T.R.
KAHRAMANMARAŞ SÜTÇÜ İMAM UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCE

DETECTION OF SHIGA TOXIN GENES (*stx*$_1$ and *stx*$_2$) IN *ESCHERICHIA COLI* O157:H7 ISOLATED FROM MEAT PRODUCTS

SOWALA MIKAEEL MERZA

MASTER THESIS

DEPARTMENT OF BIOENGINEERING AND SCIENCES

KAHRAMANMARAŞ, TURKEY 2014

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M.Sc. thesis entitled "DETECTION OF SHIGA TOXIN GENES (*stx*$_1$ and *stx*$_2$) IN *ESCHERICHIA COLI* O157:H7 ISOLATED FROM MEAT PRODUCTS" and prepared by *Sowayla Mikaeel Merza,* who is a student at Bioengineering and Sciences Department, Graduate School of Natural and Applied Sciences, Kahramanmaraş Sütçü İmam University, was certified by all the majority of jury members, whose signatures are given below.

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Sowayla Mikaeel MERZA

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ET ÜRÜNLERİNDEN İZOLE EDİLEN *ESCHERICHIA COLI* O157:H7'LERDEKİ SHIGA TOXIN 1 VE SHIGA TOXIN 2 (*stx* 1 and *stx* 2) GENLERİNİN TESPİTİ

(YÜKSEK LİSANS TEZİ)

Sowayla Mikaeel Merza

ÖZET

Bu çalışma, et ürünlerinde kültürel metot ve Multiplex PCR ile *Escherichia coli* O157:H7 izolasyonu ve bu bakterideki *stx1*and *stx2* virülans genlerinin tespit edilmesi için yapılmıştır. Bu amaçla 180 et ürünü örneği (50 kebap, 50 kıyma, 40 shawarma-döner ve 40 hamburger) Duhok- Irak'tan temin edilmiştir. Örnekler novobiocin içeren Modified Trypticase Soy Broth'ta ön zenginleştirmeye tabi tutulmuş daha sonra BCIG (5- bromo4-chloro-3-indoxyl- D-glucuronide) ve cefixime içeren sorbitol MacConkey Agar'a ekilmiştir. İzolatlatdan *E. coli* O157:H7 tespiti için Eosin Methylene Blue (EMB) ve Haemorrahgic Colitis (HC) Agar'a ekim yapılmıştır. Et ürünlerinden 48 tanesinde (%26,6) sorbitolü fermente edemeyen şüpheli koloniler tespit edilmiştir. Bu koloniler API E 20 testi ve RapID one sistemi ile doğrulanmıştır. Doğrulama işlemi sonunda 35 örnekte (%19, 9) *E. coli* O157:H7 tespit edilmiştir. PCR ile sonraki doğrulama işleminde 7 örnekte (%3, 8) O157 antijeni, 9 örnekte ise (%5) H7 geni bulunmuştur. Ayrıca bakteri izolatlarında PCR spesifik primerlerle Shiga toxin genlerinin (*stx1* ve *stx2*) varlığı araştırılmış ve 6 adet *E. coli* O157:H7'de *stx1* geni, 1 tanesinde ise *stx2* geni tespit edilmiştir.

Key Words: *Escherichia coli* O157:H7, *stx1* ve *stx2* geni, kültürel teknik, et ürünleri, PCR.

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DETECTION OF SHIGA TOXIN GENES (*stx*$_1$ and *stx*$_2$) IN *ESCHERICHIA COLI* O157:H7 ISOLATED FROM MEAT PRODUCTS

(M.Sc. THESIS)

Sowayla Mikaeel Merza

ABSTRACT

This study was conducted to identify the prevalence of the *Escherichia coli* O157:H7 and determine the virulence factors of *stx1*and *stx2* genes in meat products by culture and multiplex PCR methods. One-hundred eighty meat products (50 kebab, 50 ground meat, 40 shawarma, 40 hamburger) were sampled from Duhok province of Iraq. Samples were enriched in novobiocin containing Modified Tryptic Soy Broth and plated onto BCIG (5- bromo4-chloro-3-indoxyl- D-glucuronide) and cefixime supplemented Sorbitol MacConkey agar. Then typical colonies were plated on EMB and HC agar for isolating the *E. coli* O157:H7. There were 48 (26.6%) suspected non-sorbitol fermenting (NSF) colonies in meat products. Those suspected colonies were then confirmed with biochemical tests (API E 20 test and RapID one system). The results showed that 35 (19.9%) samples were found to be positive for *E. coli* O157:H7. These results were then confirmed with PCR. These results showed that there were 7 (3.8%) and 9 (5%) samples found to be positive for O157 and H7 antigen genes, respectively. The bacterial isolates were also identified for the presence of *stx1* and *stx2* genes. The *stx1* gene was detected in 6 isolates while the gene *stx2* was detected in 1 isolate of *E. coli* O157:H7.

Key Words: *Escherichia coli* O157:H7, *stx1* and *stx2* genes, cultural technique, meat products, PCR.

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LIST OF SYMBOLS ABBREVIATIONS

EIEC:	Enteroinvasive *E. coli*
EAEC:	Enteroaggregative *E. coli*
EHEC:	Enterohemorrhagic *E. coli*
DNA:	Deoxyribonucleic acid
ELISA:	Enzyme-linked immunosorbent assay
EPEC:	Enteropathogenic *E. coli*
ETEC:	Enterotoxigenic *E. coli*
g:	Gram
h:	Hour
HC:	haemorrhagic colitis
HUS:	hemolytic uremic syndrome
STEC:	Shiga toxin producing *E. coli*
Stx:	Shiga toxin
VT:	Verocytotoxin
VTEC:	verocytotoxin-producing *E. coli*
µ:	Micro
dNTP:	Deoxy nucleoside triphosphate
PCR:	Polymerase Chain Reaction
SMAC:	Sorbitol Mac Con key
ST:	Heat-stable toxin
Taq:	Thermus aquaticus
bp:	Base pair
FAO:	Food and Agriculture Organization
WHO:	World Health Organization
TBE:	Tris boric acid EDTA buffer
EMB:	Eiosine Methylene Blue
EDTA:	Ethylene Diamin Tetraacetic Acid
UV:	Ultra violet
mcg:	Micrograms
NSF:	Non-Sorbitol Fermenting
BCIG:	5- bromo4-chloro-3-indoxyl- D-glucuronide

1. INTRODUCTION

Meat and meat products are considered excellent sources of high quality protein, vitamins (B complex), and certain minerals, such as iron (Gracey *et al*., 1986). Meat is a nutrient-rich substrate that can support the growth of a wide range of microorganisms (Erdoğrul, 2005). This is due to the many factors which meat has, such as moisture, high nitrogenous compounds, and bountiful supply of minerals. In addition, other growth factors on meat, such as hot and cold temperatures, organic acids, water activity, salt, oxidation-reduction potential, fat and glycogen along with favorable pH may provide an excellent media for most of the enteric microorganisms (Bhandare *et al*., 2007; Podpecan *et al*., 2007). Meat products may be contaminated with microorganisms from meat handlers, which carry pathogenic microorganisms during the processes of manufacturing, packing and marketing. Furthermore, improper cooking, refrigeration or storage may lead to meat borne illness.

Microbial contamination of fresh meat has important implications for food safety and product shelf-life (Erdogrul, 2005). Therefore, contaminated raw meat is one of the main sources of food borne illnesses (Bhandare *et al*., 2007; Podpecan *et al*., 2007).

The Enterobacteriaceae are the most challenging bacterial contaminants for raw and processed meat products worldwide. In such *Salmonella*, *Klebsiella*, *Proteus*, *Yersinia* and *Escherichia* are the most predominant species in all food poisoning cases, where these have been linked to number of cases for human illness associated with meat products (Mohammed, 2011).

Escherichia coli are part of the normal large-bowel flora of the human and animal's intestinal tract. Generally, *E. coli* strains are in the human bowel and live as commensals. However, few strains are pathogenic and cause distinct diarrhea syndromes (Wong, 2009). The classification of pathogenic *E. coli* is based on their unique virulence and antigenic factors. This can only be identified by traits that are mostly implicated from water and food borne diseases outbreaks.

E. coli was first incriminated as an enteropathogen in 1945, responsible for an outbreak of infantile diarrhea. Enteropathogenic *E. coli* (EEC) has been associated with diarrhea in developing countries due to poor hygiene. In the developed countries, EEC

has been historically associated with infantile diarrhea, but it was later recognized that adults may also suffer from the illness. *E. coli* are also enterepathogenic in animals (Wasteson *et al*., 1988).

The presence of *E. coli* in food is a matter of concern because some strains may be pathogenic or can be propagated with other pathogenic organisms (Bingöl *et al.,* 2012). Those strains that cause enteric infections are generally called diarrheagenic *E. coli* or Enterohemorrhagic *E. coli* (EHEC) O157:H7, and their pathogenesis is associated with a number of virulence attributes, which may vary according to the path type (Vidal *et al*., 2005; Xiaodong *et al*., 2010).

E. coli O157:H7 serotypes are identified as enterohemorrhagic *E. coli* and are acknowledged as major pathogens causing food borne diseases in human. Human and bovine *E. coli* O157:H7 elaborate two potent phage encoded cytotoxins known as Shiga-toxins 1 and 2 (*stx1* and *stx2*) or verotoxins 1 and 2 (VT1 and VT2) (EFSA, 2007; Jamshidi *et al*., 2008). The stx and vtx are important factors in disease pathogenesis and responsible for severe complications, such as hemorrhagic colitis and the hemolytic uremic syndrome (HUS) (O'Loughlin et al., 2001; Karmali et al., 2010). Besides causing significant diseases, EHEC is also major economic problem due to the cost-associated food contaminations.

The Polymerase Chain Reaction (PCR) technique is one of the molecular based detection method commonly used for rapid and reliable diagnosis. It has high sensitivity and specificity. It is necessary to run several individual PCRs with different primer pairs for detecting different diarrheagenic *Escherichia coli* strains. In order to simplify the diagnosis, various multiplex PCR methods have been developed for the simultaneous detection of several specific genes of different diarrheagenic *Escherichia coli* strains. It has been reported that the prevalence of *E. coli* O157:H7 in meat and poultry products with conditional PCR in Northern Iraq was 9 and 4% for meat and poultry products, respectively (Altaee, 2012). In the present study, we performed the *E. coli* O157:H7 prevalence and shiga-toxin genes (*stx1* and *stx2*) using culture method and multiplex PCR system, respectively. The experimental samples were retail red meat and meat products (kebab, shawarma, ground meat and hamburger).

2. LITERATURE REVIEW

2.1. *Escherichia Coli*

Escherichia coli were first described by the German pediatrician Dr. Theodor Escherich as *Bacterium coli commune*. It was present in the stools of each patient he examined when he attempted to isolate the etiological agent of cholera in 1885 (Sussman, 1985). Even today, *E. coli* is the most plentiful facultative anaerobic specie in normal human enteric microbiota of feces (Bettelheim *et al*., 2003). This symbiosis supplies nutritious environment for bacteria and offers essential nutrients to the intestinal epithelium. It also supports healthy immune responses in the host. In addition, *E. coli* affects the synthesis of vitamin K in the host. Certain *E. coli* strains might also serve as an important factor for inhibition of enteropathogen's growth (Kruis, 2004). Moreover, *E. coli* is also competent of surviving in the environment (water, food) and spreading efficiently (Faiella-Tommasino *et al*., 2002).

E. coli is Gram-negative, non-sporulating facultative anaerobic rod-shaped, possessing both respiratory and fermentative metabolism pathways (Wang, 2011). Most *E. coli* strains are motile with peritrichous flagella, while a number of non-motile variants also exist. *E. coli* appears as colorless, translucent round colony with entire margin and smooth surface on solid culture media.

Generally, *E. coli* strains are regarded as harmless in healthy people and animals. Additionally, it is a good indicator organism reflecting the possible fecal contamination in food and water. It has similar characteristics with other major food-borne pathogens, such as reservoir, transmission route, and growth speed (Wang, 2011). Some strains of *E. coli* are pathogenic and capable of causing diseases ranging from mild diarrhea to lethal complications (Brooks *et al*., 2005).

E. coli was first convicted as an enteropathogen in 1945, and considered to be an outbreak of infantile diarrhea (Wang, 2011). It was documented as a food-borne pathogen in 1971 when imported cheeses contaminated with enteroinvasive strain caused illness in nearly 400 individuals. At least five other food-borne outbreaks were also reported in other countries, such as in England in 1974.

As human pathogen, evidences suggested that it was observed as a cause of infant diarrhea as early as in 1700s. While the meat-borne outbreaks in the United States

occurred in 1982 and 1993, the status of this bacterium as a food-borne pathogen was unquestioned (Jay *et al*., 2005).

The most pathogenic *E. coli* type is Enterohemorrhagic *E. coli* (EHEC) that can be distinguished from other diarrheagenic *E. coli* by its highest incidence in food-borne outbreaks of hemorrhagic colitis (HC) and life-threatening hemolytic uremic syndrome (HUS) in the United States and worldwide (Brooks *et al*., 2005; Pennington, 2010). As nearly, all EHEC strains produce Shiga toxins (encoded by *stx1* or *stx2*). *E. coli* O157:H7 is the most widely recognized serotype among STEC up to date, and causing major food safety and public health concerns (Pennington, 2010).

Generally, the incidence and prevalence of EHEC strains in meat, poultry, and seafood products are highly variable. The first published study for the prevalence of meat EHEC strains showed that tested *E .coli* O157:H7 strain distributed 3.7 (n= 164), 1.5 (n= 264), 1.5 (n= 263) and 2.0% (n= 205) of the surveyed beef, pork, poultry, and lamb samples, respectively (Doyle and Shoeni, 1987).

Most *E. coli* strains as well as *E. coli* O157:H7 can survive below a broad range of temperatures between 4ºC and 46ºC, with the finest temperature usually about 37ºC. Studies on heat resistance of *E. coli* O157:H7 in ground beef exposed that it is thermal sensitive under high temperature conditions. Heating is generally adopted in food industry and clinical settings as an easy and effective treatment for elimination (Wang, 2011). *E. coli* O157:H7 can proliferate over a wide range of pH values as well, though its acid-resistant ability varies according to many intrinsic and extrinsic factors, including genetic profiles, strain serotype, food type, acid type, and environmental conditions. For instance, *E. coli* O157:H7 can live in fermented sausage (pH 4.5) for up to 2 months at 4ºC (Glass *et al*., 1992), and in apple cider (pH 3.6-4.0) for 10 to 31 days at 8ºC (Zhao *et al*., 1993).

2.2. Classification of *Escherichia coli*:

E. coli is a member of the Enterobacteriaceae family and widely used as an indicator of the hygienic state of fresh foods. (Holt *et al.,* 1994). Based on disease syndromes, certain characteristics and serological groupings, five virulence groups of *E. coli* are recognized. These are Enteroaggregative (EAggEC), strain serotype (EHEC), Enteroinvasive (EIEC), Enteropathogenic (EPEC), and Enterotoxigenic (ETEC). Table 2.1 describes the type of disease caused by different groups (Murray *et al*., 2009).

Table 2.1. Virulence groups and diseases of *E. coli*

Organism	Site of action	Disease	pathogenesis
Enterotoxigenic *E. coli* (ETEC)	Small intestine	Traveler's diarrhea; infant diarrhea in developing countries; watery diarrhea, vomiting cramps, low grade fever	Plasmid-mediates, heat-stable and /or heat-labile enterotoxins
Enteropathogenic *E. coli* (EPEC)	Small intestine	Infant diarrhea in undeveloped countries; watery diarrhea and vomiting, non-bloody stools	Plasmid-mediated A/E histopathology with disruption of normal microvillus structure
Enteroaggregative *E. coli* (EAEC)	Small intestine	Infant diarrhea in undeveloped countries; traveler's diarrhea; persistent watery diarrhea with vomiting, dehydration, and low-grade fever	Plasmid-mediated aggregative adherence of rods ("stacked bricks") with shortening of microvilli, mononuclear infiltration, and hemorrhage; decreased fluid absorption
Enterohemmorrhic *E. coli* (EHEC)	Large intestine	Initial watery diarrhea followed by grossly bloody diarrhea (Hemorrhagic colitis) with abdominal cramps; little or no fever; may progress hemolytic uremic syndrome (HUS).	Mediated by cytogenic shiga toxin (stx-1, stx-2), which disrupt protein synthesis; A/E lesions with destruction of intestinal microvilli, resulting in decreased absorption
Enteroivasive *E. coli* (EIEC)	Large intestine	Disease in developing countries; fever, watery diarrhea; may progress to dysentery with scant, bloody stools.	Plasmid-mediated invasion and destruction of epithelial cells lining colon.

2.3 Serotype of *E. coli*

The classification of the *E. coli* strains is based on the epidemiologic analysis. The first evidence shows that certain serological types of *E. coli* were associated with the epidemics of enteritis during 1945-1950 (Orskov and Orskov 1984). It was recognized that *E. coli* express three principal antigens (O, K, and H). Identification of these *E. coli* surface structures is related to components of somatic, lipopolysaccharide (LPS)-associated O-polysaccharide (O-antigen), capsular polysaccharide (K), and flagellar (H)-antigen. The O grouping can be performed in many clinical laboratories, whereas the H antigen can be determined in a limited number of laboratories (Wain *et al*., 2001). However, the K antigens are even less amount (Scheutz *et al*., 2004). Currently, there are 176 distinct *E. coli* O groups available (Scheutz *et al*., 2004, Olesen *et al*., 2005).

Serotypes known to contain EHEC include *E. coli* O157:H7. The non-motile organism of *E. coli* O157:H- and members of other serogroups include O26, O103, O111, O145, O91, O104, O113, O117, O118, O121, and O128. *E. coli* O157:H- is closely related to *E. coli* O157:H7, but it is not simply a nonmotile version of this organism. It also has a distinctive combination of phenotypic and virulence features (OIE, 2009).

The somatic (O) antigens are polymorphic outer part of LPS, which encompass the repeats of an O unit having two to seven sugars. Each strain expresses only a particular O-antigen form (Thomson, 2001, Feng *et al*., 2005). The O antigens are heat stable, unaffected by heating for 2.5 hrs at 100C°. They are alcohol resistant, with a standing treatment of 96% ethanol at 37C° for 4 hrs. They are also unaffected by 0.2% of formaldehyde (Eklund, 2005).

Strains of *E. coli* posses acidic polysaccharide capsule (K antigens). These are assumed to be virulence factors and thought to act by protecting the cell against the bactericidal action and phagocytes. Although 70 different K antigens have been identified in *E. coli,* only a few are frequently found and associated with invasive strain.

The H antigen of *E. coli* is specified by a single structural subunit (flagellin) encoded by the *fliC* gene. For the species as a whole, there are 56 recognized antigenic types (H types) and among those *E. coli* strains are motile and possess flagella. The flagellum of the bacteria has a complex structure consisting of three main structural

regions. These are basal body, the hook, and the filament (Macnab, 1992). The variability of the H-antigen is associated with the flagellin amino acid sequence and its structural gene (*fli*C). Several sequences of the *fli*C gene have been published (Schoenhals and Whitfield, 1993). The significant sequence conservation of distal parts of *fli*C allows the easy amplification of this gene. Restriction of the amplified flagellar genes has been used for the identification of H-types in different bacteria. Non-motile strains generally possess the structural gene but are unable to build up a functional flagellum. *E. coli* H-type 7 has been determined by limitation of the amplified flagellin gene to facilitate the identification of enterohemorrhagic serotype O157:H7 whether it is motile or non-motile (NM) (Abbadi and Strockbine, 2007).

2.4 Enterohemorrhagic *E. coli* EHEC O157:H7

EHEC was first discovered in 1977 by the production of cytotoxin and verotoxin (VT), which led to these pathogens called verocytotoxigenic *E. coli* (VTEC) (Konowalchuk *et al*., 1977). Serotype O157:H7 is the most known serotype of the Enterohemorrhagic *Escherichia coli* (EHEC). It is recognized worldwide as zoonotic pathogen responsible for the majority of severe cases of human (EHEC) disease (Rahimi *et al*., 2012a). In the United States, it is estimated that these bacteria cause 73,000 infections and 60 deaths each year. In addition, there are more than 30 countries where the *E. coli* O157:H7 infections are responsible for human cases. EHEC disease is most common in warm months, and the highest incidence is in the children younger than 5 years old (OIE, 2009).

Many outbreaks of the disease are linked to the consumption of beef, because the main source of infection is cattle (Jamshidi *et al*., 2008). Many studies reported that the occurrence rates of *E. coli* O157 or *E. coli* O157:H7 in ground beef meat and other ruminants ranged from 0.1 to 50% (Rahimi *et al*., 2012a; Abong'o and Momba, 2009; Cadrirci and Siriken, 2010). Several authors suggested that the *E. coli* O157:H7 may be common in livestock based foods (Harris *et al*. 1985; Martin *et al*. 1986; Borezyk *et al*. 1987; Doyle and Schoeni, 1987; Orskov *et al*. 1987; Hocken *et al*. 1988; Mabrouk, 2001)

2.5 Shiga Toxin and Verotoxins Taxonomy

Kiosha Shiga described the agent of epidemic bacterial dysentery, Shigella dysenteriae type 1 (Shiga's bacillus) in 1898. Later it was found that Shiga's bacillus produce Shiga toxins. Keusch et al. (1972) found that Stx alone caused fluid accumulation and enteritis in rabbit intestines, revealing that Stx can contribute to bloody diarrhea. Konowalchuck et al. (1977) showed a critical finding that certain diarrheagenic *E. coli* stains create a cytotoxin that can kill Vero cells (cells derived from the kidney epithelial cells of the African green monkey). Therefore, the name of verotoxin was come up. O'Brien and Leveck (1983) reported that a Shiga-like toxin was produced by the *E. coli* O157:H7 that caused an outbreak of hemorrhagic colitis in the United States. As a result, the pathway researches on shiga and verotoxins were combined. Following to these significant findings (Leeper, 2009), two main categories of Shiga toxins have been distinguished. *E. coli* shiga toxin 1 (stx1) is almost identical to the Shiga toxin of *Shigella dysenteriae* in amino acid sequence. The Shiga toxin 2 (stx2) is less related to the Shiga toxin of *Shigella* and not neutralized by antibodies of either stx1 or Shiga toxin of *S. dysenteriae* (Boerlin et al., 1999).

Shiga-Like Toxin 1 also called Verotoxin 1 possesses the same subunit structure as shiga toxin (Noda *et al*., 1987). VT1 consists of two subunits (A and B) which have molecular weights of 29.000 to 31.000 and 5.000 to 6.000, respectively, and has an isoelectric point of 7.03 (O'Brien and Leveck, 1983). More than 100 *E. coli* serotypes have been reported to produce cytotoxins known as shiga toxin 1 (*stx1*) and shiga toxin 2 (*stx2*) (WHO, 1997).

2.6 Food Poisoning

2.6.1 Bacterial food poisoning

The term of food poisoning refers to acute illnesses caused by the ingestion of foods that contain harmful agents. These agents may be biological or chemical in origin (Quinn *et al.,* 2004). The incidence of food poisoning depends on a specific set of circumstances, of which some or all of the following causes may be present:

1- The food is contaminated with bacteria capable of causing food poisoning from human, animal or environmental sources. Cross-contamination from raw or cooked food can be the source.

2- The bacterium itself must produce toxins or has other virulence factors that cause clinical signs of food poisoning. It must also have the ability of surviving in the food during manufacture, storage, distribution and preparation. This survival is due to poor cooking techniques.
3- The food must be suitable for bacterial multiplication so that the levels of bacteria or bacterial toxins in the food are sufficient to cause food poisoning.
4- Susceptible humans must eat food containing critical levels of bacteria and/ or bacterial toxins (Quinn *et al.,* 2004).

There are two primary types of food related diseases:

1. There are intoxications resulting from the consumption of pre-formed toxins in foods. The responsible bacteria are *Staphylococcus aureus, Bacillus cereus, Clostridium botulinum, Clostridium perfringens,* Enterotoxogenic *Escherichia coli* and some *Streptococci*.

2. There is food-borne infection where bacteria has multiplied in food and ingested in relatively large number. They may also multiply *in vivo* producing enterotoxin in the intestinal tract. The bacteria causing these acute food-borne infections include *Salmonella, Vibrio parahaermolyticus, Campylobacter jejuni, Yersinia entrocolitica, Aeromonas hydrophila, Listeria monocytogens* and *Escherichia coli* (Quinn *et al.,* 2004; Prescott *el al.,* 2005).

2.6.2. *E. coli* O157:H7 related food poisoning

E. coli O157:H7 was first distinguished as a food-borne pathogen in 1982 during the investigation of an outbreak of hemorrhagic colitis (bloody diarrhea) related with the consumption of contaminated hamburgers (Riley, *et al*., 1983). The shiga toxin (*stx*) produced by *E. coli* O157:H7 was identified as the real culprit. Within 10 years after 1982 outbreak, approximately 30 *E. coli* O157:H7 outbreaks were documented in the United States (Griffin and Tauxe, 1991). The actual numbers that occurred were probably much higher because *E. coli* O157:H7 infections did not become a reportable disease (required to be reported to public health authorities) until 1987 (Keene *et al*., 1991). Consequently, the most geographically concentrated outbreaks would have garnered enough attention to further investigations (Keene *et al*., 1991).

E. coli are bacteria that live in human and animal intestines. Shiga toxin-producing strains of *E. coli*, or STECs, are responsible for most food-related *E. coli* infections. *E. coli* O157:H7 and other STECs like *E. coli* (O145 and O121:H19) produce a toxin called Shiga toxin, which causes illness in humans. *E. coli* bacteria do not make illness in animals such as livestock and deer, which harbor the bacteria in their intestines.

Meat is contaminated during slaughter, when infected animal intestines or feces are contacted with the carcass. Ground or mechanically tenderized meats are considered riskier than intact cuts of meat because *E. coli* bacteria can be mixed throughout the meat in the grinding process or during tenderization (Jay *et al*., 2005). So that any food contaminated with *E. coli* through cross-contamination is becoming at risk. One can also get *E. coli* bacteria from feces of infected animals or people. The breakdown sources of *E. coli* bacteria from 1998-2007 were as follows: food (69%), water (18%), animals or their environment (8%), person-to-person (6%) (Jay *et al*., 2005).

The CDC has estimated that 85% of *E. coli* O157:H7 infections are food-borne origin (Mead and Griffin, 1999). Actually, consumption of any food or beverage that becomes contaminated by animal manure (especially cattle) can result in contracting the disease. Foods that have been identified as sources of contamination include ground beef, sausages and non-cooked dried salami (Cody *et al*., 1999; Marler *et al*., 2011).

Table 2.2. Studies of *E. coli* O157:H7 occurrence from meat (WHO, 2011)

Subject	Country	Occurrence of STEC	Reference
Retail meat and meat products, survey of fresh and processed meat products from supermarkets and butchers shops	Netherlands	O157 STEC (VTEC) isolated from (1.1) raw minced beef (0.5) raw minced beef and pork mixed. (0.3) cooked or ready-to-eat meats	Heuvelink *et al.,* 1999
Healthy cattle: rectal swabs from healthy animals from 10 dairy farms (n= 121) and 4 beef farms (n= 60) and 2 samplings from 1 slaughterhouse (n= 16)	Brazil	(53) beef cattle positive for Shiga toxin gene sequences (1.5) *E. coli* O157:H7 isolated 10 non-O157 Shiga toxin-producing *E. coli* also isolated.	Cerqueira *et al.*, 1999
Cattle: Slaughter house samples (fecal samples from rectum after evisceration)	Denmark	(22.5) fecal samples positive for *E. coli* O157, (15.5) of which produced verocytotoxin.	Boel, 2000
Meat products from butchers shops Raw beef products Mixed meat products	UK	(1.1) (0.8) Positive *E. coli* O157, verotoxin positive and carried the *eae*A gene	Chapman *et al.*, 2000

2.6.3 Epidemiology

2.6.3.1. Incidences of STEC and *E. coli* O157:H7

The National Laboratory of Enteric Pathogens (NLEP), Laboratory Center for Disease Control and Health in Canada have been collecting information on STEC incidence since 1970's. They have defined an outbreak as two or more linked cases (Spika *et al.*, 1996). From 1993 to 1995, *E. coli* O157:H7 was the most commonly isolated STEC (93%) identified by the NLEP. The incidence of *E. coli* O157:H7 in the population of Canada from 1990 to 1999 ranged from 4.1 to 7.1/100000 with an average of 1407 cases reported yearly (Hallewell, 2005)

2.6.3.2 Outbreaks of *E. coli* O157:H7 and sources of bacteria

E. coli O157:H7 was first investigated after several outbreaks occurred with consumption of contaminated hamburgers in Oregon and Michigan in 1982 (Wells *et al*., 1983; Riley *et al*., 1983). The *E. coli* O157:H7 strain EDL933 was isolated from a Michigan originated ground beef outbreak. After that the entire genome sequence of this pathogen was determined (Perna *et al*., 2001). Several large outbreaks of *E. coli* O157:H7 occurred in Japan between May and December of 1996. One of the largest outbreaks occurred in Sakai City of Japan in July 1996 where 6000 individuals were infected with gastrointestinal illness, and 1000 people were hospitalized. Among the hospitalized ones, there were 100 cases of HUS and 3 deaths. Many of the victims were school children and the source of the infection was believed to be white radish sprouts served at a school cafeteria (Watanabe *et al*., 1996; Michino *et al*., 1999).

The EHEC strains are found generally in the gastrointestinal tracts of cattle, sheep and goats but also found in swine, cats and dogs. They can be transmitted to humans via contaminated food, direct contact with animals or person to person spread in human populations (Beutin *et al*., 1995; Smith *et al*., 1996; Koch *et al*., 2001). Ground beef is the most common form of contamination in North America. The outbreaks have also been linked to improper cooking of meats such as roast beef, salami and venison. The most contamination occurs during slaughter and meat processing (Griffin, 1996). The EHEC can also be spread to vegetables, fruits and other plants through irrigation water or soil contamination with fecal material (Meng and Doyle, 1996; Besser *et al*., 1999).

The most important source of human infections is cattle (beef, dairy products, bovine fecal contamination). Data on the prevalence of STEC O157 and non-O157 vary widely for both dairy cattle (0.4-74%) and beef cattle (2.1-70.1%) among different countries. Cattle often carry multiple serotypes. Some of them do not appear to be high risk for humans since they do not express any important virulence factors. The STEC are not deemed pathogenic to ruminants, except when infections occur in young animals before weaning (involved in neonatal diarrhea) (Blanco *et al*., 2003). A possible source of infection for cattle is food or drink contamination with feces of infected animals (Azucena *et al*., 2011).

2.6.4 Clinical signs and pathogenesis

The low infectious dose of EHEC bacterial (1-100 cells) strains from sero-group of O157 have been reported to cause infection (Griffin, 1998; Jaeger and Acheson, 2000). This infectious dose enables the spreading of this pathogen via food, water or animal transmission. Person to person transmission of EHEC bacteria has been also reported (Bell, 2002). The high mortality rate related to EHEC infection has differentiated this organism from the infections caused by EPEC, ETEC, and EAEC (Law, 2000). Humans can be infected asymptomatically or they may develop watery diarrhea caused by EHEC ranges from mild, uncomplicated diarrhea to hemorrhagic colitis with severe abdominal pain and bloody diarrhea. Initially, diarrhea with abdominal pain develops in patient after 3 to 4 days of incubation. Vomiting is observed approximately in half of the patients, but high fever is generally absent. Within 2 days of onset, disease in 30 to 65% of the patients progresses to bloody diarrhea with severe abdominal pain. Complete resolution of symptoms typically occurs after 4 to 10 days in most untreated patients. Hemolytic Uremic Syndrome (HUS), a disorder characterized by acute renal failure, thrombocytopenia and microangiopathic hemolytic anemia, is a complication in 5 to 10% of the infected children younger than 10 years-old. Resolution of symptoms occurs in uncomplicated disease after 4 to 10 days in most untreated patients; however, death can occur in 3 to 5% of patients with HUS. The severe symptoms (renal impairment, hypertension, CNS manifestations) can also occur in 30% of HUS patients.

The dose of EHEC is very low to cause infections (1 to 100 CFU). This is a much lower dose than for most of the other pathogens in the intestines (Paton and

Paton, 1998a). The ability of adhering to intestinal epithelial cells and colonizing in the human gut are the most main determinants of EHEC causing disease in humans. The pathogenesis involves the organization of bacteria in the gut, where it has to compete for space and nutrients with other microorganisms of the normal intestinal micro-flora. The most important virulence characteristic of the organism, once established, is its ability to produce 1 or more phage encoded verocytotoxins (VT1 and VT2) (Paton and Paton, 1998a). Toxin production mediates both local and systemic disease. Local intestinal effects cause the development of bloody diarrhea when the toxin internalizes the cells of the gut where it blocks the cellular protein synthesis and may lead to apoptosis. The HUS outcomes from micro vascular disease when the toxins enter the blood stream and bind to the receptors of endothelial cells abundant in kidneys and brain (Nataro and Kaper, 1998).

In addition to these toxins, the presence of eaeA gene encoding 'intimin' protein improves the virulence of STEC causing attachment to the intestinal epithelial cells. Moreover EHEC-hlyA gene encoding enterohaemolysin has synergistic effect on virulence (Mohd *et al*., 2013).

2.7 Route of Transmission

The transmission of STEC may be foodborne, waterborne, or during direct contact with infected persons, animals, or surfaces (UDOH, 2011). The routes of STEC transmission are summarized in Figure 2.1. Outbreaks and sporadic cases of STEC infections have been generally associated with foodborne transmission. The CDC estimates that STEC contaminated food sources cause 68% of O157:H7 illnesses and 82% of non-O157 infections (Pihkala, *et al*., 2011). The STEC illness is commonly linked to consumption of undercooked contaminated meat, particularly ground beef (Pihkala, *et al*., 2011). The US beef industry has recalled over 23 million pounds of STEC contaminated beef since 2006 (Vu, 2012).

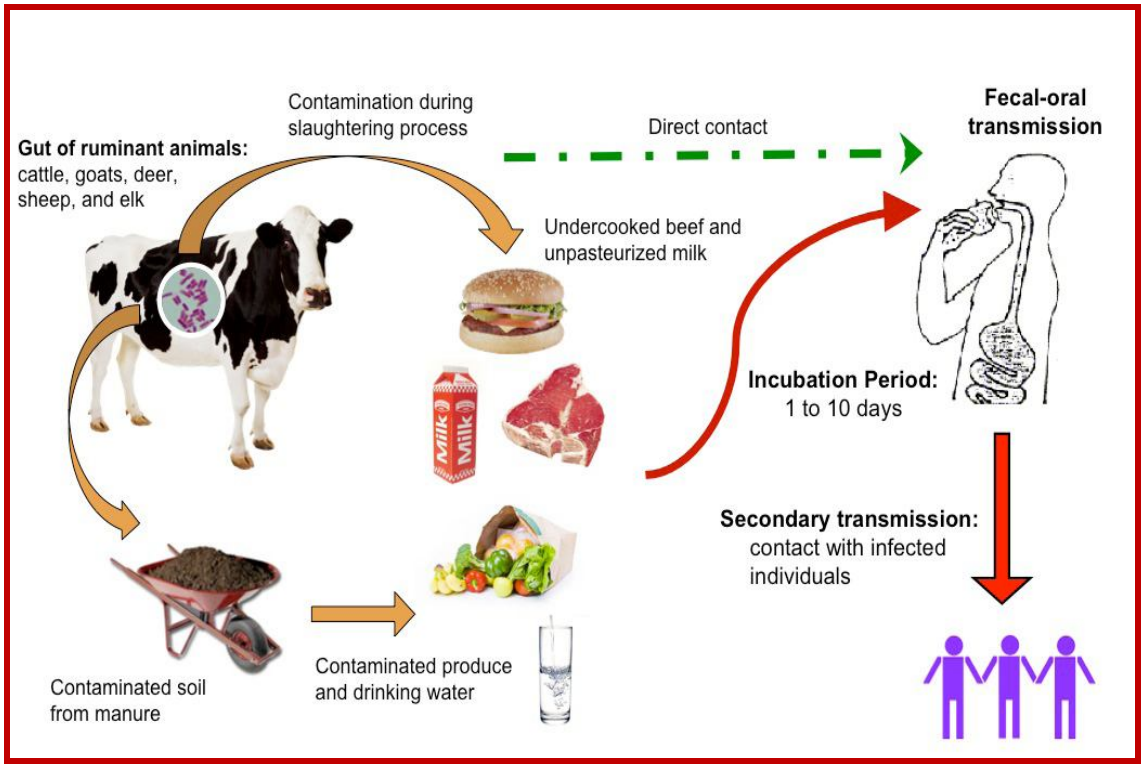

Figure 2.1. STEC Routes of Transmission (Vu, 2012).

A study reported the incidence rate of STEC O157 in ground beef ranged from 0.1 to 54.2% (Hussein, 2006). However, it is important to note that aside from beef production contamination, STEC infections can also be spread through food preparation (CDC, 2011). This may include cross contamination from raw beef juices dripping on to other foods or from reusing utensils that have touched raw meat (CDC, 2011). In addition to beef, other food sources have been bonded to STEC infection including contaminated products, such as spinach, sprouts, lettuce, fruits, and unpasteurized milk or cheeses (Hussein, 2006). Secondary transmission of STEC (spread from person to person) has also been well documented in sporadic cases and during outbreaks (Paton and Paton, 1998b; Pihkala, *et al*., 2011). Secondary transmission accounted for 19% of the cases in a study estimating O157:H7 outbreaks (Pihkala, *et al*., 2011). In secondary transmission, fecal shedding of STEC plays a significant role and found to occur in both symptomatic and asymptomatic patients (Pihkala, *et al*., 2011). An analysis of O157 infections showed that a mild duration of fecal shedding in STEC was two to three weeks (Paton and Paton, 1998b). Also the study found that 13% of patients shedding STEC were over a month, and the maximum duration of shedding was 124 days (Paton and Paton, 1998b). Information about patient risk factors, such as food history,

environmental and animal exposure, and contacting with infected individuals, play a very important role during the case and outbreak investigations (Vu, 2012).

2.8 Treatment and Prevention

Treatment of hemorrhagic colitis is supportive and may include fluids and bland diets. Antibiotics are controversial and usually avoided. They do not appear to reduce symptoms, prevent complications or reduce shedding, and they may increase the risk of HUS. Using anti-motility (anti-diarrheal) agents in hemorrhagic colitis seems to increase the risk for developing HUS. Patients with complications may need intensive care including dialysis, transfusion and/or platelet infusion. Patients who develop irreversible kidney failure may require a kidney transplant (OIE, 2009).

Recurrent hand washing, especially before eating or preparing food, and good hygiene are important factors for preventing transmission from animals and their environment. Hand washing facilities should be available in petting zoos and other areas where the public may contact with livestock, and eating and drinking should be avoided at these sites. To protect children and other household members, people who work with animals should keep their work clothing, including shoes, away from the main living areas and launder these items separately. Techniques for decreasing the microbial contamination during slaughter and meat processing can reduce the risk of EHEC from these sources. Screening and controlling programs have been established for EHEC O157:H7 in meat. Consumers should wash their hands, counters, cutting boards, and utensils thoroughly after they contact with raw meat for avoiding cross-contamination during food preparation. Raw meat, meat products, unpasteurized milk or other dairy products and unpasteurized juices should be cooked or processed thoroughly for killing *E. coli* (OIE, 2009).

2.9 Importance of Pathogen Detection in Food

Microorganisms causing food spoilage and food-borne illness have always elevated concerns of food quality and safety. Efficient detection methods with high sensitivity and speed may be a powerful tool for identifying the problem source and outlining solutions. Recently, numerous technologies have been developed to enumerate the groups of microorganisms and detect the specific pathogens and toxins in foods (Ge and Meng, 2009). Traditional methods generally rely on appropriate selective and differential agar to detect the specific microorganism in food. Although the agar

technique has the highest sensitivity among all detection methods, the progress has been made to improve the formulation of the enrichment agar and differential medium. This method is still time-consuming and labor intensive. Advanced techniques including convenience, antibody, and molecular-based assays have successfully reduced the process to several hours with high specificity. In addition, such assays may also serve as the effective tool to provide the comprehensive genetic or metabolic profiles of the organism.

2.9.1 Techniques used for the study of *E. coli* O157:H7 in food samples

Techniques that are used to test *E. coli* O157:H7 in food samples are shown in Table 2.3. (Prager *et al.*, 2005).

Table 2.3. Methods for the detection of EHEC bacteria

Diagnostic Principle	**Test method**
Phenotypic methods	
Detection of *E. coli* O26, O91, O111, O103,O111, O145, O157 antigens in bacterial culture	Latex agglutination, immunomagnetic separation (IMS)
Selective isolation of *E. coli* O157 bacteria based on color reaction on agar plate	Chromogenic agars ECD (*Escherichia coli* direct)
Detection of enterohemolysin production of bacterial culture on agar plate	Enterohemolysin agar HC agar
Screening for Stx toxin or O157 antigen in bacterial culture or stool	Enzyme immuno-assays (EIA)
Screening and separate detection of Stx1 and Stx2 toxins in bacterial culture or stool	Reverse passive latex agglutination (RPLA)
Genotypic methods	

Detection of genes encoding Stx, O specific regions or accessory virulence factors in bacterial culture or stool	PCR, PCR combined with restriction fragment length polymorphism (PCR-RFLP),PCR combined with hybridization, colony hybridization, immunoblotting

2.9.1.1 Culture-based methods

The first step for isolation of *E. coli* O157:H7 is the sample enrichment. Modified *E. coli* (mEC) broth supplemented with novobiocin has been reported to increase the selection of *E. coli* O157:H7 (Oberst *et al.,* 1998). Besides the modified *E. coli* (mEC) broth, single-tube screening medium for *E. coli* O157: H7, which contains sorbitol and flagella antigen H7antisera, is also an alternative media for the isolation of *E. coli* O157:H7. Both GN Broth Hajna and tryptic soy broth supplemented with cefixime and vancomycin have been used with success for the isolation of these bacteria (Karch *et al.,* 1996). Tryptic soy broth (TSB) supplemented with phosphate buffered saline (PBS) in combination with bile salt (pH 7.5) and novobiocin have shown positive results (Doyle and Schoeni, 1984). Buffered peptone water made into a selective enrichment broth by the addition of vancomycin, cefixime, and cefsulodin have also been used in the enrichment step of *E. coli* O157:H7 isolation (Malihe and Kadir, 2000). After the enrichment step, the enriched samples are plated onto selective agar media. The agar media commonly used for the isolation of *E. coli* O157:H7 include Sorbitol MacConkey Agar (SMAC) (March and Ratnam, 1986), Sorbitol MacConkey Agar supplemented with cefixime and tellurite (CTSMAC) (Elder *et al.,* 2000; Bopp *et al.,* 2003) and Enterohaemolysin Agar (Beutin *et al.,* 1989). Rainbow Agar O157 (Manafi and Kremsmaier, 2001; Ingrid *et al.,* 2003) and Fluorocult *E. coli* O157 Agar (Szabo *et al*., 1986) have also been used. The selectivity of SMAC Agar has also been improved by the addition of cefiximerhamnose resulting in the formulation of Cefixime-Rhamnose-Sorbitol MacConkey (CRSMAC) Agar (Chapman *et al.,* 2000) and 4-methylumbelliferyl-b-D-glucuronide (MSAMUG) Agar (Szabo *et al.,* 1986). The above media contain sorbitol replacing the lactose of the standard MacConkey medium. Unlike other *E. coli* strains, isolates of serotype O157:H7 do not ferment D-sorbitol within 24 h since they are lack of glucuronidase activity and do not grow at 45.5°C.

Sorbitol non-fermenting colonies, indicative of the typical *E. coli* O157:H7, are normally colorless on this medium. However, many organisms other than *E. coli* O157:H7, especially other serogroups of *E. coli* and *Proteus spp.* may not ferment sorbitol, and thus may be confused with *E. coli* O157:H7. Hemorrhagic Colitis Agar is another medium that has been used. It is a differential media and has been used in direct plating to isolate O157:H7 from foods (Gülmez *et al.,* 2004). It includes an enrichment step and is a new method developed sequel to food borne outbreaks (Sanderson *et al.,* 1995). Other media have also been developed for the isolation of *E. coli* O157:H7. These include Biosynth Culture Media O157:H7, (BCM O157:H7) and CHROM agar O157 (Manafi and Kremsmaier, 2001). While *E. coli* O157:H7 can grow with blue-black colonies, *E. coli* non-O157 can grow with green-yellow colonies on Biosynth Agar (Restaino, 1996; Restaino *et al.,* 1999).

2.9.1.2 Biochemical reactions

Further confirmation of *E. coli* is accomplished by using series biochemical tests. The more sensitive the result the more biochemical tests that are performed on a suspect colony.

The VTEC O157:H7 differs from the other members of *E. coli* genus in that it does not ferment sorbitol and is β-glucuronidase-negative. (Wells *et al*., 1983; March and Ratnam, 1986). They are facultative anaerobic, oxidase-negative and usually produce gas from glucose. Some strains show atypical biochemistry. For instance, they are indole-negative, non lactose-fermenting or urea-positive. Some VTEC O157 strains have been found to be fermenting sorbitol and be β-glucuronidase-positive. Further differential testing may be accomplished by examining the presence of bacterial urease, citrate, indole, methyl red and Voges Proskauer (Songer and Post, 2005).

Multi-test strip identification systems are available to confirm the identification of Enterobacteriacae for increasing accuracy. Some of these systems require overnight incubation (API 20E, Biomerieaux) while others provide rapid results as fast as in 4 hours (RapID one system, Remel). The laboratory procedures for detection and identification of *E. coli* by conventional methods are laborious and time consuming (Kumar *et al*., 2008). Some other methods have been reported for the detection of food-borne pathogens, such as chromogenic media, bacterial agglutination, fluorescent antibody technique, enzyme linked immunosorbent assay (ELISA), immunomagnetic

assay (Brigmon *et al*., 1992; D' Souza and Jatkus, 2003), PCR (Whyte *et al*., 2002) and complement fixation test (Quinn *et al*., 2004).

2.9.1.3 Molecular-based methods

The breakthrough on DNA amplification theory and subsequent discovery of highly efficient and thermal-tolerant DNA polymerase enable the emergence and evolvement of molecular-based pathogen detection assay. Methods such as PCR and real-time PCR are widely used in detection, diagnosis, genetic characterization, and other biological research area due to their high speed, sensitivity, specificity and reproducibility. PCR can exponentially generate thousands to millions of copies of a particular DNA sequence *in vitro* across several orders of magnitude (Mullis *et al*., 1986).

This method relies on two key components, primer and DNA polymerase, along with the repeated cycles of heating and cooling of the reaction to enable the selective and repeated DNA amplification. Normally, a gel electrophoresis is coupled to examine the PCR products under the UV light; however, this drawback has been overcome by the development of real-time PCR. Meanwhile, it is easy to interpret the PCR results compared to the traditional culture-based methods, where the non differentiable color changes may occur. The other desirable feature of PCR is that more than one pair of primers can be incorporated in the single test therefore it is possible to simultaneously detect two or three targets in one run (Claustres *et al*., 1989). It is noteworthy that some inhibitors in food matrix may interfere with the PCR by affecting the DNA polymerase activity. So that, it is critical to design the internal control and separate the target organism from enrichment culture completely (Hoorfar *et al*., 2003). Real-time PCR, also named as quantitative PCR (Q-PCR or qPCR), is an advanced PCR assay. It provides instantaneous amplification and detection at the same time. Different from conventional PCR, this fluorescence-based method is using a DNA-binding dye or hybridization probes to quantify the nucleic acid input by measuring the number of thermal cycles required to reach a certain level of product. The identity of tested organism is reflected by the sequence-dependent melting temperature or target-specific probe. It is generally agreed that real-time PCR is more sensitive than traditional one, along with the less running time. Both PCR assays now can detect the live cell after the incorporation of EMA or PMA as a dead DNA eliminating agent (Nocker and Camper,

2006; Wagner *et al*., 2008; Chen *et al*., 2011). Meanwhile, the real-time PCR is unaffordable equipment in small business.

2.9.1.4 Multiplex-PCR

Multiplex PCR is the simultaneous amplification of two or more target sequences (Henegariu *et al*., 2007). The described reaction protocol may change the required modification of the uniplex PCR design due to the nature of the reaction. It includes extension temperature and time, annealing temperature and time, primer concentration, and dNTP concentration (Henegariu *et al*., 2007). Therefore, multiplex PCR must be optimized from standard PCR conditions to give the best chances for amplification of multiple targets and clear product visualization. Furthermore, the false positive results may appear in multiplex PCR due to the increased use of primer pairs. This problem must be counteracted by designing the primer pairs with no amplify sequences along with high specificity (Chamberlain and Chamberlain 1994).

Multiplex PCR methods for EHEC strains are numerous and having variety of virulence and pathogenesis markers. Pollard *et al*. (1990) used four primers based on the sequences of SLT-1 and 2 and tested them on 40 SLT producing *E. coli* strains. However, two strains that produced SLT-2 variant scored as positive for SLT production by cytotoxicity assay also gave positive results with multiplex-PCR. Gannon *et al*. (1992) also described a method for the detection of SLT genes in *E. coli* with obtaining the final sensitivity of 1 CFU/g. Cebula *et al*. (1995) used primers for *uidA* gene, encoding β-glucruonidase, and the SLT's in a mismatch multiplex PCR. Multiplex PCR methods for the detection of *eaeA* gene and conserved regions of the SLT's have also been devised to control against the false positive results that may occur with using single primer pair (Fratamico *et al*.,1995; Reischl *et al*., 2002). It was demonstrated that a multiplex-PCR for *E. coli* O157:H7 could be differentiated from *E. coli* O55 strains (Meng *et al*., 1997). Gannon *et al*. (1997) also amplified a portion of the flagellar (*fliC*) gene along with SLT and *eaeA* genes. Venkateswaran *et al*. (1997) used a filtration technique to remove PCR-inhibitory food particles prior to a multiplex-PCR.

3. MATERIALS AND METHODS

One hundred eighty samples of meat products (50 kebabs, 50 ground meats, 40 shawarma, and 40 hamburgers) were collected from the markets and slaughter units in Duhok province of Iraq. The sampling period was from July to September 2013. For to study the prevalence of *Escherichia coli* O157:H7and shiga-toxin genes (*stx1* and *stx2*) in *E. coli* O157 and non O157 in meats products.

3.1 Materials

3.1.1. Culture media

3.1.1.1. Liquid media

1. Pepton Buffered Water (LabM, UK).
2. Modified Tryptic Soy Broth + Novobiocin. (Merck, Gremany, Oxoid, UK).
3. Brain Heart Infusion Broth (LabM, UK).

3.1.1.2. Solid media

1. Sorbitol MacConkey agar (SMA) with BCIG (LabM, UK).
2. Eosin Methylene Blue (LabM, UK).
3. Haemorrahagic Colitis Agar (Sifin, Germany).
4. Nutrient Agar (LabM, UK).

3.1.1.3. Biochemical solutions and reagents

1. Analytical Profile Index (API 20 E System) (Biomerieux, UK)
2. RapID One System (Remel, USA)
3. RapID one reagent (Remel, USA)
4. Indole reagent
5. Kovac's reagent

3.1.2 Equipment and apparatus

Table 3.1. Equipment and apparatus used in the study

Equipment	Company
Autoclave	Daikyo, Japan
Eppendorf centrifuge	Dragon lab, Germany
Compound Light Microscope	Zeiss, Germany
Centrifuge	Sigma, Germany
Incubator	Prodit, Italy
Gel Electrophoresis apparatus	Ammersham, USA
Hotplate magnetic stirrer	Stuart, UK
Microcentrifuge	Hettich, Germany
Micropipettes	Eppendorf, Germany
Microshaker	Camlab, UK
Oven	Memmert, Germany
pH meter	Hanna, Portugal
Power Supply	Ammersham, USA
Spectrophotometer-U.V. visible	Helious Lnican, UK
Transilluminater (UV. light source)	Ammersham, USA
Thermal reactor	M.W.G.Biotech, UK
Water Distillate	Aquateron, France
Nanodrop	Thermo scientific, Germany
Water bath	Grant, UK
Vortex	Sigma, Germany

3.1.3 Chemicals

Agarose (Biolab product); Boric acid, Glycerol (BHD); Disodium ethylene diamine tetra acetic acid (EDTA), (Sigma-Aldrich); Ethanol 99.9%; Chloroform (α-Alpha); Ethidium Bromid (Ammersham); Tris-Base (Roche).

3.1.4. Sampling

The samples were brought to the laboratory of Duhok Veterinary Directorate directly. The kebabs were collected from Duhok restaurants, ground beef samples were collected from local Duhok markets, shawarma samples were collected from cafeteria and local factories, and hamburger samples were collected from local markets of Duhok (Table3.2.).

Table 3.2. Sampling procedure

Type of samples	Source of sample	No. of Sample
Ground Beef meat	Local Duhok markets	50
Kebab	Restaurants	50
Shawarma	Cafeteria, local factories	40
Hamburger	Local Duhok markets	40
Total		180

3.2. Methods

3.2.1. Bacterial isolation

The samples were brought to the laboratory immediately in ice pack aseptically. Twenty-five g of each sample was homogenized in 225 mL of Modified Tryptic Soy Broth (mTSB) supplemented with novobiocin (20 mg/L) and incubated at 37°C for 18-24h. The enriched broth culture was plated on sorbitol MacConkey agar (SMA with BCIG) and incubated at 37°C for 24 h for isolation of *E. coli* O157:H7. The suspected colonies (colourless, sorbitol negative) were sub-cultured at Eosin Methylene Blue agar at 37C° for 18-24 h. Colonies appearing green metallic sheen color were sub-cultured on Hemorrhagic Colitis (HC) agar for more conformation. This agar was used for the identification of *E. coli* O157. This agar was also used for the differentiation of *E. coli* O157:H7 based on the lack of sorbitol utilization and the absence of β-D-glucuronidase. The colonies of *E. coli* O157:H7 on HC agar appear colorless and sorbitol negative, and MUG negative under UV light. However, some strains are MUG positive under UV light. For these reasons, biochemical tests were applied as shown in Figure 3.1.

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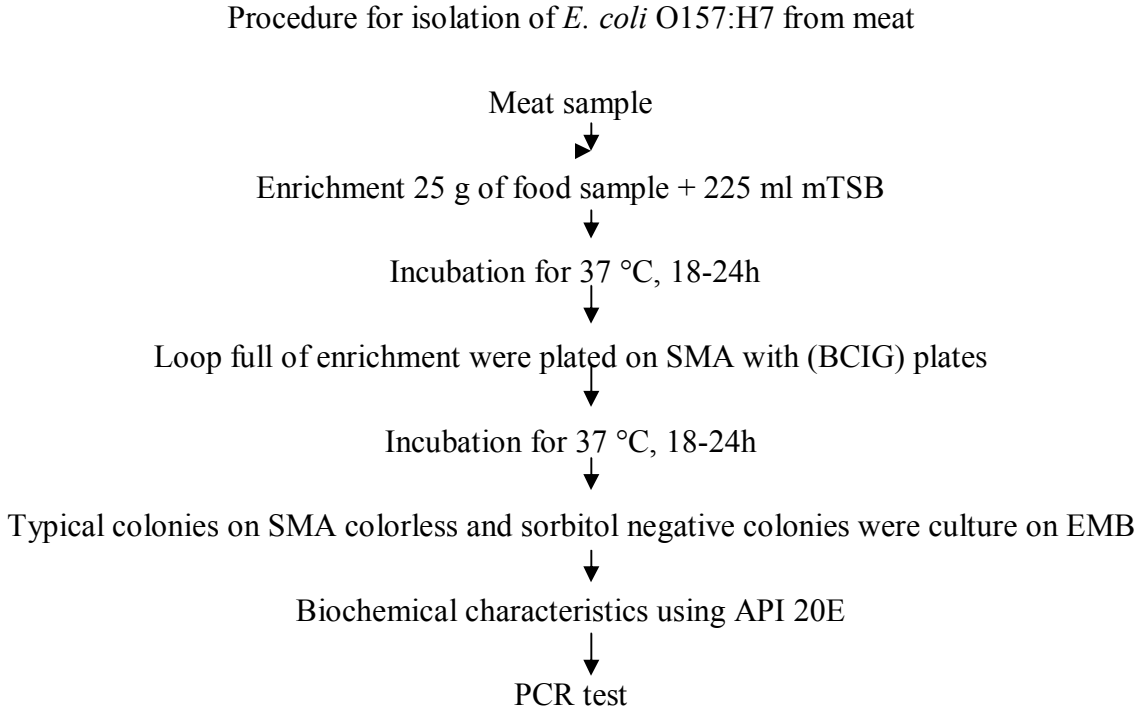

Figure 3.1. The steps involved for performing the method of *E. coli* O157:H7 isolation from meat products

3.3. Morphological and Biochemical Characterization of Isolates

In order to isolate *E. coli*, morphological characters of the positive colonies were considered. These characters were colony (color, shape, margin surface) and cell morphology (shape, arrangement, Gram reaction, and indole and oxidase tests) (Mabrouk, 2001). The biochemical characterization was based on the indole test (API 20 E system, RapID one system). The PCR technique was used for the detection of *stx1* and *stx2* genes.

3.3.1 Biochemical characterization using API 20 E system

The API 20 E system is a miniaturized version of conventional tests. It was used for the identification of the Enterobacteriaceae family and the other gram negative oxidase negative bacilli. This system utilizes a plastic strip with 20 separate compartments. Each compartment consists of a depression, or copula, and a small tube that contain a specific dehydrated medium.

A single, well isolated suspected colony of the bacterium was made into a homogeneous suspension of 5 ml sterile distilled water and mixed with a vortex. The tubes of the API 20 E strip were inoculated with the suspension. The pipette should be held against the side of the tubes when delivering the inoculums for entering bubbles

into the test solution. The API 20 E strip was placed in its incubation box with less water at the bottom for preventing dehydration. It was then incubated at 37 ℃ for 18-24 h.

3.3.2 RapID one system

It is a qualitative micro-method employing conventional and chromogenic substrates for the identification of Enterobacteriaceae and other selected oxidase negative, gram negative bacilli. This system is comprised of RapID one panels and its reagent. Each panel has 18 reaction cavities containing dehydrated reactants that give 19 test scores and the tray allows the simultaneous inoculation of each cavity with the inoculums. Test organism was grown in an Eosin Methylene Blue (EMB) for 18-24 h at 37C° and examined by an oxidase test prior to use in the system. By using inoculation loop sufficiently, the EMB growth was suspended in RapID inoculation fluid (2ml) for achieving inoculums with visual turbidity equal to 2 McFarland standards. Then the suspension was mixed with a vortex. The lid of the panel was peeled back by pulling the tab marked up and to the left. The entire content of the inoculation fluid tube was gently transferred into the upper right-hand corner of the panel by using pipette. Then the resealing of the inoculation port was performed with pressing the tab back in its place. The panel was tilted back away from the reaction cavities at a 45 degree angle. Then the panel was rocked gently from side to side to evenly distribute the inoculums along the rear baffles. The panel was slowly tilted towards to the reaction cavities until the inoculums followed the baffles into the reaction cavities. The inoculated panel was incubated at 37 C° for 4 h. After 4 h of incubation, the lid over the reaction cavities was pulled off and then 2 drops of RapID one reagent was added to cavities of 15 (PRO) through 17 (PYR). Then cavities from 1 (URE) through 18 (ADON) were scored and recorded in the report form by using test codes. After that, 2 drops of RapID One spot indole reagent was added to cavity 18 (ADON/IND), and allowed to 10 seconds for colour development. The code numbers for (+) reaction in each triplet of biochemical reactions were added together. This gave a 7-digit profile number for the bacterium. The genus name can be obtained by entering the 7-digit profile number into the computerized software provided with RapID One kit (Altaee, 2012).

3.4 Molecular Method

3.4.1 Components of PCR

The conventional PCR reaction mix consist of target DNA, two primers, heat-stable DNA polymerase, deoxynucleotide triphosphates (dNTPs including dATP, dCTP, dGTP and dTTP), and a buffer usually containing Mg^{2+}. Primers are short (20-30 base pairs) oligonucleotides of known sequence that are complementary to the two 3-ends of the target DNA (Nadder and Langley, 2001). The specificity of the primers determines the accuracy of the PCR assay as poor quality nucleic acids or non-target background DNA can influence the specific annealing of the primers. This can result in nonspecific amplification and possible misinterpreted results (Weile and Knabbe, 2009).

3.4.2 Preparation of stock solutions for PCR- amplification

All stock solutions were prepared according to Sambrook *et al*., (1989). The tris HCl (1M, pH 8.0) was prepared by dissolving 60.5 g of tris-base in 400 ml of distilled water; pH was adjusted to 8.0 by adding 10 N HCl. The volume was made up to 500 ml by adding distilled water. 0.5 M of EDTA (pH 8.0) was prepared by dissolving 93.6 g of EDTA in 400 ml of distilled water and the pH was adjusted to 8.0 by adding 10 M NaOH. The volume was made up to 500 ml by distilled water.

10XTris-Boric acid-EDTA buffer (TBE) was prepared by dissolving 121.14 g Tris base, and 55 g boric acid added to 40 ml EDTA (0.5 M, pH 8.0) and dissolved in 800 ml distilled water. The pH was adjusted to 7.8 by 10 N HCl. Then the volume was made up to 1 L by adding distilled water.

6X loading buffer (LB) were prepared by dissolving 0.25 g of Bromophenol blue with 30 ml of glycerol in 50 ml of distilled water. The pH was adjusted to 8.0 by using 10 N NaOH; the volume was made up to 100 ml by distilled water and kept in 4 ºC. Tris-

EDTA buffer (TE) was comprised of 1 mM of EDTA and 10 mM of Tris-HCl (pH 8.0). The volume was made up to 100 ml by adding distilled water.

3.4.3 DNA extraction

The DNA of *E. coli* was extracted from the sweep of few colonies grown on Mac Conkey's Agar plates by boiling method. One loop of *E. coli* from agar plates was suspended in 100 ml of sterile de-ionized water in an eppendorf tube and a bacterial suspension was vortexed. The bacterial suspension was boiled at 100 ºC for 10 min and centrifuged at 10,000x *g* for 1 min. The supernatant was used as DNA template for PCR (Nessa *et al*., 2007). Purification of DNA was achieved by using a genomic DNA purification kit (Qiagen, Germany) according to the manufacturer's instruction. The total DNA was measured by Nanodrop (2000).

3.4.4 Primers

Primers were provided by IDT (Tegrated DNA technology) in lyophilized forms and dissolved by de-ionized distill water to produce 10 pmol/µl of final concentration. Four primers were used in the study and their sequences are listed in Table 3.3.

E. coli grown on EMB agar was used for obtaining DNA templates for PCR assay. A whole-cell suspension was prepared by suspending a NSF bacterial colony from EMB agar in sterile distilled water. The bacterial DNA was extracted and purified by using genomic DNA purification kit (Fernentas, Germany).

Table 3.3. Primers of *E. coli* O157:H7, *stx1* and *stx2*

Primer	Sequence (5 –3)	Product size (bp)	Reference
O157	CGGACATCCATGTGATATGG TTGCCTATGTACAGCTAATCC	259	Paton and Paton (1998b)
fliC H7	GCGCTGTCGAGTTCTATCGAGC CAACGGTGACTTTATCGCCATTCC	625	Gannon et al. (1997)
stx1	CGCTGAATGTCGCTCTGC CGTGGTATAGCTACTGTCACC	302	Rahimi et al. 2012a
stx2	CTTCGGTATCCTATTCCCGG CTGCTGTGACAGTGACAAAACGC	516	Rahimi et al 2012a

The PCR reactions were performed in a final volume of 25 µl. It contained 12.5 µl PCR Master Mix (2X) having PCR buffer, dNTPs, Taq DNA polymerase + Mg^{+}, 1 µl of each primer (10 pmoL/µl), 8.5 µl of distilled water and 2 µl of Template DNA (5.4-12 ng/µl).

Thermal cycling was performed using a thermo-cycler (Eppendrof, Germany). The cycling was composed of an initial denaturation step at 95 ºC for 4 min, followed by 35 cycle of denaturation at 95 ºC for 30 s, annealing at 56 ºC for 30 s and extension at 72 ºC for 60 s, a final extension step at 72 Cº for 10 min and a hold step at 4 ºC until the samples were removed as show in Table 3.4.

Table 3.4. PCR conditions for amplifying O157, *fliC* H7, *stx1*and *stx2* genes

Initial denaturation		Denaturation	Annealing	Extension	Final extension
Temperature	95 ºC	95 ºC	56 ºC	72 ºC	72 ºC
Time	4 min	30 sec	30 sec	1 min	10 min
Cycle	1		35		1

3.4.5 Electrophoresis

The amplified PCR products were run on agarose gel (1.5%). The agarose gel was prepared by heat-dissolving 1.5 g of agarose (Invitrogen, Germany) in 100 ml of TBE buffer using a microwave oven. After cooling the agarose, 10 µl of ethidium bromide (Merck, Darmstadt German) was added, the solution was then gently swirled and poured in to a small gel casting tray fitted with a 6 well comb. After the agarose gel was polymerized for 20-30 min, it was transferred to the electrophoresis. The samples were loaded at 12 µl containing 10 µl PCR products plus 2 µl of 6x loading dye (10 mM Tris-HCl, pH 7.6, 0.03% (v/v), bromophenol blue, 0.03% (v/v), xylene xyanol FF, 60% (v/v) glycerol, 60 mM EDTA). As a standard 5 µl molecular marker was used (GeneRuler 1Kb plus DNA, Code SM1331; Fermentas, Germany). The electrophoresis was carried out at 70 Volts with a voltage source in 1x TBE buffer for 1 h. The amplified product was visualized as a single compact band of expected size under UV light and documented by the gel documentation system (SynGene, Gene Genius BioImaging System, and UK; Al-Naqshabandy, 2012).

3.4.6 Multiplex PCR

The multiplex PCR condition was done for the detection of four genes (157, H7, *stx1* and *stx2*) designed by Paton and Paton (1998b). The PCR contained 25 µl of PCR buffer and 1.0 µl of 10 pmol primer *stx*2F, *stx*2R, *stx*1F and *stx*1R, 157 F, 157 R, H7F, and H7R. In addition, 15 µl of sterile ultrapure deionized water and 2.0 µl of 100 ng DNA templates were also used. Amplification was performed in thermal-cycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 2 min, annealing at 57°C for 1 min and polymerization at 72°C for 2 min. Final elongation was at 72°C for 10 min. The amplification products were analyzed by electrophoresis in a 1.0% agarose in 0.5X TBE (0.1 M Tris, 0.1 M Boric acid, and 0.1 mM EDTA) at 90 V for 40 min. Gels were stained with ethidium bromide. The amplified fragments were visualized with UV trans-illuminator (Syngene, USA). The 100 bp DNA ladder (Fermentas) was used as a DNA size marker (Paton and Paton, 1998b).

4. RESULTS AND DISCUSSION

The present study identified the prevalence of *E. coli* O157:H7 and the main virulent genes of *stx1* and *stx2* in meat products by using culture, biochemical, and molecular methods.

4.1 Microbiologic Examination of *Escherichia coli* in Meat Products

The microbiologic results from 180 meat products are presented in Figures 4.1, 4.2, and 4.3 respectively. Colonies were isolated from 48 samples after enrichment on selective media modified tryptic soy broth. The results are presented in Table 4.1.

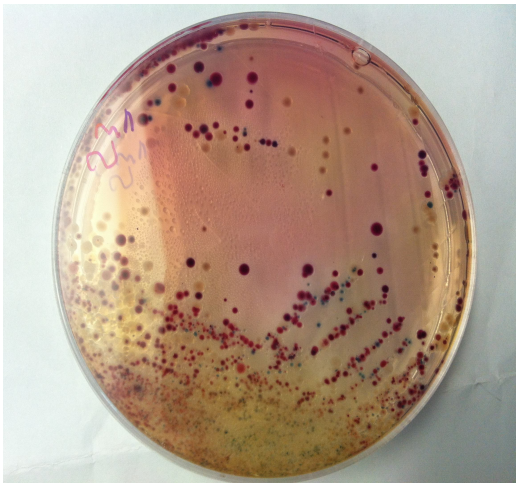

Figure 4.1. Non Sorbitol Fermenting (NSF) Colorless colonies on Sorbitol MacConkey with BCIG agar (SMA)

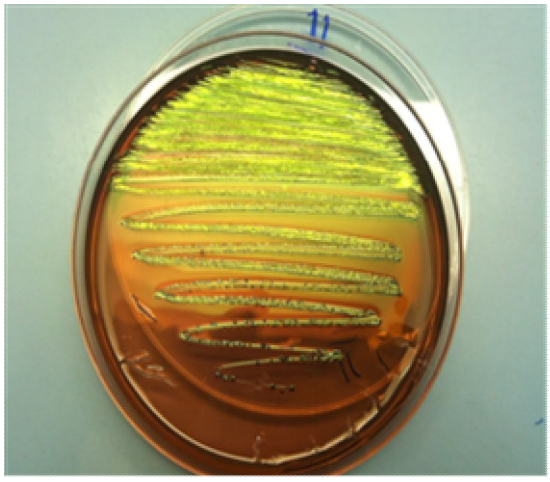

Figure 4.2. Metallic shine colonies on Eosin Methylene Blue (EMB) agar

Figure 4.3. Positive colonies without MUG on Hemolytic colitis (HC) agar

4.2 Biochemical Methods

4.2.1 API 20 E test

The positive samples that have been resulted from previous methods have also been tested by biochemical methods of API 20E test. The code numbers for (+) reaction in each API test were added together. This gave a 7-digit profile number for the bacterium. The positive code of tested *E. coli* isolates by API test was 5040552 as shown in Table 4.2 for the somatic O157 antigen. The results showed that there were 35 positive samples. Table 4.1 shows the results of cultural and biochemical methods.

Table 4.1. The results of cultural and biochemical methods

Product Type	No of sample	Number of positive sample by	
		Culture method	Biochemical method
Ground meat	50	17 (34%)	13 (26%)
Hamburger	40	11 (27.5%)	9 (22.5%)
Shawrma	40	10 (25%)	7 (17.5%)
Kebab	50	9 (18%)	6 (12%)
Total	180	48 (26.6%)	35 (19.4%)

Table 4.2. Biochemical results in API20E system

Test	Symbol	Results
Ortho-nitro-phenyl-galactoside	ONPG	+
Arginine dehydrolase	ADH	-
Lysine decarboxylase	LDC	+
Ornithine decarboxylase	ODC	-
Citrate utilization	CIT	-
Hydrogen sulfide production	H_2S	-
Urease	URE	□
Tryptophan deaminase	TDA	□
Indole	IND	+
Voges-Proskauer	VP	□
Gelatin liquefaction	GEL	□
Glucose fermentation	GLU	-
Mannitol fermentation	MAN	+
Inositol fermentation	INO	□
Sorbitol fermentation	SOR	+
Rhamnose fermentation	RHA	+
Sucrose fermentation	SAC	□
Melibiose	MEL	+
Amygdaline	AMY	□
Arabinose fermentation	ARA	+

4.2.2 RapID one system

The positive samples that have been resulted from culture method have also been tested by biochemical methods of RapID one system. The code numbers for (+) reaction in each triplet RapID one system were added together. This gave a 7-digit profile number for the bacterium. The genus name was obtained by entering the 7-digit profile number into the computerized program provided by RapID One kit. The special code for isolated *E .coli* was 4161011 as shown in Table.4.3.

Table 4.3. Biochemical results in RapID one system

Test code	Reactive ingredient	Results
URE	Urea	_
ADH	Arginine	_
ODC	Ornithine	+
LDC	Lysine	+
TET	Aliphatic thiol	□
LIP	Fatty acid ester	□
KSF	Sugar aldehyde	_
SBL	Sorbitol	+
GUR	nitrophenyle-β, D glucuronide	+
ONPG	nitrophenyl-β, D-galactoside	+
βGLU	nitrophenyl-β, D-glucoside	_
βXYL	nitrophenyl-β, D-xyloside	_
NAG	nitrophenyl-n-acetyl-β, D-glucosaminide	_
MAL	Malonate	_
PRO	Proline-β-naphthylamide	_
GGT	Glutamyl-β- naphthylamide	_
PYR	Pyrrolidonyl-β-naphthylamide	_
ADON	Adonitol	_
IND	Tryptophane	+

4.3 PCR

4.3.1 Concentration of DNA extracts

All the 180 meat samples had a promising DNA concentration (5.4-12 ng/µl) after the extraction process was performed by nanodrop. The DNAs extracted from all samples after culturing on selective agar, were used to detect the *E. coli* O157:H7 and Shiga-toxins of *stx1* and *stx2* using PCR.

4.3.2 PCR results

The PCR results are presented in Table 4.4. Out of 180 samples of meat products, the detected O157 gene (256 bp) was found to be 2 (4%), 0 (0%), 4 (10%), and 1 (2.5%) in 50 ground meat, 50 kebab, 40 shawarma and 40 hamburger, respectively. The *fliC* H7 gene (625 bp) was found to be 1 (2%), 1 (2%), 3 (7.5%), and 4 (10%) from the same number of described samples, respectively. The *stx1* gene (302 bp) was detected in 8 (16%) ground meat, 6 (12%) hamburger, 5 (12.5%) kebab and 5 (12.5%) shawarma samples. In addition, the *stx2* gene (516bp) was detected in 1 (5%) ground meat, 4 (8%) hamburger, 1 (2.5%) shawarma and 2 (5%) kebab samples. The PCR profiles of those genes are also presented in Figure 4.4. And 4.5. In addition, the results of Shiga-toxins 1 and 2 from O157 and non O157 genes are presented in Table 4.5.

Table 4.4. PCR results

Product type	No of sample	Number of positive sample by molecular test			
		O157	H7 (*fliC)*	*stx1*	*stx2*
Ground meat	50	2 (4%)	1 (2%)	8 (16%)	1 (5%)
Hamburger	40	1 (2.5%)	4 (10%)	6 (12%)	4 (8%)
Shawarma	40	4 (10%)	3 (7.5%)	5 (12.5%)	1 (5%)
Kebab	50	0 (0%)	1 (2%)	5 (12.5%)	2 (5%)
Total	180	7 (3.8%)	9 (5%)	24 (13.3%)	8 (4.4%)

Table 4.5. PCR results of Shiga toxins 1 and 2 from O157 and non O157

Type of sample	No of sample	Number of positive shiga toxin			
		stx1 of O157	*stx1* of non O157	*stx2* of O157	*stx2* of non O157
Ground meat	50	2 (25%)	6 (75%)	0 (0%)	1 (100%)
Hamburger	40	1 (16%)	5 (83%)	0 (0%)	4 (100%)
Shawrma	40	3 (60%)	2 (40%)	1 (100%)	0 (0%)
Kebab	50	0 (0%)	5 (100%)	0 (0%)	2 (100%)
Total	180	6 (25%)	18 (75%)	1 (12.5%)	7 (87%)

4.4 Multiplex PCR

In order to confirm the positive results of conventional PCR, the results were also tested by multiplex PCR. Both results were the same.

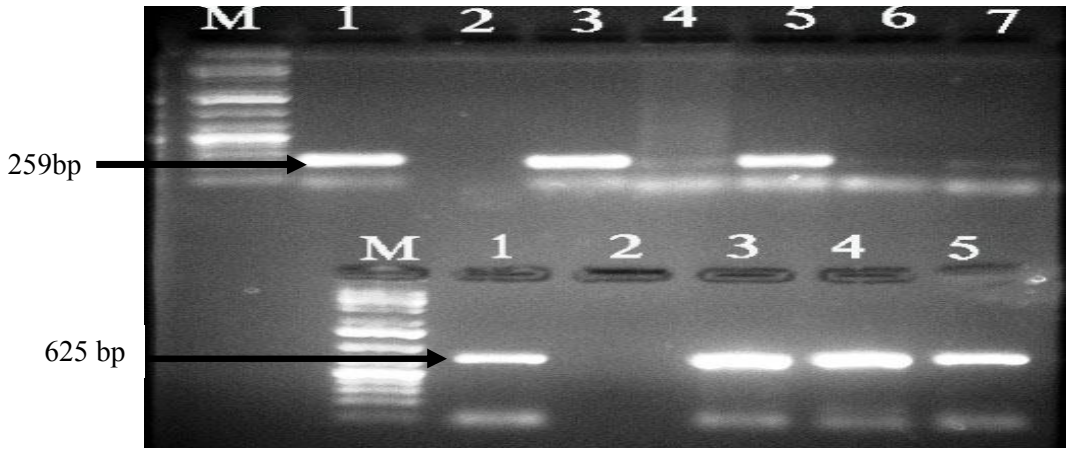

Figure 4.4. Results of the PCR assay for detection of O157and *flic* H7 genes. (Lanes 1, 3, 5 above are positive for segment of O157 (259 bp); lanes 1, 3, 4, 5 below are positive for *flic* H7 (625 bp). M represents 100bp DNA ladder.

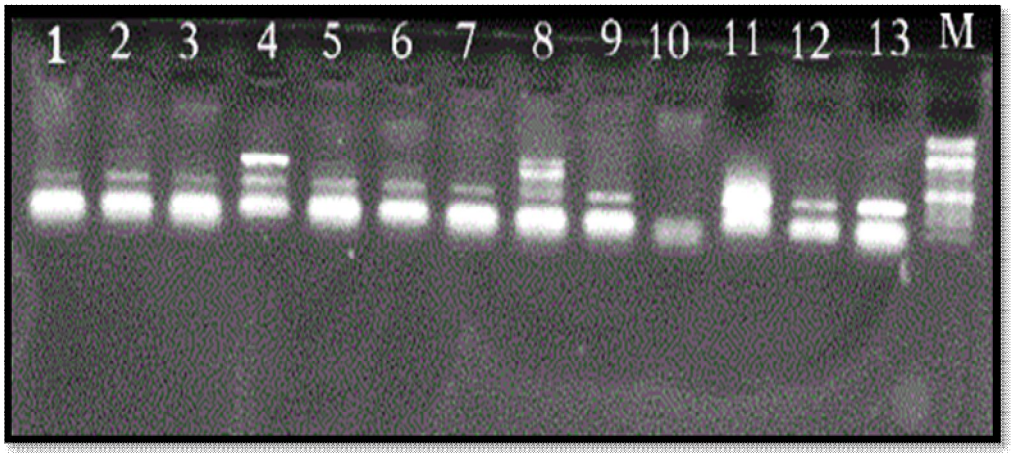

Figure 4.5. Results of the PCR assay for detection of Shiga toxin 1 and 2 genes. M represents 100bp DNA ladder. All samples except lane 10 are positive for Shiga toxin 1 gene (302bp). Only lanes 4 and 8 are positive for Shiga toxin 2 (516bp)

Discussion

Meat is the most perishable food since it contains the sufficient amount of nutrients needed to support the growth of microorganisms (Rao *et al*., 2009). Changes in eating habits, mass catering, unsafe food storage conditions and poor hygiene practices are major contributing factors for food associated illnesses. Contaminated ground beef meat and other meat products are particularly important in transmitting *E. coli* O157:H7. In 1982, more than 100 outbreaks of enterohaemorrhagic *E. coli* O157 and O157-H7 were documented in USA (Elder *et al*., 2000). Fifty-two percent of these outbreaks were attributed or linked to foods derived from cattle (Elder *et al*., 2000). So far, many studies were completed to determine the prevalence of *E. coli* O157:H7/EHEC O157 on cattle carcasses (Heuvelink *et al*., 1999; Chapman *et al*., 2000; Madden *et al*., 2001). The *E. coli* (STEC) was reported from developed countries, the United States, Canada, and other countries, such as Kenya, Turkey and Iraq (Sang *et al*., 1996; Shebib *et al*., 2003; Ulukanli *et al*., 2006). Human infections of *E. coli* O157 were attributed or linked to food products from animals (Riley *et al*., 1983; Elder *et al*., 2000).

Shatha *et al.* (2011) found the presence of O157:H7 in ground meat with 2% in Baghdad as compared to our results. This tendency for O157:H7 in ground beef was also found to be 1% (Jamshidi *et al*., 2008), 3.76% (Magwira *et al*. 2005), and 2.3% (Chinen *et al*., 2001) in Iran, Botswana, and Switzerland, respectively. Comparing the results of ground meat in our study with these three previous studies indicated that the slight differences were possibly due to improved enrichments, isolation procedures, and sample collection season, types of sample, collection procedures, and sample sizes.

Hamburgers were implicated in several outbreaks of hemorrhagic colitis due to *Escherichia coli* O157:H7 from North America (Silveira *et al*., 2000; Chapman *et al*., 2000; CDC, 2003).

In our study, the isolation rate of *E. coli* O157:H7 from hamburger samples was 2.5% with conventional PCR assay. In hamburger samples, similar results of *E. coli* O157:H7 prevalence were observed by others (Cebula *et al*., 1995, Sarimehmetoglu *et al*., 2008; Noveir *et al*., 2009) with 2.3, 2 and 2.58%, respectively. However, higher *E. coli* O157 prevalence in hamburger samples has been also reported by others (8.7% by Abdul-Raouf *et al*., 1996; 9% by Dutta *et al*., 2000). While the lower incidences of *E.*

coli O157:H7 in hamburger samples were found by other researches (Doyle *et al*., 2007; Chapman *et al.,* 2000 and Ahmad *et al*., 2012) with the rate of 1.5, 1.1, and 1.5% respectively. The higher incidence of *E. coli* O157:H7 prevalence in our study was possibly due to the samples chosen from hamburgers made locally, non-wrapped and became contacted with surrounding environment. So that it is possible that the contamination rate of handmade hamburgers with these bacteria can be higher. In addition, we used modified Tryptic Soy Broth as an enrichment medium. It has been proposed that the enrichment before plating on selective agar may increase the sensitivity of *E.coli* O157:H7 isolation compared to direct plating of test samples on selective agar (Sanderson *et al*., 1995).

Shawerma is a popular meat product consumed widely in Iraq and in many countries. In our study, the presence of *E. coli* in shawerma samples was 4%. However, the higher results were obtained by Coşansu *et al*. (2000), Köse *et al.* (2004), and Sırıken *et al*. (2009). They isolated *E. coli* O157:H7 with the rate of 6.8, 7.6 and 8.7%, respectively. Whereas high incidences of *E. coli* O157:H7 in shawerma were reported as 20 and 22% by Mohammed *et al.* (2011) %, and Vazgeçer *et al.* (2004), respectively. Lower level of *E. coli* O157:H7 prevalence rates were observed by Coia *et al.* (2001) and Noveir *et al*. (2009) with 0.17 and 0% in grilled spiced meat batches. In the present study, high incidence of *E. coli* O157:H7 prevalence in shawerma samples may be due to the contaminations during shawerma processing, such as quality of raw meat and personal hygiene in the kitchen. In addition to storage at room temperature for longer times before it is consumed; insufficient heat treatment and intermittent undercooking or improper handling of shawerma may be responsible for some unknown, sporadic or unrecognized clusters of enteric illness.

Similar to Aktaş *et al*. (2005), *E. coli* prevalence was not detected in kebab samples. However, (Hosseini *et al.*, 2013) found higher incidence of *E. coli* prevalence (10.5%) in kebab samples.

Shiga toxin is one of the exothermic factors produced by *Shigella* and *E. coli*, which initially identified in *S. dysenteriae* (Biotype 1). In addition to its destructive effect in gastrointestinal system, it can be transferred to central nervous system (CNS) via blood stream and resulted to destructive effect (Paton and Paton, 1998a). Today, more than 200 different *E. coli* O: H serotypes are known to be associated with the production of verocytotoxins. Some of them are *E. coli* sero-group O157:H7 and six

non-O157 sero-groups (O26, O45, O103, O111, O121, and O145). The genes encoding for verotoxins (*stx1* and *stx2*) are responsible for determining the virulence potential of the organism. These are essential factors for establishing the disease (Schmidt *et al*., 2001).

In our study, the *stx1* gene was detected in 6 (25%) isolates of *E. coli* O157:H7, while the *stx2* gene was detected in 1 (12.5%) isolate of *E.coli* O157:H7 from meat products. These findings are supported by several other studies (Kiranmayi and Krishnaiah, 2010; Rahimi, *et al*., 2012b). The high shiga toxin detection rate in the present study may be attributed to the use of primers designed to target *stx1* and *stx2* genes which encoded for Vt1, Vt2 toxins, respectively (Aslam *et al*., 2003).

5. CONCLUSION

Shiga toxin-producing *E. coli* (STEC) are known to cause a broad spectrum of human illness ranging from mild diarrheal to life-threatening HUS, since a large number of human illness outbreaks are traced to beef meat consumption. Worldwide testing of meat and their products revealed high prevalence rates of *E. coli* O157 and other non-O157 serotypes known to have high virulence. Thus, meat products are considered to be reservoirs for these food-borne pathogens.

Since there was no available data regarding the prevalence of *E. coli* O157 in Duhok, the aim of this study was to determine the occurrence of *E. coli* O157 in meat products. The results of current study showed that certain strains of *E. coli* O157:H7 isolated from meat samples had shiga toxin 1 and 2. Contaminated meat products with *E. coli* O157:H7 had both gene (O157 and *flic*H7) with the rates of 3.8 and 5%. The *stx1* gene was detected in 6 isolates of *E. coli* O157:H7, while the *stx2* gene was detected in 1 isolate of *E. coli* O157-H7.

In conclusion, we can recommend different solutions for future perspectives in order to reduce this type of bacterial contaminations. The governments should ensure the correct key food safety information and control for the manufacturers, retailers, professionals and training organizations etc. In addition to that, the governments should work in partnership with industry as well to coordinate the production of food sector-related international guidelines and codes in practice for eliminating the food related EHEC illness. Also, routine microbiological examinations should be adopted in meat product factories, butcher shops, groceries and other food rendering outlets. Furthermore, hygienic awareness should be applied to personnel who are involved in handling and preparing of food at factories, home or restaurants. Finally, Hazard Analysis Critical Control Point (HACCP) procedures should be adopted during all steps of manufacture, handling as well as storage of meat products to produce safe and high quality products as well as ensuring compliance with legislation.

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