantoin= ≤14; chloramphenicol= ≤12; meropenem= ≤15; imipenem= ≤13; levofloxacin= ≤13; ciprofloxacin= ≤15; trimethoprim= ≤10; tetracycline= ≤14; ceftazidime= ≤14; cephalotin= ≤14; co-trimoxazole= ≤10; tobramycin= ≤12; amikacin= ≤14; gentamicin= ≤12; streptomycin= ≤11; erythromycin= ≤13; rifampin= ≤16.

Table 3. Distribution of virulence factors in the *E. coli O157* strains isolated from raw meat samples.

No. Isolates	Source of Isolates	*iutA*	*cvaC*	*csgA*	*Cnf2*	*Cnf1*	*afa/ draBC*	*traT*	*Sfa/ focDE*	*PapG III*	*PapG II*	*papC*	*ibeA*	*PAI*	*KpsMT II*	*fimH*	*fyuA*
1	Chicken meat	+	-	+	-	+	-	-	+	-	+	-	-	+	+	+	-
2	Camel meat	-	+	+	+	-	+	+	+	-	-	+	-	+	-	+	+
3	Chicken meat	+	-	-	-	-	+	-	-	-	-	-	+	-	-	+	-
4	Bovine meat	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+
5	Turkey meat	-	+	+	-	+	-	-	+	-	-	-	+	-	-	+	-
6	Bovine meat	-	+	-	+	+	+	+	-	+	+	+	-	+	-	+	+
7	Bovine meat	+	-	-	+	+	+	+	-	+	-	+	-	-	+	+	+
8	Chicken meat	+	-	-	+	-	-	+	-	-	+	-	-	+	-	+	-
9	Camel meat	-	+	+	-	-	+	-	+	-	+	-	+	-	-	+	-
10	Ovine meat	+	-	+	-	+	+	-	+	+	+	+	+	-	+	+	+
11	Camel meat	+	+	+	-	+	+	-	+	-	+	+	-	-	-	+	-
12	Chicken meat	-	-	+	-	+	-	+	+	-	-	-	-	+	+	-	+
13	Turkey meat	-	+	-	+	-	+	-	-	+	-	+	-	-	-	+	-
14	Chicken meat	-	+	-	+	-	+	-	-	-	-	+	+	-	-	+	+
15	Chicken meat	+	-	-	-	+	-	-	+	-	+	-	-	+	+	-	-
16	Ovine meat	-	+	+	-	+	+	+	-	+	-	+	-	-	-	+	-
17	Bovine meat	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+
18	Turkey meat	-	-	-	-	-	+	-	-	-	+	+	-	-	-	+	-
Total		9	10	10	8	11	11	7	9	6	10	11	6	7	7	16	8

PAI (38.8%), *ibeA* (33.3%), *PapG III* (33.3%), and *traT* (38.8%) virulence factors were low amongst the *E. coli* O157 strains. Statistically significant differences were exhibited between types of samples and prevalence of virulence factors ($P<0.05$). The PCR results for detection of some virulence genes are shown in Figures 1 and 2.

Table 3 represents the distribution of virulence factors in the *E. coli* O157 strains isolated from the different types of samples. *FimH* (88.8%), *papC* (61.6%), *cnf1* (61.6%), *afa/draBC* (61.6%), *PapG II* (55.5%), *csgA* (55.5%), and *cvaC* (55.5%) were the most commonly detected virulence factors amongst the *E. coli* O157 strains isolated from the different types of samples. Prevalence of *KpsMT II* (38.8%), *PAI* (38.8%), *ibeA* (33.3%), *PapG III* (33.3%), and *traT* (38.8%) virulence factors were low amongst the *E. coli* O157 strains. Statistically significant differences were exhibited between types of samples and prevalence of virulence factors ($P<0.05$). The PCR results for detection of some virulence genes are shown in Figures 1 and 2.

DISCUSSION

Escherichia coli O157 is one of the Shiga toxin–producing types of *E. coli*. *It* is transmitted to humans primarily through consumption of contaminated foods such as raw or undercooked ground meat products and raw milk. Fecal contamination of water and other foods, as well as cross-contamination during food preparation (with beef and other meat products, contaminated surfaces, and kitchen utensils) will also lead to infection (Lim *et al.,* 2010).

E. coli O157 infection often causes severe acute hemorrhagic diarrhea (although nonhemorrhagic diarrhea is also possible) and abdominal cramps. Usually little or no fever is

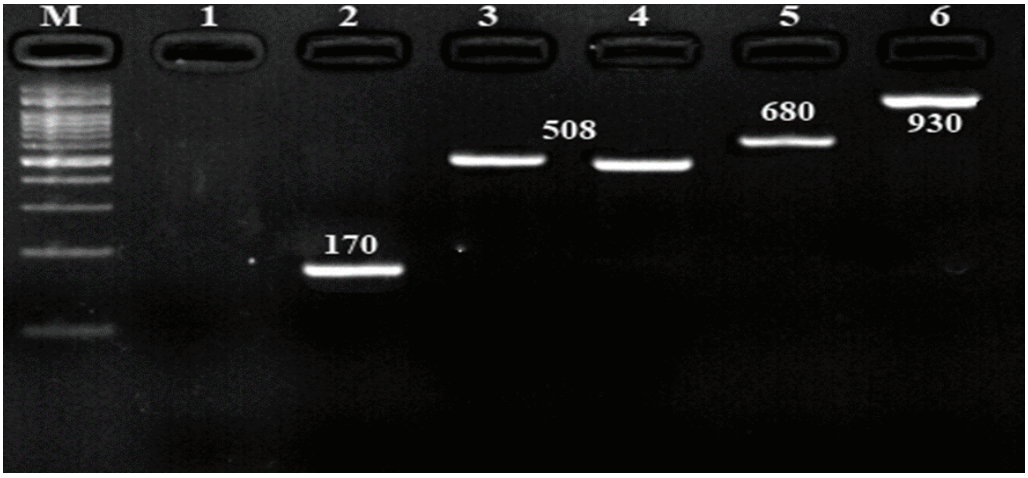

Figure 1. Agarose gel electrophoresis of PCR products amplified with a Multiplex PCR method for the *ibeA* (170 bp), *fimH* (508 bp), *cvaC* (680 bp), and *PAI* (930 bp) genes from *E.coli* O157 strains isolated from raw meat samples.

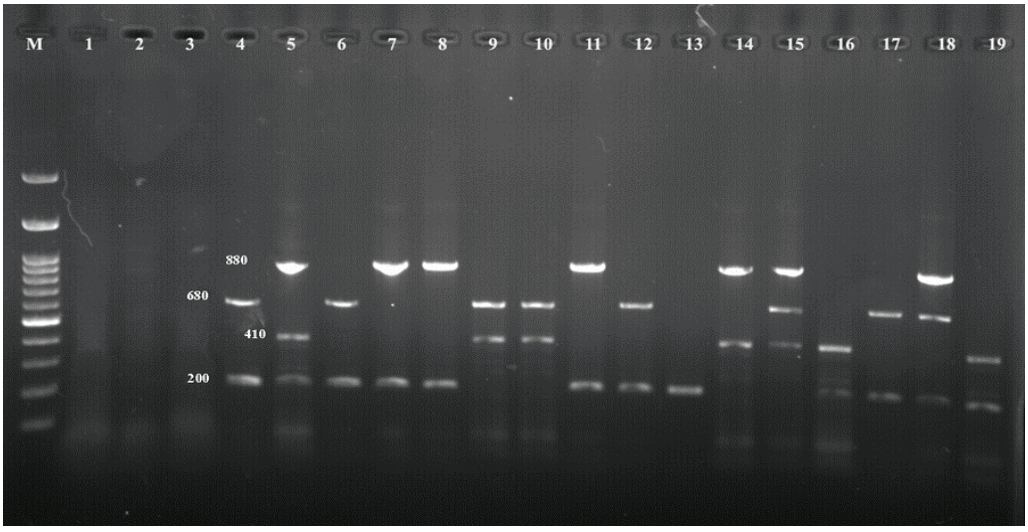

Figure 2. Agarose gel electrophoresis of PCR products amplified with a Multiplex PCR method for the *csgA* (200 bp), *sfa/focDE* (410 bp), *cvaC* (680 bp), and *fyuA* (880 bp) genes from *E.coli* O157 strains isolated from raw meat samples.

is present and the illness is resolved in five to ten days (Ciccarelli et al., 2013). It can also sometimes be asymptomatic (Roos et al., 2006).

In some people, particularly children under five years of age, persons whose immunology are otherwise compromised, and the elderly, the infection can cause hemolytic uremic syndrome (HUS). About two to seven percent of infections lead to this complication. In the United States, HUS is the principal cause of acute kidney failure in children and most cases of HUS are caused by E. coli O157 (Gould et al., 2009; Nielsen et al., 2014).

The present study was done to assess the antibiotic resistance pattern and distribution of virulence factors in the *E. coli* O157 strains isolated from different types of samples. Findings of the present study showed that *E. coli* O157 strains isolated from animal samples had the high prevalence of resistance against gentamicin, co-trimoxazole, tetracycline, and trimethoprim antibiotic agents. This high prevalence of resistance against diverse types of antibiotic agents can be explained by unauthorized and illegal prescription of human and veterinary medicine antibiotics. Schroeder *et al.* (2002) reported that the prevalence of resistance of *E. coli* O157 strains isolated from meat samples (cattle, swine, humans, and food) against tetracycline, sulfamethoxazole, cephalothin, and ampicillin were 27%, 26%, 17%, and 13% respectively. Highest prevalence of resistance occurred among swine meat isolates where 74% were resistant to sulfamethoxazole, 71% were resistant to tetracycline, 54% were resistant to cephalothin, and 24% were resistant to ampicillin.

Reuben and Owuna (2013) reported that all the isolates were resistant to multiple antibiotics. All (100%) of the isolates were resistant to two antibiotics (penicillin and tetracycline), 94.7% to erythromycin, 84.2% to amoxicillin, oxacillin, and sulphamethoxazole/trimethoprim, 68.4% to chloramphenicol, and 42.1% to streptomycin while 78.9% and 89.5% of the isolates were sensitive to ciprofloxacin and gentamicin. Meng et al. (1998) mentioned that the highest prevalence of antibiotics resistance occurred among cattle isolates (34%) where 70% were resistant to streptomycin-sulfisoxazole tetracycline. All strains showed multiple resistance to antibiotics. Goncuoglu *et al.* (2010) reported that among 11 cattle *E. coli* O157 isolates, four isolates (36.36 %) were resistant to cephalothin, one (9.09 %) isolate was resistant to streptomycin, and one (9.09 %) to nalidixic acid. In addition, among 14 sheep *E. coli* O157 isolates, two (14.28 %) isolates were resistant to sulphamethoxazole, one (7.14 %) isolate was resistant to sulphonamide compounds, and one (7.14 %) to streptomycin.

Various virulence genes, including genes encoding adhesion factors, bacteriocin production, and iron-chelating factors, are involved in bacterial pathogenesis, thus, an additional distinguished finding of this study is the high prevalence of certain virulence factors in the *E. coli* O157 strains isolated from animal samples. The most commonly detected virulence genes amongst these *E. coli O157* isolates were *FimH* (type 1 fimbriae), *papC* (P fimbriae), *cnf1* (cytotoxic necrotizing factors), *afa/draBC* (fimbriae adhesion and Dr antigen family), *PapG II* (P fimbriae), and *csgA* (curli fibers). These virulence factors are predominant in colonization, adhesion, and invasion to host cells. Prevalence of non-adhesive virulence factors including *iutA* **(**aerobactin**)** and *cvaC* (colicin V) were also high. This finding is in agreement with those of previous investigations.

Bahalo *et al.* (2013) described that the distribution of *fimH* virulence factor amongst the *E. coli* strains isolated from hospitalized patients was 30%. Rashki and Ali Abdi (2014) stated that the distribution of *cnf1* virulence factor amongst the *E. coli* strains that were collected from patients with urinary tract infection in Zabol was 28%, which was much lower than the results of this study.

In conclusion, the antibiotic resistance pattern and profile of virulence factors of *E. coli* O157 strains isolated from animal raw meat in Iran was assessed. High prevalence of resistance against tetracycline, trimethoprim, co-trimoxazole (55.5%), and gentamicin (55.5%) antibiotic agents and high distribution of *fimH*, *papC*, *cnf1, afa/draBC, papG II, csgA,* and *cvaC* virulence factors were the most important findings of the present study. Results exhibited that all bovine, ovine, caprine, and camel meat samples can be reservoirs of the virulent and antibiotic resistant *E. coli* O157 strains. Lower prevalence of resistance against tobramycin, meropenem, imipenem, and azithromycin in all studied strains showed their higher therapeutic effects on the *E. coli* O157 strains. Some of the *E. coli* O157 strains harbored simultaneous presence of some virulence factors together which indicated their high pathogenicity. Moreover, it was concluded that meat is also an important source of strains resistant to some of the categories of antimicrobials used to treat infections caused by *E. coli* O157. Additional studies are required to assess the role of resistant strains in the dissemination of resistance genes.

The presence of clinically important virulent species and multidrug-resistant strains in meat may be a threat to public health considering that meat may provide a vector for the spread of these opportunistic pathogens into both the community and hospital settings or environment.

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STATEMENT ON COMPETING INTEREST

The authors declare that they have no competing interests.

AUTHOR'S CONTRIBUTION

HM and ML carried out the molecular genetic studies, participated in the primers sequence alignment and drafted the manuscript. ML and ET carried out the sampling and culture method. HM and ET participated in the design of the survey, performed the statistical analysis and writing the manuscript. All authors read and approved the final manuscript.

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