MOLECULAR EVALUATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING OF *ESCHERICHIA COLI* ISOLATES COLLECTED FROM VARIOUS FOOD PRODUCTS IN TURKEY

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

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ABSTRACT

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Even though *Escherichia coli* (*E. coli*) is part of the intestinal microflora of healthy human beings and warm blooded animals, some strains of *E. coli* can be important food borne pathogens that can cause a wide spectrum of diseases, ranging from self-limiting to life threatening intestinal and extra-intestinal illnesses. Pathogenic *E. coli* that affect the intestines of humans have been grouped into six main pathotypes: Shiga-toxin-producing *E. coli* (STEC); of which enterohaemorrhagic *E. coli* (EHEC) is a pathogenic sub-group; enteropathogenic *E. coli* (EPEC); enterotoxigenic *E. coli* (ETEC); enteroaggregative *E. coli* (EAEC); enteroinvasive *E. coli* (EIEC); and diffusely adherent *E. coli* (DAEC). In this study, random assessment of the most common subtypes of *E. coli* that were collected from street foods in Van, Turkey was done. Out of 37 food samples, 28 *E. coli* isolates (i.e., 76% of food samples) were isolated. 28 *E. coli* isolates were screened for indicator genes for pathogenic subgroups using Polymerase Chain Reaction (PCR). For STEC screening, we used 6 genes (i.e., *stx1*, *stx2*, *eae*, *fliC*, *hlyA* and *rfbE*), while we used one gene for each pathogenic subgroup; *lt*/*st* genes for ETEC *bfpA* gene for EPEC, *aggR* gene for EAEC, *ipaH* gene for EIEC and *daaD* gene for DAEC. Further genomic

characterization of *E. coli* isolates was done by pulsed field gel electrophoresis (PFGE) with restriction enzyme *Xba*I. In addition, phenotypic characterization of *E. coli* isolates was performed by disk diffusion method for determining their antimicrobial resistance profiles. PCR screening of indicator genes for pathogenic *E. coli* subgroups revealed that *E. coli* isolates, used in this study, did not belong to any pathogenic subgroups. Molecular characterization of 28 *E. coli* isolates by PFGE detected 25 distinguishable PFGE patterns. Only 2 PFGE patterns (i.e., pattern 2 and pattern 3) were shared by more than one isolate; three isolates from raw milk and two isolates from herby cheese had the same pattern. Apart from them, each isolate had a unique band pattern. Antimicrobial susceptibility testing by disc diffusion method revealed that, 53.5% of the *E. coli* isolates showed resistance to ampicillin, 46.4 % of the *E. coli* isolates showed resistance both sulphafurazole and tetracycline. 15 isolates were multidrug resistant (i.e., resistant to more than 2 antimicrobials) which represent about 53.5% of the total isolates used. This study provides baseline information on antimicrobial susceptibility of *E. coli* isolates from various foods in Turkey that can serve as a benchmark for future research. Moreover, the prevalence of *E. coli* in Turkish foods should be monitored periodically because of its large diversity. *E. coli*'s large diversity and multiple resistance to a wide range of antibiotics might affect public health regarding to emerging pathogenic *E. coli* isolates with antimicrobial resistance. Stakeholders must be informed and public health education about personal hygiene should be intensified to avoid future outbreaks.

Keywords: *Escherichia coli*, STEC, ETEC, EPEC, EAEC, EIEC, DAEC, Pulsed Field Gel Electrophoresis, Antimicrobial susceptibility.

ÖZ

TÜRKİYE'DE BULUNAN ÇEŞİTLİ GIDA MADDELERİNDEN TOPLANAN *ESCHERCHIA COLI* İZOLATLARININ MOLEKÜLER DEĞERLENDİRİLMESİ VE ANTİMİKROBİYAL SUSEPTİBİLİTE TESTİ

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Sağlıklı insanların ve sıcakkanlı hayvanların bağırsak mikroflorasının parçası olmasına rağmen, *Escherchia coli* (*E.coli*)'nin bazı suşları; kendini sınırlayandan hayatı tehdit edene, intestinal hastalıklardan ekstraintestinal hastalıklara kadar, geniş bir hastalık yelpazesine sebep olabilen, önemli gıda kaynaklı patojenler olabilmektedirler. İnsan bağırsaklarında etkili olan patojenik *E. coli* altı ayrı patotipe gruplandırılmıştır: bir alt grubu enterohemorajenik *E. coli* olan Şiga-toksin üreten *E. coli* (STEC); enteropatojenik *E. coli* (EPEC); enterotoksijenik *E. coli* (ETEC); enteroaggregatif *E. coli* (EAEC); enteroinvasif *E. coli* (EIEC); ve difüz aderans *E. coli* (DAEC). Bu çalışmada, Türkiye'nin Van şehri sokaklarında satılan bazı gıdalarda bulunan *E.coli*' nin en çok rastlanan alt türlerinin rastgele değerlendirilmesi yapılmıştır. 37 gıda örneğinin, 28 tanesinde (yaklaşık olarak % 76') *E. coli* izole edilmiştir28 *E. coli* izolatı, patojenik alt gruplarının belirlenmesi için indikatör genleri Polimeraz Zincir Reaksinonu (PZR) ile görüntülenmeştir. STEC analizi için, 6 gen (*stx1, stx2, eae, fliC, hlyA* ve *rfbE*) kullanılmış ve bunlardan her biri ayrı bir patojenik altgrubu belirlemektedir: ETEX için *lt*/*st* geni, EPEC için *bfpA* geni, EAEC için *aggR* geni, EIEC için *ipaH* geni ve DAEC için

daaD geni. *E. coli* izolatlarının genomik karakterizasyonu, *Xba*I restriksiyon enzimi ile vuruşlu alan jel elektroforezi gerçekleştirilmiştir. İlaveten, *E. coli* izolatlarının, antimikrobiyal dirençlilik profillerini belirlemek amaçlı fenotipik karakterizasyonu da disk difüzyon tekniği ile belirlenmiştir. Patojenik *E. coli* izolatlarının altgruplarını belirleyen indikatör genlerinin PZR taramaları sonucunda, patojenik altgruba ait izolat bulunmamıştır. 28 *E. coli* izolatının PFGE ile moleküler karakterizasyonu ile 25 ayırt edilemez PFGE modeli bulunmuştur. Sadece 2 PFGE modeli (model 2 ve 3) birden fazla izolat tarafından paylaşılmıştır; üç çiğ süt izolatı, ve iki otlu peynir izolatını aynı modele sahiptir. Bu izolatlar dışındaki diğer her izolat kendine özgü bir bant modeline sahiptir. Disk difüzyonu metodu ile antimikrobiyal duyarlılık testi, *E. coli* izolatlarının % 53.5'inin ampisiline, % 46.4'ünün ise hem sulfakzazol ve hem de tetrasikline karşı dirençli olduğunu göstermiştir. 15 izolatta çoklu ilaç dirençliliği (2 veya daha çok antimikrobiyale dirençlilik) görülmüş ve bu izolatlar, tüm izolatların yaklaşık % 53.5'ünü temsil etmektedir. Bu çalışma, Türkiye'deki çeşitli gıdalardan elde edilen *E. coli* izolatlarının antimikrobiyal duyarlılıkları üzerine ileriki araştırmalar için referans işlevi görebilecek temel bilgiyi sağlamıştır. Ayrıca, büyük çeşitliliği yüzünden, Türk gıdalarındaki *E. coli* prevalansı periyodik olarak gözlemlenmesi gerektiği görülmüştür. Yeni ortaya çıkabilecek antimikrobiyal dirençliliğe sahip patojenik *E. coli* izolatları göz önüne alındığında, *E.coli*' nin büyük çeşitliliği ve çok çeşitli antibiyotiklere karşı çoklu direncinin, halk sağlığını etkilemesi olasıdır. Gelecekteki salgınları engellemek için, ilgili merciler bilgilendirilmeli ve kişisel hijyen hakkındaki halk sağlık eğitimleri yoğunlaştırılmalıdır.

Anahtar kelimeler: *Escherichia coli*, STEC, ETEC, EPEC, EAEC, EIEC, DAEC, Vuruşlu alan jel elektroforezi (PFGE), Antimikrobiyal duyarlılık.

To My Parents

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

CHAPTER 1

INTRODUCTION

1.1 *Escherichia coli*

Escherichia coli (*E. coli*) is a very common Gram negative bacteria, which can be found in the intestinal tract of healthy humans and warm-blooded animals (Farrokh et al., 2012). Taxonomically, *Escherichia* is in the Enterobacteriaceae family (Freney et al., 1990). The morphology of *Escherichia* is a straight cylindrical rod and its size is about 1.1-1.5 μm x 2.0-6.0 μm (Maluta et al., 2012). They can be found in singles or in pairs (Nataro et al., 2011). There are six species in genus *Escherichia*; *albertii*, *blattae*, *coli*, *fergusonii*, *hermannii*, and *vulneris* (Farmer, 1999, and Huys et al., 2003). *Escherichia* includes both motile and non-motile forms (Aprion and Watson, 1977) that can also undergo both aerobic and non-aerobic respiration (Portnoy et al., 2010). *Escherichia coli* uses mixed-acid fermentation in anaerobic conditions producing lactate, succinate, ethanol, acetate and carbon dioxide (Wang et al., 2009). All *Escherichia coli* can ferment D-glucose, and produce gas from the fermentation of this substrate (Changhao et al., 2008). For body temperature, 37 ^{0}C, is *E. coli*'s optimum growth temperature but some laboratory strains can multiply at temperatures of up to 49 ^{0}C (Nataro et al., 2011).

E. coli was discovered by Theodor Escherich, a German pediatrician and bacteriologist in 1885 (Shulman et al., 2007). In most occasions, *E. coli* resides in the intestines. At other times, they can survive briefly outside the body. When a new baby is born, *E. coli* is able to conquer the gastrointestinal tract of the new baby within some few hours (Bettelheim et al., 2009).

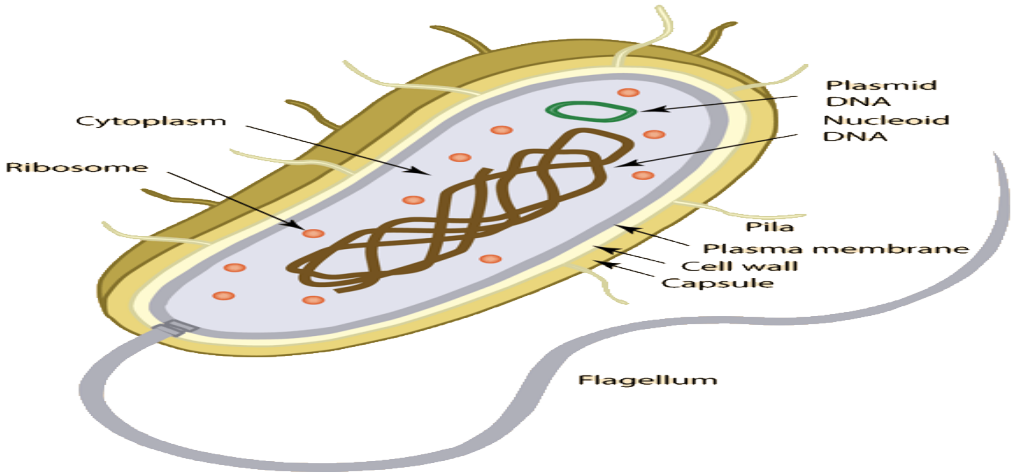

Figure 1.1. A typical *Escherichia coli* cell (Taken from Enger and Ross, 2003)

E. coli is the pronounced microorganism in the tract, and they continue to stay there in a mutually beneficial association with their host. *E. coli* in the large intestines, helps the body in the production of Vitamin K, processing of waste and in the absorption of monomers from food (Rendon et al., 2007). Over the years, researchers in various biological fields, use *E. coli* as a model bacteria for scientific and research studies, because of its ability to undergo a very easy growth and the simplicity of its genetics. Also, *E. coli* is one major bacterium which is used as an indicator microorganism for fecal contamination. In the assessment of food safety, indicator microorganisms are use as the standard tests. The presence of an indicator microorganism suggests the possibility of a microbial hazard (WHO, 2004). For example, if *E. coli* is present in drinking water, it gives you an indication that, there is a possible fecal contamination and therefore, the potential presence of enteric microorganisms that might include pathogens (Hackney et al., 1994). Presently, the most widely used microbial indicator for fecal contamination is *E. coli* (Weil et al., 2013). However, it is important to note that, the failure to detect *E. coli* in a food, does not mean that, there is no enteric pathogens. On the average, between 100 billion and 10 trillion *E. coli* bacteria shed in the feces of one person per day. (Shulman et al., 2007). Although most of the *E. coli* strains are generally not pathogenic to humans, there are some pathogenic subgroups of *E. coli* species. Transmission and infestation of

E. coli to human beings can be in several ways. The main routes of transmission of *E. coli* are; (i) through contaminated food or water, (ii) from person-to-person contact, contact with animals, (iii) contact with environments, or (iv) fomites contaminated fecal matter (Willshaw et al., 2001). Sometimes, *E. coli* may exist in the food processing plant environment and recontaminate processed foods (Frank, 2001).

1.2 Pathogenic *E. coli*

As mentioned above, *E. coli* is a common part of the microbiota of the intestinal tract of humans and warm-blooded animals, however several strains called pathogenic *E. coli* can cause diseases (Newell et al., 2010). The diseases cause by pathogenic *E. coli* vary; some pathogenic strains are fatal while others can cause mild infections. These diseases range from self-limiting diarrhea to life threatening diseases such as urinary tract infection and meningitis (Mainil, 2012). The virulence factors acquired by these pathogens are able to release powerful toxins. These toxins have the ability to inhibit protein synthesis in host cells and therefore elicit some disease responses (Chmielewski et al., 2013). Pathogenic *E. coli* that affect the intestines of humans have been grouped into six main pathotypes: (i) Shiga-toxin-producing *E. coli* (STEC; also called verocytotoxin-producing *E.coli* or VTEC), of which enterohaemorrhagic *E. coli* (EHEC) is a pathogenic sub-group; (ii) enteropathogenic *E. coli* (EPEC); (iii) enterotoxigenic *E. coli* (ETEC); (iv) enteroaggregative *E. coli* (EAEC); (v) enteroinvasive *E. coli* (EIEC); and (vi) diffusely adherent *E. coli* (DAEC) (Farrokh et al., 2012). These pathotypes have different mechanisms to cause diseases. In the United States (US), the most significant group based on frequency and severity of illness is the enterohaemorrhagic *E. coli* (EHEC) (Nataro and Kaper, 1998). Also, the infectious dose for diarrheagenic *E. coli* differs among different strains and pathotypes. For example, for *E. coli* O157:H7, an EHEC, the infectious dose ranges from 10 to 100 bacteria (Feng and Weagant, 1998).

1.2.1 Enteropathogenic *E. coli* (EPEC)

EPEC was the first pathotype of *E. coli* to be described. It is the most common cause of infant diarrhea (Nataro and Kaper, 1998). This pathogenic group has led to many deaths of infants in every year, especially in the developing countries (Kaper et al., 2004; Chen and Frankel, 2005). The original definition of EPEC is "diarrheagenic *E. coli* belonging to serogroups epidemiologically indicted as pathogens, but whose pathogenic mechanism has not been proven to be related to either enterotoxins, or Shigella-like invasiveness" (Chen and Frankel, 2005). However, EPEC have been proven to induce attaching and effacing (A/E) lesions in cells, to which they adhere and can invade host epithelial cells. The EPEC serotypes typically show a distinct pattern of localized adherence to HeLa and HEp-2 cells (Vial et al., 1990). The disease is reliant on EPEC interaction with enterocytes lining the small intestine. This is able to spark the localized loss effacement of absorptive microvilli and accumulation of host cytoskeletal proteins into pedestal-like structures beneath the adherent non-invasive bacteria (Dean et al., 2005).

The ability of this pathogen to induce these responses is dependent on the 35 kb locus of enterocyte effacement (LEE), which encodes, among other things, a type three secretion system (TTSS), EPEC-secreted proteins (Esp), injected 'effector' proteins and the surface protein, intimin (Chen and Frankel, 2005). EPEC previously was also associated with outbreaks of diarrhea in nurseries in developed countries (Doyle and Dolores, 1996). The major O serogroups associated with illness include O55, O86, O111ab, O119, O125ac, O126, O127, O128ab, and O142 (Table 1.1). Humans are an important reservoir for EPEC (Doyle et al., 2001). These serotypes usually demonstrate actin aggregation in the fluorescent actin stain test, which correlates with the attaching-and-effacing (A/E) lesion in vivo (Sergio et al., 2011). The term 'typical EPEC' has been suggested for those organisms harboring both the EAF plasmid and the LEE pathogenicity islands. Symptoms of severe, prolonged, and non-bloody diarrhea, vomiting, and fever in infants or young toddlers are characteristic of EPEC illness (Nataro and Kaper, 1998). Typical (EPEC) strains produce bundle-forming pili (BFP), which

have been implicated in EPEC virulence, and therefore the *bfpA* gene is used for the molecular detection of EPEC (Blank et al., 2000).

1.2.2 Enteroinvasive *E. coli* (EIEC)

EIEC was first isolated in 1946 from the feces of a British prison inmate, who developed dysentery (Brenner et al., 1973). Taking a critical look at their biochemistry, genetics and pathogenicity, EIEC closely resemble *Shigella spp*. (Lan et al., 2004). EIEC strains are generally lysine decarboxylase negative, nonmotile and lactose negative (Andrade et al., 2002). EIEC causes non bloody diarrhea and dysentery just like the way Shigella *species* do by invading and multiplying within colonic epithelial cells (Torres et al., 2005). The current model of Shigella and EIEC pathogenesis comprises (i) epithelial cell penetration, (ii) lysis of the endocytic vacuole, (iii) intracellular multiplication, (iv) directional movement through the cytoplasm, and (v) extension into adjacent epithelial cells (Goldberg and Sansonetti, 1993). The invasive capacity of EIEC is associated with the presence of a large plasmid (ca. 140 MDa) which encodes several outer membrane proteins (Fratamico and Smith, 2006). Genes necessary for invasiveness are carried on a 120-MDa plasmid in *Shigella sonnei* and a 140-MDa plasmid in other *Shigella* serotypes and in EIEC (Baudry et al., 1987). The presence of EIEC is detected by targeting the invasion plasmid antigen H (*ipaH* gene). In addition, *ipaH* can be used as a genetic marker for this group of pathogenic *E. coli* (Beld and Reubsaet, 2012).

The principal site of bacterial localization is the colon, where EIEC invades and proliferates in epithelial cells, causing cell death (Ramanathan, 2010). The incidence of EIEC in developed countries is low, but there are occasional foodborne outbreaks involving hundreds of people. For example, an outbreak associated with EIEC involved 370 people in Texas, the US (Gordillo et al., 1992). The identification of EIEC presents the laboratory with a number of problems. Most of the serogroups to which EIEC commonly belong are antigenically related to various serogroups of Shigella (Taylor et al., 1988). For

these reasons laboratories may mistakenly identify these strains as Shigella. Humans are a major reservoir, and the serogroups most frequently associated with illness include O28ac, O29, O112, O124, O136, O143, O144, O152, O164, and O167 (Table 1.1). Among these serogroups, the most commonly encountered serogroup is O124 (Doyle et al., 2001).

1.2.3 Enterotoxigenic *E. coli* (ETEC)

One of the main pathogens that cause diarrhea in humans is enterotoxigenic *Escherichia coli* (ETEC) (Black, 1990). Heat-labile toxin (LT) and heat-stable toxin (ST) are the main enterotoxins associated with ETEC-associated diarrhea. Phenotypic detection of one or both toxins or the genes (*lt* and *st* genes) encoding the toxins in *E. coli* isolates is used to diagnose the infection (Sack, 1980). ETEC is also another important cause of infantile diarrhea and a major cause of death in children under five years in developing countries (Quadri et al., 2005). ETEC is also the most frequently responsible for travelers' diarrhea (Doyle et al., 2006). Most ETEC outbreaks are linked to consumption of contaminated food or water. ETEC outbreaks are not frequent in advanced countries like the U.S., but infections are more common among travelers who go to foreign countries especially in areas with poor hygienic standards (Gross and Rowe, 1985).

Illness caused by ETEC is usually self-limiting (Yoder et al., 2006). The onset of symptoms is usually 26 hr after ingestion, but can range from 8 to 44 hr. Symptoms include a few days of bowel movements that look like rice-water, cramps, a low fever, nausea, and malaise (Beatty et al., 2004). Infants and immunocompromised hosts can develop a more severe illness like cholera, which lasts up to 19 days (Hart and Shears, 2003). Severe dehydration can result in the loss of more fluid from diarrhea that upsets the chemical balance of the blood, which can lead to heartbeat disturbances and may even lead to death (Naimi et al., 2003). ETEC colonizes the proximal small intestine by fimbrial colonization factors (e.g. CFA/I and CFA/II) and produces heat-labile or heat stable enterotoxin that elicits fluid accumulation and a diarrheal response (Gross and

Rowe, 1985). The most frequent ETEC serogroups include O6, O8, O15, O20, O25, O27, O63, O78, O85, O115, O128ac, O148, O159 and O167 (Table 1.1). Humans are the principal reservoir of ETEC strains that cause human illness (Doyle et al., 2001).

1.2.4 Enteroaggregative *E. coli* (EAEC)

EAEC recently have been associated with persistent diarrhea in infants and children in several countries worldwide. These organisms are uniquely different from other types of pathogenic *E. coli*, because of their ability to produce a characteristic pattern of aggregative adherence on HEp-2 cells. EAEC adhere in an appearance of stacked bricks to the surface of HEp-2 cells (Bhargava et al., 2009). EAEC strains characteristically enhance mucus secretion from the mucosa, with trapping of the bacteria in a bacterium-mucus biofilm (Hicks et al., 1996). It should be noted, however, that not all EAEC strains elicit cytotoxic effects on intestinal mucosa. Such strain heterogeneity may account for the inconsistent association of EAEC with diarrhea in epidemiologic investigations and volunteer studies (Nataro and Kaper, 1998). A growing number of studies have supported the association of EAEC with diarrhea in developing populations, most prominently in association with persistent diarrhea (more than 14 days). In several of these studies, EAEC cultured from the stool during the first few days of diarrhea is predictive of a longer duration of illness (Donnenberg et al., 1992). For example, researchers in Fortaleza, Brazil, Guerrant and colleagues have demonstrated a consistent association between EAEC and the persistent diarrhea syndrome (Fang et al., 1995). Serogroups associated with EAEC include O3, O15, O44, O77, O86, O92, O111, O104, and O127 (Neto et al., 2003) (Table 1.1). EAEC infection is diagnosed definitively by the isolation of *E. coli* from the stools of patients and the demonstration of the AA pattern in the HEp-2 assay (Sheikh et al., 2002). A gene (*aggR* gene) probe derived from a plasmid associated with EAEC strains has been developed to identify *E. coli* of this type; however, more epidemiologic information is needed to elucidate the significance of EAEC as an agent of diarrheal disease (Fang et al., 1995).

In 2011, Germany experienced the largest outbreak of shigatoxin producing EAEC cases ever recorded. A total of 3,842 cases were reported, including 2,987 cases of laboratory-confirmed *E. coli* gastroenteritis with 18 deaths and 855 cases of hemolytic uremic syndrome (HUS) that led to 35 fatal outcomes (Altmann et al., 2011). After thorough investigations, *E. coli* O104:H4, a multidrug resistant pathogen was implicated as the cause of the outbreak. *E. coli* O104:H4 strains were resistant to all penicillins and cephalosporins (Bielaszewska et al., 2011). German O104:H4 outbreak strain includes two different mobile elements, a phage and a plasmid, contributing to the essential virulence genes to the pathogen (Aurass et al., 2011). According to the whole-genome sequencing analysis, the O104:H4 strain is genetically closer to enteroaggregative *E. coli* (EAEC) than shiga-toxin producing *E. coli* (EHEC), (BGI, 2011). After ingestion of this pathogen, its adhesion and virulence factors are activated and lead to colonization of the terminal ileum and the epithelium (Phillips, 2000). The German O104:H4 epidemic is an example of a combination of relatively well-investigated virulence genes derived from two distinct pathogens which, when assembled into one organism, led to new disease manifestations that took the medical community by surprise (Muniesa et al., 2012).

1.2.5 Diffusely Adherent *E. coli* (DAEC)

The term "diffusely adherent *E. coli"* was initially used to refer to any HEp-2-adherent *E. coli* strain that did not form EPEC-like microcolonies (Scaletsky et al., 2002). With the discovery of EAEC, most authors now recognize DAEC as an independent category of potentially diarrheagenic *E. coli*. DAEC strains are defined by the presence of the DA pattern in the HEp-2 adherence assay (Schmidt et al., 1994). DAEC have been associated with diarrhea primarily in young children who are older than infants. The relative risk of DAEC-associated diarrhea increases with age from 1 year to 5 years (Ochoa et al., 2009). The most commonly found serotypes of DAEC are O1, O2, O21, and O75 (Abe et al., 2008). Typical symptom of DAEC infection is mild diarrhea without blood or fecal leukocytes (Mercado et al., 2011). DAEC generally does not elaborate heat

labile, heat stable, or elevated levels of shiga toxin, nor do they possess EPEC adherence factor plasmids or invade epithelial cells (Jallat et al., 1993). *daaD* gene is generally use for the molecular detection of DAEC (Guion et al., 2008). DAEC strains are defined by a pattern of diffuse adherence (DA), in which the bacteria uniformly cover the entire cell surface. DAEC strains use a fimbrial adhesive sheaths (Afa) as a colonization factor for intestinal tract infection (Giron et al., 1991).

1.2.6 Shiga toxin-producing *E. coli* (STEC)

Subgroups of *E. coli* that has the ability to produce cellular cytotoxins like toxins produced by *Shigella* is called shiga toxin-producing or verotoxin producing *E. coli* (Karmali, 1989). Sometimes, the terms shiga toxin and verotoxin can be interchanged because of their same cytopathogenic effect on vero cells (African green monkey kidney cells) (Roberts et al., 2001). Most of the harmful pathotypes of *E. coli* for example, enterohemorrhagic *E. coli* (EHEC) are included in STEC. STECs produce one or more cytotoxins, called shiga toxin 1 (*stx1*) and Shiga toxin 2 (*stx2*). It must be noted that, not all STEC strains are pathogens and are therefore not capable of causing diseases but some sub-groups under STEC like EHEC are all pathogenic (Karmali et al., 2003). The diseases caused by STEC range from diarrhea to hemorrhagic colitis (HC) and the life-threatening complication hemolytic uremic syndrome (HUS) (Verstraete et al., 2012). For molecular determination of STEC, PCR detection of one or more of six major virulence genes are generally used for confirmation. These genes are *stx1* (Shiga toxin 1), *stx2* (Shiga toxin 2), *eae* (intimin), *hlyA* (hemolysin), *rfbE* (O157 antigen), and *fliC* (flagellar antigen) (Chapman, 2000).

1.2.6.1 Enterohemorrhagic *E. coli* (EHEC)

Enterohemorrhagic *E. coli* (EHEC) strains involves a subgroup under STEC that cause bloody diarrhea (Neill, 1997). All EHEC strains are considered to be pathogenic, because they are associated with specific clinical implications. EHEC strains are characterized by the presence of a ca. 60-MDa plasmid, expressing shiga toxins, and having the ability to cause attaching and effacing (AE) lesions on epithelial cells (Nataro and Kaper, 1998). In 1982, *E. coli* O157:H7 was identified as causing two outbreaks of hemolytic colitis. That was the first time EHEC was observed as a human pathogen. Since then, many other serotypes of *E. coli* such as O26, O111, and sorbitol fermenting O157: NM have also been associated with cases of hemorrhagic colitis and have been classified as EHEC (O' Brien et al., 1992).

The main controversy which makes researchers to pay particular and continuous attention to EHEC infections from a clinical perspective is the high rate of serious complications associated with this infection, especially in children (Carter et al., 1987). The pathogenesis of EHEC is a multistep process, involving interactions between several additional bacterial and host factors (Welinder-Olsson and Kaijser, 2005). Interestingly, the incidence of EHEC infection in humans is relatively low. By comparing it to cattle, the main principal reservoir, which has an appreciable number of EHEC and can produce a lot of shiga toxins (Alam and Zurek, 2006). This suggests that shiga-like toxin production alone may not be sufficient for EHEC infection. Additional factors that appear necessary for virulence of EHEC O157 are the locus for enterocyte effacement (LEE) and the large plasmid pO157 (Newton et al., 2009). Polymerase Chain Reaction (PCR) can be used to characterize EHEC isolates and provides detailed information about its genetic variability. The infectious dose of EHEC is very low, between 1 and 100 CFU, which is a much lower dose than for most other pathogens of the intestines (Paton and Paton, 1998).

Among the pathogenic EHEC groups, the most dangerous serotype is *E. coli* O157:H7. In North America and Europe, this EHEC serotype (O157:H7) is the pathogen, which has been implicated in a lot of diseases. Each year, an estimated

73000 cases of illness and 60 deaths are caused by O157 STEC in the US (Mead et al., 1999). It was first identified as a food borne pathogen in 1982. There had been prior isolation of the organism, identified retrospectively among isolates at the Centers for Disease Control and Prevention (CDC); the isolate was from a Californian woman with bloody diarrhea in 1975 (Griffin and Tauxe, 1991). *E. coli* O157:H7 causes illness that behave as mild non bloody diarrhea, severe bloody diarrhea (hemorrhagic colitis), or Hemolytic Uremic Syndrome (HUS). Other symptoms of *E. coli* O157:H7 infection include abdominal cramps and lack of high fever. The probability that patients with O157 STEC diarrhea will develop HUS is 4/10 or sometimes even more than that (Rangel et al., 2005). The extent of human illness of *E. coli* O157:H7 infection includes nonbloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (Su and Brandt, 1995). Some persons may be infected but asymptomatic, that is neither causing nor exhibiting symptoms of a disease but typically for a short period of time (less than 3 weeks) (Griffin et al., 1988). Ingestion of the bacterium is followed typically by a 3- to 4-day incubation period which ranges from 2 to 12 days) during which colonization of the large bowel occurs (Besser et al., 1999). Illness begins with nonbloody diarrhea and severe abdominal cramps for 1 to 2 days and then progresses in the second or third day of illness to bloody diarrhea that lasts for 4 to 10 days (Tarr et al., 2005).

O157 STEC can be found in a lot of foods, but ground beef from dairy and beef cattle, has caused more O157 STEC outbreaks than any other vehicle of transmission (Rangel et al., 2005), and because of this, cattle have been the focus of many studies on their role as a reservoir of *E. coli* O157:H7. Other known foods, which can easily transmit O157:H7 are raw milk, sausage, roast beef, unchlorinated municipal water, apple cider, raw vegetables, and sprouts (FDA, 2007); especially when these foods are exposed to water contaminated by bovine manure. O157 EHEC spreads easily from person to person, because the infectious dose is low (less than 200 CFU). Humans can also transmit *E. coli* O157:H7 through their feces. The significance of fecal carriage of *E. coli* O157:H7 by humans is the potential for person-to-person transmission of the pathogen at its extraordinarily low infectious dose, estimated at less than 100 cells; possibly as

few as 10 can produce illness in highly susceptible populations. Inadequate attention to personal hygiene, especially after using the bathroom, can transfer the pathogen to other persons through contaminated hands, resulting in secondary transmission (Orr et al., 1994). Outbreaks associated with person to person spread have been seen in schools, long term care institutions, families and day care facilities (Grant et al., 2011). Other domestic animals and wildlife related to *E. coli* O157:H7 infections are sheep, goats, deer, dogs, horses, swine, cats, chickens (Beutin et al., 1993), water buffaloes (Galiero et al., 2005), wild birds, rodents (Nielsen et al., 2004) and guanacos (Mercado et al., 2004).

All age groups can be infected with *E. coli* O157:H7, but the children and elderly most frequently experience severe illness with complications. HUS largely affects children, for whom it is the leading cause of acute renal failure. The risk that a child younger than 10 years with a diagnosed *E. coli* O157:H7 infection will develop HUS is about 15% (Blackball and Marques, 2004). The malady is characterized by three main features: acute renal insufficiency, microangiopathic hemolytic anemia, and thrombocytopenia (Besbas et al., 2006). Significant pathological changes include swelling of endothelial cells, widened subendothelial regions, and hypertrophied mesangial cells between glomerular capillaries (Chandler et al., 2002). These changes combine to narrow the lumina of the glomerular capillaries and different arterioles and result in thrombosis of the arteriolar and glomerular microcirculation. Complete obstruction of renal microvessels can produce glomerular and tubular necrosis, with an increased probability of subsequent hypertension or renal failure (Tarr et al., 2005).

Apart from *E. coli* O157:H7, some non-O157 STEC strains have also been linked to outbreaks and sporadic cases of illness in different parts of the world (Brandt et al., 2011). The prevalence of illnesses linked to these non-O157:H7 is getting higher in the world (Tarr and Neill, 1996). The dangerous part of it is, some of the non-O157 STEC illness appear to be as severe as cases associated with O157 (Scheutz and Strockbine, 2005). However, generally, non O157 cases are less severe (Mathusa et al., 2010). Of more than 400 serotypes isolated, less than 10 serotypes cause the majority of STEC-related human illnesses (Mathusa et al.,

2010). Foods associated with non-O157 STEC illness include sausage, ice cream, milk and lettuce. Examples of non-O157 serotypes that are able to cause severe diseases and outbreaks are O26, O103, O111, O121, O145, O91 and O113 (Gyles, 2006).

Active surveillance of infections attributed to non-O157 STEC began in 2001 in the United States (CDC, 2008). The number of non-O157 STEC infections reported in the United States increased from 171 to 501 cases. There have been at least 22 outbreaks attributed to non-O157 STEC strains in the United States since 1990 (Eblen, 2007). In Europe, non-O157 STEC infections have caused 10% to 30% of sporadic cases of hemolytic uremic syndrome (HUS) in Germany (Bitzan et al., 1991), Italy (Caprioli et al., 1992), and the United Kingdom (Kleanthous et al., 1990). Moreover, HUS outbreaks have been associated with STEC O111 in Italy (Caprioli et al., 1994) and France (Boudaillez et al., 1997).

Table 1.1 Pathogenic *E. coli* groups and their common serotypes, (Nataro and Kaper, 1998)

Pathogenic group	**Serotypes**
EHEC	O157:H7, O26:H11, O113:H21, O117:H14, O145:NM
EPEC	O128:H12,O111:H2, O142:H6, O86:H34,O127:H6,NM
DAEC	O21:H21, O1:H1, O86:H18, O153:H2
ETEC	O15:H11, O128:H7, O11:H7
EIEC	O124:H30,NM, O144:NM, O164:NM
EAEC	O3:H3, O15:H18, O44:H18, O77:H18

1.3 Pathogenic *E. coli* related foodborne outbreaks

There have been more outbreaks caused by pathogenic *E. coli* than the reported ones, since most of the outbreaks have not been reported because of lack of

surveillance system. Nevertheless, few of the developed countries have followed the outbreaks and report them. *E. coli* O157:H7 has been the cause of many major outbreaks of severe illness worldwide. At least, 30 countries on 6 continents have reported *E. coli* O157:H7 infection in humans (Doyle et al., 2006). The precise incidence of *E. coli* O157:H7 foodborne illness in the United States is not known because infected persons presenting mild or no symptoms and persons with nonbloody diarrhea are less likely to seek medical attention; hence, such cases would not be reported. Foodborne Diseases Active Surveillance Network (FoodNet;//www.cdc.gov.ncidod/dbmd/foodnet/) reports that the annual rate of *E. coli* O157 infection at several surveillance sites in the United States has ranged from 0.9 to 2.8 cases per 100,000 population. Between 2000 to 2004, there has been a decline in *E. coli* O157:H7 infections from 2.0 cases per 100000 to 0.9 case per 100,000, respectively. In 1999, the CDC estimated that, *E. coli* O157:H7 causes 73480 illnesses and 61 deaths annually in the US and non-O157 STEC account for an additional 37740 cases with 30 deaths (Mead et al., 1999). 85% of these cases are attributed to foodborne transmission. However, with the dramatic reduction in cases reported by FoodNet during the past 5 years, these estimates are likely to be revised downward.

In other countries like Canada, Japan and the United Kingdom, large outbreaks of *E. coli* O157:H7 infections involving hundreds of cases have also been reported. The largest multiple outbreaks reported worldwide occurred in May to December 1996 in Japan, involving more than 11000 reported cases. In the same year, 21 elderly people died in a large outbreak involving 501 cases in Central Scotland. A large epidemic involving several thousand cases of *E. coli* O157:H7:non-motile infection occurred in Swaziland and South Africa, following the consumption of contaminated surface water (Rangel et al., 2005).

The main food product for *E. coli* infections is ground beef. When cattle are slaughtered and processed, *E. coli* bacteria in their intestines can get on the meat. Ground beef combines meat from many different animals, increasing the risk of contamination. Unpasteurized milk is another important food product for *E. coli* infections. *E. coli* bacteria on a cow's udder or on milking equipment can easily

get into raw milk to cause infections when the milk is drank. In addition, fresh produce can also cause *E. coli* infections. Runoff from cattle farms can contaminate fields where fresh produce is grown. Certain vegetables, such as spinach and lettuce, are particularly vulnerable to this type of contamination.

Non-O157:H7 STEC strains vary in their ability to cause severe human illness and outbreaks, but there is evidence that many are associated with cattle and other ruminants as is *E. coli* O157:H7 (Renter et al., 2005). In 2004, 110 non-O157 STEC infections were identified in the ten states surveyed in the US by FoodNet with serogroups O111, O103, and O26 most frequently detected (CDC, 2006). Other outbreaks in the US include: a 1994 outbreak in Montana associated with milk in which serotype O104 was implicated (Moore et al., 1995), a 1999 outbreak in Connecticut associated with lake water (serogroup O121) (McCarthy et al., 2001), a 1999 outbreak in Texas associated with a salad (serogroup O111) (Brooks et al., 2004), a 2000 outbreak in Washington associated with punch (serogroup O103), a 2001 outbreak in South Dakota in a day care (serogroup O111), and a 2001 outbreak in Minnesota associated with lake water (serogroup O26) (Misselwitz et al., 2003). In Australia, *E. coli* O157:H7 is not as commonly isolated in cases of bloody diarrhea and HUS as are other serotypes. Serogroup O111 was identified in a 1995 outbreak associated with contaminated sausage (Paton et al., 1996) and a 2003 outbreak thought to result from person to person transfer (Combs et al., 2003). Other Australian outbreaks caused by serotype O86:H27 (Morgan et al., 2005) and O48:H21 (Goldwater and Bettelheim, 1995) were from contact with cattle. Non-O157 STEC have also caused outbreaks in several European countries and Argentina (Schimmer, 2006).

In Turkey, the true incidence of pathogenic *E. coli* infections is not known (Erdoğan et al., 2008). Individual case reports and a few small case series of persons travelling to Turkey have been reported in literature (Smith-Palmer et al., 2005; Eklund et al., 2001). The recent one was about some French tourists getting sick in Turkey. According to this report, French tourists had *E. coli* O104:H4 infection. In their findings, two cases of (HUS) were caused by *E. coli* O104:H4, "genetically similar but not indistinguishable" from the *E. coli* O104:H4 outbreak

strain in France and Germany. From this study, we can conclude that shiga toxin-producing *E. coli* (STEC) serogroup O104 circulates in Turkey and therefore public health authorities and clinicians should be vigilant for possible STEC O104 infection in individuals returning from Turkey who present with post-diarrheal symptoms (Jourdan-da Silva et al., 2012). Several researchers have cited the incidence of *E. coli* O157:H7 in humans in Turkey as varying from 0% to 4% of the total *E. coli* isolates (Yildiz et al., 2005; Kaleli et al., 1999; Tolun et al., 2001; Aydoğan et al., 2001; Hasçelik et al., 1991), but only one of these studies was accompanied by shigatoxin detection (Tolun et al., 2001). Also, little data are available regarding the presence of *E. coli* O157 in cattle and other ruminants.

1.4 Subtyping of *E. coli*

Subtyping is grouping and characterization of bacterial isolates beyond the species and subspecies level. Subtyping of *E. coli* is putting *E. coli* in different groups based on certain common characteristics they share (Bender et al., 1997). There are over 100 subspecies of *E. coli* (Fukushima et al., 2002). Since not all *E. coli* strains are pathogenic, it is very important to differentiate the pathogenic ones from their non-pathogenic counterparts. By so doing, it will be very easy for scientist and medical doctors to give proper diagnostic tools for their treatment. There are two main methods for *E. coli* subtyping; phenotypic subtyping and genetic subtyping. Whereas the phenotypic subtyping focuses on the physical characteristics of the bacteria in classifying them, the genetic subtyping uses the DNA of the bacteria in classification. Subtyping of *E. coli* can be used for different purposes; to detect and to trace some foodborne disease outbreaks. Moreover, for proper identification of bacterial pathogens, subtyping methods are very fast, precise and very effective for foodborne surveillance system. In addition, subtyping methods have helped us to track the source of contamination in an outbreak. Also, with subtyping methods, researchers have been able to better understand the biology of *E. coli* strains (Weidmann, 2002).

It is crucial to find the source of an outbreak as soon as possible to protect public health. Subtyping methods have been used in investigation of outbreaks. Researchers choose an appropriate subtyping method based on different reasons like the type of research they do, the goal of the research, and the materials available for them to use. Other criteria which can be taken into consideration when you are choosing a subtyping method are: its discriminatory ability, that is how powerful and the extent it can reach in giving the differences, the cost involved, standardization, that is whether it can be repeated in a different laboratory with the same conditions, ease of use and automation, how fast it can be and finally its applicability to the type of bacterial species you are working with (Weidmann, 2002).

1.4.1 Phenotype based subtyping methods

The most commonly used phenotypic subtyping methods for *E. coli* strains are serotyping, biotyping, phage typing and multilocus enzyme electrophoresis (Nielsen et al., 2000). Although phenotype-based subtyping methods are commonly used, they may lack a good discriminatory power and also in most cases, are not reproducible (Weidmann, 2002). It must be noted that, notwithstanding these demerits, some phenotype based methods have been used in a lot of outbreaks and have been very useful in the characterization of some *E. coli* isolates (Olive and Bean, 1999).

1.4.1.1 Serotyping of *E. coli*

Serotyping is a very popular phenotype-based subtyping method for typing of *E. coli* (Foley et al., 2006). In any infection which involves pathogenic *E. coli*, one important step to focus on is to try to identify its serogroup and this knowledge will help in a long way in how to diagnose it. The main feature which is used in serotyping lies on the antigens which are carried on cell surface (Wang et al., 2003). Different strains of *E. coli* differ in the antigens they carry on their cell

surfaces. These surface antigens can be detected by antibodies and antisera reactions (Anjum et al., 2006). Before specific virulence factors in pathogenic *E. coli* strains were determined, serotypic analysis was the leading means by which pathogenic strains were differentiated (Engleberg, 1984).

In 1994, Kauffman proposed a scheme for the serological classification of *E. coli* and the modified form of this scheme is still in used today (Nataro and Kaper, 1998). According to the Kauffman scheme, *E. coli* isolates are serologically based on three major surface antigens. These are: the O (somatic), H (flagella), and K (capsule) antigens. Only some few international reference laboratories are able to conduct complete serotyping of *E. coli*. In serotyping *E. coli* which can cause diarrhea, determination of only the O and H antigens is laudable. Serogroups of *E. coli* can be associated with certain clinical syndromes, but it is not in general the serologic antigens themselves that confer virulence. Rather, these serotypes serve as readily identifiable chromosomal markers that correlate with specific virulent clones. The O antigen gives the serogroup of a strain whist the H antigen gives its serotype. Serotyping can be used to differentiate isolates associated with diarrheal disease since specific serogroups often fall into one group of pathogenic *E. coli*. However, some serogroups such as O55, O111, O126, and O128 fall to more than one category. Diarrheagenic *E. coli* isolates are categorized into specific groups (pathotypes) based on virulence properties, mechanisms of pathogenicity, clinical syndromes, and distinct O: H serotypes. More than 600 serotypes of STEC have been identified, including approximately 160 O serogroups and 50 H types, and the list continues to grow (www.microbionet.com.au). Table 1.1 gives a list of pathogenic *E. coli* and some of their common serotypes.

1.4.1.2 Phage typing

Another commonly used phenotypic subtyping method is phage typing. Phage typing characterizes bacterial isolates by their susceptibility to a standard set of phages. Bacteria are susceptible to viral infections. By exposing a bacterial isolate to a standard set of antithetical phages (bacterial viruses), a pattern will developed

incidentally upon its susceptibility or resistivity of the culture under study. By this procedure, numerous types of strains can be differentiated within a given species (Khakhria et al., 1990). Bacteriophages are specific in action. A particular phage may be very specific in that it will infect only a few strains of a certain bacterial species. On the other hand, another phage may infect strains of two or more species of a particular genus (Levin, 1994). Susceptibility to be lysed by a particular phage may be the only apparent phenotypic difference between two bacterial strains and may be the only means by which a strain causing an outbreak of disease can be recognized.

Phage typing of *E. coli* has been useful in some countries. For example, in Canada, the phage typing scheme for verotoxigenic (VT) *E. coli* 0157:H7 has been successfully used in epidemiological investigations of *E. coli* 0157: H7 infections (Frost et al., 1989). Phage typing is fast and very effective, but a standardized reference phage must be acquired, so that you can compare your results with other laboratories. This makes it a little bit difficult (Olive, 1999). In Europe, the most widely used conventional sub-typing method for *E. coli* O157 is bacteriophage typing. However, this technique is not applicable to non-O157 STEC. The susceptibility of each isolate to be lysed by a panel of 16 bacteriophages is determined and the lytic patterns obtained usually allow typing into one of 82 possible types (Demczuk et al., 2003). Bacteriophage typing does not usually provide the level of discrimination required for epidemiological and outbreak investigations, as the number of different types identified routinely may not be sufficient for confident interpretation of results (Ahmed et al., 2000).

1.4.2 Genetic subtyping methods

Unlike phenotypic methods, genetic subtyping methods further differentiate bacterial strains at the molecular level or the DNA level. Genetic subtyping methods are highly reproducible. Phenotypic subtyping methods might be affected by research conditions and results might vary between different laboratories, but genetic subtyping methods are highly standardized and reproducible (Wiedmann,

2002). Moreover, genetic subtyping methods are able to provide a more sensitive strain discrimination. For example, there are some *E. coli* pathotypes which can only be determined by using genetic subtyping methods whereas phenotypic subtyping methods cannot identify them (Cai et al., 2002). Some common examples of genetic subtyping methods for *E. coli* include Pulsed Field Gel Electrophoresis (PFGE), Multilocus Sequencing Techniques (MLST), Ribotyping, Plasmid Profiling and Random Amplification of Polymorphic DNA (RAPD) (Wiedmann, 2002).

1.4.2.1 Polymerase Chain Reaction (PCR)

PCR is one of the most efficient analytical methods for confirmation of virulence genes in pathogenic bacteria. In PCR, a single segment of DNA can be amplified to produce several copies. There are 3 major steps in PCR; denaturation of DNA template, primer annealing and extension of the annealed primers. These 3 steps are repeated for a number of times depending on the reaction protocol. The results is exponential amplification of a specific target DNA sequence (Chapman, 2000).

1.4.2.2 Pulsed field gel electrophoresis (PFGE)

Pulsed Field Gel Electrophoresis (PFGE) is a reliable and highly discriminating genetic method for subtyping foodborne pathogens (Barrett et al., 1994). In this method, the DNA of the bacteria is digested with a restriction enzyme. After digestion of whole DNA, different band patterns of DNA with different size will be produced (Swaminathan et al., 2001), since restriction enzyme will cut at recognition sites on DNA. By so doing, we can group or classify the bacteria by size of digested DNA fragments. If we compare and observe that two DNA from different isolates share the same DNA pattern, we conclude those two isolates might be the same strain (Georing, 2004). One remarkable advantage of PFGE over the other genetic subtyping methods is that, large genomic DNA fragments can be compared (Struelens et al., 2001). Restriction enzymes are chosen such

that, they can hardly cut DNA to yield between approximately 8 and 25 large DNA bands which range from 40 to 600 kb (Wiedman, 2002).

Standard gel electrophoresis cannot separate these large fragments of DNA, and therefore, a different and unique electrophoresis technique using alternating fields is used to separate the DNA fragments (Lai et al., 1989). DNA banding patterns for different bacterial isolates are compared to differentiate distinct subtypes (Georing et al., 2004). PFGE subtyping shows a high level of discrimination for many foodborne bacterial pathogens and thus is often considered the current gold standard for discriminatory ability, and it has been considered a gold subtyping method for foodborne pathogens by state public health laboratories such as Center for Disease Control (CDC) to determine the outbreaks as well as source of outbreaks (Boxrud et al., 2010). The CDC and state health departments in the United States have developed a national network (PulseNet) to rapidly exchange standardized PFGE subtype data for isolates of foodborne pathogens (Swaminathan et al., 2001). It is important to realize, however that PFGE (as well as other subtyping methods) may also sometimes detect small genetic differences that may not be epidemiologically significant (Tenover et al., 1995).

1.4.2.3 Multilocus Sequencing Techniques (MLST)

Multilocus Sequencing Technique (MLST) refers to a molecular subtyping approach that uses DNA sequencing of multiple genes or gene fragments to differentiate bacterial subtypes and to determine the genetic relatedness of isolates. MLST often includes sequencing of seven housekeeping genes, (Spratt, 1999), but sequencing of multiple virulence genes can also be used to increase the discriminatory power (Wiedmann, 2002). Housekeeping genes are essential genes for cell functions (Phillips, 2008). For each housekeeping gene used in MLST, the different sequences present within a bacterial isolate are assigned as distinct alleles, and for each isolate, the combination allelic types define the sequence type (Losada et al., 2011).

A major advantage of this approach is that sequence data are considerably less ambiguous and easier to interpret than banding pattern-based subtyping approaches described above. The development of web based databases, for example MLST (http://www.mlst.net), includes databases for MLST of several microorganisms that facilitates global, large-scale surveillance and tracking of bacterial foodborne pathogens (Spratt, 1999). DNA sequencing data also provides an opportunity to reconstruct ancestral and evolutionary relationships among bacterial isolates, allowing scientist to further probe the evolutionary biology and ecology of foodborne pathogens. MLST- based approaches for subtyping of bacterial foodborne pathogens are still in the early developmental stages and optimal target genes are still being defined for the different bacteria of interest (Cai et al., 2002). MLST also has the capability of studying the population biology of pathogenic microorganisms including *E. coli* and it helps us to understand the population structure of the pathogens (http://web.mpiib-berlin.mpg.de). In the northwestern part of England, MLST was used for the genetic characterization of uropathogenic *E. coli* strains and to investigate the population biology of uropathogenic *E. coli* strains circulating within the region. They used 88 uropathogenic *E. coli* isolates, including 68 isolates from urine and 20 isolates from blood. In the results, MLST identified an important genetic lineage of *E. coli*, designated sequence type 131 (ST-131), represented by 52 of these isolates, 51 of which were resistant to extended-spectrum cephalosporins (Lau et al., 2008).

1.4.2.4 Ribotyping

Ribotyping is another DNA-based subtyping method in which bacterial DNA is initially cut into fragments using restriction enzymes (Parveen et al., 1997). Whereas PFGE uses restriction enzymes that cut the bacterial DNA in very few large pieces, the initial DNA digestion for ribotyping cuts DNA into many (greater than 300-500) smaller pieces (approximately 1-30 kb) (Bidet et al., 2000). These DNA fragments are separated by size through agarose gel electrophoresis and a subsequent southern blot step uses DNA probes to specifically label and

detect those DNA fragments that contain the bacterial genes encoding the ribosomal RNA (rRNA). The resulting DNA banding patterns are thus based on only those DNA fragments that contain the rRNA genes (Grimont, 1986). A completely automated, standardized system for ribotyping (the RiboPrinter Microbial Characterization system) has been developed by Qualicon-DuPont and is commercially available (Bruce, 1996). Scott et al., sampled *E. coli* isolates from humans, beef cattle, dairy cattle, swine, and poultry from locations in northern, central, and southern Florida. These isolates were subjected to ribotyping analysis to determine if ribotype profiles are capable of discriminating the source of *E. coli* at the host species level and if the resulting fingerprints are uniform over an extended geographic area or if they can be applied only to a specific area. Their results indicated that, using a single restriction enzyme (*Hind*III), ribotyping is not capable of differentiating *E. coli* isolates from the different animal species however, the procedure can still be used effectively to differentiate *E. coli*, isolates from different geographic regions (Scott et al., 2002).

1.5 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing is a procedure that is performed on bacterial isolates to find out which antimicrobial substances might be effective in the treatment of bacterial infections. An antibiotic is any natural, semisynthetic or synthetic molecule that is used in the treatment or prevention of diseases. In most cases, they are designed to target cellular processes such as cell wall synthesis, DNA replication, RNA transcription and messenger RNA translation. Even though there are hundreds of antibiotics which have been developed, researchers are still developing new ones, because many antibiotics have lost much of their effectiveness as bacteria develop resistance to them (Davis and Davis, 2010). It has reached a point that the World Health Organization (WHO) now regards antibiotic-resistance as a "global threat" (WHO, 2011).

Different antibiotics have different ways of fighting the bacteria. Penicillins like ampicillin is able to inhibit a number of bacterial enzymes called Penicillin-

Binding Proteins (PBPs). These PBPs are essential for peptidoglycan synthesis and results to the formation of deficient cell wall types (Kohanski et al., 2010). The cephalosporins are similar to the penicillins. They act by binding to PBPs of susceptible organisms, thereby interfering with synthesis of peptidoglycan of the bacterial cell wall (Neu and Gootz, 1996). Cephalothin and ceftriazone are some examples of cephalosporins that generally are more active against *E. coli*. However many strains of *E. coli* are susceptible to cephalothin (Sayah et al., 2005). Another group of antibiotics with a very wide spectrum is the carbapenems, which include ertapenem and imipenem. Carbapenems, cephems and phenems are all part of a big group called ß-lactam antibiotics. They also work by binding to PBPs of bacteria to cause cell elongation and lysis (Goffin and Ghuysen, 2002). Amikacin, gentamicin, streptomycin and kanamycin are common examples of aminoglycosides. Aminoglycosides are group of bactericidal agents that inhibit bacterial protein synthesis by binding irreversibly to the bacterial 30S ribosomal subunit. The aminoglycoside-bound bacterial ribosomes then become unavailable for translation of mRNA during protein synthesis, thereby leading to cell death. The folate pathway inhibitors interfere with the folic acid pathway. Sulphamethoxazole/trimethoprim is a combination of two folate pathway inhibitors. Such a combination provides a broad spectrum of activity. The quinolones such as nalidixic acid target DNA gyrase, an enzyme essential for DNA replication, recombination and repair (Pohlhaus et al., 2008).

Tetracyclines act against susceptible microorganisms by inhibiting protein synthesis. Many *E. coli* isolates are susceptible to tetracyclines. Tetracyclines are used mainly for the treatment of acute, uncomplicated urinary tract infections due to *E. coli*, and as effective prophylactic therapy for traveler's diarrhea caused by enterotoxigenic *E. coli*. Chloramphenicol is a bacteriostatic agent that inhibits protein synthesis by binding reversibly to the peptidyltransferase component of the 50S ribosomal subunit and preventing the transpeptidation process of peptide chain elongation (Morita et al., 2013). In the laboratory, susceptibility testing is usually performed by either the disk diffusion method or the minimal inhibitory concentration method. Standards that describe these methods are published and frequently updated by the Clinical and Laboratory Standards Institute (CLSI).

In Turkey, almost all the antimicrobial susceptibility testing of *E. coli* are from clinical cases (Aslan et al., 2012). Yilmaz et al., tested 4,534 *E. coli* strains that were obtained from outpatients with urinary tract infection with 9 antibiotics with the disc diffusion method. The antibiotics they used include ampicillin, amoxicillin-clavulanic acid, cefuroxime, cefotaxime, piperacillin-tazobactam, amikacin, gentamicin, trimethoprim and ciprofloxacin. They found out that a considerable proportion of the studied *E. coli* isolates were resistant to most of them except amikacin which was found to be resistant in 8.3% of isolates in their study (Yilmaz et al., 2009). Ipek and her colleagues also conducted similar research to determine the antimicrobial patterns of uropathogens collected from Turkish children. They found out that, *E. coli* was the leading uropathogen and resistance to ampicillin, amoxycillin-clavulanate, cefazolin and trimethoprim-sulfamethoxazole was remarkable. They went on further to propose a country wide antibiotic susceptibility testing (Ipek et al., 2011). There have not been any studies of antimicrobial resistance on *E. coli* isolates from food in Turkey.

1.6 Aims of this study

Turkey exports food products like hazelnuts, pistachios, figs, pulses, citrus, melons, vegetables, tomato products, poultry meat and cereals to many parts of the world especially Europe. It is also a major cheese exporter, particularly of white goat and sheep cheeses preserved with salt or brine. Semi-hard cheeses are also made and some foreign style cheeses for export (Export promotion center of Turkey, 2009). The main aim of this study was to find out the prevalence of pathogenic *E. coli* isolates especially *E. coli* O157:H7 in Turkish food products in Van, a pilot region. Secondly, to determine the most common subtypes of *E. coli* isolates found in food by using the gold standard method, pulsed field gel electrophoresis. In addition, to determine the antimicrobial susceptibility of *E. coli* isolates using the disc diffusion method. The last but not the least was to provide data on *E. coli* isolates collected from Turkey to a publicly available database. Establishing a surveillance system entails doing in-depth research into the occurrence of microorganisms which cause foodborne diseases, the frequency and

pattern of occurrence of those diseases and the areas which are prone to those diseases.

CHAPTER 2

MATERIALS AND METHODS

2.1. Buffers and solutions

All the buffers and solutions that were used through the analyses are listed with their suppliers in Appendix B.

2.1.2 Growth media

Appendix C describes the preparation of growth media that were used for the analyses.

2.2 Isolation of *E. coli*

Between 1st February and 31st May of 2011, 37 food samples were collected for *E. coli* isolation (Table 2.1). These samples included raw chicken and products, raw milk and cheese, pistachio, raw patty meat, red pepper, minced meat and lahmacun, a traditional Turkish food which looks like a thin piece of dough topped with minced meat and minced vegetables. These food samples were taken from the market center in the Van region of Turkey. Isolation was done by Dr. Dilek Avşaroğlu in Food Microbiology Laboratory of 100. Yil University in Van, Turkey, using Food and Drug Administration Organization Bacterial Analytical Manual, FDA isolation method for *E. coli*. Right after collection, the samples were quickly sent to the laboratory and 25 g of each was aseptically weighed with a weighing balance (≥ 2 kg with 0.1 g sensitivity). After weighing, they were put into 225 ml of buffered peptone water to enrich the *E. coli*. It was then

homogenized with a stomacher. The homogenate was incubated for 10 min at room temperature with periodic shaking so as to allow the sample to settle by gravity. The medium was gradually poured into a sterile container and incubated for 3 hr at 35°C to resuscitate injured cells. With a micropipette, 20 µl of each homogenate was sub cultured on Endo agar. Endo Agar is a slightly selective and differential medium for the isolation, cultivation and differentiation of Gram-negative microorganisms. *E. coli* produces pink to rose-red colonies when cultured on this medium. After 20 hr of incubation at 44.0°C, 29 out of the 37 food samples produced red colonies and were assumed to be *E. coli*. 29 suspected *E. coli* isolates were sent to Food Engineering Department of Middle East Technical University (METU) for further confirmation and subtyping.

Table 2.1 Food sample distribution

Food samples	**Number of food samples**
Raw chicken drumstick	15
Raw chicken wing	1
Raw turkey wing	1
Raw milk	6
Herby cheese	4
Salted cheese	2
Minced meat	2
Raw patty meat (Cig kofte)	3
Lahmacun	1
Pistachio	1
Red pepper	1
Total	**37**

2.3 Confirmation of *E. coli* isolates

The 29 pure cultures were transported to the food safety laboratory in the Food Engineering Department of Middle East Technical University for biochemical and molecular confirmation.

2.3.1 Biochemical Confirmation of *E. coli*

All the *E. coli* isolates, which were transported in the cold chain, condition that is without any contamination, were grown on petri dishes which contained eosin methylene blue (EMB) media. EMB media is a selective media for Gram negative bacteria against gram positive bacteria. Differentiation of enteric bacteria is possible due to the presence of the sugars lactose and sucrose in the EMB and the ability of certain bacteria to ferment lactose in the medium. Lactose fermenting gram negative bacteria (like *E. coli*) acidify the medium, and under acidic conditions, the dyes produce a dark purple complex, which is usually associated with a green metallic sheet. When grown on the EMB agar, *E. coli* should appear very dark, almost black color (Cheeptham, 2007). 5 µl of each isolate was dispensed on a medium and streaked gently with an inoculating loop. The EMB agar plates were incubated overnight at 37°C for 24 hr.

2.3.2 Molecular Confirmation of *E. coli* Isolates

The *rpoB* gene region, used in our study, is a specific region for the *E. coli* species (Table 2.2). It is used for the detection or quantification of total *E. coli* populations. It is used as a marker for *E. coli.* (Rantsiou et al., 2012). It was used to determine whether biochemically confirmed *E. coli* isolates are *E. coli* or not.

2.3.2.1 DNA Isolation

A sterile inoculating loop was used to pick a single colony per isolate of *E. coli* from BHI (Brain Heart Infusion Agar) and was put into a tube containing 95 µl of sterile water. It was gently mixed inside the water to ensure uniform mixing and also to prevent the single colony from attaching to the walls of the tube. The prepared mixture was exposed to heat by microwaving it for about 30 s to lyse the *E. coli* cell. This breaks down the bacterial cell wall and expose the internal contents which contain the DNA. They were then kept under room temperature ready to be used for PCR.

2.3.2.2 Polymerase Chain Reaction (PCR) of *rpoB* gene region

In a total volume of 20 µl, the PCR reagents and conditions used for *rpoB* gene detection have been given in table 2.2 and the method as described by Rantsiou et al., 2012 was used.

Table 2.2 PCR reagents and conditions used for *rpoB* gene detection, (Rantsiou et al., 2012)

Master mix reagents	**Vol (µl)**	**PCR conditions**
dH_2O	9.1 µl	95°C for 10 min [1x] 50 cycles of the following: 95°C for 30 s 58°C for 30 s 72°C for 30 s 72°C for 5 min [1X] 4°C∞ [1X]
10x Buffer	4 µl	
$MgCl_2$ [7.5mM]	3 µl	
dNTPs [0.6mM]	1.2 µl	
0.4 µM primer (rpoB)- F	0.8 µl	
0.4 µM primer (rpoB)-R	0.8 µl	
2.5 units of native Taq DNA pol DNA Polymerase	0.1 µl	
DNA template	1 µl	
Total	20 µl	
rpoB F1- GTATGTCCAATCGAAACCCC **rpoBR1- GGTAGTGAATTTCGTCAGTTACA**		

2.3.2.3 Agarose gel electrophoresis of the amplicons

After the amplification was done, the PCR products were run on an agarose gel to determine the presence of the gene region. 0.5 X TBE buffer was prepared from a stock solution of 5 X TBE (54 g Tris base 15.5 ml 85% phosphoric acid [1,679 g/ml] 40 ml 0.5 M EDTA [pH:8]) by 1:10 dilution. After that, 1.8 g of Agarose (AppliChem) was dissolved into a glass beaker containing 150 ml 0.5X TBE buffer. It was then microwaved until all the agarose was completely dissolved. The Agarose/TBE solution was allowed to cool down to about 55°C for 5 min at room temperature. The cooled gel was poured slowly into the tray. Any form of

air bubbles was avoided by making use of a disposable tip. The gel was left for about 30 or more min to solidify at room temperature. The electrophoretic chamber consists of 0.5X Tris Boric EDTA (TBE) as a running buffer, positive and negative electrodes for supplying the current connected to an electric voltmeter which produces the current. The PCR products were loaded to gel. 10 µl of each each PCR product, which had been mixed with 1.5 µl of 6x loading dye (ThermoScientific) were pipetted into each well. DNA marker was loaded into first and last well of the gel. The electrophoresis was run at 120 V for 45 min.

2.3.2.4 Staining and destaining of agarose gel

For staining, ethidium bromide solution was used. Ethidium bromide must be handled with care, since it is carcinogenic. To prepare the stock solution, the ethidium bromide was dissolved in water at 10mg/ml to give a red solution. This was done in a light-protected container at room temperature. 20 µl of ethidium bromide stock solution (10 mg/ml) was put into 100 ml of distilled water. The agarose gel was put into the 300 ml ethidium bromide solution and stained for 5 min. Moreover, the gel was transferred into distilled water for 30 min to be destained. Finally, gel picture was taken under UV light in a BIORAD Universal hood II (Biorad, SN 76 S, Milan, Italy). Amplification of gene appears as sharp distinct white bands.

2.3.3 Labeling and freezing of confirmed *E. coli* isolates

Confirmed *E. coli* isolates, were then transferred to another medium called Brain Heart Infusion (BHI) medium. BHI Agar is an enriched non-selective medium for the isolation and cultivation of most bacteria. The *E. coli* isolates were labeled according to the labeling standards of METU Food Safety Laboratory in ascending order. The first isolate was labeled as MET K1-001. The rest continued as followed. Appendix A gives a detailed description of how the labeling was done.

A single bacterial colony was picked from the BHI agar with an inoculating loop. It was immersed in a fresh 5 mL BHI broth and mixed thoroughly. Inoculated cultures were incubated overnight for approximately 8-12 h. On the following day, 150 µl of autoclaved sterile glycerol was pipetted into an empty freezing tube. With a new tip, the micropipette was used to transfer 850 µl of the bacterial culture to the same freezing tube. The freezing tube was capped and inverted several times to thoroughly mix the glycerol and bacteria. They were then stored in a special -80°C freezer.

2.4 Molecular detection of pathogenic subgroups

For molecular detection, primers from literature were used to detect pathogenic groups of *E. coli*. PCR enables the DNA of pathogenic bacteria to amplify with specific primers. Table 2.3 gives a list of the pathogenic primers used. Table 2.4 gives the PCR conditions of amplification of the primers of pathogenic indicator regions and the functions of the genes. The reference strains for the pathogenic subgroups of *E.coli* were kindly provided by the Turkish Ministry of Health Laboratory. These strains represented ETEC, including *est* (i.e., original number for this isolate is A1851), EIEC, including *ipaH* 1(i.e., original number for this isolate is 583), EAEC, including *aggR* (i.e., original number for this isolate is 2059) and EPEC, including *eae* (i.e., original number for this isolate is 8064). They were labeled and frozen down as MET K1-042, MET K1-045, MET K1-051 and MET K1-054, respectively.

2.4.1 Further screening for STEC

The DNA of the isolates were screened to check if they contain STEC genes by utilization of STEC primers. The PCR conditions of the STEC indicator gene region primers and the functions of their target genes that were used for this study can be found in table 2.5. The reference strain *E. coli* O157:H7 was kindly provided by Prof. Dr. Kadir Halkman (Food Engineering Department at Ankara

University) and labeled as MET K1-029. This strain possessed all the six virulence genes (*fliC*, *stx1*, *stx2*, *eae*, *hlyA* and *rfbE*) and was used as the positive control. The presence and absence were detected by agarose gel electrophoresis procedure described above in subsection 2.

Table 2.3 Primer information about STEC and pathogenic subgroups used in this study

Primers	**Primer sequence (5'-3')**	**Target gene (product size)**	**Reference**
fliC-F	AGC TGC AAC GGT AAG TGA TTT	*fliC* (949 bp)	Bai et al. (2010)
fliC-R	GGC AGC AAG CGG GTT GGT C		
stx 1-F	TGT CGC ATA GTG GAA CCT CA	*stx 1* (655 bp)	Bai et al. (2010)
stx 1-R	TGC GCA CTG AGA AGA AGA GA		
stx 2-F	CCA TGA CAA CGC ACA GCA GTT	*stx 2* (477 bp)	Bai et al. (2010)
stx 2-R	TGT CGC CAG TTA TCT GAC ATT C		
eae-F	CAT TAT GGA ACG GCA GAG GT	*eae* (375 bp)	Bai et al. (2010)
eae-R	ACG GAT ATC GAA GCC ATT TG		
rfbE-F	CAG GTG AAG GTG GAA TGG TTG TC	*rfbE* (296 bp)	Bai et al. (2010)
rfbE-R	TTA GAA TTG AGA CCA TCC AAT AAG		
hlyA-F	GCG AGC TAA GCA GCT TGA AT	*hly A* (199 bp)	Bai et al. (2010)

Table 2.3 Primer information about STEC and pathogenic subgroups used in this study (Cont'd)

Primers	Pathogenic Subgroup	Primer sequence (5'-3')	Target gene	Reference
st-F	ETEC	5'-ATTTTTATTTCTGTATTATCTT-3'	*st* 190 bp	Stacy-Philips et al., (1995)
st-R		5'-CACCCGGTACAAGCAGGATT-3'		
lt-F		5'-GGCGACAGATTATAC CGTGC-3'	*lt* 450 bp	
lt-R		5'-CGGTCTCTATATTC CCTGTT-3'		
aggr-F	EAEC	5'-CGAAAAAGAGATTATAAAAATTAAC-3'	*aggr* 100 bp	Guion et al., (2008)
aggR-R		5'-GCTTCCTTCTTTTGTGTAT-3'		
ipaH-F	EIEC	5'-GTTCCTTGACCGCCTTTCCGATACCGTC-3'	*ipaH* 619 bp	Guion et al., (2008)
ipaH-R		5'-GCCGGTCAGCCACCCTCTGAGAGTAC-3'		
bfpA-F	EPEC	5′-AATGGTGCTTGCGCTTGCTGC-3′	*bfpA* 326 bp	Botkin et al., (2012)
bfpA-R		5′ -GCCGCTTTATCCAACCTGGTA-3′		
daaD-F	DAEC	5'-TGAACGGGAGTATAAGGAAGATG-3′	*daaD* 444 bp	Guion et al., (2008)
daaD-R		5'- GTCCGCCATCACATCAAAA-3′		

Table 2.4 PCR conditions for pathogenic *E. coli* groups and the functions of their target genes

Pathogenic subgroup	PCR conditions	Target gene and function	Reference
EPEC bfpA-F/R (10 mM)	94°C for 5 minutes [1X] 40 cycles of the following: 94°C for 30 seconds 59°C for 1 minute 30 seconds [40X] 72°C for 40 seconds 72°C for 10 minutes 4°C ∞ [1X]	*bfpA*: produces bundle forming pili which sparks the effacement of absorptive microvilli.	Botkin et al., (2012)
DAEC daaD-F/R (20 mM)	98°C for 50 seconds [1X] 40 cycles of the following: 60°C for 20 seconds 72°C for 30 seconds [40X] 75°C for 1 second 72°C for 7 minutes 4°C ∞ [1X]	*daaD*: Use fimbrial adhesive sheath as a colonization factor and form a pattern of diffuse adherence to cover the entire cell surface of the small intestines	Guion et al., (2008)

Table 2.4 PCR conditions for pathogenic *E. coli* groups and the functions of their target genes (Cont'd)

Pathogenic subgroup	PCR conditions	Target gene and function	Reference
EIEC ipaH-F/R (10 mM)	98°C for 50 seconds [1X] 25 cycles of the following: 60°C for 20 seconds 72°C for 30 seconds 75°C for 1 second [25X] 77°C for 7 minutes 4°C ∞ [1X]	*ipaH*: Invades and multiplies within colonic epithelial cells to cause cell death	Guion et al., (2008)
ETEC st-F/R lt-F/R (10 mM)	95°C for 2 minutes [1X] 50°C for 5 minutes 40 cycles of the following: 50°C for 45 seconds 70°C for 30 seconds [40X] 72°C for 12 minutes 4°C∞ [1X]	*st* and *lt*: Colonize the small intestines and produce heat-labile or heat stable enterotoxins that elicits diarrhea	Stacy-Philips et al., (1995)
EAEC aggR-F/R (20 mM)	98°C for 50 seconds [1X] 95°C for 5 minutes 25 cycles of the following: 60°C for 20 seconds 72°C for 30 seconds 75°C for 1 second [25X] One hold at 72°C for 7 minutes 4°C ∞ [1X]	*aggR*: Produces a characteristic pattern of aggregative adherence on HEp-2 cells to elicit cytotoxic effects on intestinal mucosa	Guion et al., (2008)

Table 2.5 PCR conditions for STEC and the functions of their target genes

STEC primers	**PCR conditions**	**Target gene and function**	**Reference**
fliC-F/R	Master Mix Reagents dH2O- 9.1 µl 10x Buffer- 4 µl $MgCl_2$ (7.5mM)- 3 µl dNTPs (0.6mM)- 1.2 µl primer- F (0.4 µM)- 0.8 µl primer-R (0.4 µM)- 0.8 µl 2.5 units of Native Taq DNA pol- 0.1 µl DNA template- 1 µl Total volume- 20 µl 94°C for 5 minutes [1X] 25 cycles of the following: 94°C for 30 seconds 65°C for 30 seconds [25X] 68°C for 75 seconds One hold at 68°C for 7 minute 4°C∞ [1X]	*fliC*: Encodes the flagellar proteins which help the bacteria to move and bind to epithelial tissues	Modified from Wang et al., (2000)
stx 1-F/R		*stx* 1: Produce lytic shiga-like toxins to inactivate repressor proteins and cause cell damage in small intestines	Bai et al., (2010)
stx 2-F/R		*stx* 2: Produce toxins that cross the intestinal epithelial barrier and gain access to systemic circulation to inhibit metabolic cell processes.	Bai et al., (2010)
eae-F/R		*eae*: Produces intimin for intimate attachment to epithelial cells	Bai et al., (2010)
rfbE-F/R		*rfbE*: Provides expression of the O157 antigen of E. coli O157:H7 and promotes the adherence of E. coli O157:H7 to epithelial cells	Bai et al., (2010)
hlyA-F		*hlyA*; Produces hemolysin. It lyses cells by creation of pores in the target cell membrane and affects erythrocytes, leucocytes and renal tubular cells.	Bai et al., (2010)

2.5 Pulsed Field Gel Electrophoresis (PFGE)

PFGE was performed according to PulseNet (CDC PulseNet, 2013). DNA fragments were separated in a PFGE CHEF-DR III system (Bio-Rad Laboratories, Hercules, US). PFGE procedure involves 3 steps; plug preparation, restriction digestion and electrophoresis

2.5.1 PFGE plug preparation

The *E. coli* isolates were streaked on BHI agar with an inoculating loop. After streaking, they were incubated at 37°C for 18 hr. 5 ml of Cell Suspension buffer, (100 mM Tris, 100 mM EDTA [pH 8.0]) were put in red capped tubes. A sterile cotton swab which has been moistened with the cell suspension buffer was used to genltly rub the bacterial cultures on the BHI agar and transferred to the 5ml CSB red capped tubes. They were then mixed thoroughly in the capped tube with the swab. 800 µl of the bacterial and CSB solution were transferred into a cuvette. The cuvette was put in a spectrophotometer to determine the absorbance of the solution at a wavelength of 610 nm. Ideal absorbance should be between an optical density of 1.3 and 1.4. 400 µl of adjusted cell suspension was put into labelled eppendorf tubes. They were then incubated in 37°C water bath for 10 min. A freshly prepared 20 mg/mL proteinase K solution was added directly to each eppendorf tube. It was mixed by gently flicking it several times with fingers. 1% SKG Agarose and 1% Sodium dodecyl sulfate were prepared. These were used for the plugs. For the preparation of the SKG Agarose, 0.25 g of SKG was added to 23.5 ml of TE Buffer in a 300 ml flask. They were put in a microwave to ensure that they have been mixed well. The Agarose mixture was allowed to cool in a water bath at 55°C for 10 min. Before the 20% SDS was added, it was also pre-warmed in 55°C water bath for at least 10 min. 1.25 ml of the 20% SDS was added to the Agarose and mixed gently. 400 µl of the equilibrated agarose mixture was added to each cell suspension and mixed gently by pipetting up and down two to three times before immediately dispensing into the wells of reusable or disposable PFGE plug molds. The plugs were allowed to cool so that they can

solidify at room temperature for 15 min. They were taken from the molds and placed in 5 ml of Cell lysis Buffer/Proteinase K solution contained in falcon tubes. Cell Lysis Buffer contains (CLB; 50 mM Tris, 50 mM EDTA [pH 8.0] and 1% Sarcosyl). They were then incubated at 54°C at a rate of 170 rpm for 2 hr with constant and vigorous agitation. After lysis of the cells embedded in agarose, the lysis buffer was poured away and they were quickly washed with double distilled water for 10 min. This was done at 50°C at a rate of 70 rpm. The same procedure (of washing with distilled water) was repeated again under the same conditions. They were washed with TE buffer at 50°C and 70 rpm but for 15 min. This same procedure (of washing with TE Buffer) was repeated three times. All these washing were done to remove the residual lysis buffer coating the plugs and the inside of the walls of the tubes. 5 ml of sterile TE buffer (room temperature) was added to each tube to serve as storage media for the plugs. The plugs were restricted immediately or stored in TE buffer at 4°C until needed. They were now ready to undergo restriction digestion with *Xba*1 restriction enzyme.

2.5.2 Restriction digestion with *Xba*I

Restriction of DNA was done using *Xba*I enzyme in restriction buffer, H buffer. In each of the labelled eppendorf tubes, 200 µl of the H buffer was added. The H buffer solution contains 20 µl of the H buffer and 180 µl of double distilled water. The plugs were cut into 2 mm slices with a single edge razor blade or scalpel and immersed into the H buffer. The agarose plug DNA size standard strain (*Salmonella* ser. Braenderup H9812; Hunter et al., 2004) was also cut into 3 or 4 slices and immersed into H buffer. They were then incubated in a 37°C water bath for 10 min. The *Xba*1 enzyme solution was prepared. This consists of 175 µl of double distilled water, 20 µl of H buffer and 5 µl of the *Xba*1 enzyme. The restriction buffer was poured away and replaced with 200 µl of the *Xba*1 enzyme solution to each of the slices. The slices were incubated in the enzyme solution in a 37°C water bath for 2 hr. After incubation, the restriction digestion was stopped by the addition of 200 µl of TE Buffer to the slices. Digested DNA samples were then ready for electrophoresis.

2.5.3 Casting of agarose gel and electrophoresis conditions

This stage was began by first preparing the SKG gel. The 1% SKG agarose gel was prepared using a 15-well comb in the wide or long casting stand (Bio-Rad) as seen in figure 2.1. 1.5 g of SKG was added to 150 ml of 0.5X TBE buffer and it was microwaved until it was boiled. After they were well mixed, the agarose was cooled in a 55°C water bath for 10 min. It was cooled at room temperature for at least 5 min. The agarose was cast in a very clean gel mold. The gels were allowed to solidify for approximately 30 min at room temperature. A running buffer which consists of 110 ml 10 X TBE and 2090 ml of autoclaved double distilled water was poured into the chamber (Fig. 2.1). The pump speed and the cooler were set at 70 ms^{-1} and 14°C respectively. The TE buffer was removed from the slices and 200 µl of 0.5 X TBE buffer was added to the slices. A sealing agarose which had been cooled in a 55°C water bath for 10 min was added. The slices were then loaded into the gels in their respective lanes. 836 µl of 10 mg/ml thiourea solution was added to the running buffer. The resulting electrophoresis conditions which were used are in table 2.6.

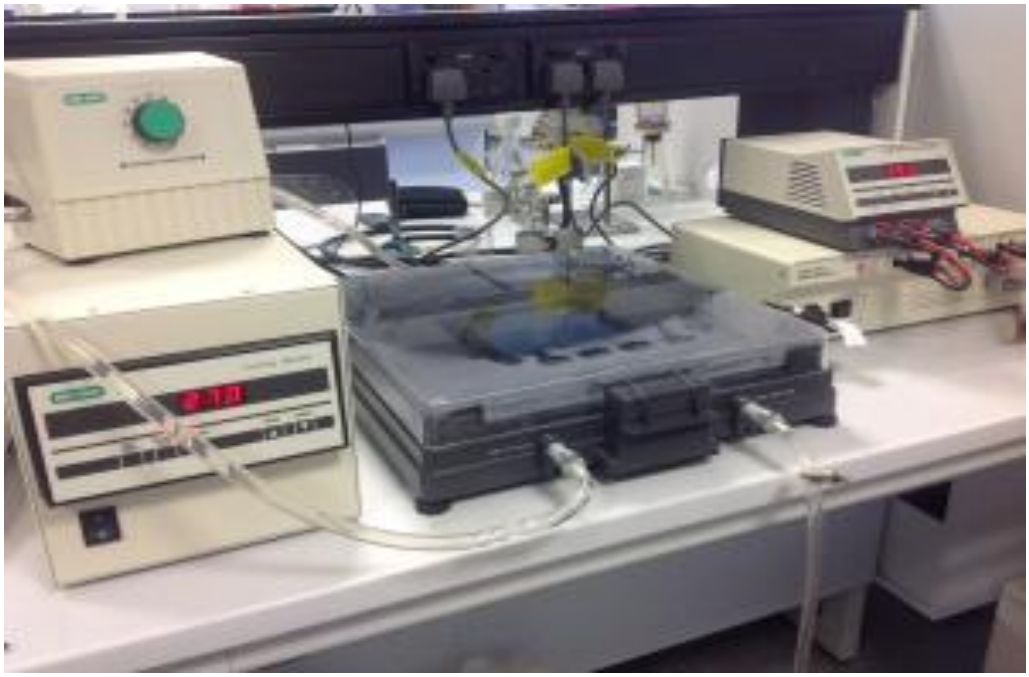

Figure 2.1 Pulsed Field Gel Electrophoresis Set-up

Table 2.6 Electrophoretic conditions used in PFGE

Low KB	30 KB
High KB	700 KB
% Agarose	1%
Gradient	6.0 V/cm
Run time	19 h
Included Angle	120°C
Initial switch time	2.23 s
Final switch time	1.03 min 80 s
Pump speed	70

2.5.4 Image acquisition

After the electrophoresis was completed, the gels were stained with 400 ml of Ethidium bromide solution (40 g/mL) for 40 min with gentle shaking. The gels were then de-stained with 400 ml of deionized water for 30 min by shaking. The banding patterns were observed under ultraviolet (UV) illumination and a digital image using the Gel Doc system (Bio-Rad) following the settings and integration parameters recommended by the manufacturer.

2.6 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of the isolates was performed using the Kirby–Bauer disc diffusion method and Mueller–Hinton agar (Merck, Germany) according to CLSI guidelines (CLSI, 2007).

2.6.1 Disc Diffusion Method

The concentrations of the antibiotics tested were between 10-300 μg/mL. Table 2.7 gives information about the types of antibiotics that were used in this research, their concentrations and their symbols.

The *E. coli* isolates were sub-cultured in a fresh BHI agar. After 18 hr in 37°C incubation, the plates were removed. Single colonies from each isolate was picked with an inoculating loop and suspended in Mueller-Hinton Broth and they were put into a 37°C incubator again for 2 hr. Tubes were taken out and the inocula were adjusted to a turbidity that is equivalent to 0.5 FTU McFarland standard. This adjustment was done by placing the tubes in front of a white paper with black lines. If the turbidity of 0.5 FTU and that of the suspensions were in parallel to each other to the naked eye, they were deemed to be equal. Those which were out of range in this standard, were adjusted again by diluting them with Mueller-Hinton Broth and re-checked again. Then after, Mueller Hinton Agar (MHA) was prepared and poured into plates.

Figure 2.2 Multichannel disc dispenser used for disc diffusion

To maintain a proper depth, about 4 mm of the MHA was poured into each plate. The innocula which had been adjusted were vortexed again. A fresh sterile cotton swab was dipped into the suspension and was streaked thoroughly on the MHA.

After 15 min, the antimicrobial agents containing discs were placed firmly on the MHA Agar with a multichannel disc dispenser (Fig 2.3). Finally they were incubated at 37°C for 18 hr. Plates were removed and examined closely after incubation with a standard metric rule. The zone diameters around the discs were measured and compared with the break points of CLSI. ATCC 25922 *E. coli* was used as a reference strain.

Table 2.7 List of antibiotics and concentrations CLSI, 2007

Class of Antibiotic	**Antibiotic**	**Symbol**	**Content**
Penicillins	Ampicillin	AMP	10 µg/disk
Beta-lactam	Amoxycillin/ Clavulanic Acid	AMC	30 µg/disk
Cephems	Ceftiofur	EFT	30 µg/disk
Carbapenems	Ertapenem	ETP	10 µg/disk
	Imipenem	IMP	10 µg/disk
Cephems(parenteral)	Cefoxitin	FOX	30 µg/disk
	Cephalothin	KF	30 µg/disk
	Ceftriaxone	CRO	30 µg/disk
Sulfonamide	Sulphafurazole	SF	300 µg/disk
Folate pathway inhibitors	Sulphamethoxazole/ Trimethoprim	SXT	25 µg/disk
Quinolones	Nalidixic Acid	NA	30 µg/disk
Tetracyclines	Tetracycline	TE	30 µg/disk
Aminoglycosides	Amikacin	AK	30 µg/disk
Aminoglycosides	Gentamicin	CN	10 µg/disk
Aminoglycosides	Streptomycin	S	10 µg/disk
Aminoglycosides	Kanamycin	K	30 µg/disk
Phenicols	Chloramphenicol	C	30 µg/disk

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Isolation of *E. coli*

Food sampling and isolation of suspected *E. coli* isolates were done in 100. Yil University, Van, Turkey, under the supervision of Dr. M. Dilek Avşaroğlu Erkan. The prevalence of *E. coli* in food samples was assessed by random sampling of 37 food samples. In terms of food sample numbers, some food samples such as raw chicken drumsticks (15) were more than red pepper (1). Prevalence of *E. coli* was based on the number of food samples from which E. coli was obtained to the total number of food samples tested. For example, from 15 raw chicken drumsticks, *E. coli* was isolated from 15 of them, making the prevalence 100%. Likewise, only one raw chicken wing was sampled and it had *E. coli*. This prevalence was also 100%. Out of 2 salted cheese samples, *E. coli* was isolated from 1 of them, making its prevalence 50 %. The highest prevalence of *E. coli* was found in raw chicken drumsticks (100%), chicken wing (100%), raw turkey wing (100%), raw milk (100%) and herby cheese (100%). No *E. coli* was found in minced meat (0%), lahmacun (0%), pistachio (0%) and red pepper (0%) (Table 3.1). The prevalence of *E. coli* in salted cheese was 50% and 33% in raw patty meat (cig kofte). This shows that most of the foods were contaminated. *E. coli* is an indicator microorganism and its presence suggests a possibility of a microbial hazard (WHO, 2004). The most common transmission way is fecal contamination because personal hygiene is limited. Contaminated ground beef consumption causes the highest illnesses due to pathogenic *E. coli*. Other foods like unpasteurized (raw) milk and juice, poultry products, and soft cheeses can get contaminated and transmit *E. coli* to humans. Studies worldwide have shown that *E. coli* are often present in fresh meat and poultry (Todd, 1997). In our study, *E.*

coli was isolated from all the raw chicken drumsticks, raw chicken wing and turkey wing. However, no *E. coli* was isolated from lahmacun, pistachio and raw pepper. The prevalence of *E. coli* from fruits, vegetables and nuts like green pepper and pistachio are generally very low as compared to foodstuffs with animal origin (EFSA, 2013).

Table 3.1 Distribution of *E. coli* among different food samples

Food samples	**Number of food samples collected**	**Number of *E. coli* positive samples**
Raw chicken drumsticks	15	15
Raw chicken wing	1	1
Raw turkey wing	1	1
Raw milk	6	6
Herby cheese	4	4
Salted cheese	2	1
Minced meat	2	0
Raw patty meat (cig kofte)	3	1
Lahmacun	1	0
Pistachio	1	0
Red pepper	1	0
Total	**37**	**29**

3.2 Biochemical and molecular confirmation of *E. coli*

The presumptive *E. coli* isolates were sub cultured successively onto EMB agar for presumptive identification of *E. coli*. Out of 29 suspected *E. coli* isolates, only

one isolate from herby cheese did not form greenish-black metallic sheath. The other 28 isolates with greenish-black metallic sheath colonies (Fig. 3.1) were presumptively selected as *E. coli*. EMB agar provides a rapid and accurate method of distinguishing *E. coli* from other gram-negative bacteria pathogens. *Escherichia coli* can be identified with (EMB) agar based on the occurrence of a green-metallic sheen. EMB agar contains peptone, lactose, sucrose, and the dyes eosin Y and methylene blue. The dye methylene blue in the medium inhibits the growth of Gram-positive bacteria. Lactose-fermenting Gram-negative bacteria like *E. coli* acidify the medium, and under acidic conditions the dyes produce a dark purple complex which is usually associated with a green metallic sheen. This metallic green sheen is an indicator of vigorous lactose and/or sucrose fermentation ability. EMB agar is a reliable, simple and inexpensive medium for biochemical confirmation of *E. coli* (Leininger et al., 2001).

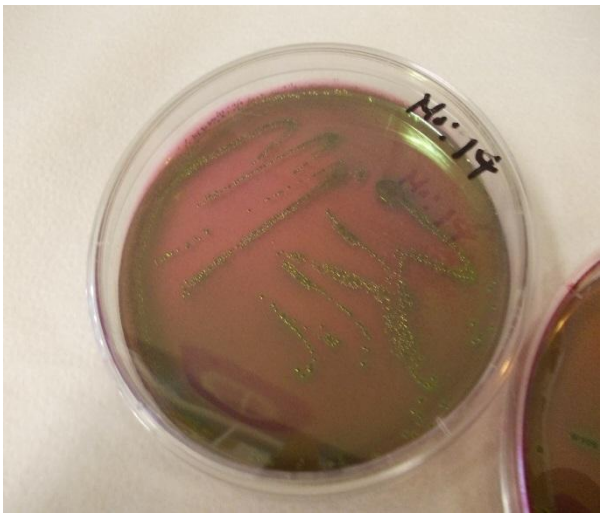

Figure 3.1 Pure cultures of isolated *E. coli* on EMB. After 24 hours of incubation, cultures showed metallic green sheath colonies, which indicate the presence of *E. coli*.

Biochemically confirmed suspected *E. coli* isolates were used as template for amplification of *rpoB* gene using polymerase chain reaction (PCR). The PCR amplification using *Taq* DNA polymerase led to the presence of single bands in

Agarose gel. At the end of the PCR, *rpoB* gene amplified all the 28 isolates at 130 bp, which confirmed that the DNA of the isolates were *E. coli*. The *rpoB g*ene encodes the β subunit of RNA polymerase. It is used for detection and quantification of total *E.coli* populations. The amplification for the *rpoB* gene is specific for the *E. coli* species (Rantsiou et al., 2012). All the 28 isolates amplified rpoB primers, revealing DNA amplicons at 130 bp on 1.5% Agarose gel (Fig. 3.2). This result confirmed that, the DNA of presumptive *E. coli* isolates were *E. coli.*

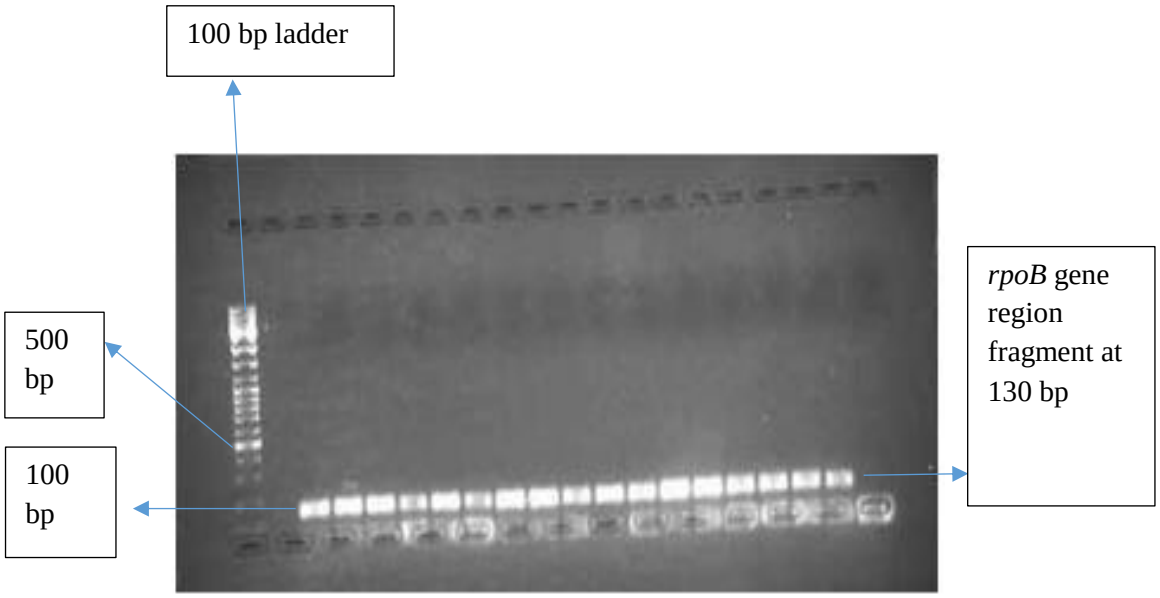

Figure 3.2 Agarose gel picture of electrophoresis of PCR products of *rpoB* gene region amplification

3.3 Screening of shigatoxin producing *E. coli* (STEC) genes by PCR

A total of twenty eight *E. coli* isolates were screened for the presence of genetic markers associated with STEC. Six STEC genes (*stx1*, *stx2*, *eae*, *fliC*, *hlyA* and *rfbE*) were used in this research. Only the *fliC* gene was present in three samples (raw milk (2), and raw patty meat) which represents 10.71% of the total number of food samples. None of the *E. coli* isolates in our isolate set belonged to STEC subgroup.

The H antigen of *E. coli* is specified by a single structural subunit (flagellin) encoded by the *fliC* gene. The flagellar protein constitutes the subunit of the helical filament which forms the flagellar organelle. The flagellar protein carries the antigenic determinant(s) for the H antigen. *E. coli* H-type 7 has been determined by restriction of the amplified flagellin gene to facilitate identification of enterohemorrhagic serotype O157:H7 whether it is motile or nonmotile (Reid et al., 1998). It was found that all O157:NM producing shiga toxin carried the H7 antigen. Studies have been conducted on the adhesive properties of H7 flagella and the abilities of STEC O157:H7 flagella to bind bovine mucus, mucin proteins, and ECM proteins. They have revealed that H7 flagella and the flagellin monomers can bind mucins and also freshly isolated bovine mucus. Deletion of the *fliC* gene STEC O157:H7 caused the bacteria to become significantly less adherent to bovine intestinal tissue than the parental wild-type strain, which may indicate that H7 flagella possess adhesive properties (Erdem et al., 2007).

Our study shows that, the three isolates, i.e., MET K1-001 from raw milk, MET K1-006 from raw patty meat, and MET K1-023 from raw milk have flagellar protein encoding genes (Fig. 3.3). Flagellar can help them to move and bind to epithelial tissues. However, it has been proposed that flagella may not be essential for STEC pathogenesis in humans, because, for example, nonmotile STEC strains were associated with up to 40% of HUS cases in Germany and are an emerging problem in Europe overall (Karch and Bielaszewska 2001).

Research regarding STEC has so far mainly focused on isolates from patients who have suffered a STEC infection. Information regarding the prevalence and distribution of STEC in foodstuffs is only recently becoming available. Studies conducted so far (Brooks et al., 2001; Doyle and Schoeni, 1987; Fantelli and Stephan, 2001; Pradel et al., 2000), particularly those investigating STEC strains with different virulence genotypes (Pradel et al., 2008; Slanec et al., 2009), highlight the need for determination of a possible association of certain STEC genotype profiles with specific food types in order to assess their possible risk to human health.

The prevalence and true incidence of *E. coli* O157:H7 and other pathogenic *E. coli* strains in humans as well as Turkey's food products are not well-known. Cases are likely to be under diagnosed owing not only to improper laboratory diagnostic methods but also to a lack of awareness of the epidemiologic significance (Erdogan et al., 2008). Individual case reports and a few small case series of people travelling to Turkey have been reported in literature (Smith-Palmer et al., 2005). Several researchers have cited the incidence of *E. coli* O157:H7 in humans in Turkey as varying from 0% to 4% in cases of gastroenteritis (Yildiz et al., 2005), but only one of these studies was accompanied by shigatoxin detection (Tolun et al., 2001). In our research, none of the *E. coli* isolates was found to be pathogenic. Although much is not known on the prevalence of *E. coli* in food products in Turkey, little data is available regarding the presence of *E. coli* O157 in cattle and other ruminants. Aslantas and colleagues reported that *E. coli* O157 was isolated in 77 of 565 clinically healthy cattle samples (13.6%) in the Hatay province (Aslantas et al., 2006). Other studies have proved the prevalence of *E. coli* O157:H7 from raw milk to be quite low and varied between 0% and 10%. (Padhye and Doyle, 1991). All these studies are consistent with the results of this research considering the number of samples they used and this study.

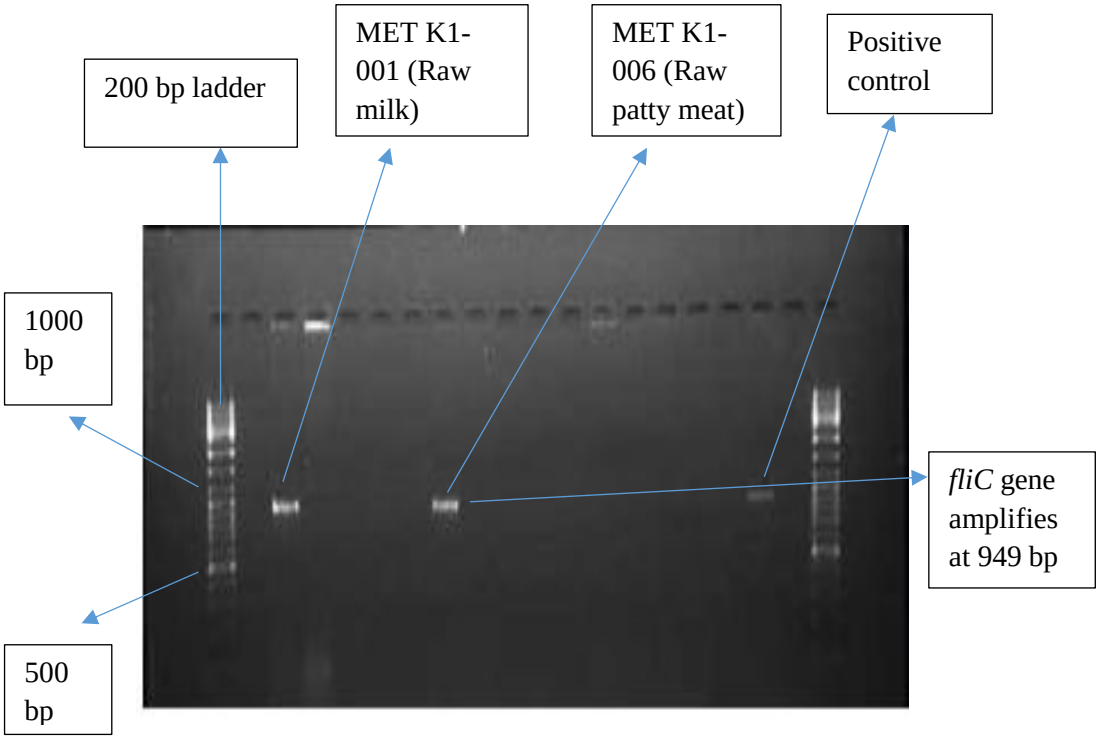

Figure 3.3 Agarose gel picture of electrophoresis of PCR products of *fliC* gene region amplification

3.4 Screening of NON-STEC pathogenic subgroup genes by PCR

PCR reactions for pathogenic subgroups of *E. coli* genes *st*, *lt*, *aggR*, *ipaH*, *bfpA*, *daaD* representing ETEC, EAEC, EIEC, EPEC and DAEC, respectively were also conducted. None of these primers were able to amplify the *E. coli* isolates found in this study. This shows that, none of the 28 isolates were pathogenic. From literature, the prevalence of pathogenic *E. coli* in food isolates is very low or sometimes cannot be found as compared to animal isolates and clinical infections (Holvoet et al., 2013). Prevalence of commensal *E. coli* isolates from food has been extensively studied in the developed countries (Allocati et al., 2013) but very little research has been conducted in developing countries, for example Turkey (Cengiz et al., 2012).

3.5 PFGE typing

A total of 28 *E. coli* food isolates, were also characterized by PFGE to provide a better understanding of diversity among the isolates. The 28 *E. coli* isolates represent 25 different genotypes by PFGE. All the bands from isolates were within the range of 20 kb and 600 kb (Fig. 3.5). Three isolates (MET K1-003, MET K1-004 and MET K1-027) shared the same PFGE pattern, pattern 3 (Fig. 3.6). All of them were obtained from raw milk. Apart from them, two isolates from herby cheese (MET K1-002 and MET K1-026) also shared the same PFGE pattern, pattern 2 (Fig. 3.7). The rest of the isolates had distinguishable PFGE patterns (Fig. 3.4 and 3.5).

PFGE remains the method of choice for epidemiological typing of *E. coli*. It is more discriminatory than other methods, plus all isolates are typeable, and good reproducibility is obtained. The dendogram (Fig. 3.5) shows a great diversity of *E. coli* because of the different band patterns which was produced. Using various DNA fingerprinting techniques such as PFGE and ribotyping for microbial source tracking, several researchers have reported the high genetic diversity of commensal *E. coli* in literature (Goto and Yan, 2011). The enormous diversity of *E. coli* is manifested by the presence of numerous distinct *E. coli* genotypes (Snipen et al., 2009). PFGE of 46 representative ETEC and STEC isolated from pigs with post weaning diarrhea revealed 36 distinct patterns (Khac et al., 2006). From this study, we can deduce that, if there is an outbreak in this community, researchers have to elicit different measures to tackle and control pathogens because of the wide genetic differences between the isolates. This may include using different antibiotics to control. The PFGE profiles of the isolates were saved in our database and can be used for tracking outbreaks when needed.

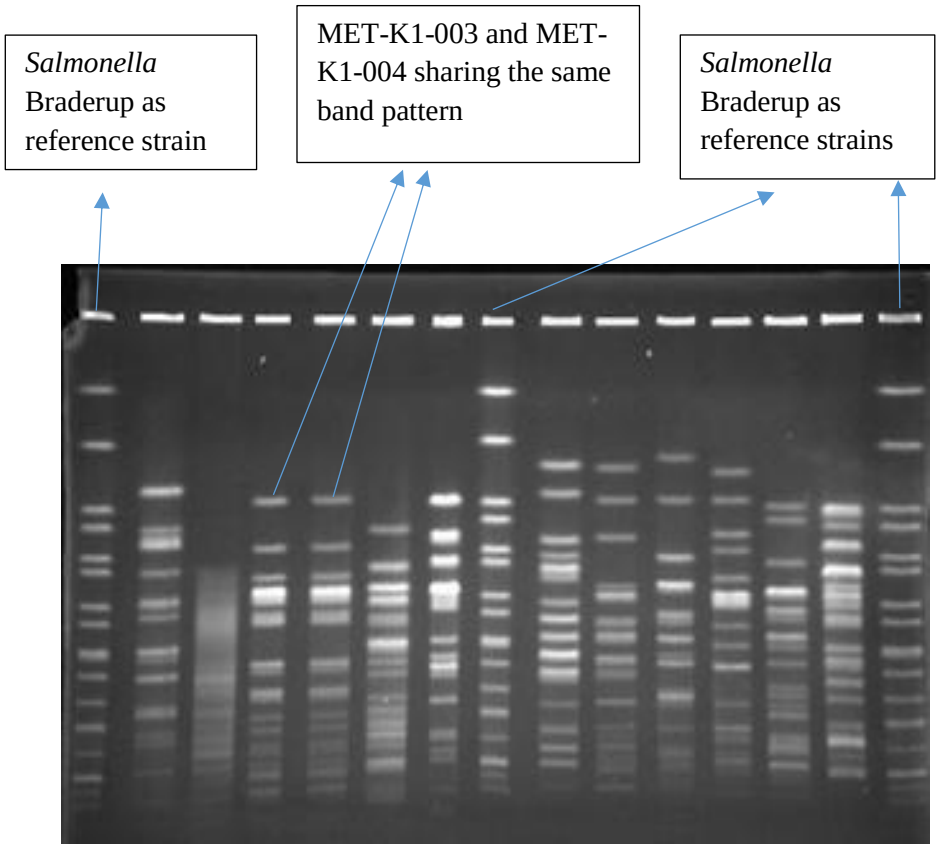

Figure 3.4 PFGE gel picture of 12 *E. coli* isolates.

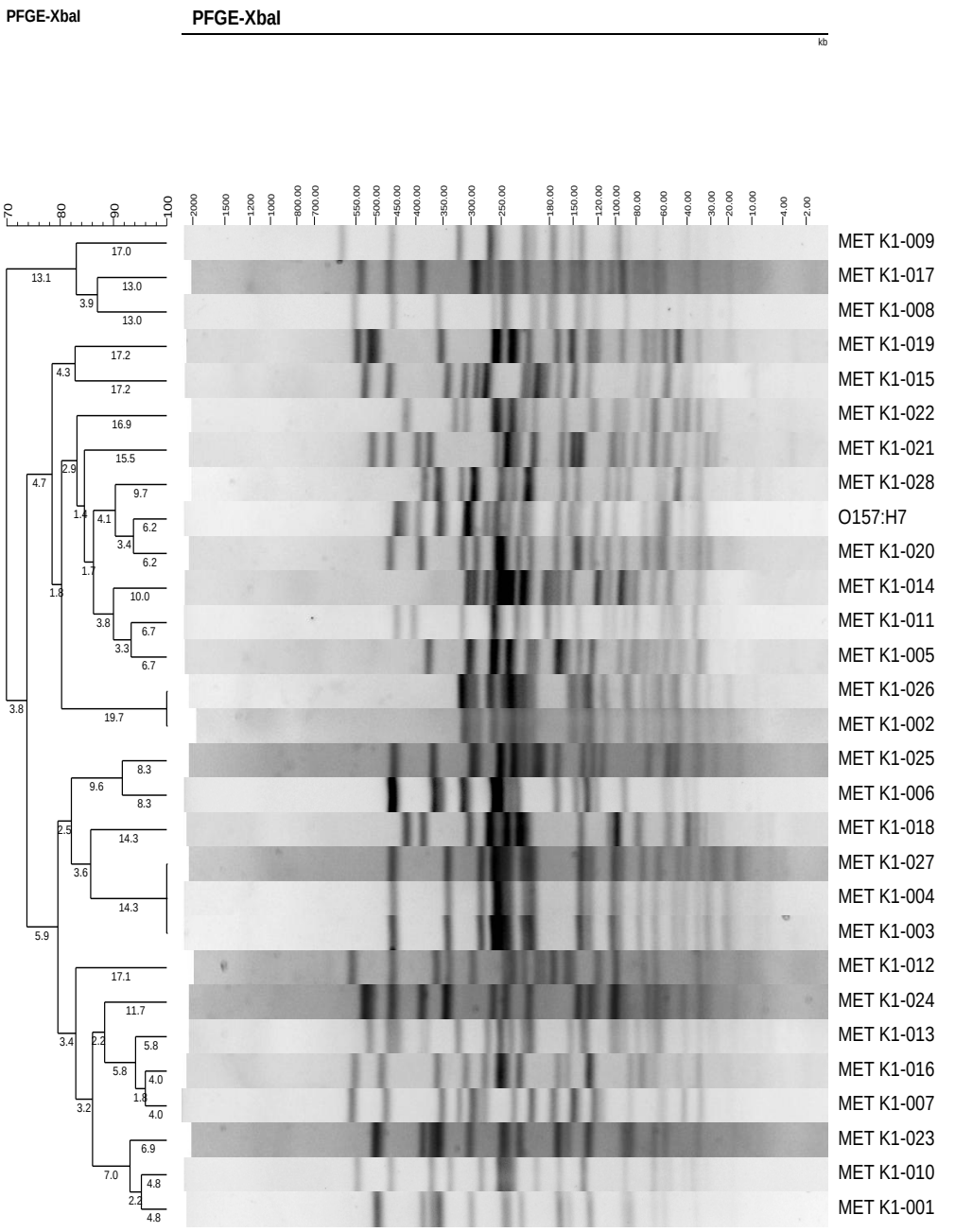

Figure 3.5 A dendogram showing different PFGE band and cluster patterns of *E. coli* isolates

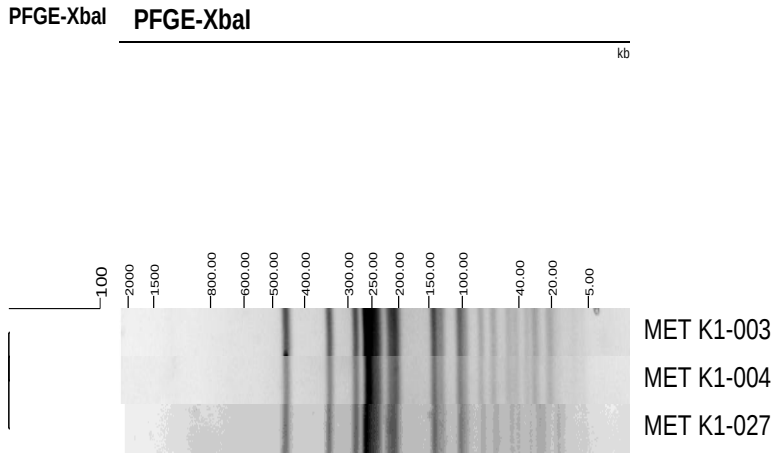

Figure 3.6 A dendogram showing three isolates from raw milk, MET K1-003, MET K1-004, and MET-K1-027, sharing the same PFGE pattern, pattern 3.

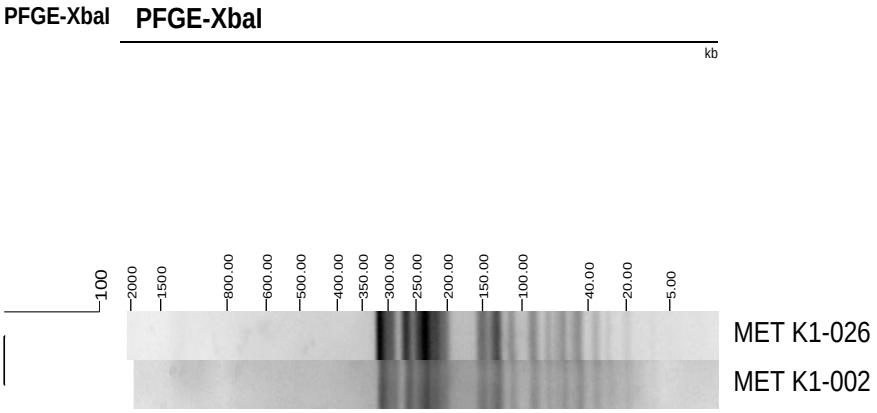

Figure 3.7 A dendogram showing two isolates from herby cheese, MET K1-002 and MET K1-026 sharing the same PFGE pattern, pattern 2.

3.6 Results of Antimicrobial Susceptibility Testing

The antibiotics tested is found in table 2.7. The results revealed a 100% susceptibility to ceftiofur, ertapenem, ceftriazone, amikacin and imipenem. However, there were high antimicrobial resistances to ampicillin, sulphafurazole and tetracycline (Fig. 3.8). The lowest level of sensitivity was recorded for ampicillin. Tetracycline and sulphafurazole were also low. *E. coli* resistivity to penicillins (ampicillin), aminoglycosides (streptomycin, gentamycin and kanamycin) were high. These results correlate with the European Antibiotic Resistance Surveillance Report (EARS-Net, 2012). From literature, the antibiotic with the highest resistivity of the *E. coli* isolates was ampicillin. 15 of the *E. coli* isolates were resistant to ampicillin (Fig. 3.8). In Enterobacteriaceae, resistance to ampicillin is mainly due to ß-lactamases (Brenwald et al., 2006). The isolates generally showed lower resistance to the cephems (cefrtriazone, cephalothin and cefoxitin).

Ceftiofur is only used in animals such as cattle and pigs to treat bacterial infections. Aminoglycosides such as gentamicin and kanamycin are used to treat bacterial infections in humans but people use them in farm animals such as cattle. FDA has warned farmers to stop using gentamicin in cattle. Ceftriazone is used only in humans for the treatment of severe Gram negative bacterial infections in young children. C, IMP, S, AK, TE, NA, SXT, KF, and AMC are used both in humans as well as animals. Ertapenem and cefoxitin are also use for treatment of bacterial infections in humans. They are effective against both Gram positive and Gram negative bacteria including *E. coli* (EFSA, 2011). The presence and frequency of tetracycline resistance in *E coli* in this study agree with findings of other studies on antibiotic resistance in *E coli*. (Sayah et al., 2005). 15 *E. coli* isolates from chicken wings, chicken drumsticks, turkey wings, and herby cheese were multidrug resistant. This represents about 53.5% of the total isolates used. It ranges from a minimum of 2 antibiotic resistance isolates to a maximum of 9 antibiotic resistance isolates, with an average of resistance at 7-8 antibiotics (Fig. 3.8). Resistance of isolates from chicken drumsticks were the highest. Apart from two chicken drumstick isolates which were not resistant to any of the antibiotics,

all the other isolates from chicken drumstick were showing resistivity to at least 4 antibiotics. The isolate which showed the highest number of resistance i.e. 9 antibiotics was from chicken drumstick. However almost all of the isolates from milk were susceptible to all the 17 antibiotics used. The only exception was one milk isolate which was resistant to gentamycin. *E. coli* isolates from 2 out of 3 herby cheese were susceptible to all the antibiotics but one was resistant to about 7 antibiotics. Isolates from chicken wings were resistant to 3 antibiotics. The isolate from turkey wings was also resistant to 2 antibiotics whereas that from salted cheese was susceptible to all the antibiotics (Fig. 3.8). The long-term use of antimicrobials for therapy and growth promotion in animals selects for drug resistance in *Escherichia coli*. Antibiotic usage is possibly the most important factor that promotes the emergence, selection, and dissemination of antibiotic-resistant microorganisms. This acquired resistance occurs not only in pathogenic bacteria but also in the endogenous flora of exposed individuals or populations (Marshall and Levy, 2011).

The emergence of multidrug resistance in *E. coli* has become a concern in the world. From the results, it has been clearly shown that most of the *E. coli* isolates found in Turkish food products have multi resistance to some antibiotics. Most multidrug resistance *E. coli* strains are acquired in the community (Collignon, 2009). In most cases, they are acquired through the diet (food and water) and their numbers keep on increasing with time (de Kraker et al., 2012). Johnson et al., revealed that food animals especially poultry contribute to most of the drug resistance *E. coli* carried by people (Johnson et al., 2007). As it was found in this study, chicken drumstick contained the highest number of multidrug resistance properties.

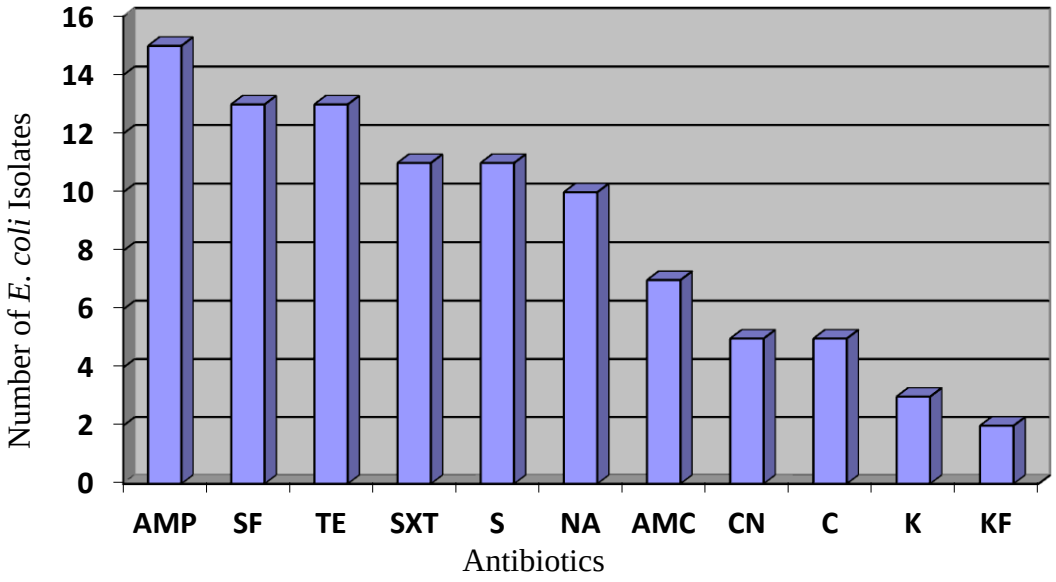

Figure 3.8. Number of *E. coli* isolates showing resistance to antibiotics

The massive use of antimicrobial agents in the poultry industry has supported their production by facilitating earlier weaning, higher animal densities among others. However, these gains have come at a great cost. These antibiotics have resulted in the propagation of resistant bacteria. Thus, the levels and patterns of resistance observed in food animals to a wide extent reflect the patterns of drug usage (Alhashash, 2013). The resistance pattern most frequently observed in the isolates was resistance to ampicilin, tetracycline and sulpharazole. The higher levels of multidrug resistance in *E. coli* might be attributed to several factors: One possibility is that resistant bacteria may be readily transferred from one food animal to the other. This is of great concern because these *E. coli* are found in foods. Even though they are not pathogenic, a foodborne outbreak that might occur in the area will be very serious because a more intensive multiple antibiotic strategy will be needed to cure *E. coli* infections.

Table 3.2 *E. coli* isolates, their sources, PFGE pattern, pathogenic genes, and antibiotics resistance

METU IDs	Specific food source	Pathognic gene found	PFGE pattern	Antibiotics resistant to
MET-K1-001	Raw milk	*fliC*	Pattern 1	NR
MET-K1-002	Herby Cheese	None	Pattern 2	NR
MET-K1-003	Raw milk	None	Pattern 3	Gentamycin
MET-K1-004	Raw milk	None	Pattern 3	NR
MET-K1-005	Raw milk	None	Pattern 4	NR
MET-K1-006	Raw patty meat	*fliC*	Pattern 5	NR
MET-K1-007	Chicken wings	None	Pattern 6	AMP, FOX, and NA.
MET-K1-008	Salted cheese	None	Pattern 7	NR
MET-K1-009	Chicken drumstick	None	Pattern 8	AMP, AMC, SF, SXT, TE, CN, and S.
MET-K1-010	Chicken drumstick	None	Pattern 9	AMP, AMC, SF, SXT, TE, CN, and S.
MET-K1-011	Chicken drumstick	None	Pattern 10	AMP, SF, SXT, NA, TE, CN, and S.
MET-K1-012	Turkey wings	None	Pattern 11	AMP and TE.
MET-K1-013	Chicken drumstick	None	Pattern 12	NR
MET-K1-014	Chicken drumstick	None	Pattern 13	AMC, SF, SXT, NA, TE, S, and K.
MET-K1-015	Chicken drumstick	None	Pattern 14	AMP, AMC, SF, SXT, NA, TE, and S.
MET-K1-016	Chicken drumstick	None	Pattern 15	AMP, AMC, SF, SXT, NA, TE, and S.
MET-K1-017	Chicken drumstick	None	Pattern 16	AMP, AMC, SF, SXT, NA, and TE.
MET-K1-018	Chicken drumstick	None	Pattern 17	AMP, SF, SXT, NA, TE, S, K, and C.
MET-K1-019	Chicken drumstick	None	Pattern 18	AMP, KF, SF, SXT, NA, TE, CN, and C.
MET-K1-020	Chicken drumstick	None	Pattern 19	NR
MET-K1-021	Chicken drumstick	None	Pattern 20	AMP, SF, SXT, TE, and S.
MET-K1-022	Chicken drumstick	None	Pattern 21	AMP, KF, SF, SXT, NA, TE, S, K, and C.
MET-K1-023	Raw milk	*fliC*	Pattern 22	NR

Table 3.2 *E. coli* isolates, their sources, PFGE pattern, pathogenic genes, and antibiotics resistance (Cont'd)

METU IDs	Specific food source	Pathognic gene found	PFGE pattern	Antibiotics resistant to
MET-K1-024	Herby cheese	None	Pattern 23	AMP, AMC, SF, NA, TE, S, and C.
MET-K1-025	Chicken drumstick	None	Pattern 24	AMP
MET-K1-026	Herby cheese	None	Pattern 2	NR
MET-K1-027	Raw milk	None	Pattern 3	NR
MET-K1-028	Chicken drumstick	None	Pattern 25	AMP, SF, S, and C.

AMP: Ampicillin, AMC: Amoxycilllin/Clavulanic Acid, SF: Sulpafurazole, SXT: Sulphamethoxazole/Trimethoprim, TE: Tetracycline, CN: Gentamicin, S: Streptomycin, NA: Nalidixic Acid, K: Kanamycin, C: Chloramphenicol, KF: Cephalothin, NR: Not Resistant

CHAPTER 4

CONCLUSION

We found a great genetic diversity of *E. coli* isolates from randomly selected food product set investigated in our study. Even though no pathogenic strain was identified, the rate of fecal contamination is alarming. This is a serious issue and public health education about personal hygiene should be intensified to avoid future outbreaks. Apart from the three isolates (MET K1-003, MET K1-004 and MET K1-027) from milk and the two isolates (MET K1-002 and MET K1-026) from herby cheese, which had the same PFGE band patterns, none of them was the same. More work involving a larger sample size need to be conducted to identify *E. coli* diversity in foods in Turkey. This research buttresses the need to conduct an intensive surveillance system of *E. coli* in Turkey because of its wide genetic diversity. All these food samples were taken from one locality. Turkey is an important exporter of food products so from this research, it shows that there is more work to be done in finding out the quality of the foods which are exported to other countries. Our database is publicly available and has been saved in the Food Engineering Department at Middle East Technical University for further reference and research. Another remarkable results of this research is the prevalence of multidrug resistance *E. coli* found in Turkish foods. Drug-resistant *E. coli* are readily acquired through water, food and especially food animal). When people eat sterile food, there is a rapid and substantial fall in the numbers of drug-resistant *E. coli* these people carry (Corpet, 1988). There is widespread use of antibiotics in food animals (often for inappropriate practices such as growth promotion or ongoing mass prophylactic medication). If huge numbers of people who live in developing countries are infected with multidrug-resistant *E. coli*, they might not be able to effectively treat them. Moreover, people who travel to Turkey have a risk factor in acquisition of antibiotic-resistant strains of *E. coli*.

This is also an issue for food exported to other countries, because these can contain multidrug resistant strains of *E. coli*. People should be educated on the severity of using antibiotics that have not been prescribed for both human and animals especially in the poultry industry because we can get a lot of negative consequences from these habits.

CHAPTER 5

RECOMMENDATIONS

From the results of this research, it is highly recommended to educate the public and citizens to beware of street foods and the importance of personal hygiene. This will help citizens to be clean so that they can prevent foodborne diseases and outbreaks in the future. Also, intensive surveillance studies about *E. coli* in Turkey should be conducted because of the wide genetic diversity of the microorganism.

For future studies, it is highly recommended that, food sample size should be increased and should be extended to many locations to find out the prevalence of the *E. coli* isolates. Moreover, a relatively few antibiotics were used. An expansion to involve more antibiotics would help to get highly convincing and significant findings.

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APPENDIX A

APPENDIX A. COLLECTION OF FOOD SAMPLES AND ISOLATION OF *E. COLI*

Table A.1 *E. coli* isolates, their food source and sampling dates

METU ID	Previous ID	Specific source of isolate	Exact date of sample collection	City collected
MET K1-001	E1002-Q001	Raw milk	2/15/2011	Van
MET K1-002	E1004-Q002	Herby cheese	2/21/2011	Van
MET K1-003	E1007-Q004	Raw milk	2/27/2011	Van
MET K1-004	E1008-Q004	Raw milk	2/27/2011	Van
MET K1-005	E3-Q06	Raw milk	2/27/2011	Van
MET K1-006	E1-Q05	Raw patty meat	3/6/2011	Van
MET K1-007	E4-Q07	Chicken wings	3/6/2011	Van
MET K1-008	E2-Q013	Salted cheese	3/23/2011	Van
MET K1-009	E-18-1	Chicken drumstick	5/27/2011	Van
MET K1-010	E-19-1	Chicken drumstick	5/27/2011	Van
MET K1-011	E-20-1	Chicken drumstick	5/27/2011	Van
MET K1-012	E-21-1	Turkey wings	5/27/2011	Van
MET K1-013	E-22-1	Chicken drumstick	5/27/2011	Van
MET K1-014	E-23-1	Chicken drumstick	5/27/2011	Van
MET K1-015	E1-Q24	Chicken drumstick	5/31/2011	Van
MET K1-016	E1-Q25	Chicken drumstick	5/31/2011	Van
MET K1-017	E1-Q26	Chicken drumstick	5/31/2011	Van
MET K1-018	E1-Q27	Chicken drumstick	5/31/2011	Van
MET K1-019	E1-Q28	Chicken drumstick	5/31/2011	Van
MET K1-020	E1-Q29	Chicken drumstick	5/31/2011	Van

Table A.1 *E. coli* isolates, their food source and sampling dates (Cont'd)

MET K1-021	E1-Q30	Chicken drumstick	5/31/2011	Van
MET K1-022	E1-Q31	Chicken drumstick	5/31/2011	Van
MET K1-023	E1001-Q001	Raw milk	2/15/2011	Van
MET K1-024	E1005-Q002	Herby cheese	2/21/2011	Van
MET K1-025	E1003-Q001	Chicken drumstick	2/15/2011	Van
MET K1-026	E1006-Q002	Herby cheese	2/21/2011	Van
MET K1-027	E1009-Q004	Raw milk	2/27/2011	Van
MET K1-028	E6-Q012	Chicken drumstick	3/23/2011	Van

APPENDIX B

COMPOSITION OF BUFFERS AND SOLUTIONS

Table B.1 Composition of Buffers and Solutions

0,25 N HCl Solution

Formula	
5 N HCl	12.5 mL
Sterile dH_2O	247.5 mL

0,5 M EDTA, pH 8

Formula	
EDTA	93,05 g
Sterile dH_2O	450 mL
NaOH	12 g

0,5 N NaOH Solution

Formula	mL
5 N NaOH	25
Sterile dH_2O	225

1 M Tris-HCl, pH 8

Formula	
Trizma-base	24.22 g
Sterile dH_2O	200 mL

10X Tris-Borat-EDTA (TBE) Stock Solution

Formula	
(0.9 M Trizma-base, 0.9 M Boric acid, 0.02 M EDTA)	
Tris-Base	108 g
$Na_2EDTA.2H_2O$	9.3 g
Boric acid	55 g

20 % SDS Solution

Formula	
SDS	2 g
Sterile dH2O	10 mL

Cell Lysis Buffer Solution

Formula	
1 M Tris-HCl, pH 8	25 mL
0,5 M EDTA, pH 8	50 mL
Sarcosyl	5 g
Sterile dH_2O	425 mL
Proteinase K (20 mg/mL)	2.5 mL

Cell Suspension Buffer Solution

Formula	
(100 mM Tris-HCl, 100 mM EDTA, pH 8)	
1 M Tris-HCl, pH 8	10 mL
0.5 M EDTA, pH 8	20 mL
Sterile dH2O	70 mL

Seakem Agarose (1 %)-SDS

Formula	
Seakem Agarose	0.25 g
Tris-EDTA solution (TE)	23.5 mL
20 % SDS solution	1.25 mL

Seakem Agarose (1%)-TBE

Formula	
Seakem Agarose	1 g
0.5X TBE solution	100 mL

Tris-EDTA (TE) Buffer Solution

Formula	
(10 mM Tris-HCl, 1 mM EDTA, pH 8)	
1 M Tris-HCl, pH 8	10 mL
0,5 M EDTA, pH 8	2 mL
Sterile dH2O	988 mL

APPENDIX C

COMPOSITION OF MEDIA

Table C.1 Composition of Media

Brillant Green Agar (Modified) - (BGA), (Oxoid Ltd., UK -CM0329)

Typical Formula	**gm/litre**
`Lab-Lemco' powder	5.0
Peptone	10.0
Yeast extract	3.0
Disodium hydrogen phosphate	1.0
Sodium dihydrogen phosphate	0.6
Lactose	10.0
Sucrose	10.0
Phenol red	0.09
Brilliant green	0.0047
Agar	12.0
pH 6.9 ± 0.2 @ 25°C	

Rappaport-Vassiliadis Soy Broth, (Oxoid Ltd., UK -CM0866)

Typical Formula	**gm/litre**
Soya peptone	4.5
Sodium chloride	7.2
Potassium dihydrogen phosphate	1.26
Di-potassium hydrogen phosphate	0.18
Magnesium chloride (anhydrous)	13.58
Malachite green	0.036
pH 5.2 ± 0.2 @ 25°C	

Brain Heart Infusion Broth, (Oxoid Ltd., UK -CM1135)

Formula	**gm/litre**
Brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5
pH 7.4 ± 0.2 @ 25°C	

Brain Heart Infusion Agar, (Oxoid Ltd., UK)

Formula	gm/litre
Brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Sodium chloride	5.0
Glucose	2.0
Disodium phosphate	2.5
Agar Bacteriological, OXOID UK (LP0011)	15.0
pH 7.4 ± 0.2 @ 25°C	

Tryptone Soy Agar

Formula	gm/litre
Agar Bacteriological, OXOID UK (LP0011)	15g
Tryptone Soy Broth, (Lab M Ltd., UK)	30g

Tryptone Soy Broth, (Lab M Ltd., UK)

Formula	gm/litre
Tryptone (casein digest U.S.P)	17.0
Soy Peptone	3.0
Sodium Chloride	5.0
Dipotassium hydrogen phosphate	2.5
Dextrose	2.5

E C Agar, (LAB M Ltd., UK)

Formula	gm/litre
Enzymatic Digest of Casein	20.0
Lactose	5.0
Bile Salts Mixture	1.5
Dipotassium Phosphate	4.0
Monopotassium Phosphate	1.5
Sodium Chloride	5.0
Agar Bacteriological, LAB M, UK (LP0011)	15.0
pH 6.9 ± 0.2 @ 25°C	

Eosin Methylene Blue Agar, (Oxoid Ltd., UK)

Formula	gm/litre
Peptone	10.0
Lactose	10.0
Dipotassium hydrogen phosphate	2.0
Eosin Y	0.4
Methylene blue	0.065
Agar Bacteriological, OXOID UK	15.0
pH 6.8± 0.2 @ 25°C	

ENDO Agar Base, (Oxoid Ltd., UK)

Formula	gm/litre
Peptone	10.0
Lactose	10.0
Di-potassium phosphate	3.5
Sodium sulphate	2.5
Agar Bacteriological, OXOID	10.0

APPENDIX D

STEC PROFILES OF *E.COLI* ISOLATES WITH STEC GENES

Table D.1 Results of PCR amplification of STEC genes with *E. coli* isolates

METU IDs	**Source**	***Stx 1***	***Stx 2***	***eae***	***rfbE***	***fliC***	***hlyA***
MET K1-001	Raw milk	Absent	Absent	Absent	Absent	**Present**	Absent
MET KI-002	Herby cheese	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-003	Raw milk	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-004	Raw milk	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-005	Raw milk	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-006	Raw patty meat	Absent	Absent	Absent	Absent	**Present**	Absent
MET K1-007	Chicken wings	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-008	Salted cheese	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-009	Chicken drumstick	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-010	Chicken drumstick	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-011	Chicken drumstick	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-012	Turkey wings	Absent	Absent	Absent	Absent	Absent	Absent
MET KI-013	Chicken drumstick	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-14	Chicken drumstick	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-015	Chicken drumstick	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-016	Chicken drumstick	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-017	Chicken drumstick	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-018	Chicken drumstick	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-019	Chicken drumstick	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-020	Chicken drumstick	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-021	Chicken drumstick	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-022	Chicken drumstick	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-023	Raw milk	Absent	Absent	Absent	Absent	**Present**	Absent
MET K1-024	Herby cheese	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-025	Chicken thigh	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-026	Herby cheese	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-027	Raw milk	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-028	Chicken drumstick	Absent	Absent	Absent	Absent	Absent	Absent
MET K1-029 (POSITIVE CONTROL)	Dr. Hallemans lab. (Ankara Univ.) O157:H7	Present	Present	Present	Present	Present	Present

APPENDIX E

AMPLIFICATIONS OF POSITIVE CONTROLS OF STEC PRIMERS ON AGAROSE GELS

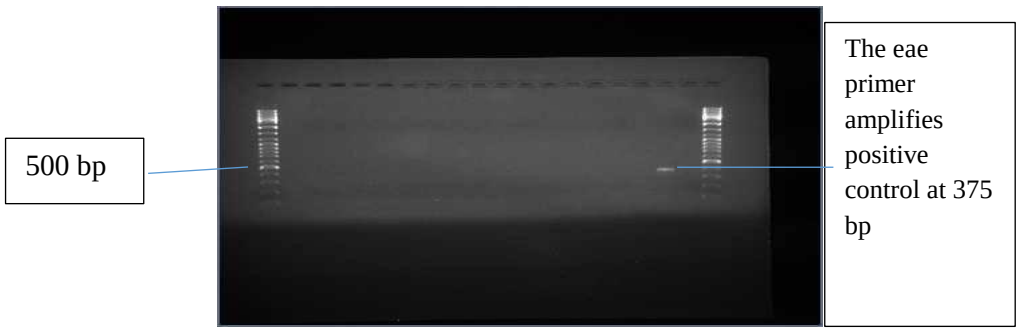

Figure E.1 *E. coli* isolate which was used as positive control amplifies at 375 bp with *eae* gene

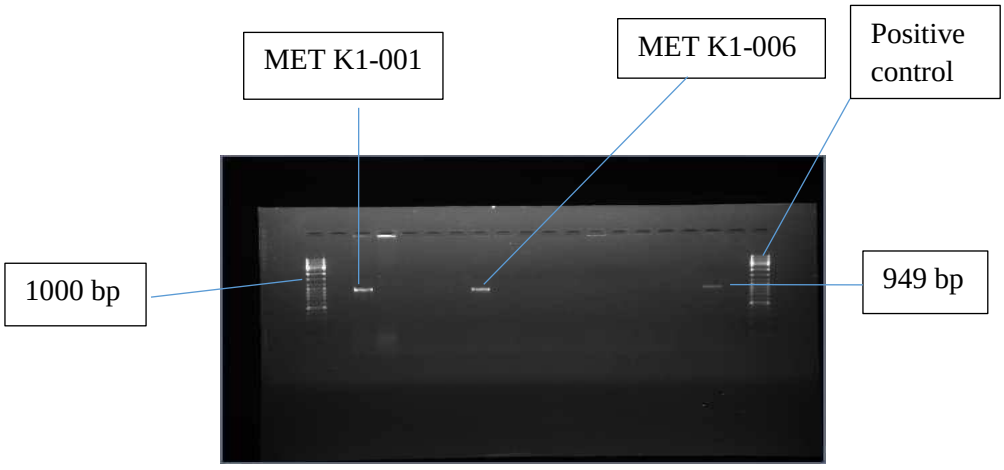

Figure E.2 Amplification of two isolates (MET K1-001 and MET K1-006) with the positive control of *fliC* gene on Agarose gel

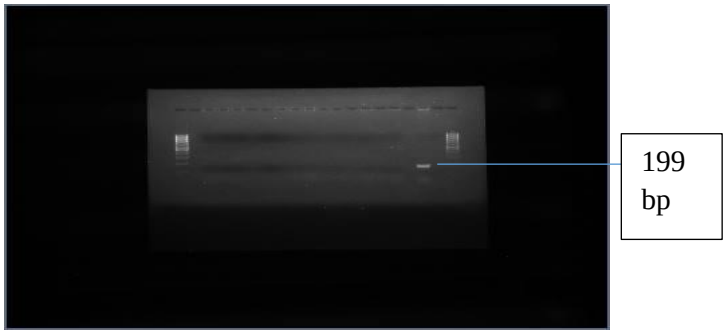

Figure E. 3 Amplification of the positive control of STEC gene *hlyA*

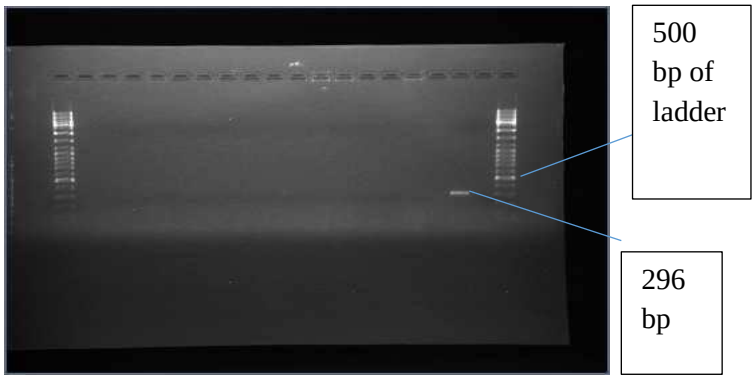

Figure E. 4 Amplification of the STEC gene *rfbE* positive control

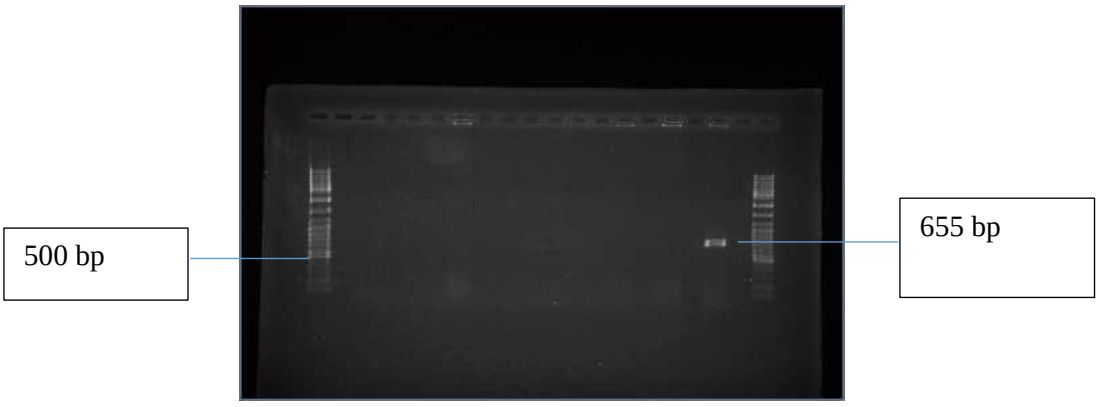

Figure E. 5 Amplification of *stx 1* gene on Agarose gel

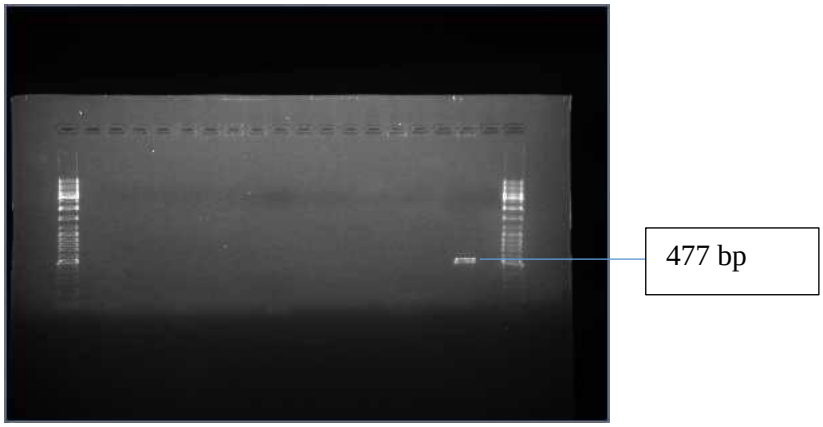

Figure E. 6 Amplification of the positive control of stx 2 primer on Agarose gel

APPENDIX F

AMPLIFICATIONS OF POSITIVE CONTROLS OF NON-STEC PATHOGENIC SUBGROUPS OF *E. COLI*

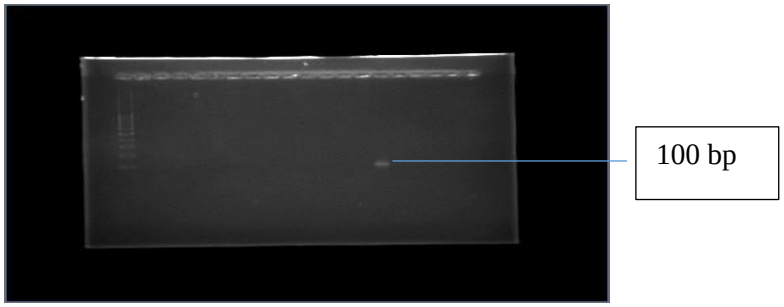

Figure F. 1 Amplification of *aggR* gene for EAEC

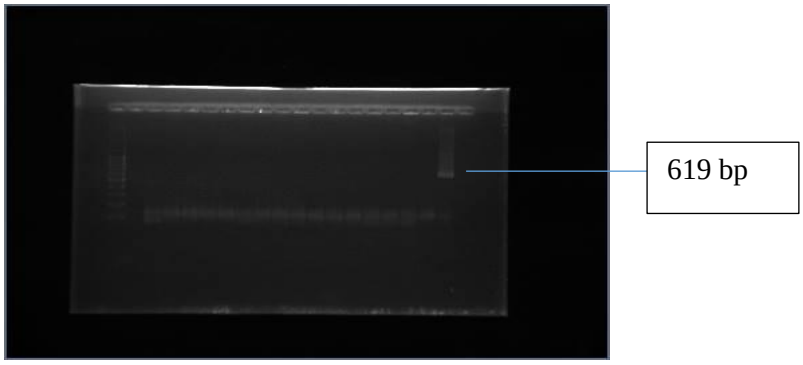

Figure F.2 Amplification of *ipaH* gene for EIEC

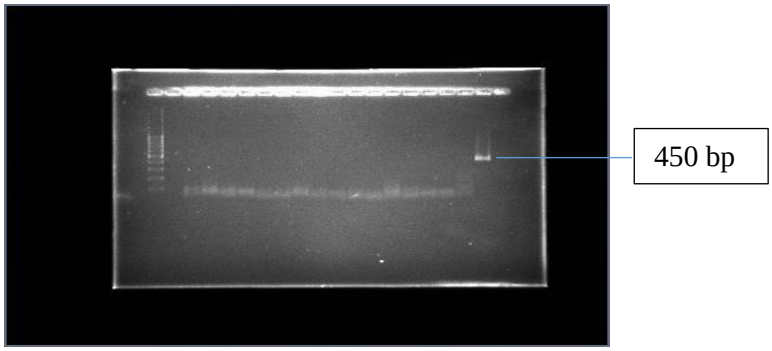

Figure F.3 Amplification of *lt* gene for ETEC

APPENDIX G

PFGE BAND PATTERNS ON AGAROSE GEL

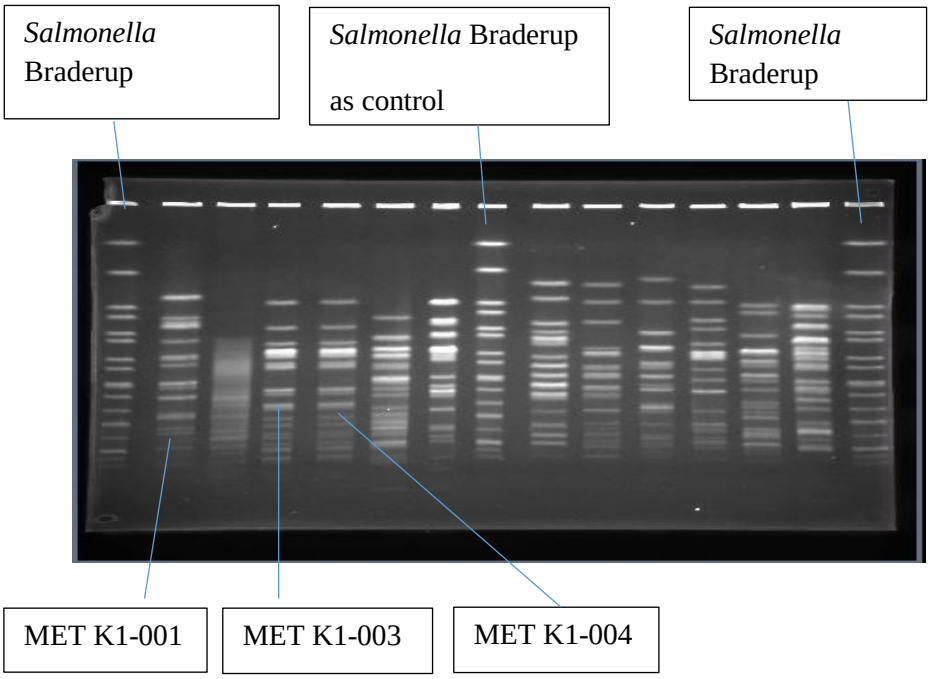

Figure G. 1 PFGE patterns of *E. coli* Isolates

APPENDIX H

ANTIMICROBIAL SUSCEPTIBILITY RESULTS

Table H.1 Zone diameters of *E. coli* isolates with first set of antibiotics

	Antibiotics and zone diameter in mm					
Sample Number	**Ampicillin (AMP)**	**Amoxycillin/ Clavulanic Acid (AMC)**	**Ceftiofur (EFT)**	**Cefoxitin (FOX)**	**Cephalothin (KF)**	**Ertapenem (ETP)**
E. coli Reference range ATCC25922	16-22	18-24	26-31	23-29	15-21	29-36
E. coli ATCC25922	21	21	26	26	15	34
1A	22	22	28	27	18	34
2A	20	23	26	25	15	34
3A	22	24	26	26	20	33
4A	21	24	26	26	19	34
5A	20	23	26	26	19	34
6A	21	23	27	26	22	35
7A	0	19	21	0	15	35
8A	20	22	28	25	16	31
9A	0	20	25	24	17	34
10A	0	18	25	24	19	31
11A	0	17	25	25	16	32
12A	8	20	25	26	17	33
13A	20	23	27	26	20	35
14A	19	21	27	26	17	33
15A	0	19	26	24	17	32
16A	0	20	22	26	15	32

Table H.1 Zone diameters of *E. coli* isolates with first set of antibiotics (Cont'd)

17A	0	18	26	27	17	32
18A	0	17	27	24	15	32
19A	0	15	27	24	14	31
20A	21	24	28	26	19	33
21A	0	18	25	25	15	30
22A	0	18	26	25	14	32
23A	17	19	25	23	15	32
24A	0	12	24	24	16	31
25A	17	20	24	23	14	31
26A	17	22	26	24	18	31
27A	21	23	27	27	18	32
28A	0	22	28	24	16	34

Table H. 2 Interpretation of the zone diameters according to CLSI standards

	Antibiotics Results														
Sample Number	**Ampicillin (AMP)**			**Amoxycillin (AMC)**			**Ceftiofur (EFT)**			**Cefoxitin (FOX)**			**Cephalothin (KF)**		
E. coli Reference Range ATCC25922	16-22			18-24			26-31			23-29			15-21		
Interpretative Criteria	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
	≥ 17	14 - 16	≤ 13	≥ 18	14 - 17	≤ 13	≥ 21	18 - 20	≤ 17	≥ 18	15 - 17	≤ 14	≥ 18	15 - 17	≤ 14
1A	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		

Table H. 2 Interpretation of the zone diameters according to CLSI standards (Cont'd)

2A	Susceptible	Susceptible	Susceptible	Susceptible	Intermediate
3A	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
4A	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
5A	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
6A	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
7A	Resistant	Susceptible	Susceptible	Resistant	Intermediate
8A	Susceptible	Susceptible	Susceptible	Susceptible	Intermediate
9A	Resistant	Resistant	Susceptible	Susceptible	Intermediate
10A	Resistant	Resistant	Susceptible	Susceptible	Susceptible
11A	Resistant	Intermediate	Susceptible	Susceptible	Intermediate
12A	Resistant	Susceptible	Susceptible	Susceptible	Intermediate
13A	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
14A	Susceptible	Resistant	Susceptible	Susceptible	Intermediate
15A	Resistant	Resistant	Susceptible	Susceptible	Intermediate
16A	Resistant	Resistant	Susceptible	Susceptible	Intermediate
17A	Resistant	Resistant	Susceptible	Susceptible	Intermediate
18A	Resistant	Intermediate	Susceptible	Susceptible	Intermediate
19A	Resistant	Intermediate	Susceptible	Susceptible	Resistant
20A	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
21A	Resistant	Susceptible	Susceptible	Susceptible	Intermediate
22A	Resistant	Susceptible	Susceptible	Susceptible	Resistant
23A	Susceptible	Susceptible	Susceptible	Susceptible	Intermediate
24A	Resistant	Resistant	Susceptible	Susceptible	Intermediate
25A	Susceptible	Susceptible	Susceptible	Susceptible	Resistance
26A	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
27A	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
28A	Resistant	Susceptible	Susceptible	Susceptible	Intermediate

Table H. 3 Zone diameters of *E. coli* isolates with second set of antibiotics

	Antibiotics and zone diameter in mm				
Sample Number	**Ceftriazone (CRO)**	**Sulphafurazole (SF)**	**Sulphamethoxazole/ Trimethoprim (SXT)**	**Nalidixic Acid (NA)**	**Tetracycline (TE)**
E. coli Reference range ATCC25922	29-35	15-23	23-29	22-28	18-25
E. coli ATCC25922	29	18	23	25	25
1B	30	22	29	23	24
2B	29	22	28	22	24
3B	30	23	27	24	26
4B	30	21	27	21	25
5B	32	24	27	24	26
6B	30	22	24	23	25
7B	30	23	29	0	27
8B	30	23	28	23	24
9B	29	0	0	24	0
10B	27	0	0	22	8
11B	29	0	0	0	0
12B	27	19	27	22	10
13B	31	25	28	22	26
14B	29	0	0	0	0
15B	28	0	0	0	0
16B	27	0	0	0	0
17B	27	0	0	0	9
18B	31	0	0	0	9
19B	30	0	0	0	0
20B	29	24	23	23	25
21B	26	0	0	20	9
22B	29	0	0	0	0
23B	29	16	24	21	24
24B	26	0	19	0	8
25B	28	21	25	20	24
26B	29	22	28	23	26
27B	29	23	25	22	25
28B	30	0	22	24	25

Table H. 4 Interpretation of the zone diameters of the second set of antibiotics according to CLSI standards

	Antibiotics Results														
Sample Number	**Ceftriazone (CRO)**			**Sulphafurazole (SF)**			**Sulphamethoxazole/ Trimethoprim (SXT)**			**Nalidixic Acid (NA)**			**Tetracycline (TE)**		
E. coli Reference Range ATCC25922	29-35			15-23			23-29			22-28			18-25		
Interpretative Criteria	S ≥ 23	I 20-22	R ≤ 19	S ≥ 17	I 13-16	R ≤ 12	S ≥ 16	I 11-15	R ≤ 10	S ≥ 19	I 14-18	R ≤ 13	S ≥ 15	I 12-14	R ≤ 13
1B	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
2B	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
3B	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
4B	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
5B	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
6B	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
7B	Susceptible			Susceptible			Susceptible			Resistant			Susceptible		
8B	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
9B	Susceptible			Resistant			Resistant			Susceptible			Resistant		
10B	Susceptible			Resistant			Resistant			Susceptible			Resistant		
11B	Susceptible			Resistant			Resistant			Resistant			Resistant		
12B	Susceptible			Susceptible			Susceptible			Susceptible			Resistant		
13B	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
14B	Susceptible			Resistant			Resistant			Resistant			Resistant		
15B	Susceptible			Resistant			Resistant			Resistant			Resistant		
16B	Susceptible			Resistant			Resistant			Resistant			Resistant		
17B	Susceptible			Resistant			Resistant			Resistant			Resistant		
18B	Susceptible			Resistant			Resistant			Resistant			Resistant		
19B	Susceptible			Resistant			Resistant			Resistant			Resistant		
20B	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
21B	Susceptible			Resistant			Resistant			Susceptible			Resistant		
22B	Susceptible			Resistant			Resistant			Resistant			Resistant		
23B	Susceptible			Intermediate			Susceptible			Susceptible			Susceptible		
24B	Susceptible			Resistant			Susceptible			Resistant			Resistant		
25B	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
26B	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
27B	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
28B	Susceptible			Resistant			Susceptible			Susceptible			Susceptible		

Table H. 5 Zone diameters of *E. coli* isolates with last set of antibiotics

	Antibiotics and zone diameter in mm					
Sample Number	**Amikacin (AK)**	**Gentamicin (CN)**	**Streptomycin (S)**	**Kanamycin (K)**	**Imipenem (IPM)**	**Chloramphenicol (C)**
E. coli Reference range ATCC25922	19-26	19-26	12-20	17-25	26-32	21-27
E. coli ATCC25922	19	19	16	20	27	26
1C	21	20	14	21	27	25
2C	19	18	16	19	27	24
3C	19	0	15	21	27	26
4C	21	19	16	20	25	25
5C	22	19	15	22	24	24
6C	22	19	16	21	25	24
7C	24	19	15	22	25	27
8C	20	17	15	19	26	25
9C	21	20	10	19	24	26
10C	21	18	7	20	26	24
11C	22	18	0	20	28	25
12C	22	18	14	20	25	6
13C	23	19	15	22	27	26
14C	22	19	0	0	29	22
15C	21	20	0	18	24	26
16C	23	19	7	18	26	25
17C	22	20	12	19	26	21
18C	19	19	9	6	27	6
19C	22	10	13	17	25	10
20C	22	19	15	20	26	26
21C	19	15	7	18	24	25
22C	20	16	0	0	25	0
23C	22	20	16	21	26	23
24C	20	20	0	19	25	0
25C	21	18	14	24	25	26
26C	21	19	17	19	25	25
27C	21	19	18	21	25	26
28C	24	14	7	15	29	0

Table H. 6 Interpretation of the zone diameters of the last set of antibiotics according to CLSI standards

				Antibiotics Results													
Sample number	**AK**			**CN**			**S**			**K**			**IPM**			**C**		
E. coli reference range ATCC25922	19-26			19-26			12-20			17-25			26-32			21-27		
Interpretative criteria	S ≥ 17	I 15 - 16	R ≤ 14	S ≥ 15	I 13 - 14	R ≤ 12	S ≥ 15	I 12 - 14	R ≤ 11	S ≥ 18	I 14 - 17	R ≤ 13	S ≥ 23	I 20 - 22	R ≤ 19	S ≥ 18	I 13 - 17	R ≤ 12
1C	Susceptible			Susceptible			Intermediate			Susceptible			Susceptible			Susceptible		
2C	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
3C	Susceptible			Resistant			Susceptible			Susceptible			Susceptible			Susceptible		
4C	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
5C	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
6C	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
7C	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
8C	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
9C	Susceptible			Resistant			Resistant			Susceptible			Susceptible			Susceptible		
10C	Susceptible			Resistant			Resistant			Susceptible			Susceptible			Susceptible		
11C	Susceptible			Resistant			Resistant			Susceptible			Susceptible			Susceptible		
12C	Susceptible			Susceptible			Intermediate			Susceptible			Susceptible			Susceptible		
13C	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
14C	Susceptible			Susceptible			Resistant			Resistant			Susceptible			Susceptible		
15C	Susceptible			Susceptible			Resistant			Susceptible			Susceptible			Susceptible		
16C	Susceptible			Susceptible			Resistant			Susceptible			Susceptible			Susceptible		
17C	Susceptible			Susceptible			Intermediate			Susceptible			Susceptible			Susceptible		
18C	Susceptible			Susceptible			Resistant			Resistant			Susceptible			Resistant		
19C	Susceptible			Resistant			Intermediate			Intermediate			Susceptible			Resistant		
20C	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
21C	Susceptible			Susceptible			Resistant			Susceptible			Susceptible			Susceptible		
22C	Susceptible			Susceptible			Resistant			Resistant			Susceptible			Resistant		
23C	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
24C	Susceptible			Susceptible			Resistant			Susceptible			Susceptible			Resistant		
25C	Susceptible			Susceptible			Intermediate			Susceptible			Susceptible			Susceptible		
26C	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
27C	Susceptible			Susceptible			Susceptible			Susceptible			Susceptible			Susceptible		
28C	Susceptible			Intermediate			Resistant			Intermediate			Susceptible			Resistant		