

Detection of some virulence genes by PCR in *Escherichia coli* isolates from some local food in Erbil City

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ABSTRACT

The present study was aimed to isolate and characterize *Escherichia coli* pathogenic types from raw red meat, poultry and ready to eat food sources. Enrichment, selective plating, biochemical tests as well as vietik kit system have been applied for isolation and identification of pathogenic *E. coli* from collected samples. Out of 200 food samples, 180 (90%) were contaminated with *E. coli*, the highest contaminated foods were red meat 74 (92.5%), while the poultry 55 (92%), and 51 (85%) of ready to eat foods. The prevalence of *E. coli* O157:H7 found in red meat is 20% and 18% in poultry while 14% of the ready to eat food samples were contaminated with this bacterium. The isolates were screened for some virulence genes using PCR assays; from the total (128) isolates, 98 (76.5%) possessed *uidA* gene as detected in 40 (74%) red meat, 30(81%) and 28(75.5%) in poultry and ready to eat food respectively, while 37 (28.9%) of the isolates possessed *It* gene as follows; 10(18.5), 15 (40.5) and 12(32.4) in red meat, poultry and ready to eat food, respectively. The results showed the detection *flicH7* gene specific for *E. coli* O157:H7 were 7 (38.8%), 5 (35.7%) and 4(26.6%) of the total 47 isolates were possessed the *flicH7* gene found in red meat, poultry and ready to eat food respectively. Our results showed that *E. coli* isolates presence in high percentage in raw red meat, poultry and ready to eat foods (kebab, shawerma and hamburger) and virulence genes; *udiA*, *It* and *flicH7* genes detected by PCR in the isolates and may be a source of potentially pathogenic *E. coli* for humans.

Keywords: Pathogenic, *E. coli*, virulence genes, PCR.

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INTRODUCTION

Pathogenic *Escherichia coli* are generally regarded as part of the normal flora of the human intestinal tract and animals. Most of *Escherichia coli* strain are non-pathogenic, however some of them are major food borne pathogen of public health importance and responsible for watery and bloody diarrhea, infantile diarrhea, traveler's diarrhea, hemolytic uremic syndrome (Croxall et al., 2011; Bélanger et al., 2011). *E. coli* divided into diarrheal pathogens causing diarrhea (DPEC) and extra intestinal pathogenic *E. coli* (Croxall et al., 2011; Abdalla et al., 2009; Croxen and Finlay, 2010; Mataragas et al., 2008).

Based on their pathogenic phenotypes and the diseases that they cause, diarrhoeagenic *E. coli* have

been classified into 6 groups: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and Shiga toxin producing *E. coli* (STEC)/enter hemorrhagic *E. coli* (EHEC)/verocytotoxin-producing *E. coli* (VTEC) (Kaper et al., 2004). Epidemiology, treatment, pathogenesis, and clinical manifestations can be used to preliminary identify each pathotype (Huang et al., 2006). Moreover, researchers using molecular methods such as polymerase chain reaction can identify EPEC. EPEC has been sub-divided into typical and atypical EPEC depending on their adhesion mechanisms in human

epithelial cells (Trabulsi et al., 2002). The presence of locus of enterocyte effacement has been used to identify atypical EPEC. This locus codes for the gene of attaching and effacing, *eae* gene enables the bacteria to adhere and destroy human intestinal enterocytes (Campellone, 2010). Enteroaggregative *E. coli*, have mechanisms that promotes diarrhea in humans are coded by *eagR*, the master regulator of EAEC virulence, controls the expression of adherence factors, a dispersing protein, and a cluster of genes encoded on the EAEC chromosome. EIEC is characterized by the destruction of the colonic epithelium caused by the inflammatory response induced upon invasion of the mucosa by EIEC (Parsot 2005) using the *ial*; marker for invasively gene. ETEC are important causes of total diarrheal incidents worldwide. These strains cause diarrhea through the action of enterotoxins: the heat-labile (LT) and heat-stable (ST) enterotoxins. Some strains may express LT only, or ST only, or both LT and ST coded by *lt* and *st* in the plasmid. *E. coli* strains that are capable of producing both LT and ST toxins (ETEC) can cause severe diarrhea (Piekrard et al., 1997). Enterohaemorrhagic *E. coli* (EHEC) is associated with foodborne outbreaks in the developed world and can cause bloody diarrhea, hemorrhagic colitis (HC) and the Hemolytic Uremic Syndrome (HUS) due to the elaboration of Shiga toxin (Stx). Other virulence factors such as membrane protein intimin, encoded by the *eae* gene, *fliCH7* encoding flagella antigen H7 and enterohaemolysin, encoded by the *HlyA* gene (Stephan et al., 2008; Hagan and Mobley, 2007) have been identified. Uropathogenic *E. coli* strains have been identified by detecting Discriminating various *E. coli* pathotypes from cattle and their products have been greatly studied in the last two decades with molecular techniques, they are sensitive and specific (Belanger et al., 2002; Chapman et al., 2001; Rappelli et al., 2001). However, in the presence of diarrhoeagenic infection, little is known about the prevalence, distribution and associated virulent genes of *E. coli* pathotypes in foods of animal origin in Erbil city. Thus this study was designed with the following objectives; Evolutions the incidences of *E. coli* pathotypes in different types of local food in Erbil city, determination of virulence gene of isolated *E. coli* by PCR.

MATERIALS AND METHODS

Sample collection and Isolation of pathogenic *E. coli*

Two hundred local food samples were collected from Erbil city markets as illustrated in Table 1.

Conventional isolation method

Twenty five grams of each of food samples were minced and an adequate amount was transferred into a pre-labeled test tube of buffered peptone water (BPW) and incubated at 37°C for 24 h. After

Table 1. Type and numbers of collected samples.

Food types	Number of samples
Red meat	80
Poultry	60
Ready to eat food	60
Total	200

incubation, a loopful of the sample was streaked in MacConkey agar (MCA) plate and incubated at 37°C for 24 h. Pink round isolated colonies in MCA were streaked in Eosin Methylene Blue (EMB) agar incubated at 37°C for 24 h. The characteristic green metallic sheen growth of colonies is a presumptive identification for *E. coli*. Purification of the isolates was performed and after Gram staining, biochemical tests were done for confirmation. The biochemical tests include the following: IMViC (Indole, Methyl Red, Voges-Proskauer and Simmons Citrate) and sugar; xylose, inositol, maltose, trehalose, lactose, arabinose, glucose, sorbitol and mannitol (Levinson and Jawetz, 2000). For isolation of *E. coli* O157; colonies was streaked on Sorbitol MacConkey agar (SMAC) which contains cefixime (50 mg/L) and potassium tellurite 2.5 mg/L, and incubated at 40°C for 24 h (Dontorou et al., 2003).

Detection of virulence genes of *E. coli* by PCR

The sequencing of primer which used for the detection appeared in Table 2.

DNA extraction

DNA was extracted from identified *E. coli* and from a positive control strains for *E. coli* (ATCC 8739, ESC 20) purchased from center media for medical analysis in Erbil city. DNA extraction was prepared as briefly, single colony of confirmed *E. coli* grown overnight at 37°C on MacConkey agar plates were picked, suspended in 200 µl of sterile distilled water, vortexes for 2 min. The cells were then centrifuged for 10 min at 13,000 rpm (Thermo Fisher Scientific, Germany). Five hundred microliters distilled water was pipetted into the eppendorf tubes, vortexes and the cells were lysed using a heat on water bath for 15 min at 100°C (Nessa et al., 2007). The cell debris was removed by centrifugation at 10,000 rpm for 5 min using a Mini Spin centrifuge (Thermo Fisher Scientific). The supernatant was used as a template in the PCR assay immediately after extraction.

Detection of virulence genes in *E. coli* isolates

PCR assay was carried out in a 25 µl reaction volume containing a 24 µl of 2xmaster mix (containing 0.05 U/µl of *Taq* DNA polymerase, reaction buffer, 4 mM MgCl₂, and 0.4 mM of each dNTP) and 1 µl of template DNA. Oligonucleotide primers targeting the *uidA* gene were used to confirm the isolates EPEC, *fliCH7* gene encoding for structural flagella antigen H7 in Enterohemorrhagic *E. coli*, *lt* gene encoding for heat labile toxins found in Enterotoxigenic *E. coli* were used in following the conditions:

1. *E. coli uidA* an initial 2 min of denaturation at 94°C followed by 25 cycles of 94°C for 1 min, 58°C for 1 min and final extension temperature 72°C for 2 min (Tsai et al., 1993).
2. EHEC *fliCH7*: Initial denaturation at 95°C for 15 min followed by 35 cycles of heat denaturation at 94°C for 45 s, primer annealing at

Table 2. Primers sequencing.

Target gene	Target isolate	Primer Sequence (5'–3')	Product Size (bp)	Reference
<i>uidA</i>	<i>E. coli</i>	AAAACGGCAAGAAAAAGCAG ACGCGTGGTTAACAGTCTTGCG	147	Tsai et al., 1993
<i>flicH7</i>	EHEC	TACCATCGCAAAGCAAC TCC GTCGGCAACGTTAGTGATAC	230	Wang et al., 2002
<i>Lt</i>	ETEC	GCACACGGA GCTCCTCAGTC TCCTTCATCCTTTCAATGGCT	218	Wang et al., 2002

55°C for 45 s and DNA extension at 68°C for 2 min, and final extension temperature at 72°C for 5 min (Wang et al., 2002).

3. ETEC *Lt*: An initial denaturation step at 94°C for 5 min, followed by 36 cycles of 94°C for 35 s, annealing at 62°C for 30 s and extension at 72°C for 1 min. final extension step at 72°C for 5 min (Wang et al., 2002).

Gel electrophoresis

The PCR products were analyzed after electrophoresis in 1.5% agarose gel to detect specific amplified product by comparing with standard molecular weight marker (Toma et al. 2003). One percent agarose gel was prepared by melting 3.0 g agarose in 200 ml of diluted TBE buffer using a microwave oven. The melted agarose was allowed to cool to about 50°C and 20 µl ethidium bromide was mixed, shacked and was poured into gel tray; combs were placed. After solidification of the gel, the comb was removed. During electrophoresis, the gel was placed in a horizontal electrophoresis apparatus containing TBE buffer and ethidium bromide. Electrophoresis was carried out at 100 volts for 35 min (Adwan et al., 2015).

RESULTS AND DISCUSSION

Isolation of *E. coli*

Two hundred samples were collected from various local food for examined pathogenic *Escherichia coli*, the samples included as appeared in Table 3; red meat (80), poultry (60) and ready to eat foods (60); Out of 200 food samples, 180 (90%) were contaminated with *Escherichia coli*, and according to the results the highest contaminated foods were red meat 74(92.5%), while the poultry 55 (92%), and 51 (85%) of ready to eat foods were contaminated with *Escherichia coli* as shown in Table 3.

Morphological characteristics of colonies (size, shape, elevation, form, pigmentation and opacity) developed after incubation on MacConkey agar plate was appeared similar to that characterized by Kaper et al. (2004). The growth *E. coli*, colonies were black with a metallic green sheen caused by the large quantities of acid that is produced and precipitates out the dyes onto Eosin Methylene blue (EMB) agar plate corresponding to that appeared in Cappuccino and Sherman (2011).

Isolation of *Escherichia coli* O157H:7

The results appeared in figure 1 the prevalence of *E. coli* O157: H7 as follow: this bacteria found in red meat as 20 and 18% in poultry while 14% of the ready to eat food samples were contaminated with this bacteria.

Escherichia coli serotype O157:H7 was recognized human pathogen associated with hemorrhagic colitis. Unlike most *E. coli* strains, *E. coli* O157:H7 does not ferment sorbitol. Therefore, the efficacy of MacConkey agar containing sorbitol (SMAC medium) instead of lactose as a differential medium for the detection of *E. coli* O157:H7 in foods cultures was determined in comparison with MacConkey agar, most *E. coli* O157:H7 lack the capacity to ferment sorbitol; this trait has also been useful because it distinguishes this pathogen from the majority of other *E. coli* strains (Strockbine et al., 1998).

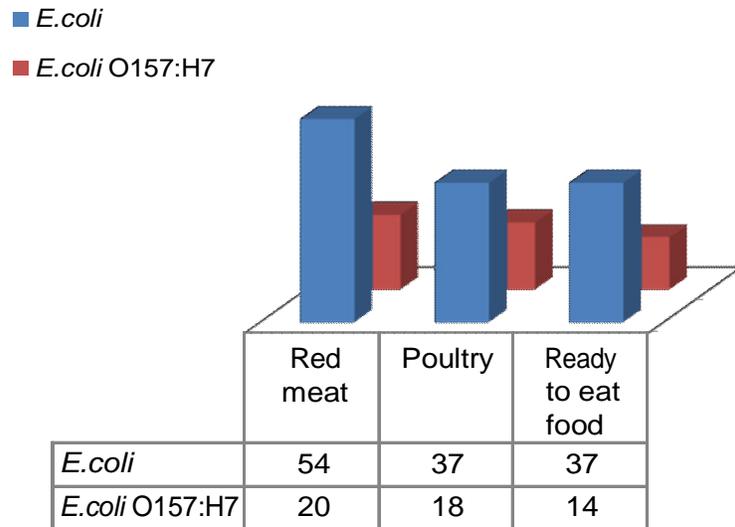
Distribution of *E. coli* in local food samples

Satisfactory result on all biochemical tests and colony characteristic on differential agar was confirmed that all 180 isolates were *E. coli*. Divided (52) isolates was *E. coli* O157:H7 which had colourless colony when cultured on MacConkey sorbitol agar which isolated 20 from red meat, 18 from poultry and 14 from ready to eat food, on the other hand (128) isolates of *E. coli* isolated as 54 from red meat and 37 from each poultry and from ready to eat food as shown in Figure 1.

Meat is considered as an important source of protein, essential amino acids, B complex vitamins and minerals. So, it offers a highly favorable environment for growth of pathogenic bacteria (Abdalla et al., 2009; Zakaria et al., 2018). As well as, poultry meat is an excellent substrate for the growth of a wide variety of microorganisms including pathogens and spoilage microorganisms. On the other hand, chicken and turkey are the major types of poultry meat. Chicken meats comprise about the two-thirds of the total production in the world (Mead, 2000). Meat and poultry carcasses and their parts are frequently contaminated with pathogens which reach the carcasses from intestinal feathers (Dinçer and Baysa, 2004).

Table 3. Number and percentage of contaminated food with *E. coli*.

Type of food	Total no. of examined samples	No. and percentage of positive sample	
		No. of samples	%
Red meat	80	74	92.5
Poultry	60	55	92
Ready to eat food	60	51	85
Total	200	180	90

**Figure 1.** Presence of *E. coli* and *E. coli* O157:H7.

Escherichia coli is commonly used as surrogate indicator, its presence in food generally indicate direct and indirect fecal contamination (Clarence et al., 2009).

The incidence of *E. coli* in red meat, poultry meat and some of ready to eat food revealed that incidence of isolation of *E. coli* in the examined samples was 54, 37 and 37%, respectively. As well as the incidence of *E. coli* O157 in the examined samples 20, 18 and 14% in red meat, poultry and ready to eat food, respectively. These results nearly corresponding with other authors; Rawaihs (2014), Odu and Akano (2012) and Abdalhamid et al. (2013) also reported low incidence of *E. coli* in shawerma 6.67, 13.6 and 8%, respectively. Samaha et al. (2012) reported low incidence in nuggets 12%; on the other hand, lower incidence from poultry reported by Mohamed (2004) isolated *E. coli* from the examined samples of chicken breast with percentage of 7.5% and in chicken thigh with percentage of 2.5%, Marionette et al. (2009) isolated *E. coli* from the examined samples of chicken breast and thigh with percentages of 6.67 and 10%, respectively. Edris et al. (2015) isolated *E. coli* from the examined samples of chicken breast with percentage of 12% and in chicken thigh with percentage of 16%, Khaled and Hendy (2015) isolated *E. coli* from poultry with percentage rate 10%; and Riyad (2011) isolated *E. coli*

from the examined chicken samples with percentage of 8.7% in both samples. On the other side higher incidence of *E. coli* found in ready to eat food (chicken nuggets and shish taouk) reported by Al-Dughaym and Altabari (2010), Hassanin et al. (2014) isolated *E. coli* with percentage of 60 and 26.7%, respectively. Also, higher incidence reported by Mohamed-Walaa (2014), Saad et al. (2015) they isolated *E. coli* with percentage of 26.7 and 25% of examined ready to eat food, respectively. Fulden et al. (2013) failed to isolate *E. coli* O157:H7 from chicken shawerma and ready to eat food, respectively.

The variation in the results between different authors may be due to the differences in manufacture practices, handling from producers to consumers, storage and the effectiveness of hygienic measures applied during production (Tebbut, 1999). A number of studies have confirmed that the prevalence of *E. coli* O157:H7 varies among studies due to the above reasons. *Escherichia coli* O157:H7 prevalence rates were reported from Ethiopia by Hiko et al. (2008) 8% and from other countries, 8.3% in Iran (Hashemi et al., 2010). Our study reported the prevalence of *E. coli* O157 and *E. coli* there is no one-to-one correspondence with previous prevalence studies, we compared our results to those from studies using methods similar to ours in which

naturally contaminated red meat or poultry product and ready to eat food samples. The variation in prevalence among studies could be attributed to difference in sampling and isolation procedures, variability in sampled populations, diverse origins of meat, poultry and ready to eat food study design, season, conditions and sanitation with personal hygiene during processing and slaughter or during preparation.

Biochemical identification of isolated bacteria

The adopted isolates were identified using standard biochemical tests, as appeared in Table 4. All the isolates (180) 100% were Indole positive and negative for Oxidase test, VP test, Urease test, Citrate test and Gelatinase test. It was also revealed that 100% catalase positive isolates as well as were giving positive haemolytic activity (70%) on blood agar and were all motile. IMViC (Indole, Methyl red, Voges-Proskauer, and Citrate) tests are frequently employed for identification of this group of microbes *Escherichia coli* (Barnes et al., 2003). Haemolytic activity of isolates revealed that those isolates were pathogenic due to production of haemolysin, which binds with the haemolysin receptor present on the surface of RBC, that favour haemolysis (Zinnah et al., 2007). Isolates were motile due to presence of flagellum, supports for colonization (El-Housseiny et al., 2010; Lee et al., 2009). Conventional methods for bacterial pathogens detection in foods are generally based on identification of bacteria using selective culture media by their morphological, biochemical and immunological characteristics (Wang and Mustapha, 2007).

Molecular characterization of *E. coli*

Isolates that were Gram negative, oxidase negative, and catalase positive and confirmed by vietik kit were screened for *E. coli uidA* gene as well as *lt* gene using PCR assays. Total (128) isolates from food samples showed 98 (76.5%) possessed *uidA* gene (Table 5, Figure 2).

E. coli has been regarded as an indicator of fecal contamination (Odonkor and Ampofi, 2013), in industry; it is regarded as poor hygiene indicator and insufficient sanitary practices during processing (Farzan et al., 2010). Different virulence genes were isolated from *E. coli* in diarrheic stools and watery (Osode and Okoh, 2008). We based our selection of virulence genes for this study on their association with *E. coli* strains causing extra-intestinal and intestinal infections. There were no biochemical differences between *E. coli* isolates. Approximately 23.4% of the isolates in our study did not show amplification of examined genes. This might be indication that *E. coli* may non-pathogens or have another virulence gene, some studies about *E. coli* were

found that have a single virulence gene (Wenz et al., 2006). Another study have been identified several virulence factors associated *E. coli* isolates (Altalhi and Hassan, 2009).

Generally, molecular approaches especially PCR-based technique is considered as a sensitive detection method for specific pathogens. PCR assay seems to be a useful technique for rapid and specific detection of pathogens in food and has been used for the control and detection of foodborne epidemics (Kawasaki et al., 2009).

The differentiation of *E. coli* enterotoxigenic requires detection of virulence *lt* genes either by biological assays or by molecular techniques. in present study simple PCR assay, used to detect the presence of *lt* gene in 37 (28.9%) isolates of *E. coli* that belonged to the 15 (40.5%), 12 (32.4%) and 10 (18.5%), related to poultry, ready to eat food and red meat respectively as appeared in Table 5 and Figure 3.

The present study indicated that ETEC isolates were present in different examined types of food and this isolates can cause profuse, watery diarrhea by release LT, or other enterotoxins. In India, isolation of STEC has been reported from cattle (Pal et al. 1999), sheep (Wani et al., 2004; Bhat et al., 2008), human feces (Khan and Ner, 2002) and piglets (Dutta et al., 2010). Phenotypic assays are routinely used in most laboratories to characterize *E. coli* pathotypes. However, these methods alone are not sufficient to identify all five pathotypes of Diarrheagenic *Escherichia coli* (Brandal et al., 2007). The genes encoding virulence factors are extensively studied and characterized, and several PCR methods have been developed to identify the virulence genes of DEC (Kimata et al., 2005).

In this study, two distinct pathotypes of *E. coli* were detected in three types of foods in Erbil city, PCR method shown high sensitivity and specificity, using primers for the different Diarrheagenic *Escherichia coli* (DEC) pathotypes in a single reaction. EC was the most commonly isolated pathotype, followed by atypical EHEC, whereas the frequency of ETEC was low.

Detection of *fliCH7* gene in *Escherichia coli* O:157H7 isolates by PCR.

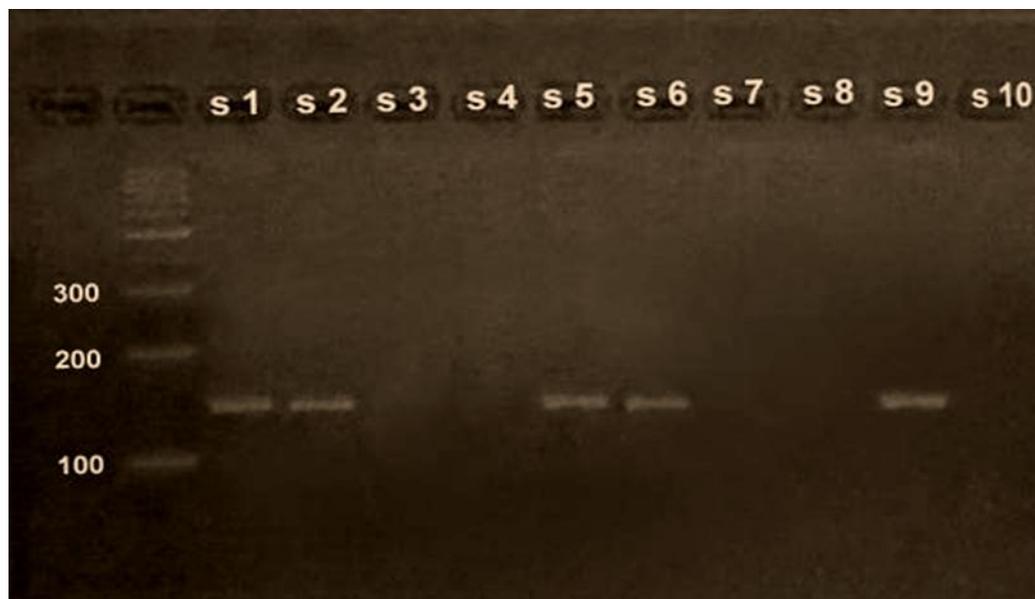
The results shown that 47 isolate of total 52 isolates of *E. coli* which grown on MacCokey sorbitol agar as a colourless colony and confirmed by vietik kit were tested for detection *fliCH7* gene the result showed that; 7 (38.8%), 5 (35.7%) and 4 (26.6%) of the total isolates were possessed the *fliCH7* gene specific for *E. coli* O157H:7. Found in red meat, poultry and ready to eat food respectively as appeared in Table 6, thus the specific gene products size 230 bp was detected as bands in gel electrophoresis appeared in Figure 4, this examination performed by *fliCH7* gene which is one of the genes used as a marker to identify *E. coli* O157:H7 (Kumer et al., 2013). These results in agreement with result of

Table 4. Biochemical tests for isolated *E. coli*.

Biochemical tests	No. of isolate (180)
	% of positive reaction
Indole	100
TSI	100
Hemolysis	70
Motility	100
Carbohydrate fermentation, Glucose, Lactose, Sucrose	100

Table 5. Detection of *LT* and *UidA* gene in *Escherichia coli* isolates by PCR technique.

Type of source	No. of isolates identified by biochemical tests	No. of isolates identified by vietik	No. and % of positive <i>uidA</i> gene	No. and % of positive <i>lt</i> gene
Red meat	54	44	40 (74)	10 (18.5)
Poultry	37	29	30 (81)	15 (40.5)
Ready to eat food	37	30	28 (75.6)	12 (32.4)
Total	128	103	98 (76.5)	37 (28.9)

**Figure 2.** Gel electrophoresis for amplification *uidA* gene (product size 230 bp) Electrophoresis was performed on 1.5% agarose gel at 70 volt for 90 min Lane: M is (1000-100bp) ladder. Lane: S1, S2, S5, S6, and S9 positive amplification for *uidA* gene. Lane: S3, S4, S7, S8 and S10 negative gene.

Kiranmayi and Krishnaiah (2010) for identification *E. coli* O157H:7 by rapid method PCR technique, this gene was detectable in 10.8% of the poultry, meat and beef and other source. Also, Osode and Okoh (2010) detected *fliCH7* gene in *E. coli* isolated from water.

CONCLUSION

The findings of the our study showed heavy contaminants

load in different meat types and ready to eat foods (like shawarma, hamburger and kebab) with pathogenic *E. coli*, from a total 200 samples: 180 (90%) were contaminated with this bacteria as well as the presence of *E. coli* O157 H:7 was detected when the isolates cultured on the selective and differential media (MacConkey sorbitol agar). This heavy contamination is measured as an indicator for non-hygienic, an safe as well as the short shelf life of meat and ready to eat foods. The detection of virulence genes by PCR allows the



Figure 3. Gel electrophoresis for amplification *It* gene (product size 218 bp). Electrophoresis was performed on 1.5% agarose gel at 70 volt for 90 min Lane: M is (1000-100bp) ladder. Lane: S1, S2, S3 and S5 positive amplification *It* gene Lane: S4, S6 to S10 negative gene.

Table 6. Identification of *Escherichia coli* H7:157 by PCR technique.

Type of source	No. of isolates	No. of isolates identified by vietik	No. and % of positive <i>flicH7</i>
Red meat	20	18 (90%)	7 (38.8%)
Poultry	15	14 (93.3%)	5 (35.7%)
Ready to eat food	17	15 (88.2%)	4 (26.6%)
Total	52	47 (90.3%)	16 (34%)



Figure 4. Gel electrophoresis for the amplification *flicH7* gene (product size 230 bp) Electrophoresis was performed on 1.5% agarose gel at 70 volt for 90 min Lane: M is (1000-100 bp) ladder. Lane: S1, S2, S4, S5, S7, S8 and S9 showed positive amplification for *flicH7* gene. Lane: S3, S6 and S10 negative gene.

determination of possibly enteropathogenic pathogen regardless of whether the isolates produces the toxin or not, for this reason, PCR important and considered sensitive methods to determine pathogenic *E. coli*. This study investigated the presences of EPEC and indicated that ETEC isolates were presents in red meat, poultry and ready to eat food different types of food and this isolates can cause profuse, watery diarrhea by release (LT) heat liable toxins.

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