DETECTION OF *ESCHERICHIA COLI* VIRULENCE GENES IN RAW BEEF MEAT FROM BEIRUT SLAUGHTERHOUSE

by

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ABSTRACT

DETECTION OF *ESCHERICHIA COLI* VIRULENCE GENES IN RAW BEEF MEAT FROM BEIRUT SLAUGHTERHOUSE

By

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Escherichia coli is the predominant facultative anaerobe of the microbiota in the human colon. Infections due to pathogenic *E. coli* may be limited to the mucosal surfaces or can disseminate throughout the whole body, with symptoms ranging from enteric diarrheal disease, to urinary tract infections, or sepsis. *E. coli* carrying virulence genes provides different pathogenic strategies that confer serious effects on human hosts, namely different types of diarrhea. Raw meat samples, potential reservoirs of *E. coli*, were chosen for sampling to detect and characterize the *E. coli* types present therein, according to their respective virulence gene(s). Therefore, 600 raw meat samples were collected from Beirut Slaughterhouse on weekly basis intervals, over a period spanning from February till September 2002. The detection of *uspA* gene was indicative of an *E. coli*-containing sample. *uspA* is a gene used by investigators to differentiate *E. coli* from other gram-negative bacteria. Eleven virulence genes were chosen on the basis of different pathogenic strategies: Shiga toxin genes (VT1, VT2, and VT2e), intimin gene (*eaeA*), cytotoxic necrotizing factors genes (CNF1 and CNF2), enteroaggregative gene (EAgg), enteroinvasive gene (EInv), heat-stable and heat-labile genes (ST1, ST2, LT1). Multiplex

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polymerase chain reaction (PCR) was adapted to detect these eleven virulence genes in combinations. *E. coli* O157:H7 presence was investigated by the detection of *uidA* gene, a mutant form of which is harbored by this particular strain. Five samples were further subtyped by pulsed field gel electrophoresis chosen particularly since they harbored *eaeA* gene in addition to at least one of the VT genes.

Of the total number of samples collected, 81.8% contained *E. coli*, of which 31.8% were virulent as indicated by the detection of the following virulence genes: CNF1 (10.2%), VT2 (8.4%), VT1 (7.1%), *eaeA* (6.7%), CNF2 (5.3%), VT2e (4.1%), ST1 (2%), EAgg (0.4%), LT1 (0.2%). None of the samples harbored the *uidA* gene. The period of May-June witnessed the highest prevalence of virulence-positive samples. On the other hand, the period spanning from mid-June till mid-July witnessed the highest prevalence of virulence-negative samples. The five samples that were positive for *eaeA* and VT genes were genetically distinct after analysis by PFGE, indicating they originated from different sources. Most of the genes detected were consistent with those reported by other investigators. The process of slaughtering was monitored closely and several sources of contamination of meat with intestinal contents were noted, many of which could be controlled and prevented. The remaining issue is the contamination process that could take place during the transport and delivery process from the slaughterhouse to retail shops, then to the consumers. Further studies should be conducted to monitor this process and find other potential sources of contamination. Additionally, the hospitalization status for *E. coli*-caused diarrhea could be monitored in concordance with the detection of contaminated meat in the slaughterhouse.

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...à Renée...

"Hope is a state of mind, not of the world. Hope, in this deep and powerful sense, is not the same as joy that things are going well, or willingness to invest in enterprises that are obviously heading for success, but rather an ability to work for something because it is good."

-Vaclav Havel

Thank God for the gifts of persistence, creativity and stubbornness.

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Last but not least, I would like to thank my family for their patience and constant support.

GLOSSARY

EAEC: Enteroaggregative *E. coli*

EHEC: Enterohemorrhagic *E. coli*

EIEC: Enteroinvasive *E. coli*

EPEC: Enteropathogenic *E. coli*

ETEC: Enterotoxigenic *E. coli*

NTEC: Necrotoxigenic *E. coli*

PCR: Polymerase chain reaction

PFGE: Pulsed field gel electrophoresis

SE buffer: Salt EDTA buffer

TE buffer: Tris EDTA buffer

Chapter 1

INTRODUCTION

Escherichia coli is a facultative anaerobe that inhabits the human gut hours after birth, where both benefit mutually. It remains harmless until the gastrointestinal barrier is violated, or upon acquiring virulent genetic characteristics; only then will it become virulent. *E. coli* inhabits the ruminant gut as well. Upon improper slaughtering, such *E. coli* could contaminate the meat. Ingestion of meat products contaminated with virulent *E. coli* has been the reason behind many of the diarrheal and septicemic disease in humans. Following a standard strategy of infection, *E. coli* colonizes the mucosal surface, evades host defenses, and then causes damage to the host (Drasar and Hill, 1974).

This study was conducted to get a clear picture of the level of *E. coli* contamination of meat at the Beirut Slaughterhouse.

Purpose of the study:

The purpose of the current study is to detect and characterize *E. coli* in raw beef meat from freshly slaughtered calves at Beirut Slaughterhouse during the period of February-September 2002:

1. Detect the *uspA* gene as an indication of *E. coli* presence in the enriched meat samples.
2. Further detect, by multiplex PCR, the following virulence genes: VT1, VT2, VT2e, *eaeA*, CNF1, CNF2, Eagg, Einv, ST1, ST2, and LT1 in order to characterize the *E. coli* types present among the *uspA*-positive samples.
3. Detect the *uidA* gene, specific to *E. coli* O157:H7 in enterohemorrhagic *E. coli*.

4. Subtype the samples positive for eaeA and at least one of the VT1, VT2 and VT2e genes by pulsed field gel electrophoresis (PFGE).

Chapter 2

LITERATURE REVIEW

2.1. General Overview of *E. coli*:

A common member of the normal microbiota of the large intestine of humans is *E. coli*. It inhabits the human gut hours after birth, and lives thereafter as a facultative anaerobe where *E. coli* and the host mutually benefit (Drasar and Hill, 1974; Baron et al., 1996). This organism remains harmless in the intestinal lumen, and causes infection when the gastrointestinal barrier is violated. When it acquires additional genetic elements encoding virulence factors, *E. coli* will invade the mucosal surface or disseminate throughout the whole body. The results of such an invasion are three general clinical symptoms: enteric/diarrheal disease, sepsis/meningitis, or urinary tract infection (Nataro and Kaper, 1998).

2.1.1. *E. coli* pathogenesis:

Being a mucosal pathogen, *E. coli* follows a standard strategy of infection: it first colonizes the mucosal site, evades host defenses, multiplies, and then causes damage to the host (Drasar and Hill, 1974).

Escherichia spp. and *Salmonella* spp. diverged from a common ancestor about 120 to 160 billion years ago. Such an observation is based on phylogenetic trees based on sequencing of 16S rRNA and 5S rRNA (Ochman and Groisman, 1994). Feng (1995) proposed a stepwise model for the evolution of a particular *E. coli* serotype O157:H7, based on the discrete evolutionary events from an EPEC-like ancestor resembling most present-day commensal *E. coli*. O157:H7 evolved sequentially from the common EPEC-like ancestor, acquiring first a locus of enterocyte effacement (LEE), and then acquiring a gene for Shiga toxin 2 by transduction (O'Brien and Holmes,

1987). Later on, the EHEC plasmid encoding hemolysins was acquired (Bilge *et al.*, 1996), followed by acquisition of Shiga toxin 1 gene (Strockbine *et al.*, 1986).

The production of stress-responding proteins by microbes has been proven to help such microorganisms to adapt to conditions usually inhabitable by enteric organisms (Völker *et al.*, 1992). Nyström and Neidhart (1992) were the first to describe one such protein that is produced by *E. coli*.

Universal stress protein (USP) is a cytoplasmic protein; synthesized mainly in the absence of nutritional substances and in the presence of toxic agents. The gene (*uspA*) encoding USP is chromosomally encoded; *uspA*. Chen and Griffiths (1998) described a polymerase chain reaction (PCR)-based procedure, where uspA gene amplification is used to differentiate *E. coli* from other gram-negative bacteria.

Diarrheagenic *E. coli* strains, being a subset of pathogenic *E. coli* possess a particular feature that is highly conserved among them, their ability to colonize the intestinal mucosal surface despite peristalsis and other microbiota's competition for nutrients. In addition to this feature, virtually all *E. coli* strains possess surface adherence fimbriae (Knutton et al., 1984). However, diarrheagenic *E. coli* strains possess additional features that enhance their ability to colonize the intestine and adhere to the small bowel mucosa. They possess specific fimbrial antigens (Fig. 1) that have an inferred, non-demonstrated role, partly due to the host specificity of most fimbrial adhesions (Levine et al., 1984).

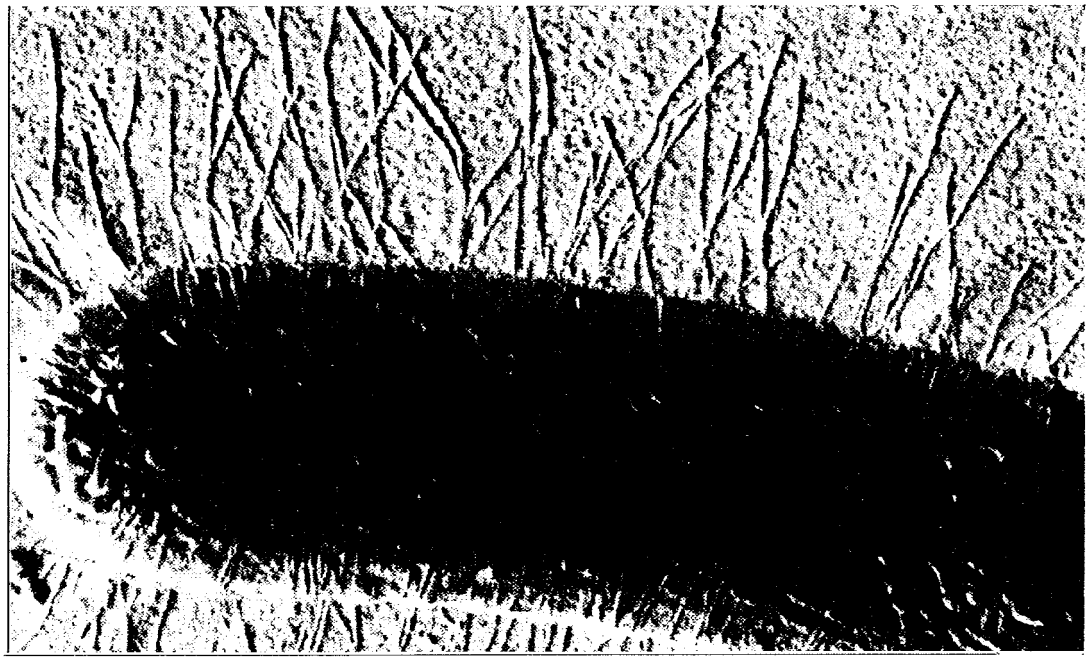

Figure 1: Surface replica of *E. coli* strain 469-3. Numerous ~7nm-diameter fimbriae project from the bacterial surface. *Infection and Immunity 44: 600.*

2.1.2. *E. coli* types:

In the case of diarrheagenic *E. coli,* after colonization is established, several different pathogenic strategies are exhibited. The enterotoxigenic (ETEC) and enteroaggregative *E. coli* (EAEC) cause severe diarrhea by enterotoxin production. Enteroinvasive (EIEC), enteropathogenic (EPEC), and enterohemorrhagic (EHEC) *E. coli* invade and/or adhere with membrane signaling (Nataro and Kaper, 1998).

Regarding non-diarrheagenic *E. coli,* necrotoxigenic *E. coli* (NTEC) are associated with intestinal and extraintestinal infections (Van Bost et al., 2003; Fournout et al., 2000). Each type exhibits a particularly unique mode of pathogenesis, as inferred from the virulence trait it carries.

2.2. ENTEROHEMORRHAGIC *E. coli*:

2.2.1. Historical Background:

Starting with EHEC, this type of *E. coli* comprises enteric pathogens that cause intestinal and renal disease. In 1983, two outbreaks in Oregon and Michigan

(February-June 1982) of unusual gastrointestinal illness were investigated. Those outbreaks were characterized by cramps and abdominal pain, initial watery diarrhea followed by glossy bloody diarrhea, with little or no fever. The results of stool cultures and serotyping of the isolates revealed a clinically distinctive gastrointestinal illness associated with a previously unreported *E. coli* serotype O157:H7 apparently transmitted by undercooked meat (Riley *et al.,* 1983). In 1983 as well, another study was published (Karmali et al., 1983), where an association was established between certain *E. coli* serotypes and cases of haemolytic uraemic syndrome (HUS). From there on, EHEC were recognized as a distinct type of pathogenic *E. coli.* This type was described by two different investigators, each used their own nomenclature. Konowalchuk et al., (1977) described Vero cytotoxin-producing *E. coli* strains (VTEC) which produced a toxin that was cytotoxic to Vero cells, whereas their proponents (Calderwood et al., 1996) described Shiga toxin-producing *E. coli* (STEC) that produced cytotoxins essentially identical at the genetic and protein levels to the Shiga toxin produced by *Shigella dysenteriae* I. The term EHEC was originally used to denote strains that cause hemorrhagic colitis (HC) and HUS, express Shiga toxin (VT/Stx), cause attaching and effacing (A/E) lesions on intestinal epithelial cells, and possess a 60-MDa plasmid (Levine, 1987).

2.2.2. EHEC pathogenesis:

A major virulence factor of EHEC is Shiga toxin gene (*stx*). Multiple Stx types are recognized, where Scotland et al., (1985) showed that anti-Stx could not neutralize the cytotoxicity of some STEC strains. Some strains produced only the anti-Stx neutralizable toxin denoted Stx1, others produced the nonneutralizable toxin (denoted Stx2), yet others produced both (Scotland *et al.,* 1985). A variant of Stx2 (denoted Stx2e) could be isolated from STEC isolates with edema disease in piglets. Marques

et al., (1987) found a different cytotoxicity pattern on HeLa cells, but the same neutralization effect upon using polyclonal anti-Stx2, thereby describing it as an Stx2 variant.

Members of the Stx type are compound toxins of two subunits: a 32-kDa catalytic A subunit and a multimeric B subunit (7.7 kDa monomers) involved in the binding to specific glycolipid receptors on the surface of target cells (O'Brien and Holmes, 1987): globotetraosylceramide (Gb_4) for Stx2 and globotriaosylceramide (Gb_3) for the rest of the Stx type members (Samuel *et al.,* 1990). Once bound to the target receptor, toxin molecules are internalized by the formation of a clathrin-coated pit within the cell membrane, pinching off to form a sealed coated vesicle with the toxin bound to its internal surface. These complexes then undergo retrograde transport via the Golgi apparatus to the endoplasmic reticulum, then translocation into the cytosol. Throughout this process, the A subunit becomes catalytically activated by membrane-bound protease action contracting RNA *N*-glycosidase activity, and cleaving a specific *N*-glycosidic bond in 28S rRNA, thus inhibiting a peptide chain elongation step of protein synthesis and ultimately causing cell death (Sandvig and van Deurs, 1996).

Shigella dysenteriae and *E. coli* harbor genes encoding Stx, the nucleotide sequences of which were determined in the 1980's (Calderwood *et al.,* 1987; DeGrandis et al., 1987). The structural genes for Stx1 and Stx2e are found on lysogenic lambdoid bacteriophages; the genes for Stx2 are chromosomally encoded. The operons have a single common transcriptional unit encoding first the A subunit followed by the B subunit. To satisfy the 1:5 A/B subunit stoichiometry of the holotoxin, the B subunit has a stronger ribosome-binding site than the A subunit, resulting in increased translation of the B subunits (Habib and Jackson, 1993). The sequence of the operon

encoding the Stx2 variant, Stx2e, was determined (Gyles et al., 1988). Its A subunit has 94% homology to the Stx2 A subunit, and the B subunit has 87% homology to the Stx2 B subunit. One thing worth mentioning is that the production of Stx1 from *E. coli* and *S. dysenteriae* is repressed by iron and reduced temperature, whereas expression of Stx2 is unaffected by these factors (Nataro and Kaper, 1998).

A/E lesions on enterocytes caused by STEC infection involve ultrastructural changes, including loss of enterocyte microvilli, intimate attachment of the bacterium to the cell surface, and accumulation of cytoskeletal components. The result of such changes is the formation of pedestals (Knutton *et al.,* 1989) (Fig. 2).

Figure 2: Characteristic EPEC A/E lesion observed in the ileum after oral inoculation of gnotobiotic piglets. *J Pediatr Gatroenterol Nutr (1983) 2: 536.*

A/E lesions were initially described in EPEC (Jerse *et al.,* 1990), but later on EHEC were shown to possess such virulence mechanisms (Sherman et al., 1988). Binding of EPEC to epithelial cells triggers intracellular signals: release of inositol triphosphate (IP_3), phosphorylation of myosin light chains, and tyrosine phosphorylation of certain epithelial cell membrane proteins (Donnenberg *et al.,* 1997). All the genes necessary for the A/E lesion production are located on a 35.5-kb "pathogenicity island" termed the locus for enterocyte effacement (LEE), which is inserted at 82' in the *E. coli*

chromosome. LEE includes a cluster of genes. They encode a type III secretion system and express EspA, EspB, and EspD, responsible for initiation of signal transduction events. Additionally, eaeA gene encodes intimin, an outer membrane protein which mediates intimate attachment to the enterocyte (Lai *et al.*, 1997). The mechanism by which STEC produce A/E lesions is homologous to that by which EPEC produce these lesions. STEC strains have a LEE homologue, and display an A/E phenotype.

O157:H7 strains commonly harbor a 60-MDa plasmid, containing genes encoding an enterohemolysin. This gene is commonly found in nearly all O157:H7 strains as well as many non-O157 (Schmidt *et al.*, 1995). Enterohemolysin belongs to the RTX type of toxins, members of which are expressed in uropathogenic *E. coli*. Enterohemolysin causes lysis of erythrocytes in vivo, releasing heme and hemoglobin and serving as a source of iron for the growth of O157:H7 (Bauer and Welch, 1996).

E. coli O157:H7 has a unique genetic characteristic. It does not exhibit β-glucuronidase activity, but carries the gene encoding such activity (*uidA* gene) (Feng *et al.*, 1991). The O157:H7 *E. coli uidA* gene has a G residue at position 92. This highly conserved base change was used to design a mismatch amplification mutation assay, which preferentially amplifies the *uidA* allele in O157:H7 strains (Cebula *et al.*, 1995).

2.2.3. Epidemiology:

The features of EHEC epidemiology include: intestinal tract of cattle and other animals serving as a reservoir, transmission by a wide variety of food items mainly contaminated beef, and a very low infectious dose.

The Centers for Disease Control and Prevention (CDC) estimate the annual *E. coli* O157:H7 burden in the United States to be 20,000 infections and as many as 250

deaths (Boyce et al., 1995). On a nationwide basis, *E. coli* O157:H7 is the pathogen most frequently isolated from stool specimens with visible blood (Slutsker *et al.*, 1997). The CDC initiated the Foodborne Disease Active Surveillance Network (FoodNet) to continually assess, at several laboratories throughout the United States, the burden of foodborne disease. The results from 1996 showed a nationwide incidence of *E. coli* O157:H7 of 3 per 100,000 (CDC, 1997). This surveillance network has been expanded to assess the burden of non-O157 EHEC as well as HUC cases (Mahon *et al.*, 1997).

Stx-producing *E. coli* can be found in a wide variety of animal fecal microbiota, including cattle, sheep, goats, pigs, cats, dogs, and chicken (Beutin *et al.*, 1993; Johnson et al., 1996). In terms of human infection, cattle are the most important reservoirs. High rates of colonization of Stx-positive *E. coli* have been found in bovine herds in many countries, ranging from 10 to 25% (Burnens et al., 1995; Griffin and Tauxe, 1991; Hancock *et al.*, 1994). The occurrence of STEC in retail meats corresponds to their widespread distribution. Doyle and Schoeni (1987) isolated *E. coli* O157:H7 from 3.7% of retail beef. Samandpour et al., (1994) however found no *E. coli* O157:H7 in raw meats from grocery stores in Seattle, but a high prevalence of non-O157:H7 isolates (23% in beef).

EHEC can be transmitted by food and water and from person to person. Most cases have been associated with ingestion of contaminated food, mainly of bovine origin. The largest outbreak reported in North America was caused by consumption of hamburgers from a fast-food restaurant chain in December 1992 and January 1993. 732 individuals were affected, 195 of which were hospitalized, and 4 died. The modern food-processing technology is partly the reason behind the contamination of the hamburgers; beef from thousands of cattle coming from different farms is ground

together in the same hamburger plant, and distributed to thousands of restaurants (Bell et al., 1994). Several other outbreaks have been associated with the consumption of mayonnaise (Griffin, 1995), unpasteurized apple juice (Besser *et al.,* 1993), and fermented hard salami (CDC, 1995). Juices and salami have a low pH permissive for *E. coli* O157:H7 growth, but not for other pathogens. Raw vegetables have also had their share of contamination, where an outbreak in Japan involving more than 9,000 cases was attributed to undercooked radish sprouts. Fruits and vegetables contaminated with cattle feces, combined with undercooking could have been the main reasons behind such an outbreak (Swinbanks, 1996). Moreover, an outbreak affecting 243 individuals causing 4 deaths in Missouri was due to leakage in the municipal water distribution system (Swerdlow *et al.,* 1992).

The low infectious dose for EHEC infection, 100 to 200 organisms, has been estimated from several outbreak reports (Bell *et al.,* 1994; Rowe *et al.,* 1994).

Since most outbreaks were attributed to the presence of *E. coli* O157:H7, one could conclude that this particular serotype is the most virulent and more transmissible than other serotypes. Nevertheless, incidence of disease due to other serotypes is considered to be on the rise (Johnson *et al.,* 1996). Mere expression of Stx is apparently not sufficient to confer virulence, but other virulence factors are necessary (Tarr and Neill, 1996). There are at least two virulence factors that can add a pathogenicity phenotype to Stx-positive *E. coli*: the A/E and enterohemolysin phenotype. Beutin *et al.,* (1993) found 1.4% of Stx-positive strains isolated from animals, positive for eae as well. On the other hand, most non-O157:H7 EHEC strains isolated from humans were eae-positive (Willshaw *et al.,* 1992). However, many non-O157:H7 *E. coli* associated with outbreaks did not harbor the eae or enterohemolysin

genes (Bokete *et al.,* 1993), indicating the presence of a yet unknown virulence factor that confers pathogenicity to such strains.

2.2.4. Clinical features of EHEC infection:

The clinical features of disease due to EHEC are nonbloody diarrhea, HC and HUS in most of the cases. Complications that might arise include cholecystitis, colonic perforation, intussusception, pancreatitis, posthemolytic biliary lithiasis, postinfection colonic stricture, rectal prolapse, appendicitis, hepatitis, hemorrhagic cystitis, pulmonary edema, myocardial dysfunction, and neurological abnormalities (Tarr, 1995). The incubation period of EHEC diarrhea is usually 3 to 4 days, associated with crampy abdominal pain, short-lived fever, and nonbloody diarrhea, as well as vomiting in some cases. The diarrhea becomes bloody after 1 to 2 days, with the abdominal pain increasing, lasting for 4 to 10 days. In most cases, bloody diarrhea will resolve without apparent sequelae, but in 10% of the patients, too young or too old, the illness will progress to HUS (Riley *et al.,* 1983). HUS clinically manifest as a triad of symptoms: hemolytic anemia, thrombocytopenia, and renal failure, with initial clinical manifestations of oliguria/anuria, edema, pallor, and sometimes, seizures (Pickering *et al.,* 1994). Treatment of disease due to EHEC is primarily supportive (Cimolai *et al.,* 1994).

2.3. ENTEROPATHOGENIC *E. coli*:

2.3.1. EPEC pathogenesis:

EPEC belongs to the diarrheagenic category of *E. coli.* One hallmark of EPEC infection is the A/E histopathology (Fig 2). This phenotype is characterized by effacement of microvilli and intimate adherence to the intestinal epithelial cell membrane. The attached bacteria sit on pedestal-like structures with polymerized filamentous actin beneath them (Moon et al., 1983). Donnenberg and Kaper (1992)

postulated the following EPEC pathogenesis strategy: localized adherence, signal transduction, and intimate adherence. The ability of EPEC strains to adhere in a localized pattern is dependent on the presence of a 60-MDa plasmid designated the adherence factor plasmid (Baldini *et al.,* 1983), and 7-nm-diameter fimbriae that allow for bundle formation, denoted as "bundle-forming pilus" (Giron *et al.,* 1991). Adherence of EPEC to epithelial cells stimulates several signal transduction pathways. The corresponding genes are located on the LEE pathogenicity island (Lai *et al.,* 1997). The activated signal transduction pathways were described before. Intimate adherence of EPEC to epithelial cells is mediated by the previously described outer-membrane protein, intimin, encoded by eae (for *E. coli* attaching and effacing) (Jerse *et al.,* 1990). At least three proteins essential for the A/E histopathology are secreted extracellularly by EPEC: EspA (Kenny *et al.,* 1996), EspB (Foubister *et al.,* 1994), and EspD (Lai *et al.,* 1997). EPEC possess as well a type III protein secretion system which is responsible for secretion and translocation of critical virulence factors. The genes encoding for this secretion system were initially named *sep* (for secretion of EPEC proteins) (Jarvis *et al.,* 1995). Mutation of *sepB*, one of the *sep* genes, abolishes secretion of EspA, EspB, and Esp, signal transduction and the A/E phenotype (Jarvis *et al.,* 1995).

The mechanism of diarrhea resulting from infection with EPEC was proposed from the genetic and cellular understanding of their pathogenesis. The incubation period in adults is 2.9 hours, between the ingestion of the organism and the onset of diarrhea. The loss of absorptive microvilli in the A/E lesion would explain the diarrhea via malabsorption. A significant decrease in the transmembrane potential in epithelial cells infected with EPEC was noted (Stein *et al.,* 1996). On the other hand, Knutton *et al.,* (1996) found EPEC to stimulate a rapid but transient increase in the short circuit

current of intestinal cells. In both studies, the ionic changes were abrogated by mutation of the *espB* gene but not by the *eae* gene.

2.3.2. Epidemiology:

The epidemiology of EPEC infection has a striking age distribution, affecting infants younger than two years. EPEC can cause diarrhea in adults only after high dose ingestion (Levine and Edelman, 1984). Transmission of EPEC is fecal-oral with contaminated hands, weaning foods, contaminated fomites as vehicles. The reservoir of EPEC is thought to be symptomatic children, asymptomatic children, and asymptomatic adult carriers (Levine and Edelman, 1984).

In the United States and the United Kingdom, EPEC once caused frequent outbreaks of infant diarrhea, community-acquired outbreaks and nosocomial outbreaks that were often explosive, up to 50% mortality (Robbins-Browne, 1987). In the developed world EPEC is of limited importance, while in the developing countries, it is the major cause of infant diarrhea. Numerous case-control studies found EPEC to be most frequently isolated from infants with diarrhea than from matched healthy controls (Levine and Edelman, 1984).

2.3.3. Clinical features of EPEC infection:

EPEC clinically causes acute diarrhea, with EPEC infection often being quite severe. Small intestine mucosal biopsy specimens often show intimately adherent bacteria and the classic A/E histopathology associated with disarrangement of the digestive-absorptive enzyme system leading to malabsorption of nutrients (Rothbaum *et al.*, 1982). Treatment of EPEC diarrhea is to prevent dehydration by correcting fluid and electrolyte imbalances (Fagundes-Neto, 1996).

2.4. ENTEROTOXIGENIC E. COLI:

2.4.1. ETEC pathogenesis:

ETEC are *E. coli* strains that harbor at least one of two enterotoxins: heat-stable (ST) and heat-labile (LT) toxins (Levine, 1987). ETEC strains colonize the surface of the small bowel mucosa and release their enterotoxins ST and LT, giving rise to a net secretory state. The heat-labile toxins are oligomeric toxins that are closely related to the cholera enterotoxin expressed by *Vibrio cholera* in structure and function (Sixma *et al.,* 1993). There are two major serogroups of LT: LT-I and LT-II. LT-I is an oligomeric toxin composed of one 28 kDa A subunit and five identical 11.5 kDa B subunits (Streatfield *et al.,* 1992); the A subunit is responsible for the enzymatic activity of the toxin. After binding to the intestinal cell membrane, the toxin is endocytosed and translocated through the cell via the trans-Golgi vesicular transport system (Lencer *et al.,* 1995). Adenylate cyclase is targeted in the basolateral membrane of intestinal epithelial cells. Since the cleaved A subunit has an ADP-ribosyltransferase activity, a series of stimulations take place resulting in Cl^- secretion from secretory crypt cells and inhibition of NaCl absorption by microvilli. Water is drawn passively due to the increased luminal ion content, resulting in osmotic diarrhea (Sears and Kaper, 1996). LT-II shows 55% homology to the LT-I A subunit, but none to the B subunit. LT-II induces cytotoxicity in a similar way as LT-I, but there is no evidence that it is associated with human or animal disease (Fukuta *et al.,* 1988).

Heat-stable toxins are small monomeric toxins compared to LT's. There are two unrelated classes of ST, different in structure and mechanism of action: ST-I and ST-II. Upon opportunistically binding its membrane receptor, ST-I activates guanylate cyclase (GC-C), leading to the stimulation of Cl^- secretion and/or inhibition of NaCl absorption resulting in a state of net intestinal fluid secretion (Mezoff *et al.,* 1992). ST-II induces the loss of villus epithelial cells and partial villus atrophy, thereby

causing histologic damage. ST-II stimulates the secretion of bicarbonate from intestinal epithelial cells (Sears and Kaper, 1996). To cause diarrhea, ETEC must adhere to the enterocytes. This adherence is mediated in human ETEC strains by colonization fimbriae, CFA's. CFA's are differentiated according to the morphological varieties they possess: rigid rods, bundle-forming flexible rods, and thin flexible wiry structures (Jann and Hoschutsky, 1991). CFA genes are usually encoded on plasmids that typically encode the ST and LT enterotoxins (De Graff and Gaastra, 1994).

2.4.2. Epidemiology:

A number of factors determine the epidemiologic pattern of ETEC infection: exposed individuals develop mucosal immunity; even immune asymptomatic individuals may shed virulent ETEC in their stool. A high infectious dose is required for pathogenesis to be initiated (DuPont *et al.,* 1971). Common vehicles for ETEC infection are food and water (Ryder *et al.,* 1976; Wood *et al.,* 1983). The period of endemic ETEC infection includes the warm and wet months of the year when multiplication of ETEC in food and water is most efficient (Levine, 1987). Predictably, ETEC traveler's diarrhea occurs most commonly in first-time travelers to the developing world during warm and wet months (Arduino and DuPont, 1993).

2.4.3. Clinical features of ETEC infection:

Illness due to ETEC infection is typically abrupt in onset with a short incubation period (14 to 50 hours), diarrhea being watery without blood, pus or mucus. However, fever and vomiting are present in a minority of affected individuals (DuPont *et al.,* 1971). The most life-threatening cases of ETEC diarrhea occur in weaning infants in the developing world (Levine *et al.,* 1977).

2.5. ENTEROAGGREGATIVE *E. coli*:

2.5.1. EAEC pathogenesis:

Many *E. coli* strains that were not of the EPEC serotypes adhered in a clearly distinguishable pattern from the usual EPEC adherence; they displayed a diffusely adherent phenotype (Scaletsky *et al.*, 1984; Nataro *et al.*, 1985). Nataro *et al.*, (1987) proposed a new category of diarrheagenic *E. coli*, the enteroadherent-aggregative *E. coli*. Based on the observation of prominent autoagglutination of the bacterial cells to each other, on the surface of HEp-2 cells (Aggregative adherence), or dispersal over the surface of HEp-2 cells with little aggregation (diffuse adherence), such a type of *E. coli* was characterized. Therefore, EAEC strains are defined as *E. coli* strains that adhere to HEp-2 cells in an aggregative adherence pattern without ST/LT secretion, the pathogenic strains of which are associated with diarrheal disease (Mathewson *et al.*, 1985). A flexible bundle-forming fimbrial structure designated aggregative adherence fimbriae I (AAF/I), has been described as a mediator of HEp-2 adherence and erythrocyte hemagglutination. AAF/II was also described by Nataro *et al.*, (1992). Nataro and Kaper (1998) hypothesized a three-stage model of EAEC pathogenesis, since a full description of the process has not yet been achieved. Stage I involves initial adherence to the intestinal mucosal layer, only after AAF/I and AAF/II facilitate initial colonization (Fig. 3). Stage II involves the enhancement of mucus production leading to the deposition of a thick mucous-containing biofilm encrusted with EAEC, and enhancing persistent colonization and nutrient malabsorption. Stage III includes the elaboration of an EAEC cytotoxin (Eslava *et al.*, 1993), resulting in damage to intestinal cells.

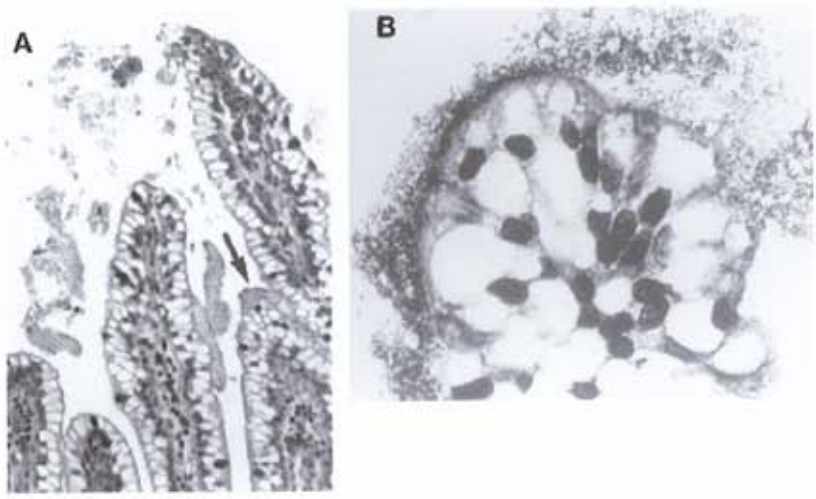

Figure 3: Interaction of EAEC with the intestinal epithelium. (A) Photomicrograph of the ileum of a gnotobiotic piglet fed EAEC strain 042. (B) High magnification of the ileal mucosa of a piglet fed EAEC strain 17-2 as in panel A. *Infection and Immunity (1992) 60: 5303.*

2.5.2. Epidemiology:

EAEC has been reportedly associated with diarrhea in developing countries, especially with persistent diarrhea of more than 14-day duration (Henry *et al.*, 1996). Several reports have described EAEC outbreaks (Eslava *et al.*, 1993; Smith *et al.*, 1994).

2.5.3. Clinical features of EAEC infection:

The clinical features of EAEC diarrhea were suggested to be a watery mucoid, secretory diarrheal illness with low-grade fever, and little or no vomiting (Bhan *et al.*, 1989).

2.6. ENTEROINVASIVE *E. coli*:

2.6.1. EIEC pathogenesis:

DuPont *et al.*, (1971) were the first to show that EIEC strains cause diarrhea. EIEC and *Shigella* spp. have virtually identical phenotypic features.

Both EIEC and *Shigella* invade the colonic epithelium, having one or more secretory enterotoxins, encoded by plasmid and chromosomal genes. The pathogenic

mechanisms of both EIEC and *Shigella* include: epithelial cell penetration, lysis of the endocytic vacuole, intracellular multiplication, directional movement through the cytoplasm, and extension into adjacent epithelial cells (Goldberg and Sansonetti, 1993; Sansonetti, 1992). Genes necessary for invasiveness, including the *mxi* and *ipa* loci, are carried on plasmids and encode a type III secretion apparatus (Allaoui *et al.*, 1995; Menard *et al.*, 1996). The Ipa proteins, which are encoded by plasmid-borne gene (*sen)*, which are either the effectors of the invasion phenotype or promoters of uptake into epithelial cells might have a role in the characteristic watery diarrhea attributed to EIEC (Sansonetti, 1992; Nataro *et al.*, 1995).

2.6.2. Epidemiology and clinical features of EIEC infection:

Epidemiologic studies of EIEC infection document foodborne and waterborne outbreaks (Snyder *et al.*, 1984; Tulloch *et al.*, 1973). EIEC most commonly causes watery diarrhea that is indistinguishable from that caused by ETEC. A minority of patients experience the dysentery syndrome, manifested as blood, mucus, and leukocytes in stool, and fever (Taylor *et al.*, 1988).

2.7. NECROTOXIGENIC *E. coli*:

2.7.1. NTEC pathogenesis:

NTEC are a pathogenic type of *E. coli* characterized by the production of cytotoxic necrotizing factors (CNF's). NTEC cause diarrhea and septicemia in humans and in domestic animals (Blanco *et al.*, 1990). Two different types of NTEC have been isolated: NTEC1 and NTEC2 (DeRycke *et al.*, 1990): NTEC1 have been isolated from extraintestinal infections in humans (DeRycke *et al.*, 1999), and from cases of enteritis in ruminants (Oswald *et al.*, 1991).

In NTEC1 strains, the virulence genes are encoded on a pathogenicity island of a chromosome. One of the genes, *cnf1*, may be associated with *pap* gene cluster, which

codes for structural subunits and transport proteins (Johnson, 1991). In NTEC2 strains, *cnf2* gene may be encoded on a Vir plasmid along with genes coding for fimbrial and afimbrial adhesions (Oswald *et al.*, 1991). The overall effect of CNF toxins is characterized by the formation of multinucleated cells. CNF2 interaction with HeLa cells led to the production of giant mononucleated cells with actin stress fibers appearing along with a block at G2/M phase of cell division. The CNF2 toxin mediates the actin cytoskeleton reorganization (Oswald *et al.*, 1994).

2.8. *E. coli* type characterization:

Differentiation of the various *E. coli* types according to their virulence genes by PCR detection of each of these virulence genes has been the concern of many investigators (Candrian *et al.*, 1991; Fratamico *et al.*, 1995; Lin *et al.*, 1993; Louie *et al.*, 1994; Mainil *et al.*, 1997; Schmidt *et al.*, 1995; Sethabutr *et al.*, 1994).

Chapter 3

MATERIALS AND METHODS

3.1. Sample Collection:

600 samples of raw meat were randomly collected over a 30-week period after securing the proper authorization from the Municipality of Beirut. The number of samples was determined according to relevant statistical criteria. This task was initiated on the last week of February 2002. Beirut Slaughterhouse was visited on a weekly basis, on either one of the following days: Monday, Wednesday or Friday. On each visit, 20-g samples were aseptically collected from the freshly slaughtered calves, precisely from the diaphragmatic insertion. Alcohol was spilled on the scalpel and pincers, and burned till it got relatively cold to hold the diaphragm and cut off a 20-g piece. The samples were individually packed in sterile urine containers, in a cool box. Among the average of 110 calves slaughtered on each day, 20 samples were randomly chosen. The meat samples were transported within 1 hour to LAU-Byblos labs where immediate analysis took place.

30-week distribution over the period of sample collection: February-September 2002.

February	1 2
March	3 4 5 6
April	7 8 9 10
May	11 12 13 14 15
June	16 17 18 19
July	20 21 22 23 24
August	25 26 27 28
September	29 30

3.2. Identification and enumeration of isolates:

5g of each collected sample were inoculated overnight in 125 ml EC broth (BBL) at 45.5°C with slight agitation in a shaking incubator (Innova 4400, New Brunswick Scientific, NJ, USA). This broth is selective for the cultivation of *E. coli* when incubated at 45.5°C. Api20E was performed to confirm that *E. coli* was enriched.

Total aerobic count (TAC) and Total Coliform count (TCC) were performed according to standard microbiological techniques, where 1g of sample was suspended in 9 ml of sterile 0.85% NaCl (saline), vortexed, then an aliquot (0.1ml) was spread by a Drigalski's spatula (bent sterile glass rod) on tryptone soy agar (TSA) and MacConkey agar (Oxoid), and incubated overnight. The next day, plates were scored and colony counts recorded.

Regarding the enriched broths, 1 ml aliquots were diluted $x10^7$, then spread by Drigalski's method on MacConkey agar, and incubated overnight at 37°C. The next day, an inoculation loop was used to mix the whole colonies together, and a loopful was incubated in nutrient broth + 10% glycerol at 37°C overnight, after which they were transferred to the deep-freeze at -70°C.

3.3. Sample re-culturing and DNA extraction:

An appropriate number of tubes containing the frozen bacteria were systematically thawed at room temperature for 1 hour, then each tube was vortexed, and a loopful was taken and streaked heavily on TSA, then incubated overnight at 37°C. The next day, a loopful of each plate was suspended in 200µl of sterile DNase-free water (ABgene®), and boiled for 15 min, with agitation. After boiling, the Eppendorff tubes were centrifuged at 13000g for 5 min, the supernatant contained DNA.

3.4. Detection of *E. coli*-containing samples:

PCR was performed to detect the *uspA* gene, harbored by *E. coli*, according to the method described by Chen and Griffiths (1998). In a total volume of 50 µl, a mastermix was prepared with final concentrations of the following:

1X PCR buffer : (50 mM KCl, 10 mM Tris HCl)

dNTP's (each) : 0.2 mM

$MgCl_2$: 3 mM

Primers (each) : 0.1 µM (sequences described in Table 1)

Taq polymerase : 1U (all PCR products are ABgene® products)

45 µl of mastermix were added to 5 µl of DNA template, and run through the following cycling conditions, in a thermal cycler (Perkin-Elmer 2400, CT USA):

94°C for 5 min

30 cycles of: 94°C for 1 min, then 53°C for 1 min, then 72°C for 1 min

72°C for 5 min

After the cycles were over, 3µl of amplicons were mixed with 3µl of gel loading buffer, and loaded in 2% NA Agarose (Amersham Pharmacia®), with Ethidium Bromide (EtBr) added, and run in a gel submarine (Pharmacia Biotech GNA 200) at a voltage of 120V, then visualized under UV for an 884-bp product.

Table 1: Primer sequences of *uspA* primers, adapted from Chen and Griffiths (1998)

uspA forward primer 5'- CCG ATA CGC TGC CAA TCA GT -3'

uspA reverse primer 5'- ACG CAG ACC GTA GGC CAG AT -3'

3.5. Multiplex PCR for the detection of *E. coli* virulence genes:

E. coli-containing samples were processed for the detection of *E. coli* virulence genes: VT1, VT2, VT2e, and eaeA (Mix 1), CNF2 and EInv (Mix2), CNF1 and EAgg (Mix 3), and ST1, ST2 and LT1 (Mix 4), as described by Pass *et al.*, (2000). In a total volume of 25 µl, a mastermix was prepared with final concentrations of the following components:

1X PCR buffer

dNTP's (each) : 0.1mM

$MgCl_2$: 3mM

Primers (Table 2) : 2.5 µl, according to Table 3

Taq polymerase : 1.25 U

22.5 µl of the mastermix were added to 2.5 µl of DNA template, then run through the following cycling conditions, in a thermal cycler (Perkin-Elmer 2400, CT USA):

95°C for 5 min

5 cycles of: 95°C for 30 sec, then 72°C for 1 min

20 cycles of: 95°C for 30 sec, then 63°C for 30 sec, then 72°C for 30 sec

72°C for 5 min

After the cycles were over, 3µl of amplicons were mixed with 3µl of gel loading buffer, and loaded in 2.5% ABgarose (ABgene®, provided by Numelab), with EtBr added, and run in a gel submarine (Pharmacia Biotech GNA 200) at a voltage of 120V, then visualized under UV for the corresponding product length (Table 2). The positive controls were kindly provided by Dr. Michael A. Pass, University of Sunshine Coast, Queensland – Australia.

Table 2: Primer sequences of the *E. coli* virulence genes, adapted from Pass *et al.*, (2000), and uidA gene sequence (Cebula *et al.*, 1995):

VT1	F: 5'- ACG TTA CAG CGT	R: 5'- TTG CCA CAG ACT GCG
121 bp	GTT GCA* GGG ATC – 3'	TCA GTA* AGG -3' *: Wobble G
VT2	F: 5'- TGT GGC TGG GTT	R: 5'- TCC GTT GTC ATG GAA
102 bp	CGT TAA TAC GGC -3'	ACC GTT GTC -3'
VT2e	F: 5'- CCA GAA TGT CAG	R: 5'- GCT GAG CAC TTT GTA
322 bp	ATA ACT GGC GAC -3'	ACA ATG GCT G -3'
eaeA	F: 5'- TGA GCG GCT GGC	R: 5'- TCG ATC CCC ATC GTC
241 bp	ATG AGT CAT AC -3'	ACC AGA GG -3'
CNF1	F: 5'- GGC GACAAA TGC	R: 5'- GAC GTT GGT TGC GGT
552 bp	AGT ATT GCT TGG -3'	AAT TTT GGG -3'
CNF2	F: 5'- GTG AGG CTC AAC	R: 5'- CCA CGC TTC TTC TTC
839 bp	GAG ATT ATG CAC TG -3'	AGT TGT TCC TC -3'
LT1	F: TGG ATT CAT CAT GCA	R: 5'- CCA TTT CTC TTT TGC
360 bp	CCA CAA GG -3'	CTG CCA TC -3'
ST1	F: 5'- TTT CCC CTC TTT	R: 5'- GGC AGG ATT ACA ACA
160 bp	TAG TCA GTC AAC TG -3'	AAG TTC ACA G -3'
ST2	F: 5'- CCC CCT CTC TTT	R: 5'- TgC TCC AgC AgT ACC
423 bp	TgC ACT TCT TTC C -3'	ATC TCT AAC CC -3'
EInv	F: 5'- TGG AAA AAC TCA	R: 5'- TTC TGA TGC CTG ATG
140 bp	GTG CCT CTG CGG -3'	GAC CAG GAG -3'
EAgg	F: 5'- AGA CTC TGG CGA	R: 5'- ATG GCT GTC TGT AAT
194 bp	AAG ACT GTA TC -3'	AGA TGA GAA C -3'
uidA	F: 5'- GCG AAA ACT GTG	R: 5'- TGA TGC TCC ATC ACT
252 bp	GAA TTG GG – 3'	TCC TG – 3'

Table 3: Final concentrations of primers according to the primer mixes:

	Primer (each)	Final Conc. pmol/µl
MIX 1	VT1	1.0
	VT2	1.0
	VT2e	1.0
	eaeA	0.5
MIX 2	CNF2	0.3
	EInv	2.0
MIX 3	CNF1	2.0
	EAgg	1.0
MIX 4	LT1	0.3
	ST1	0.5
	ST2	1.0

3.6. Detection of *uidA* gene in VT1/VT2/VT2e/eaeA-positive samples:

To amplify the *uidA* allele, specific to *E. coli* O157:H7, the procedure set by Cebula *et al.*, (1995) was adapted. In a total volume of 50 µl, a mastermix was prepared with the following final concentrations:

1X PCR buffer

$MgCl_2$: 2.5 mM

dNTP's (each) : 0.2 mM

Primers : 0.5 pmol/µl (Sequences in Table 2)

44.5 µl of mastermix was added to 5 µl of DNA template, mix was hot-started at 94°C for 5 min, before 2.5U of Taq polymerase were added, and the reaction was run

through the following cycling conditions, in a thermal cycler (Perkin-Elmer 2400, CT USA):

35 cycles of: 94°C for 1.5 min, then 64°C for 1.5 min, then 72°C for 1.5 min.

After the cycles were over, 3µl of amplicons were mixed with 3µl of gel loading buffer, and loaded in 2.5% ABgarose (ABgene®, provided by Numelab), with EtBr added, and run in a gel submarine (Pharmacia Biotech GNA 200) at a voltage of 120V, then visualized under UV for a 252-bp product.

3.7. Genotyping of samples positive for eaeA and at least 1 of the VT genes by PFGE:

The samples positive for eaeA and at least one of the VT genes were subcultured on MacConkey agar, in order to isolate the *E. coli* strains positive for these genes. DNA fingerprinting of the five *eaeA*-positive/VT-positive strains—that could be recovered that kept their genetic characteristics, was performed as described by Böhm and Karch (1992). Briefly, 1 ml of a cell suspension of each strain was pelleted by centrifugation, pellets washed and resuspended in SE buffer (NaCl 10 mM, EDTA 0.1mM). 0.5 ml of this suspension was mixed with an equal volume of melted 1.2% chromosomal grade agarose (BioRad, CA, USA), and then the mixture was dispensed into the wells of plug molds. After their solidification, cell lysis was carried out overnight, where the plugs were incubated at 56°C in 1 ml of lysis buffer containing proteinase K (Roche Diagnostics, GmbH, Germany). The next day, proteinase K was inactivated by washing the plugs several times with TE buffer. Digestion of the agarose-embedded DNA was carried out with *Xba1* for 16 hours at 37°C. After the digestion, the plugs were set in 2% Agarose NA (Amersham Pharmacia, Sweden), wells were sealed with 0.5% Agarose Prep (low melting point) (Amersham Pharmacia, Sweden). After a 24-hour run at a constant voltage of 200V, in 0.5X TBE that was kept at 14°C, 120° switch angle, pulse times of 5 to 50 seconds with linear

ramping in a Gene Navigator (Amersham Pharmacia, Sweden) using the HEX electrode, the gel was stained with 0.5 ml of ethidium bromide in 1 l of 0.5X TBE for 30 min, then destained for 30 min. A lambda ladder PFGE marker (Amersham Pharmacia, Sweden) was used in the first and last lanes, whereas lane 2 contained ATCC 35150 as a reference strain.

Chapter 4

RESULTS:

4.1. Total aerobic counts versus total coliform counts in fresh samples:

After vortexing 1g of each meat sample in 9 ml of sterile saline, and plating on TSA, an average TAC of 1240 CFUs/ml (excluding no-growth and the heavy uncountable growths). Half of the plates, on average, had growth. The plates with growth had an average count of 1240 CFUs/ml. Regarding the TCC, on average, 5 of the 20 plates contained fermenters, with an average count of 100 CFUs/ml. It is worth mentioning that some plates had *Klebsiella* spp., others *Pseudomonas* spp. as determined by Api 20E, that was performed aside from the routine experiments.

4.2. Detection of *E. coli*-containing samples:

Among the 600 samples, 491 samples out of 600 meat (81.8%) samples were *uspA*-positive (Fig. 4). These are the samples that were later subjected to the PCR-detection of virulence genes according to Pass *et al.*, (2000).

Fig. 4: Example of *uspA* detection in 8 samples, of which the first is a non- *E. coli*-containing sample, whereas the rest are *uspA*-positive *E.coli*-containing samples. The first lane is a 100bp-ladder. Note that the banding in the lanes represents genomic DNA.

4.3. Multiplex PCR for the detection of *E. coli* virulence genes:

All the *uspA*-positive samples were subjected to further processing, for the detection of the *E. coli* virulence genes according to the primer mixes (Fig. 4a). Fig. 5 indicates the number of samples positive for each particular gene, and the percentage of samples positive for those genes.

Fig. 4a: Positive controls for the *E. coli* genes: L: duplicates of the mixes 1, 2, 3, and 4 performed on positive controls; R: adapted from Pass *et al.*, 2000.

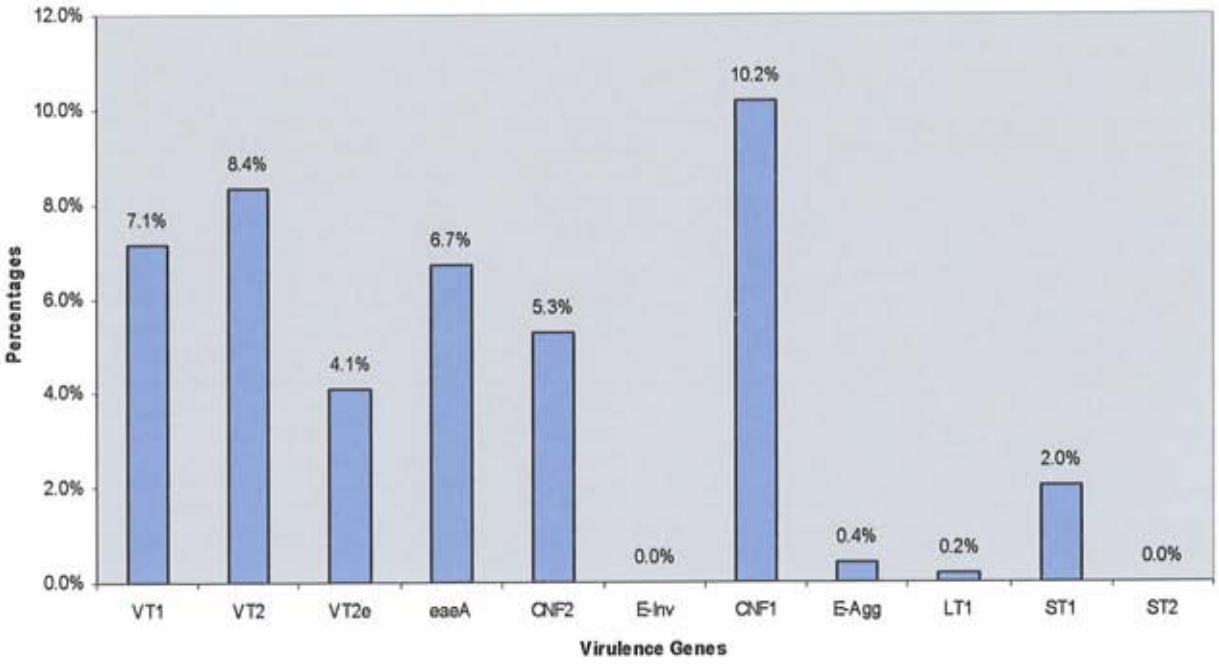

Figure 5: Percentages of sample-positives, according to the respective virulence genes:

It is worth noting that counting was done according to the gene-positive sample, irrespective of its being positive for another gene or not. Forty samples were processed with Primer Mix 1 to detect VT1, VT2, VT2e, and eaeA (Fig. 6).

Fig. 7 shows the weekly distribution of the various virulence genes, charted showing the number of virulence genes according to the weeks. Note that weeks 22 and 23 have the highest number of samples positive for virulence genes, 6 of them to be precise. On the other hand, week 25 has 5 virulence genes detected.

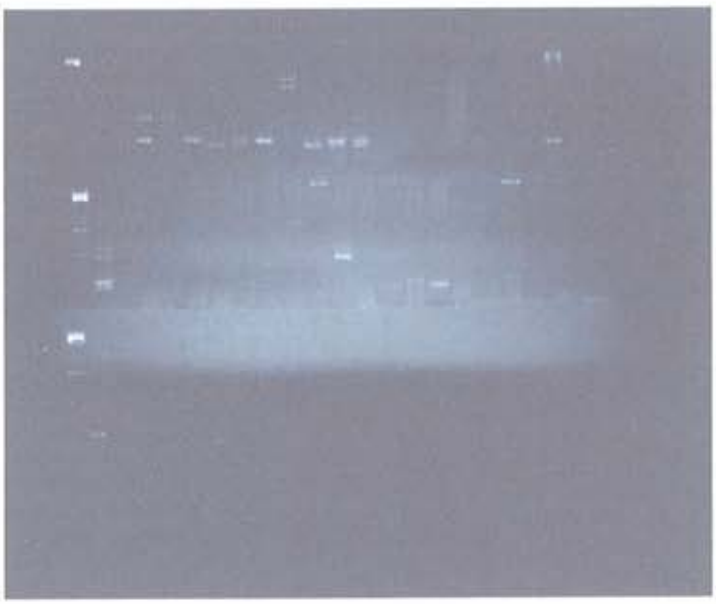

Fig. 6: Processing of 46 samples for Primer Mix 1 (VT1, VT2, VT2e, eaeA). Note that a 100 bp ladder was used (1st lane), a positive control (2nd lane), and a blank (3rd lane).

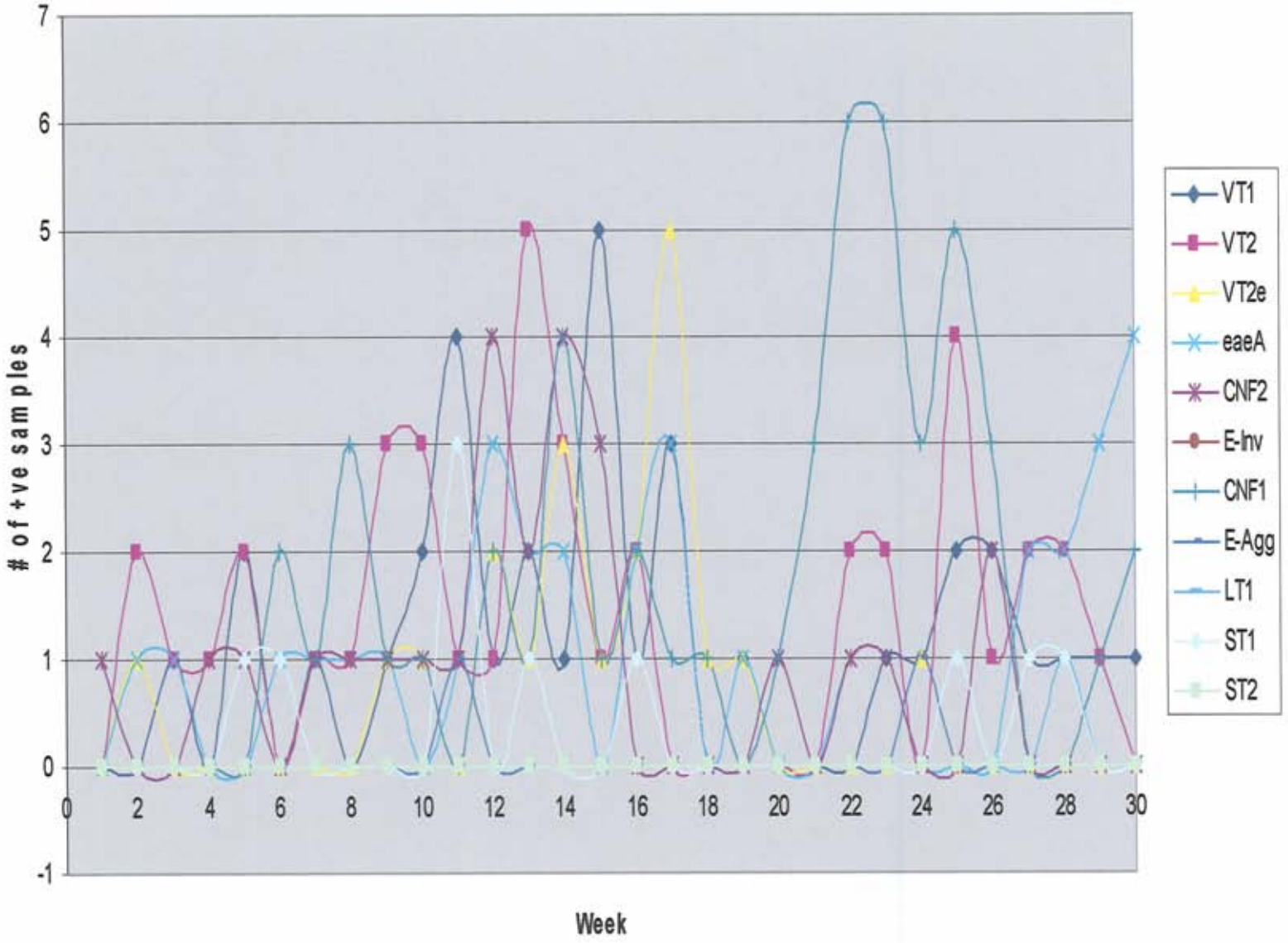

Fig. 7: Weekly distribution of the number of genes, with reference to each of them:

4.3.1. EHEC:

EHEC detected in samples positive for at least one of the VT1, VT2, VT2e, and eaeA genes, were distributed across the weeks (Fig. 8) in a fashion where most of the samples collected over the 30-week period had at least one of the EHEC genes detected. The least number of samples had 3 or 4 EHEC genes detected. It is worth noting that the period between weeks 9 and 17 (the period spanning from Mid-April till Mid-June) had the highest number of EHEC-positive genes, mainly VT2e, and to a lesser extent VT2e and eaeA.

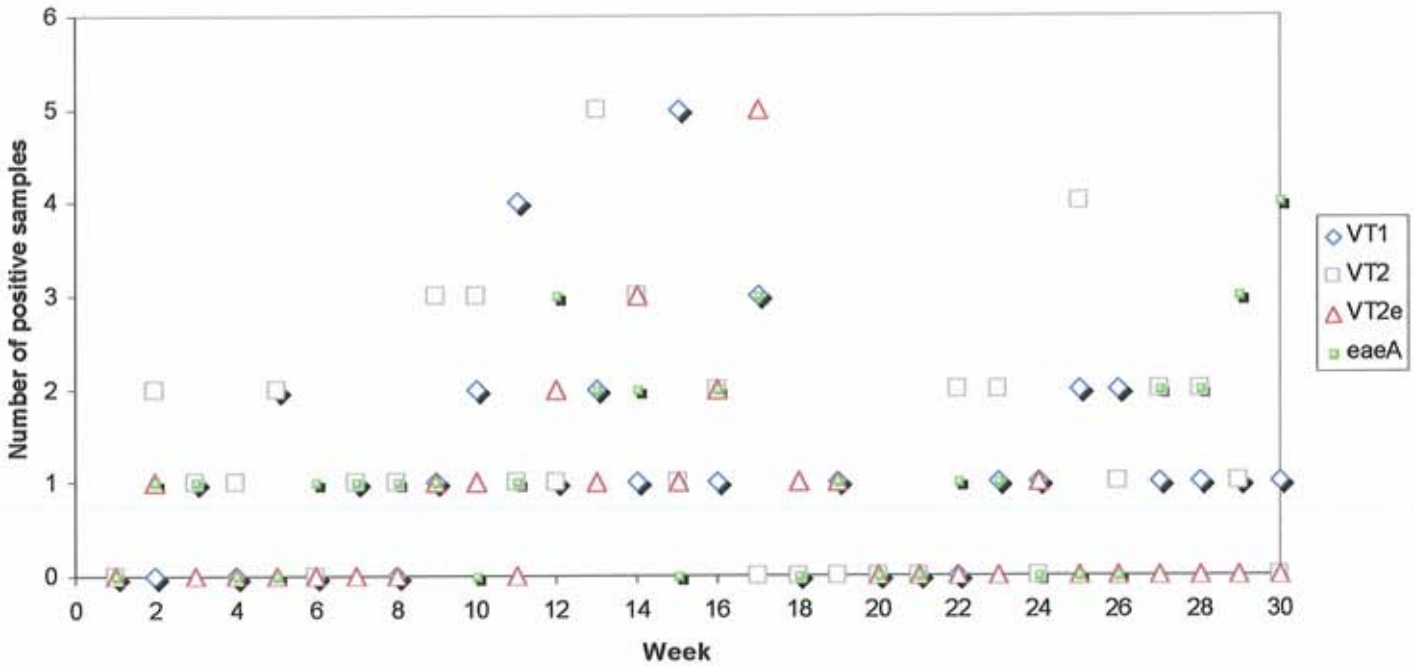

Figure 8: Distribution of EHEC (represented by samples positive for VT1, VT2, VT2e, and eaeA) across the 30-week period.

4.3.2. EPEC:

EPEC, those positive for eaeA alone had a low weekly distribution (Fig. 9), where mostly no eaeA gene was detected. On week 30 (Mid September) three samples were positive for eaeA alone. The period including weeks 6 till week 16 (late March till early June) had the highest prevalence of eaeA-containing samples.

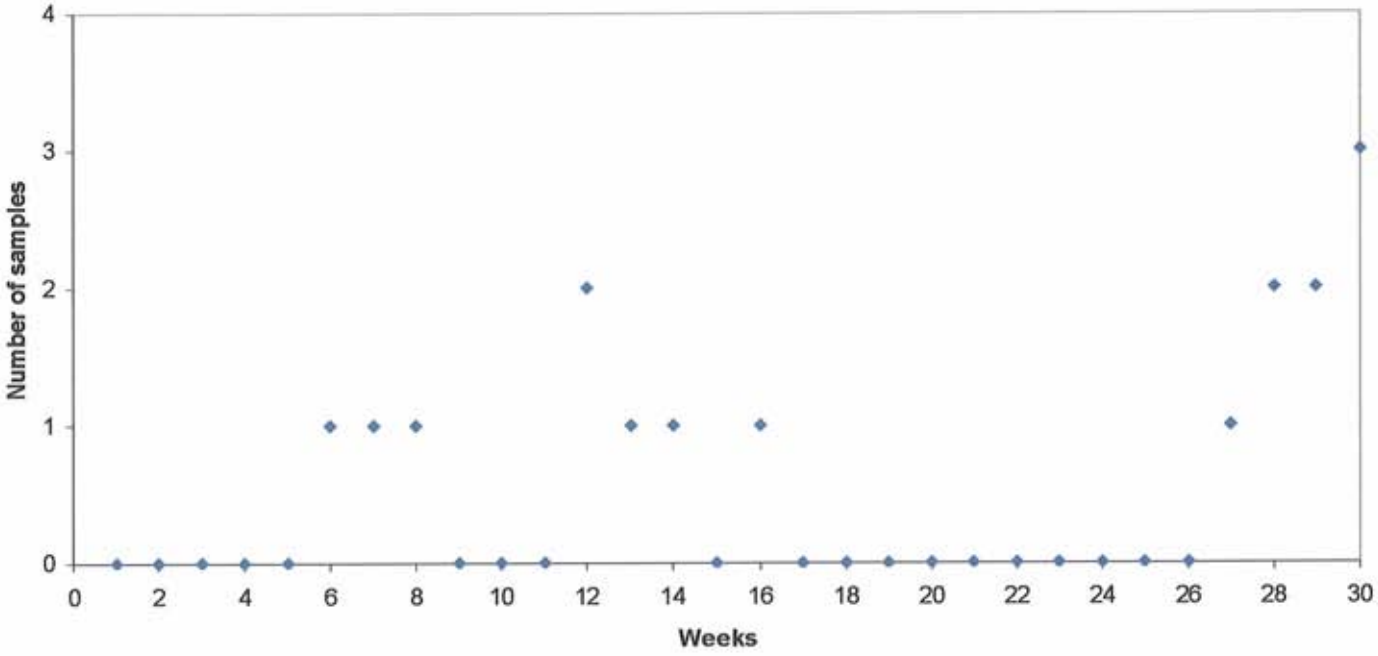

Figure 9: EPEC distribution (represented by samples positive for eaeA gene alone) across the 30-week period:

4.3.3. ETEC:

ETEC, those positive for either one of the ST1, ST2 or LT genes (Fig. 10), were mostly prevalent during weeks 11 and 16 (from early May till early June). The presence of one ST1-positive sample was very evident throughout 7 dispersed weeks, and the absence of ST2 was evident throughout the whole period of sample collection. As for LT1, it was only detected once in 1 sample during week 28 (late August).

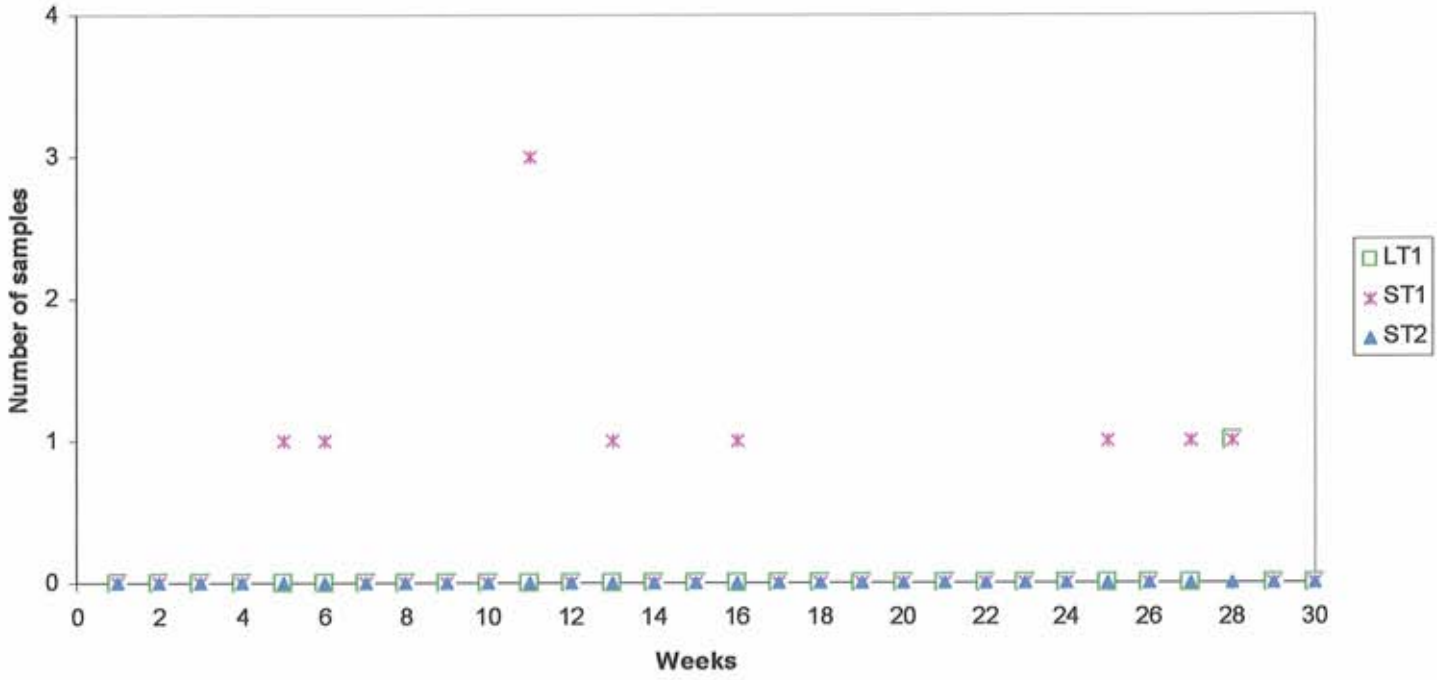

Figure 10: ETEC distribution across the weeks, according to the number of ST/LT genes detected:

4.3.4. EAEC:

EAEC, those positive for EAgg gene (Fig. 11), were only detected on weeks 11 (early May) and 24 (Late July).

EIEC, positive for EInv gene were not detected throughout the 30-week period of sample collection.

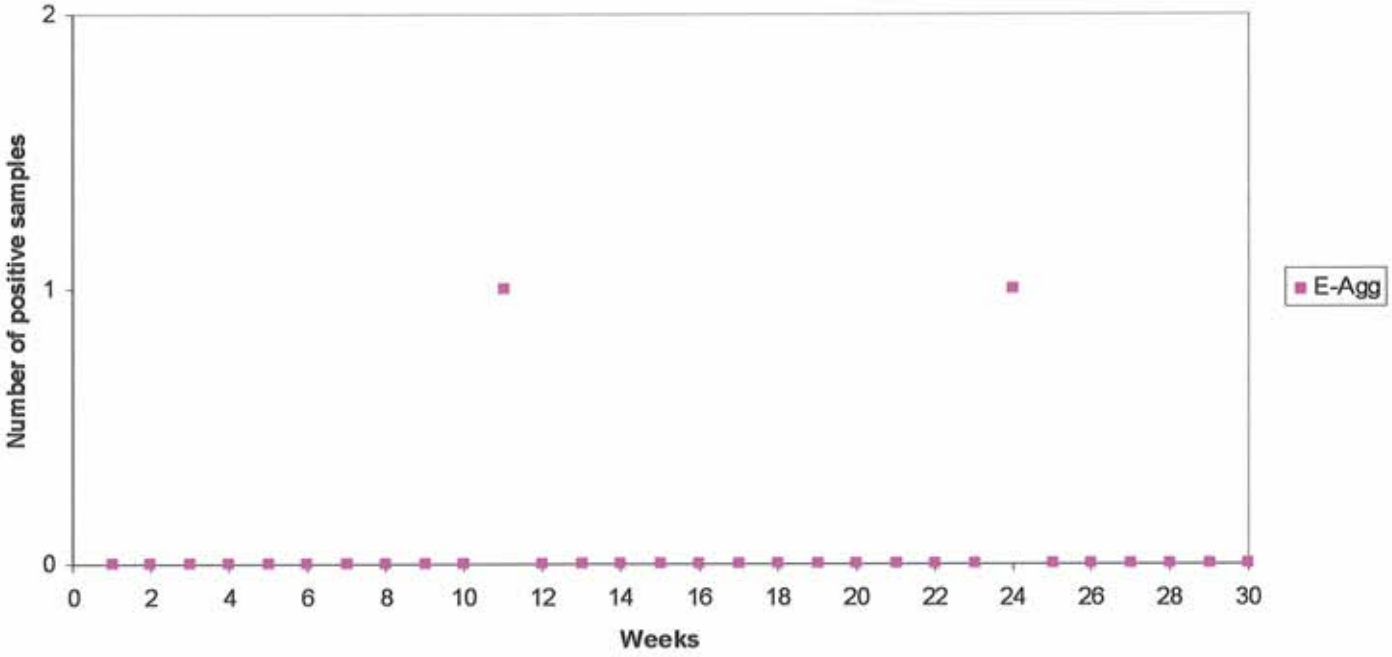

Figure 11: Weekly distribution of EAEC across the weeks, according to the number of samples positive for EAgg gene:

4.3.5. NTEC:

NTEC, positive for CNF1 and CNF2 genes (Fig. 12), were the most prevalent of the *E. coli* in question. They were detected nearly every week, excluding the first and last two weeks. The period spanning from week 22 till week 25 (mid-July till early August) had the highest number of samples positive for CNF1 gene (5 to 6 samples). Regarding the CNF2-positive samples, weeks 12 till 15 had the highest prevalence of such samples (3 to 4 samples).

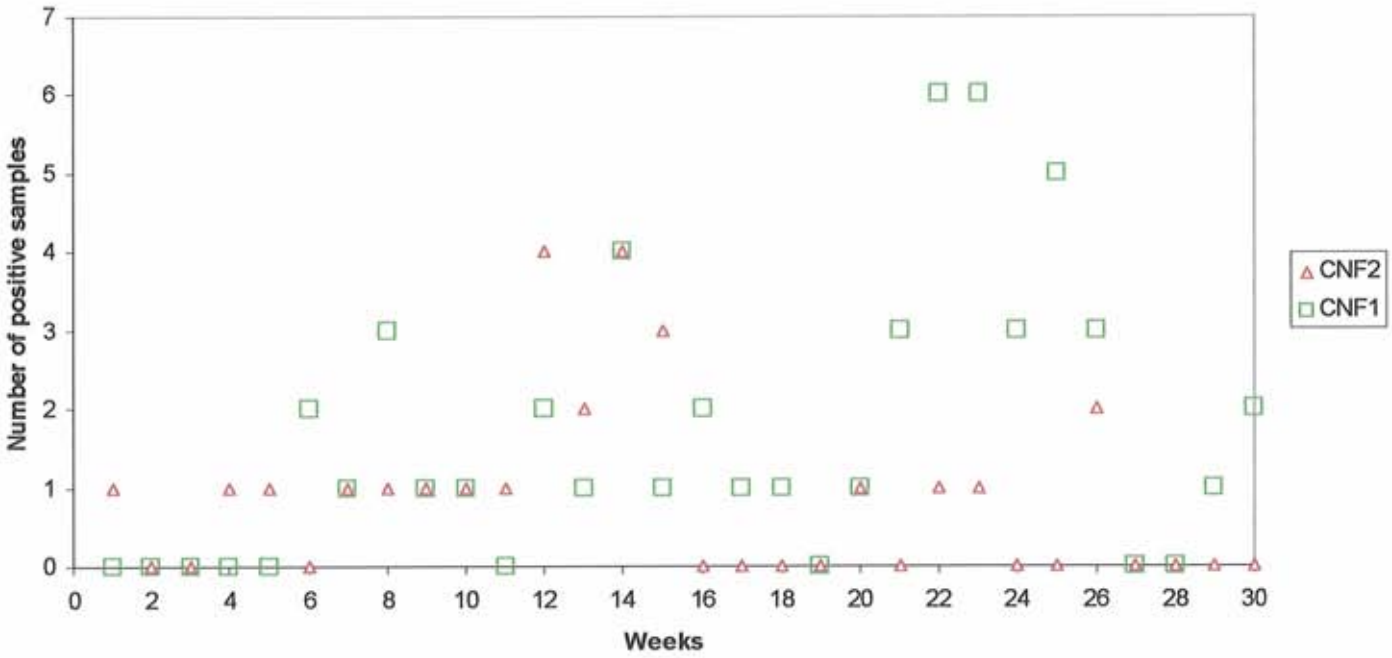

Figure 12: Weekly distribution of NTEC, according to the number of samples positive for CNF1 and CNF2 genes:

4.3.6. Combinations of *E. coli* types:

The overall distribution of *E. coli* types across the 30-week period was assessed (Fig. 13). Another issue remains that of combinations of *E. coli* types present within the same samples (Table 6), where 7 samples contained both EHEC and NTEC, and 4 samples contained both EHEC and NTEC.

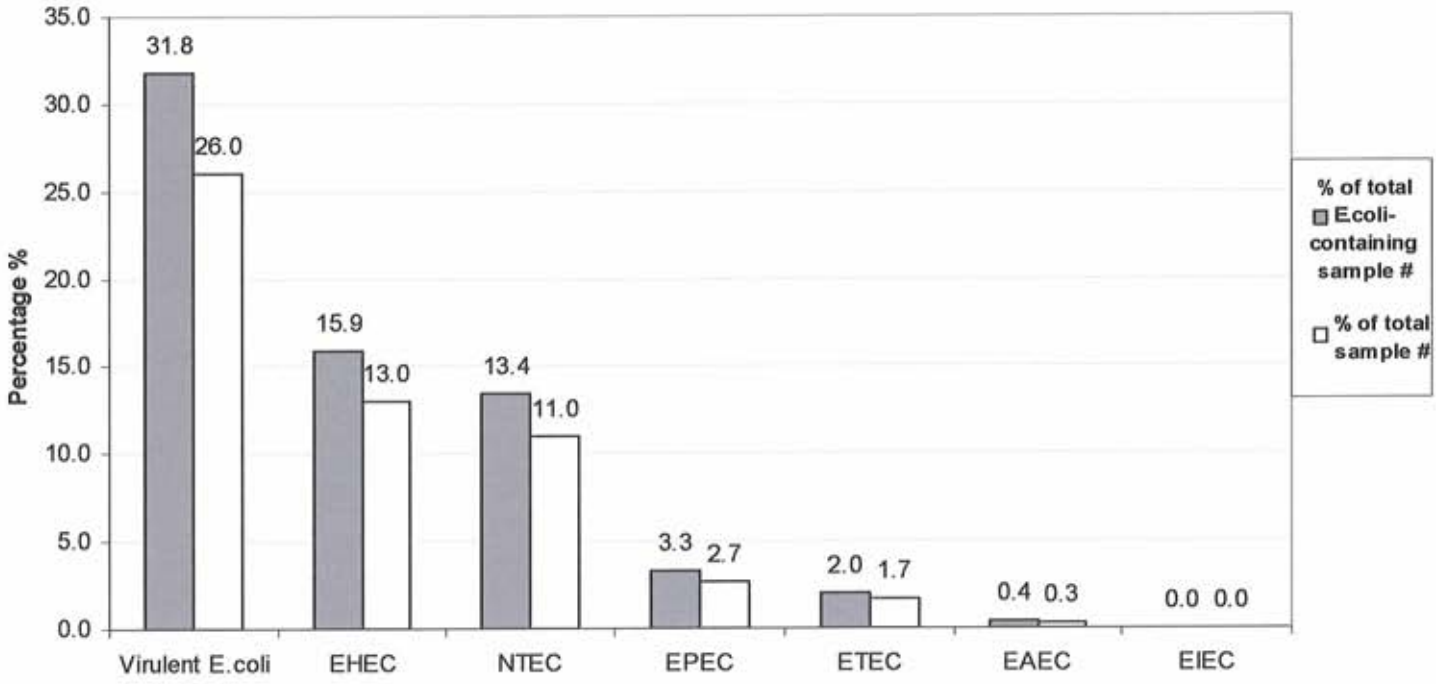

Figure 13: Percentage of virulent *E. coli* and their types of the 491 *E. coli*-containing samples, and total number of samples:

Table 4: Numbers of combinations of *E. coli* types within the same sample

TYPES	NUMBER OF SAMPLES	WEEK	NUMBER
EHEC + NTEC	7	29	1
		22	1
		16	1
		14	1
		13	1
		12	1
		9	1
EHEC + ETEC	4	27	1
		25	1
		16	1
		5	1
EPEC + NTEC	2	12	1
		7	1
EPEC + ETEC	1	28	1
ETEC + NTEC	1	11	1
NTEC + EAEC	1	24	1

4.3.7. *E. coli*-negative samples:

On the other hand, samples that did not contain any of the *E. coli* virulence genes in question, were distributed with an average of 6 to 16 samples being negative for *E. coli* virulence genes within the same week, with a range of samples containing *E. coli* between 8 and 20 (Fig. 16).

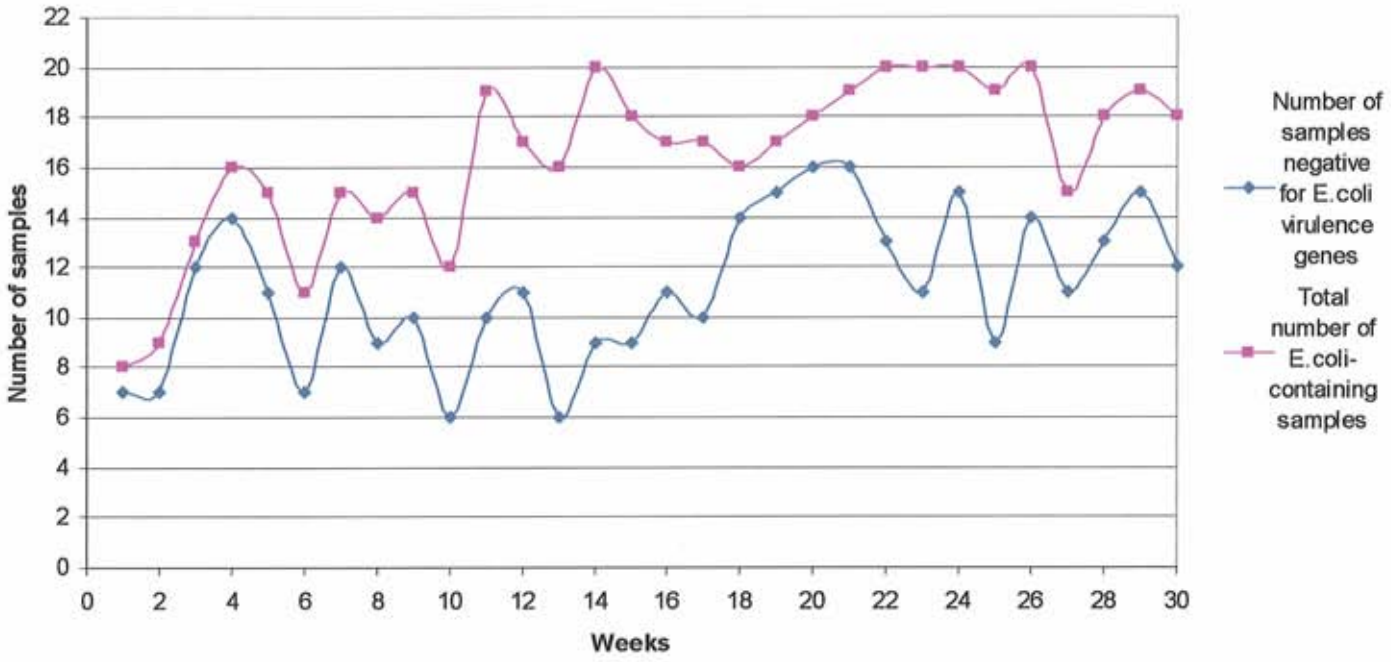

Figure 14: Weekly distribution of *E. coli*-free samples negative for *E. coli* virulence genes:

4.3.8. *E. coli* O157:H7 presence and PFGE subtyping:

As for the *uidA* gene, it was not detected in any of the EHEC-positive samples (Fig. 17). All of the isolates that tested positive for eaeA and at least one of the VT genes (Fig. 18) are genetically distinct, as denoted by a difference of more than 2 bands (≥100kb) in their PFGE patterns (Fig. 19).

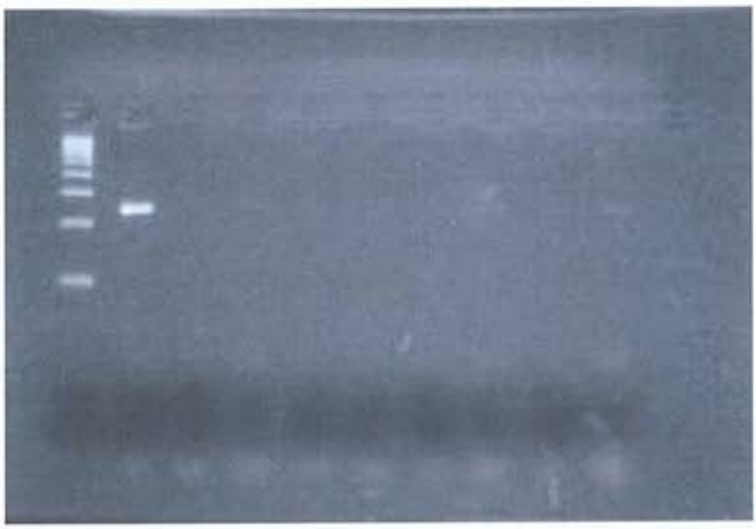

Fig. 15: EHEC-harbored uidA gene, 100b.p. ladder (1st lane), positive control (2nd lane), blank (3rd lane), samples (7 lanes):

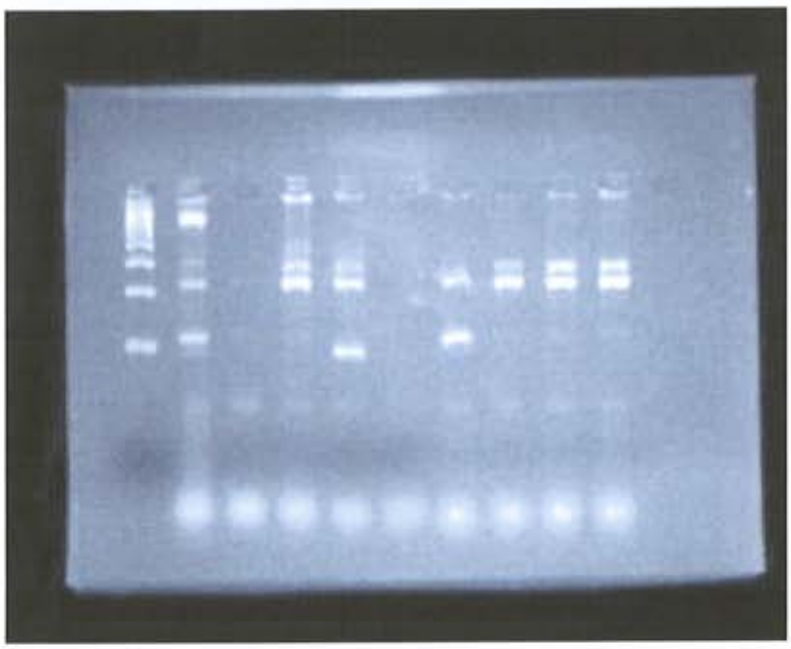

Fig. 16: Distribution of isolates positive for eaeA and one of the VT genes: 1st lane: 100bp ladder, 2nd lane: positive control, 3rd till 9th lanes: isolates

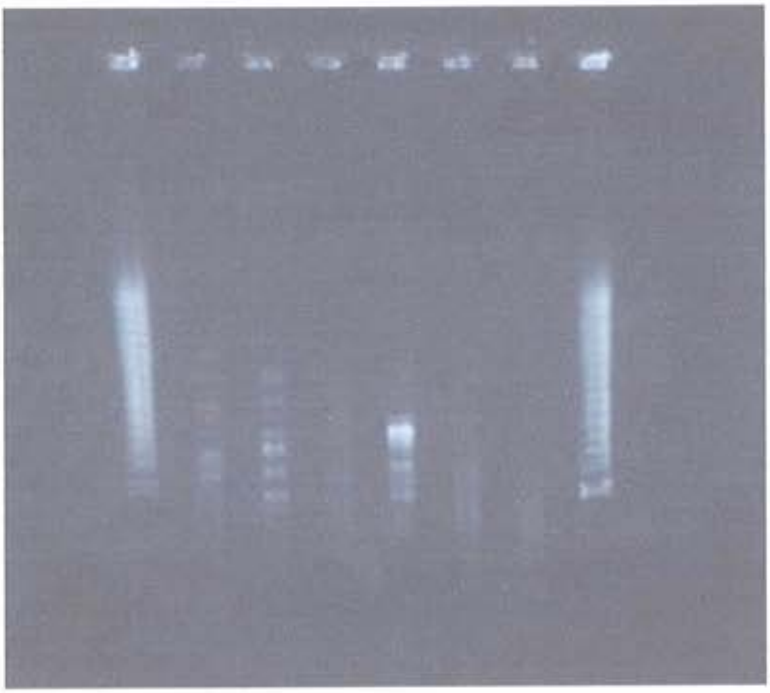

Fig. 17: PFGE patterns of the eacA-positive and VT-positive isolates: 1st and 8th lanes: λ phage ladder, 2nd lane: ATCC 35150, 3rd till 7th lanes: isolates

Chapter 5

DISCUSSION AND CONCLUSIONS

Ingestion of meat products contaminated with *E. coli* was associated with diarrheal and septicemic disease in humans. *E. coli* inhabits the human gut as a symbiotic organism. The scenario however changes when *E. coli* acquires additional genetic characteristics, such as virulence factors, resulting in diarrhea or septicemia depending on the site of infection (Nataro and Kaper, 1998). The problem gets worse when the gastrointestinal barrier is broken, resulting in multiple infections, from normal *E. coli* flora, or pathogenic strains of *E. coli*.

In our present study, *E. coli* was detected in 491 out of 600 samples (81.8%), indicating that the meat samples collected from the calves were contaminated, thereby ingested by consumers. Immunocompromised patients or patients who have a defective/injured gastrointestinal barrier, would be prone to infections with normal intestinal microbiota including those detected. The serious problem lies in those organisms that are virulent. The TACs and TCCs are indicative that meat is not incompletely free of microorganisms.

A total of 156 samples were positive for at least one of the following genes: VT, eaeA, CNF, ST, LT, EAgg, and EInv virulent genes. This represented 26% of the total number of samples analyzed. Virulent *E. coli* was detected in 31.8% of the total *E. coli*-containing samples.

EHEC was detected in 15.9% of the *E. coli*-containing samples, characterized by the presence of at least one of the VT genes. This prevalence was the highest among the rest of the types. Regarding the prevalence of the virulence genes, 7.1% of the *E. coli*-containing samples were positive for VT1 genes, 8.4% for VT2, and 4.1% for VT2e. Similarly, Samandpour *et al.*, (2002) detected 16.8% of 296 ground beef samples

from retail grocery stores, positive for Shiga-toxin genes in King County, Washington. In France, 11% of 411 beef samples were positive for Shiga-toxin genes (Pradel *et al.*, 2000). In Calcutta - India, Khan *et al.*, (2002) detected VTEC in 50% of raw beef samples, while in New Zealand, Brooks *et al.*, (2001) isolated STEC from 12% of the raw beef samples tested.

The period witnessing the highest prevalence of EHEC, 5 positive samples, is the period spanning weeks 13 till 17, i.e. mid-May till mid-June. The last 8 weeks had 2 samples positive for EHEC. The period where the highest prevalence was noted, is quite similar to that noticed by previous investigators (Chapman *et al.*, 1997; Mechie *et al.*, 1997; Chapman *et al.*, 2000). It is worth noting, that throughout the month of May, all of the VT genes could be detected. Seven samples harbored both EHEC and NTEC, whereas 4 samples harbored both EHEC and ETEC (Table 6). Early summer is characterized by more shedding of cattle flora in the feces. This factor, combined with less hygienic slaughtering skills could partly explain why throughout the period of May-June, EHEC was highly prevalent, along with other *E. coli* types.

Of all the EHEC that were detected, none harbored the *uidA* gene, i.e., no O157:H7 could be detected. Samadpour *et al.*, (1994) did not detect any O157:H7 in beef samples neither did Pierard *et al.*, (1997). As for Pradel *et al.*, (2000), they detected O157:H7 in 0.5% of the samples. Not all such investigations report a low O157:H7 prevalence; Chapman *et al.*, (1997) detected O157:H7 in 15.7% of the samples in the U.K. The detection of O157:H7 by many investigators was done using special enrichment broths, then using immunomagnetic separation (Weagant *et al.*, 1995; Vaishnavi *et al.*, 2001). The technique used in our study is the amplification by PCR of the mutant uidA gene (Cebula *et al.*, 1995), which is unique to *E. coli* O157:H7. It is worth noting that it is a specific technique, especially that the positive control used

ATCC 35150 tested positive for this gene. One possible reason for the absence of *E. coli* O157:H7 in the meat samples would have been the ineffectiveness of the EC broth to allow for growth of this particular serotype. This broth requires several modifications (novobiocin and vancomycin, O157 antibody coated magnetic beads ...) to allow for specific enrichment of *E. coli* O157:H7 growth (Weagant *et al.*, 1995; Okrend *et al.*, 1992).

Pradel *et al.*, (2000) observed a significant association between highly cytotoxic isolates from diarrheagenic patients and the presence of the *stx2* gene. Other studies have also indicated that *stx2*-producing STEC strains are commonly associated with severe human disease (Kleanthous *et al.*, 1990; Ostroff *et al.*, 1989). A possible explanation for that is that the level of transcription of *stx2* in vivo is higher than that of *stx1*. This increase is due to a combination of amplification of *stx2* as the phage DNA is replicated and an increase in *stx2* promoter activity mediated by a phage-encoded positive regulatory factor (Mühldorfer *et al.*, 1996).

Many have concluded (Karmali, 1989; Piérard *et al.*, 1997; Sandhu *et al.*, 1996; Schmidt and Karch, 1996) that no single virulence gene is sufficient for EHEC to acquire virulence and cause infection. This was the reason behind isolating 5 isolates, positive for eaeA in addition to either one of VT1 or VT2 or VT2e, and subtyping them, in order to determine whether they originate from different calves or different origins. Since more than 2 banding differences ≥100kb were observed, they were regarded as genetically distinct isolates, thereby originating from different sources (Tenover *et al.*, 1995).

EPEC were isolated from 3.3 % of *E. coli*-containing samples, representing 2.7% of the total number of samples. This type was considered as the one that harbors *eaeA* gene alone, since many EHEC-positive samples were positive for *eaeA* gene as well.

eaeA-positive samples comprised 6.7% of the *E. coli*-containing samples. Previously, *eaeA* prevalence was reported within the category of VTEC, and not by itself; in a study aiming at detecting VTEC in cattle, sheep, and various foods in Spain, *eaeA* gene was found in 17% of the non-O157 VTEC (Blanco *et al.*, 2003). Orden *et al.*, (1998) found 5.9% of the *E. coli*-containing samples to be *eae*-positive non-O157. Jenkins *et al.*, (2002) also found that 17% of the non-O157 strains isolated from cattle in Scotland were positive for the *eaeA* gene. Orden *et al.*, (2002) however found 24.3% of the VTEC positive for *eae* gene. Similar results were obtained in our study, where 17 samples out of 78 VTEC (21.8%) harbored the *eaeA* gene. Orden *et al.*, (2002) found that EPEC represented 4.2% of the *E. coli* isolated, as compared to 3.3% EPEC in our study.

NTEC were detected in 13.4% of the *E. coli*-containing samples, where those harboring CNF1 and/or CNF2 were detected in 10.2% and 5.3%, respectively of the *E. coli*-containing samples. CNF1-positive samples had the highest prevalence among the other gene-positive samples, and peaked up to 6 positive samples over the weeks 22 and 23 (mid-July). In harmony to our results, Orden *et al.*, (2002) found NTEC in 9.9% of fecal samples isolated from cattle. NTEC strains from fecal samples harboring CNF2 gene were isolated from 24% of the calves tested (Blanco *et al.*, 1998). Orden *et al.*, (1999) detected NTEC in 15.8% of fecal samples from diarrheic calves with 3.7% being CNF1-positive, and 92.6% CNF2-positive. However in our study, 77% of the NTEC were positive for CNF1, and 39.5% were positive for CNF2. Osek (2001) found NTEC in 13.6% of the calves tested, with CNF2 being detected in 6.1% of the tested samples. Regarding the distribution across the weeks, 25 out of 30 weeks had samples containing NTEC strains, peeking up to 3-6 positive samples

every week from mid-July till mid-August. NTEC could be detected along with EHEC in 7 samples, with EPEC in two samples, and ETEC in one sample.

The percentage of samples positive for ETEC was 1.7%, which represents 2% of the *E. coli*-containing samples. LT1 was detected in only one sample, on week 28 (late August). ST2 was not detected, whereas ST1 was detected in 2% of the *E. coli*-containing samples. In an effort to epidemiologically determine the source of ETEC in two reported outbreaks, Nishikawa *et al.*, (1995) detected ETEC in pickles (kimshi) and frozen seafood in two outbreaks. Blanco *et al.*, (1993) isolated ETEC from 1% of calves with diarrhea and from 4% of healthy controls. Most of the studies were performed on stools of diarrheic infants or young adults (Ratchtrachenchai *et al.*, 2004; Rao *et al.*, 2003; Subekti *et al.*, 2003).

EAEC were isolated from 0.4% of the *E. coli*-containing samples representing 0.3% of the total number of samples collected. Prevalence of EAEC was mostly assessed in patients admitted for diarrhea, ranging from 1% to 10.2% and up to 38% (Keskimaki *et al.*, 2001; Pabst *et al.*, 2003; Presterl *et al.*, 2003).

EIEC were not detected in any of the samples. EIEC were detected in 0.5% and 1% of diarrheal patients (Ratchtrachenchai *et al.*, 2004; Regua-Mangia *et al.*, 2004).

It is worth noting that LEE that encodes several pathogenicity genes is conserved among all intestinal pathogens that produce the A/E lesion, including EPEC, EHEC, *Hafnia alvei*, and *Campylobacter freundii* (McDaniel *et al.*, 1995). This conservation is consistent with a cluster of virulence genes that spread horizontally among unrelated bacteria during evolution (McDaniel *et al.*, 1995). The transfer of genetic clusters and plasmids sets forth a question: were the virulence genes detected harbored by *E. coli* or other bacteria? To answer such an inquiry requires purification of isolates, identifying them, and then detecting the virulence genes. The problem that

would arise then is the instability of the virulence genes upon subculturing to purify and isolate strains (Karch *et al.*, 1992; Piérard *et al.*, 1997).

The multiplex PCR assay that was optimized by Pass *et al.*, (2000) was noted to have a slight problem in Mix 2 (primers specific for CNF2 and EInv genes). A 200-bp non-specific band appeared in the lanes. Its presence did not affect the mix assay, since it had a different size than the two bands sought, but could have resulted in false-negatives. Its amplification would have competed with a possible correct amplification of the corresponding genes for PCR components.

The slaughtering process in Beirut Slaughterhouse was carefully observed upon the collection of samples. Throughout this process, butchers encounter several points where contamination of calves with microorganisms takes place. To start with, the calves have their fur, most of the time, coated with patches of feces, from their own, from others, or from the stables where they were. These patches contain microorganisms, shed by these calves, and can contaminate the hands of the butchers. Upon slaughtering the calf, the fur is removed with tiny sharp knives, along with some pulling and relieving pressures by the butchers; with their hands contaminated, the meat may get contaminated. The calf is held high by its hind legs using ropes, and the cutting process is initiated. Upon hanging upside down, the calf is often rinsed with water, to remove some of the blood that may have "discolored" the meat. This rinsing process can carry many microorganisms from the feces to the whole body of the calf. The intestine and stomach are then eviscerated. The problem is that the intestinal content is so huge, that the butcher has to set part of it on the side of the abdominal wall, and initiate a series of pressures on the intestine and the stomach, to shift the weight toward the outside of the abdominal wall so that they fall off. Most of the time, these intestines/stomachs drip some of their content onto the diaphragm and

interior thoracic region, resulting in contamination. After the intestine and stomach are removed, the diaphragm is cut, and the lungs, trachea, bronchi, along with the heart are removed. The abdominal wall is then cut, and the process of breaking the spine starts. When finished, the halves are rinsed with water to clean them from blood, thereby dispersing what was contaminated locally, to include the whole carcass. After the spine is cut in half, each side is further cut into two halves with the same knife used since the beginning of the process. The carcass breaks are transported afterwards for further cutting and selling. After one calf is slaughtered, some of the butchers rinse their knives with running water, to "clean" it, and start over again, all over the night, starting at around 19:00 (p.m.) till 4:00 (a.m.), slaughtering on average 120 calves per night. The origins of the calves are diverse, from around the globe. The meat samples were taken from all of the butchers present at Beirut Slaughterhouse, thereby covering the whole number of calves slaughtered. The meat samples were taken from the same region from all the calves, aseptically, and without cross-contamination among samples. The samples were taken just after the calf is cut in half and rinsed with water, and just before cutting the two halves into four carcass breaks. The timing of sampling was distributed among the covered hours of slaughtering, thereby ensuring that the calves are slaughtering both by energetic butchers and by tired butchers—to note, the slaughtering process requires too much effort, especially the part when the spine is literally broken in two; all this to ensure that no bias was taken while sampling.

As discussed previously, the time of year chosen for the sampling to take place covers the relatively warm season, since studies confirm this season as being that when cattle shed most of the intestinal microbiota (Chapman *et al.*, 1997; Mechie *et al.*, 1997).

The process of slaughtering and carcass breaking discussed above is a major route of contamination. Gill *et al.*, (2001), upon sampling from all the sides of the carcass breaks and materials used for breaking them, found that the equipment used in breaking is the source of contamination, further confirming our observation that knives were a major source of carcass contamination.

As for the contamination from butchers' hands, Gill and McGinnis (2003) found that, although washing and/or rinsing with disinfectants, for several times, apparently did not reduce the numbers of bacteria on hands and fewer bacteria were recovered from hands before than after work.

Bell (1997) investigated beef-dressing lines with special attention to: contamination associated with carcass/hide and carcass/feces contacts, the distribution of microbial contamination on carcasses, and the antimicrobial efficacy of cold water carcass washes. They found that hides contaminated by direct fecal contact or contact with fecally- contaminated hides had the highest aerobic plate count and *E. coli* content. Cold water carcass washing was ineffective in removing microbial contamination and tended to bring about a posterior to anterior redistribution. Our observation of herd contamination with feces therefore holds true.

As noted in the literature, some studies detect *E. coli* in cattle feces; others isolate them from meat itself. Our study aimed at detecting *E. coli* in meat in an attempt to get an idea of the meat product reaching the consumer, whether it is contaminated or not with *E. coli*. Fecal contamination of *E. coli* has been assessed by Chapman *et al.*, (1993), who analyzed rectal swabs and meat samples. They found *E. coli* O157 present in 30% of meat samples from rectal swab positive herds. Bonardi *et al.*, (2001) took the same approach and isolated *E. coli* O157 from 35.3% of meat samples from rectal swab-positive herds. The conclusions from such studies would point out

that meat samples are a better indicator of meat contamination than the analysis of the fecal content of *E. coli*. However, such a conclusion is dependent only upon the purpose of the study that is to be performed. In our case, the epidemiological approach mandated that we get the closest picture to what meat quality, in terms of microbial contamination, the consumer is having. The processes of slaughtering and processing analyzed in the present study are the initial steps of a whole process of meat processing and handling before reaching the consumer.

The issue of handling meat after moving out of the slaughterhouse is beyond the scope of this study, but, it is worth noting that handling meat with contaminated equipment, hands, and transport means, are all new factors to be analyzed in an attempt to give a better idea of what the consumer is getting regarding the quality of meat consumed.

Besides *E. coli*, *Salmonella* species (Rose *et al.*, 2002), *Clostridium perfringens* (Kilic *et al.*, 2002), and *Enterococcus* species (Del Grosso *et al.*, 2000) were analyzed for meat contamination, but such an analysis is beyond the scope of our study.

Many outbreaks have been associated with non-O157:H7 *E. coli* (reviewed by Johnson *et al.*, 1996), with cattle being the main reservoirs for such pathogens, as inferred from the clonal relatedness of bovine non-O157:H7 strains and isolates from HUS patients (Bielaszewska *et al.*, 2000). Characterization of isolates from cattle was correlated with clinical isolates from HUS patients. Such correlations can be made between our study and other studies. One such clinical study has been conducted in Beirut between May and October 1999 by Matar *et al.*, (2002), where 77 *E. coli* isolates from stools of diarrheagenic patients were genotyped by multiplex PCR. None of these isolates were of the EHEC type, but 5 were ETEC, 4 EIEC, 3 EAEC, and 2 EPEC. A useful study is recommended to characterize *E. coli* isolates from

patients admitted for diarrhea throughout the period when our samples were collected. With the PFGE patterns at hand, correlations between *E. coli* isolates from meat and from patients would be conducted.

According to the results of this study, we recommend the following:

1. Herds: simple hygienic practices such as cleaning the stables where cattle is kept before slaughtering, can minimize, but not eradicate *E. coli* cross-contamination of the cattle.
2. Butchers: could be provided with antiseptics that dry off easily and require minimal time for setting and washing.
3. Slaughtering equipment: could be cleansed every now and then with the simple application of alcohol on the knives and burning the blades; within a few seconds, the blade could be sterilized to minimize cross-contamination among slaughtered calves.
4. Slaughtering process: could be performed in a manner slower than usual, since gentle evisceration can minimize the spilling of the intestinal contents on meat, and thus contamination.
5. Rinsing carcasses with water: could be delayed until the carcasses are broken into the last formats; this way, no auto-contamination could occur and spread the contaminants throughout the whole carcass.
6. Transporting of carcass breaks: each could be wrapped in huge sterile nylon bags, preparing for their transport; so as the person transporting the carcasses on his back will not cross-contaminate them.

These simple practices could help in reducing the risk of meat spoilage and thus reduce the spread of disease in humans, which at times could be severe.

Proper cooking is the best solution to all the contaminations that would take place before cooking the meat. To the best of our knowledge, Lebanese do not have the custom of eating raw beef meat; instead, most types of Lebanese food require boiling meat along with special sauces, which helps in reducing or even eradicating any source of contamination.

Chapter 6

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