ABSTRACT

Title of Dissertation: PATHOGENIC *ESCHERICHIA COLI* IN RETAIL MEATS

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Pathogenic *Escherichia coli*, including diarrheagenic *E. coli* (DEC) and extraintestinal pathogenic *E. coli* (ExPEC), cause numerous human infections annually. Retail meats are generally contaminated with *E. coli* and may serve as a vehicle transmitting pathogenic *E. coli*. The objectives of this project were to detect and characterize different pathogenic *E. coli* from retail meats, and to explore their potential in causing human infections.

E. coli isolates recovered from ground beef, ground turkey, chicken breast and pork chop during 2002 to 2007 were screened for Shiga toxin genes and Shiga toxin-producing *E. coli* (STEC) were characterized phenotypically and genotypically. In addition, *E. coli* isolates recovered in 2006 were also examined for virulence genes specific for other DEC. The results demonstrated that retail meats, especially ground beef, were contaminated with diverse STEC strains and some strains contained Shiga toxin genes associated with severe human infections. The presence of atypical

enteropathogenic *E. coli* in retail meat is also of concern due to their potential to cause human infections.

Meanwhile, *E. coli* isolates recovered in 2006 were investigated for the presence of five ExPEC-defining virulence genes using multiplex PCR. Identified ExPEC isolates were characterized by serotyping and phylogenetic grouping, and their antimicrobial susceptibility data were analyzed. The findings showed that ExPEC, including antimicrobial resistant strains, were widely distributed in retail meats, especially in chicken and turkey products. This indicates that meat may be a vehicle for dissemination of ExPEC strains.

ExPEC strains identified were further examined by multiplex PCR for the presence of 21 uropathogenic *E. coli* (UPEC) virulence genes. Selected strains were further characterized by multi-locus sequence typing and studied for their interactions with human bladder epithelial cells. Data showed that most UPEC virulence genes selected could be detected in isolates from meat. And some isolates belonged to sequence types associated with clinical UPEC. Meat-source isolates exhibited lower level of adherence and invasion compared to a clinical UPEC strain. These observations suggested that a small proportion of *E. coli* isolates from retail meats resemble human UPEC in various aspects, but their potential in causing human UTI needs further investigation.

PATHOGENIC *ESCHERICHIA COLI* IN RETAIL MEATS

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joy and fun. I can't thank my parents and parents-in-law enough for their understanding, support, and love.

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List of Abbreviations

AAF	Aggregative adherence factor
APEC	Avian pathogenic *E. coli*
ATCC	American Type Culture Collection
BFP	Bundle forming pilus
CDC	Centers for Disease Control and Prevention
DAEC	Diffusively adherent *E. coli*
DEC	Diarrheagenic *E. coli*
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme linked immunosorbent assay
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
FDA	Food and Drug Administration
HC	Hemorrhagic colitis
HlyA	Hemolysin A
HUS	Hemolytic uremic syndrome
IL	Interleukin
LEE	Locus of enterocyte effacement

LT	Heat labile toxin
MLST	Multi-locus sequence typing
MOI	Multiplicity of infection
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
ST	Heat stable toxin
STEC	Shiga toxin-producing *E. coli*
STs	Sequence types
STX	Shiga toxin
TTP	Thrombotic thrombocytopenia purpura
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infection
EAF	EPEC adherence factor

CHAPTER I. LITERATURE REVIEW

Escherichia coli (*E. coli*) are commonly found in the intestinal flora of humans and animals. They were recognized as non-pathogenic until late 1950s. However, certain isolates of *E. coli* have been implicated in a wide range of diseases that inflict humans and animals. It was estimated that each year in the United States, there are approximately 269,060 foodborne illness caused by enteric *E. coli* (46). In addition, *E. coli* infections outside the intestinal tract ranged from 6.7 to 8.6 million cases at an annual cost of around 1.5-2.3 billion dollars (59). Food originating from warm blooded animals may be contaminated with *E. coli* and are well known for transmitting certain enteric *E. coli*. Strains causing non-enteric infections are generally not considered being transmitted through food, but it was recently suggested that food may play a role in spreading them to humans. This chapter reviews basic characteristics and classification of *E. coli*. Moreover, the epidemiology, pathogenesis, treatment, transmission and detection of different pathotypes of *E. coli* are described. An overview of the project is presented at the end.

General characteristics of *E. coli*

E. coli are Gram-negative, facultative anaerobic bacteria that belong to the *Enterobacteriaceae* family. *E. coli* cells are typically rod-shaped and about 2 μm long and 0.5 μm in diameter. Optimal growth occurs at 37 °C and growth could be supported by aerobic and anaerobic respiration. Some strains possess flagella which enable bacteria to move. *E. coli* can be identified with a variety of biochemical

reactions and the indole test remains the most useful method to differentiate *E. coli* from other members of the *Enterobacteriaceae*.

Classification of pathogenic *E. coli*

E. coli is a major component of the normal intestinal flora of humans and other mammals. These commensal *E. coli* strains from the normal intestinal flora are usually harmless to the host and only cause disease in immunocompromised hosts or when the gastrointestinal barriers are breached. However, some specific *E. coli* strains represent primary pathogens with an enhanced potential to cause disease after acquiring specific virulence attributes. These virulence attributes are normally encoded on genetic elements that can be exchanged between different strains or on those elements once having been mobile but later becoming fixed into the genome. Specific combinations of virulence factors form different pathotypes: a group of strains of a single species that cause a related disease using the same set of virulence factors. Based on the various human diseases which *E. coli* can cause, pathogenic *E. coli* have been broadly classified into two major categories: the diarrheagenic *E. coli* and the extraintestinal pathogenic *E. coli*. Among the diarrheagenic *E. coli*, there are currently six categories: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC)/Shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusively adherent *E. coli* (DAEC) (32). Extraintestinal pathogenic *E. coli* are phylogenetically and epidemiologically distinct from diarrheagenic *E. coli*. They could colonize a variety of anatomical locations and cause various infections outside the gastrointestinal tract, among which urinary tract infections are the most common (30).

The *E. coli* strains causing extraintestinal infections have been collectively called extraintestinal pathogenic *E. coli* (ExPEC), which includes two major pathotypes: uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC). Current understanding of different pathogenic *E. coli* except NMEC will be discussed in detail below.

EPEC

Enteropathogenic *E. coli* is a leading cause of potentially fatal infant diarrhea in developing countries and also an important cause of diarrhea in industrialized countries. EPEC infection is primarily a disease in infants younger than 2 years. Sporadic disease also occurred in some adults with compromising conditions. Once defined only on O and H serotyping, they are currently defined by pathogenic features as those diarrheagenic *E. coli* that induce attaching and effacing (A/E) lesions on intestinal cells and do not produce Shiga toxins. They could be divided into typical EPEC and atypical EPEC based on the presence of EPEC adherence factors (EAF) plasmid. Moleculard detection and differentiation of EPEC could be based on the *eae* gene (A/E lesions) and *bfp* gene (which resides on EAF plasmids and encodes bundle- forming pilus). Typical EPEC contain both *eae* and *bfp* genes, while atypical EPEC contain only *eae* gene. Typical EPEC infections are common in developing countries while atypical *E. coli* seems to predominate in the industrialized countries (8, 67). Not like typical EPEC which are found only in human so far, atypical EPEC have been isolated from a variety of animal species such as cattle, goats, sheep, chickens, pigeons and gulls (12, 50, 76).

Pathogenesis of EPEC is currently considered to include four stages: expression of adhesion factors, initial localized adherence, signal transduction and intimate contact, cytoskeletal rearrangement and pedestal formation. Initally the bacteria attach to intestinal epithelial cells by adhesive fimbriae called bundle forming pili (BFP) or EPEC adherence factor (EAF) (18). EPEC also adhere to epithelial cells by expressing intimin (encoded by *eae* gene) and surface–associated filament EspA (34). After initial binding, EPEC utilize type III secretion system to inject into host cells translocated initmin receptor (TIR) and several effector molecules, which activate cell signaling pathways and actin polymerization and depolymerization to alter cytoskeletal structure. TIR is then phosphorylated and inserted into the host cell membrane for later intimate contact. Activated host cell signal transduction pathway causes extensive rearrangement of actin, which results in the formation of the characteristic attaching and effacing lesions (33). The membrane under the bacteria forms a pedestal due to host cell cytoskeletal rearrangement. The microvilli are lost due to depolymerization of actin filament in microvilli. The effector proteins also influence membrance permeability and cause diarrhea-associated symptoms. Virulence genes in EPEC are mostly located in a pathogenic island called locus of enterocyte effacement (LEE).

EHEC/STEC

The EHEC group is also referred to Shiga-toxin producing *E. coli* (STEC) because its pathogenicity is largely attributed to the production of Shiga toxins. It should be noted that some researchers prefer to use EHEC only for those STEC containing LEE pathogenic island, while others use them exchangeably. I will use the

broader term STEC later in this project. Among six categories of diarrheagenic strains, STEC strains distinguish themselves by their ability to cause severe life-threatening complications, such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura(TTP). Other symptoms of STEC infection include bloody diarrhea and hemorrhagic colitis (HC). Children and the elderly are more susceptible to severe STEC infections than healthy adults. Outbreaks and sporadic cases of STEC infections are frequently reported worldwide, indicating the great threat that STEC could pose for human health (10, 22, 74, 78).

STEC can be disseminated through a variety of means. Most human infections are caused by consumption of contaminated foods. Domestic and wild ruminant animals, in particular cattle, are considered as the main reservoir of STEC and the main source for contamination of the food supply (1, 23, 25). Food products derived from these animals can be contaminated with STEC during slaughter and further processing. In addition, vegetables contaminated with cattle manure have been also implicated in many cases and outbreaks of STEC infections (56, 73). STEC have been also isolated from other food animals such as pigs and poultry, but whether these animals represent real hosts or are just temporarily colonized with STEC is not clear (9, 19).

The mechanisms of STEC infection in humans are not fully understood. The major virulence factors implicated in STEC infection are potent Shiga toxins, which are classified into two groups: Stx1 and Stx2. In each group, variants that differ in toxicity, toxin receptor, and amino acid sequences have been described. Few variants (Stx1c , Stx1d) were found for Stx1 (20, 82), whereas Stx2 contains several variants

including Stx2c, Stx2d, Stx2e, Stx2f, Stx2g (26, 51, 65, 66, 72). Shiga toxin types were suggested to correlate with the clinical symptoms of STEC infection. Some Stx types, such as Stx2, Stx2c and Stx2d-activatable, have been associated with high virulence and ability to cause HUS, while Stx1, Stx1c, Stx2e occurred mainly in milder diarrhea patients or asymptomatic carriers (13). In addition to Stx production, other (putative) virulence factors that could contribute to the pathogenicity have been discovered. The *eae* gene, which is located in a pathogenic island in the chromosome called the locus of enterocyte effacement (LEE), is the best characterized virulence loci other than *stx*. The *eae* gene encodes the adherence factor intimin, an outer membrane protein involved in the attachment of *E. coli* to the enterocyte. In addition, many pathogenic STEC possess a large plasmid that harbors several putative virulence factors such as EHEC-*hlyA,* which encode for a cytolytic EHEC-hemolysin. EHEC hemolysin is strongly associated with STEC isolates causing severe infections in human, but its exact role in pathogenesis is still yet to be known.

More than 400 serotypes of STEC strains have been implicated in human infections (7). Although *E. coli* O157: H7 is considered the principal STEC in the U. S., infections due to non-O157 STEC occurr and are thought to be underreported (5, 53). In some other countries, such as Germany, Australia, and the UK, non-O157 STEC infections predominate (4, 79). Globally, only a limited numbers of serotypes were frequently observed and are responsible for the majority of STEC infections.

ETEC

ETEC are defined as those *E. coli* strains that contain at least one of two defined groups of enterotoxins: heat stable toxin (ST) and heat labile toxin (LT).

ETEC is an important cause of childhood diarrhea in developing countries due to poor sanitary conditions. It is also a common cause of diarrhea in travelers to developing countries (55). It is estimated that around 650 million cases of ETEC infection occur each year in developing countries, which include 800,000 deaths mostly in young children (55). It causes watery diarrhea ranging from mild form to severe purging disease. The diarrhea persists for 3-4 days and is usually self-limiting, however, diarrhea may be fatal in young children and infants. Epidemiologic studies found that contaminated food and water serves as the most common vehicles for ETEC infections, and fecal contamination of water and food is the primary reason for high incidence of ETEC infection in the developing countries (37, 80).

Colonization factors (CF) and one or more enterotoxins that induce a secretory diarrhea are the major determinants of ETEC virulence. CFs are proteinaceous fimbrial and afimbrial structures that enable bacteria to attach to intestinal mucosa. More than 20 CFs have been identified and characterized in ETEC (75). Other adhesion factors such as TibA (an afimbrial adhesion) and Tia (an outer membrane protein) are also involved and implicated in the attachment of ETEC (41).

Having established contact with epithelial cells, ETEC can produce one or more ST or LT. ST have been divided into two distinct groups: methanol soluble STI (or STa) and methanol insoluble STII (or STb) (57). STa toxins have two genetic variants STh and STp, described originally in association with strains isolated from human and pigs, respectively. However, new studies found that both variants could be found in ETEC strains of human origin. STa binds to guanylate cyclase C receptor and activates its guanylate cyclase domain, which results in an increase in

intracellular cGMP level. Increase cGMP influences ion pumps, resulting in enhanced salt and water secretion and inhibition of Na^+ absorption. STb is most associated with porcine strains of ETEC.

LT is an oligomeric protein composed by a ring of five identical B subunits with one A subunit. The B subunits bind to a GTP binding protein (ganglioside receptor), while the A subunit is responsible for the enzymatic activity of the toxin (21). Based on type of cell surface receptor to which B subunits bind, LT could be divided into LT-I (bind to ganglioside receptor GM1) and LT-II (bind to ganglioside receptor GD1). A subunit ADP-ribosylates the alpha subunit of the GTP-binding protein Gs, leading to activation of adenylate cyclase in the enterocyte and accumulation of cyclic AMP. The increase in intracellular cAMP increases Cl^- secretion in crypt cells and decreases absorption of Na^+ and Cl^- by villus tip cells. Other toxins that may contribute to ETEC infection include a novel heat stable enterotoxin EAST1 and a serine protease autotransporter, EatA, and a pore–forming toxin, ClyA.

EIEC

EIEC strains are genetically and pathogenically related closely to *Shigella spp*. (54). EIEC infections mostly produce watery diarrhea that is indistinguishable from other *E. coli*, although they may induce invasive inflammatory colitis and dysentery. Sporadic outbreaks are common and those outbreaks are usually foodborne or waterborne (71).

EIEC invade colonic epithelial cells, followed by lysis of endocytic vesicle, intracellular multiplication, movement inside the cytoplasm, and spread among

adjacent cells. Genes required for invasions are present on a plasmid called pInv. Much of the EIEC pathogenesis can be attributed to a type III secretion system, which encodes many effector proteins such as IpaA, IpaB IpaC and IpgD. These loci mediate epithelial signaling events, cytoskeletal rearrangement, cellular uptake, lysis of the endocytic vacuole and other processes (77).

EAEC

EAEC are defined as *E. coli* that do not produce LT or ST and that adhere to HEp-2 cells in a pattern described as autoaggregative. EAEC have been increasingly recognized as an important causative agent of persistent diarrhea in children and adults in both developing and developed countries. EAEC mostly cause sporadic cases, but several outbreaks have been reported (69).

Pathogenesis of EAEC includes colonization of intestinal mucosa by adhesins followed by secretion of enterotoxins and cytotoxins (62). EAEC express aggregative adherence fimbriae (AAF) I, II and III and outer membrane adhesion proteins. Adherence is described as a stacked-brick shape. EAEC elaborate enteroaggregative heat stable toxin (EAST) and a cytotoxin that is responsible for pathological effects. Infections usually lead to mucoid stool and persistent diarrhea (often more than 14 days).

DAEC

The most recently distinguished category in DEC are the diffuse adherent *E. coli* (DAEC), strains that are capable of adhering to HEp-2 cells in a nonlocalized (diffuse) pattern. DAEC have been implicated in infantile diarrhea (63). It cause watery diarrhea without blood or fecal leukocytes.

Pathogenesis of DAEC is largely unknown. Most of DAEC produce a fimbrial adhesion called F1845, a member of the Dr family of adhesins, which is thought to be partly responsible for diffuse adherence pattern. DAEC strains elicit a cytopathic effect characterized by the development of long cellular extension which wrap around the bacteria. DAEC have also been shown to impair the activities and synthesis of brush-border-associated hydrolysase, which implies the existence of pathogenic mechanisms other than Dr adhesins (3).

ExPEC

The *E. coli* pathotypes that cause extraintestinal infections are collectively called ExPEC. They are phylogenetically and epidemiologically distinct from commensal and diarrheagenic stains. Compared to human commensal strains, which usually derive from phylogenetic groups A and B1, most of ExPEC strains belong to the B2 and D groups and harbor various virulence factors which allow them to induce diseases in both healthy and compromised hosts (59, 60). Based on the prevalence of various virulence factors in clinical *E. coli* isolates from extraintestinal infections, ExPEC were currently defined as *E. coli* isolates containing two or more of the following genes: *papA* (P fimbriae structural subunit) and/or *papC* (P fimbriae assembly), *sfa/foc* (S and F1C fimbriae subunits), *afa/dra* (Dr-antigen-binding adhesins), *kpsMT* II (group 2 capsular polysaccharide units), and *iutA* (aerobactin receptor) (31).

Unlike diarrheagenic strains, ExPEC are incapable of causing gastrointestinal disease in human. However, ExPEC can cause diverse infections at different anatomical locations outside the human intestinal tract (38, 52). They are the most

common cause of urinary tract infections and one of the leading causes of neonatal meningitis and neonatal sepsis. In addition, *E. coli* can be implicated in community-acquired bacteremia and sepsis. *E. coli* also occur in intra-abdominal infections and nosocomial pneumonia, and occasionally are involved in other extraintestinal infections such as osteomyelitis, cellulitis, and wound infections.

UPEC

Urinary tract infections are the most common bacterial infections, which account for approximately seven billion infections per year and over $1 billion cost . Uropathogenic *E. coli* (UPEC), which belong to the ExPEC, are the principal cause of community-acquired urinary tract infections (UTI) (70–95%) and a large portion of nosocomial UTIs (50%) in the U. S. (16).

It is generally accepted that a primary reservoir of UPEC isolates is within the human intestinal tract (61). Due to the proximity of urinary tract to the rectum, bacteria can be transferred to the urinary tract, where they ascend the urethra into the bladder and cause cystitis. Sometimes bacteria find their way through ureters to kidney and cause pyelonephritis. Normally UPEC cause sporadic cases and outbreaks due to the same strain are rare. But transimission of a single clonal group of UPEC isolates may occur in a community by contaminated food or other consumables (44). UPEC from UTI patients often match fecal isolates from their sex partners, suggesting that UPEC could be sexually transmitted (17).

Most UPEC strains belong to limited serogroups based on their O-antigen (O1, O2, O4, O6, O7, O8, O16, O18, O25, O75), while specific K and H antigens have a less defined pattern(43). It is known that certain O and K antigens contribute

to survival for some UPEC strains, although how the O antigen enhances UPEC virulence still needs exploring. UPEC encode various adhesive structures called pili and fimbriae, among which are type 1, P, S, F1C pili (70). One UPEC strain can harbor several different pili and be able to switch from one type of pilus to another depending on the environmental changes. To survive in a low iron environment such as the urinary tract, UPEC express different iron acquisition systems to take iron from host for their benefit. They secrete siderophores such as aerobactin, salmochelin, and yersiniabactin that can scavenge iron from the environment and then be taken back to bacteria through receptors on the bacterial cell surface. Various toxins that UPEC secrete are involved in UTI pathogenesis. More than half of UPEC isolates contain alpha-hemolysin (HlyA), which can form pores in host cells. Vacuolating autotransporter toxin (Vat) and secreted autotransporter toxin (Sat) are considered as potential virulence factors due to their ability to cause vacuolating and swelling. Another important toxin that UPEC elaborate is cytotoxic necrotizing factor 1(CNF1), which is found in one third of UPEC isolates. CNF1 can activate the Rho family of GTPases, leading to membrane ruffling, modulation of inflammatory signaling pathways and apoptosis (40). Many UPEC virulence genes are encoded on pathogenic islands, in which many virulence genes may be clustered together.

Upon arriving at the urinary tract, UPEC must first bind to host epithelial cells to avoid clearance by the flow of urine. UPEC achieve this by expressing pili or fimbriae (27). The most common pili with UPEC are type 1, S/F1C and the Dr family of adhesins. These pili are assembled by periplasmic chaperone protein and outer-membrane usher protein. The most studied pili in UPEC are type 1 and type P pili,

which are encoded by many UPEC strains. Type 1 pili, through its adhesive tip FimH, bind to mannosylated glycoprotein receptors expressed by a number of host epithelial cells and are considered the most important virulence factor in the pathogenesis of UTI. Although many commensal *E. coli* also encode type 1 pili, FimH in commensal stains only bind to trimannose receptors, while 70% of UPEC express FimH variants with higher affinity for monomannose receptors in uroepithelial cells. Type P pili are commonly associated with pyelonephritis. Their tip protein, PapG, binds to a glycolipid receptor called globotriasylceramid (GbO3) and its variants expressed on the surface of kidney cells. In addition to type 1 and P pili, other groups of pili are also found to be associated with adherence of UPEC to host cells. S pili use SfaS tip to interact with sialic acid residue of receptors on kidney epithelial cells and vascular endothelial cells and are often associated with strains causing ascending UTIs, sepsis and meningitis. F1C pili, which are homologous to S pili but with different receptor specificity, may also influence pathogenesis of many UPEC strains. Dr adhesion family include the uropathogen- associated fimbrial adhesion Dr and non-fimbrial adhesion such as AFA I to IV. Members of Dr adhesins recognize one or more of 60 amino acid short consensus repeat sequence, which are present in receptors on the uroepithelia. They are thought to contribute to ascending colonization and chronic interstitial infections of the urinary tract.

After colonizing the urinary tract through binding to host cells, UPEC invade epithelial cells. UPEC invasion of host cells was postulated to enhance bacterial survival by protecting themselves from host immune defenses and allowing them to get into deeper tissues (49). Both type 1 pili and Dr family adhesion have been

proposed as contributing factors that promote invasion of host cells by activating host cell signaling events. Within epithelial cells, UPEC are transported into acidic compartments with features like late endosomes or lysosomes. In the superficial uroepithelial cells, UPEC can break from those compartments into the cytosol and multiply rapidly to form biofilm-like intracellular bacterial communities. Within immature bladder epithelial cells which underlie the superficial epithelial cells, these apartments are often emeshed within a network of actin fibers and bacterial replication is greatly limited. It is suggested that UPEC could persist quiescently for long periods in these immature cells and could later lead to recurrent infections when induced to multiply (14).

Upon UPEC infection, host tissues respond quickly by initiating various defense pathways. The presence of bacteria or bacterial products inside the normally sterile urinary tract could trigger rapid and robust responses from the host. The most obvious response after infection is the production of cytokines and influx of neutrophiles (64). Interactions between adherent UPEC and host epithelial cells stimulate the expression of a number of pro-inflammatory molecules, including interleukin 6 (IL-6), and IL-8, two cytokines found in the urine of patients with UTI. IL-6 is considered as a proinflammatory or immunomodulatory cykotine. It may facilitate the transition from a neutrophile to a predominantly monocytic response. IL-8, which serves as chemotactic stimuli, is a key components in aiding transepithelial migration of neutrophiles. Bladder epithelial cells play an important role in the innate immune response during a UTI, as they express Toll-like receptor 4 (TLR4), a receptor for lipopolysaccharide (LPS) from Gram-negative bacteria, and

secrete IL-6 and IL-8 in response to LPS and *E. coli*. Recently, UPEC have been found to be able to evade or subvert host innate immune response although the mechanism is not clear (6). It has been shown that UPEC can suppress NF-kB signaling pathway, which stimulates the transcription of anti-apoptotic and proinflammatory genes (36). Another prominent response of host is the exfoliation of infected epithelial cells. The bladder epithelium is normally quiescent and is only renewed every few months in healthy individuals. While soon after infection by UPEC, exfoliated epithelium cells could often be found with urine in UTI patients (48). Exfoliation of infected bladder cells containing intercellular bacterial communities may provide a means for the host to clear lots of bacteria with the flow of urine. However, this process also leaves the underlying layers of immature bladder epithelial cells exposed and more susceptible to UPEC infection.

Treatment of *E. coli* infections

Diarrhea caused by various diarrheagenic *E. coli* normally are self-limiting and do not require antibiotic treatment. For infants and children who are at high risk for dehydration, fluid replacement would be given in the case of fluid loss caused by ETEC, EPEC and EAEC. Antibiotics could also be given to those travelers who suffer from severe diarrhea to reduce the duration of the symptoms (35). But for infections caused by STEC, antibiotics are not recommended due to the potential risk of inducing more toxin production and making the disease worse (81). For severe HUS caused by STEC, compensation for possible kidney failure with dialysis is the current treatment strategy. In contrast to diarrheagenic infections, extraintestinal infections caused by ExPEC, ranging from mild infection such as cystitis to more

severe and potentially fatal infection such infant meningitis, are generally treated with antibiotics. Commonly prescribed antibiotics for extraintestinal infections include trimethoprim-sulfamethoxazole (TMP-SMX), fluoroquinolones, generations of cephalosporin and nitrofurantoin. Although antibiotics have been very useful in treating ExPEC infections in the past, the increase in antibiotic resistance in *E. coli* make treatment of *E. coli* infections complicated. For example, the emergence of fluoroquinolones resistant and extended-specturm beta-lactamse (ESBL) producing strains have caused great concern due to limited therapeutic options for infections with those *E. coli* strains (15, 47). Considering this situation, researche on developing effective vaccines and identifying risk factors for ExPEC infections are going on with an aim to prevent ExPEC infections (58).

Detection methods

Generally, detection of *E. coli* contamination in food samples is performed by pre-enrichment and enrichment utilizing appropriate enrichment and selective media. Suspected isolates are subjected to a series of biochemical tests. Further characterization can be done by immunological methods to determine serotype and toxins. Specific cell cultures or animal models are used to assess their pathogenic potential. For example, the HEp-2 cell adherence assay is still the gold standard for detection of EAEC and DAEC. Although cell culture and animal models could provide direct evidence about the presence of pathogenic *E. coli*, most of them are time-consuming and require cumbersome work, especially when there are numerous samples for analysis. In addition, sometimes there are no corresponding animal

models that can be used since some pathogenic *E. coli* can only cause disease in humans. Accumulating knowledge about genetic determinants that are responsible for *E. coli* pathogenesis makes molecular-based methods more and more popular in detecting and characterizing *E. coli*. Compared to phenotypic methods, molecular methods are generally more sensitive, quicker and easier for automation. Among all molecular methods, Polymerase Chain Reaction (PCR) is the most commonly used method to identify specific virulence genes in *E. coli* strains. PCR is especially advantageous when dealing with food samples, where a relatively low number of cells is present. The occurrence of real time PCR makes it possible for simultaneous quantification and detection of *E. coli*. Recently, great efforts have been taken to design methods which can examine a strain for all known *E. coli* virulence genes to assess its virulence potential (2, 39). Such broad range virulence gene detection systems, especially microarray system, would be very useful for differentiating pathogenic strains from non-pathogenic ones and identification of specific pathogroups.

E. coli contamination in retail meat

The intestinal tracts of food animals are natural habitats of different *E. coli* strains. Food products derived from these animals may be contaminated with *E. coli* during slaughter and further processing. *E. coli* contamination in retail meats has been found to be very common in various studies (42, 68). Considering different pathogenic potential of different pathotypes of *E. coli* , most of these studies concentrated on O157 STEC, while other serogroups of STEC were not extensively assessed (24). Even fewer data exist about the occurrence of other groups of diarrheagenic *E. coli*

strains in retail meats (11). Although meat is generally considered as vehicles to transmit diarrheagenic *E. coli*, recent researche showed that retail meat could be contaminated with various ExPEC strains and may represent an important means to disseminate ExPEC strains (28, 29). In addition, there was epidemiological evidence that meat consumption was associated with urinary tract infection (45). Although the existence of ExPEC strains in meats was determined in some researche, those studies didn't go further to assess the potential of those ExPEC from meat to cause disease such as urinary tract infections.

Project overview

Pathogenic *E. coli* can cause a wide variety of diseases in humans. There are many routes by which *E. coli* can be transmitted, including food and water, person-to-person contact, and animal-to-person contact. Consumption of food (especially meats from food animals) contaminated with various DEC serves as a very common cause of human enteric infections with *E. coli*. In addition, researche suggests that foods might serve as a source of extraintestinal *E. coli* which are responsible for various extranitestinal infections. Those findings imply that ExPEC may represent an important group of foodborne pathogens. The goal of this study was to explore if *E. coli* from retail meat pose a significant human health threat as an important diarrheagenic pathogen as well as a potential source for ExPEC strains causing UTI. Three specific objectives of this project were as follows: 1) To detect and characterize different pathotypes of diarrheagenic *E. coli* in retail meats; 2) To determine the occurrence and antimicrobial resistance of ExPEC in retail meats; and 3) To explore the potential of ExPEC from retail meat to cause urinary tract infections. In the

subsequent chapters (II to IV), three research studies are described that were performed to accomplish each objective respectively.

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CHAPTER II. PRESENCE AND CHARACTERIZATION OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* AND OTHER POTENTIALLY DIARRHEAGENIC *E. COLI* IN RETAIL MEATS

Abstract

To determine the presence of Shiga toxin-producing *Escherichia coli* (STEC) and other potentially diarrheagenic *E. coli* in retail meats, 7,258 *E. coli* isolates collected by the U. S. National Antimicrobial Resistance Monitoring System (NARMS) retail meat program from 2002 to 2007 were screened for Shiga toxin genes. In addition, 1,275 of the *E. coli* isolates recovered in 2006 were examined for virulence genes specific for other diarrheagenic *E. coli*. Seventeen isolates (16 from ground beef and 1 from pork chop) were positive for *stx* genes, including five for both stx_1 and stx_2, two for stx_1 and 10 for stx_2. The 17 STEC belonged to 10 serotypes: O83:H8, O8:H16, O15:H16, O15:H17, O88:H38, ONT:H51, ONT:H2, ONT:H10, ONT:H7 and ONT:H46. None of the STEC isolates contained *eae*, whereas seven carried EHEC-*hlyA*. All except one STEC isolate exhibited toxic effects on Vero cells. DNA sequence analysis showed that stx_2 from five STEC isolates encoded mucus-activatable Stx2d. Subtyping of the 17 STEC isolates by PFGE yielded 14 distinct restriction patterns. Among the 1, 275 isolates from 2006, 11 atypical enteropathogenic *E. coli* (EPEC) isolates in addition to three STEC were identified.

This study demonstrated that retail meats, mainly ground beef, were contaminated with diverse STEC strains. The presence of atypical EPEC strains in retail meat is also of concern due to their potential to cause human infections.

Introduction

Escherichia coli is an important component of the intestinal microflora of humans and warm-blooded mammals. While typically *E. coli* harmlessly colonize in the intestinal tract, several *E. coli* clones have evolved the ability to cause a variety of disease within the intestinal tract and elsewhere in the host. Those strains that cause enteric infections are generally called diarrheagenic *E. coli*, and their pathogenesis is associated with a number of virulence attributes that vary according to pathotypes (54). Currently, diarrheagenic *E. coli* is classified into six main pathotypes based on their distinct virulence determinants and pathogenic features, including: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC)/ Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusively adherent *E. coli* (DAEC) (37).

Among diarrheagenic *E. coli*, STEC are distinguished by their ability to cause severe life-threatening complications, such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (30). Other symptoms of STEC infection include watery diarrhea, bloody diarrhea and hemorrhagic colitis (HC). STEC that cause HC and HUS are also called EHEC. Although individuals of all ages are at risk of STEC infection, children younger than five years and the elderly are more likely to experience severe complications (51). Outbreaks and sporadic cases of STEC infections have been reported frequently worldwide.

The pathogenesis of STEC infection in humans is not fully understood. The major virulence factors implicated in STEC infection are potent Shiga toxins, which are classified into two groups: Stx1 and Stx2 (23). Additional factors that contribute to virulence have also been described, including intimin (encoded by *eae* gene), an outer membrane protein involved in the attachment of *E. coli* to the enterocyte, and EHEC-hemolysin (encoded by EHEC-*hlyA*), which acts as a pore-forming cytolysin and cause damage to cells (41).

The first O157 STEC infections were reported in 1982 when *E. coli* O157:H7 was involved in outbreaks associated with two fast food chain restaurants in the United States (44). Since then, ever-increasing numbers of cases and outbreaks due to O157 STEC have been reported worldwide. Although non-O157 STEC have also been associated with human cases and outbreaks, few laboratories have been looking for them and their potential in causing human infections may be underestimated (2). But recently, the significance of non-O157 STEC as human pathogens is becoming more recognized. In the United States alone, there were 23 reported outbreaks of non-O157 STEC infection between 1990 and 2007 (10).

Shiga toxin-producing *E. coli* can be transmitted through different routes, including food and water, person-to-person contact and animal-to-person contact (9). Most human infections are caused by consumption of contaminated foods (16). Domestic and wild ruminant animals, in particular cattle, are considered the main reservoir of STEC and the main source for contamination of the food supply. Retail meats derived from animals could potentially act as transmission vehicles for STEC and other diarrheagenic *E. coli* strains. However, there is limited information about

STEC contamination in retail meats; and fewer data exist about the presence of other diarrheagenic *E. coli* in retail meats. In the present study, we investigated 7,258 *E. coli* isolates from four types of meat samples (beef, chicken, pork and turkey) collected during 2002-2007 to assess STEC contamination in retail meats. In addition, the presence of other potentially diarrheagenic *E. coli* was examined by detecting specific virulence determinants among *E. coli* isolates collected in 2006.

Materials and methods

Bacterial strains. A total of 7,258 *E. coli* isolates (1,806 from ground beef, 2,106 from ground turkey, 2,179 from chicken breast, and 1,167 from pork chop) from the retail meat program of the U.S. National Antimicrobial Resistance Monitoring System (NARMS) were investigated. The detailed information of sampling, isolation, and identification could be found at http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/default.htm. Briefly, retail meats were collected monthly from grocery stores in four states (Georgia, Maryland, Oregon and Tennessee) from 2002 to 2007. For chicken and pork samples, one piece of meat was examined; for ground beef and ground turkey, 25 g of product was processed. Portions from each sample were placed in separate bags with 250 ml of buffered peptone water, and the bags were vigorously shaken. Fifty milliliters of the rinsate was mixed with 50 ml of double strength MacConkey broth and the content was incubated with shaking at 35°C for 24 h. One loop of the cultures was streaked onto an Eosin Methylene Blue (EMB) agar plate and incubated at 35°C for 24 h. The plate

was examined for typical *E. coli* colonies (colonies with a dark center and a green metallic sheen), and one typical, well-isolated colony was streaked onto a blood agar plate and incubated at 35°C for 24 h. Indole positive and oxidase negative isolates were presumptively identified as *E. coli* and confirmed as *E. coli* using the Vitek 2 Compact microbial identification system (bioMérieux, Hazelwood, MO). All *E. coli* isolates were stored in Tryptic Soy Broth containing 15% glycerol at – 80°C until use.

DNA template preparation. *E. coli* were recovered from –80°C frozen culture and streaked onto blood agar plate and incubated overnight at 37°C. DNA of each isolate was extracted using boiling method previously described with modification (46). Briefly, approximately 10 to 20 colonies were taken by cotton swabs and were suspended in 500 µl distilled water. The mixture was then boiled at 100°C for 10 min. After centrifugation at 18,000 × g for 5 min, supernatants were transferred to a new tube and stored in – 20°C until use in PCR amplification.

Identification of Shiga toxin genes. STEC were identified by the presence of *stx*$_1$ and /or *stx*$_2$ genes. All isolates were subjected to a multiplex PCR (assay 1) which targeted *stx*$_1$ and *stx*$_2$ and most of their variants (except *stx*$_{1d}$ and *stx*$_{2f}$, due to their considerable sequence divergence from classical *stx*$_1$ and *stx*$_2$, respectively) (Table II-1). PCRs were performed in a 25 µl reaction containing 2 µl of DNA template, 2.5 µl of 10×PCR buffer, 2 µl of a 1.25 mM mixture of deoxynucleoside triphosphate, 2.5 µl of 25 mM $MgCl_2$, and 0.25 µl of 5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ) and 0.5 µl (25 pmol) of each oligonucleotide primer (Invitrogen, Carlsbad, CA). Thermocycling protocol included an initial denaturation at 94 °C for 10 min, followed by 30 cycles of denaturation (94

°C for 30 s), annealing (58 °C for 30 s), and extension (72 °C for 30 s), and a final extension at 72 °C for 3 min. PCR products (10 µl) were resolved by electrophoresis with a 1.5% (wt/vol) agarose gel at 100mV for 30 min. Gels were then stained with ethidium bromide (0.5 µg /ml) and the DNA bands were visualized and photographed under UV illumination. *E. coli* EDL 933 containing stx_1 and stx_2 and K-12 were used as positive and negative control, respectively. For initial screening, DNA templates of five isolates were pooled and mixed thoroughly, and the mixture was used as template for PCR. When the mixture was tested positive for *stx* gene(s), DNA of individual isolates was then tested separately to identify *stx* positive isolate(s).

DNA sequence and phylogenetic analysis of *stx* genes. Shiga toxin genes were amplified and sequenced using primers listed in Table 1. The stx_1 genes were amplified with primer Paton 1 to generate a 1,470 bp product which covered both A and B subunit of stx_1. Two overlapping DNA fragments (779 and 714 bp), which together cover the entire stx_2 gene, were amplified with primer sets Stx2-1 and Stx2-2, respectively. The PCR products were purified using 96-well multiscreen filter plates (Millipore Corp., Billerica, MA) and purified amplicons were sequenced on both strands. Sequencing reactions were performed using Big-dye terminator cycle sequencing ready reaction DNA sequencing kit and electrophoresed on AB3730 DNA sequencer (Applied Biosystems, Foster City, CA). DNA sequence data were compiled and analyzed using DNA Sequencher 4.0 software (Gene Codes Corp., Ann Arbor, MI). Nucleotide phylogenetic trees were generated by multiple sequence alignment and neighbor-joining analysis of the alignment using MAFFT program (http://align.bmr.kyushu-u.ac.jp/mafft/software/). Reference DNA and amino acid

sequences for Shiga toxins were obtained from GenBank and indicated in the phylogenetic tree by their accession numbers.

Vero cell cytotoxicity assay. The STEC isolates were examined for their cytotoxicities on Vero cells according to the protocols published before (20, 55). First, 96-well microtitre plates were seeded with approximately 10^4 Vero cells/well and incubated at 37°C for 24 h in the presence of 5% CO_2. The tissue culture medium (EMEM containing 10% fetal bovine serum; ATCC, Manassas, VA) was aspirated and replaced with 100 µl of fresh medium prior to the addition of bacterial supernatant dilutions. The bacterial isolates were inoculated into 5 ml LB broth and incubated overnight at 37°C with shaking. The cell concentration of the overnight bacterial culture was adjusted with LB broth to approximately 10^9 CFU/ml (OD_{600} = 1). The culture was centrifuged at 10,000 × g for 10 min and the supernatant was filtered through a 0.45µm–pore-size membrane filter. The filtrate was serially diluted (1:5) in tissue culture medium. One hundred microliters of each dilution was inoculated to triplicate wells of the 96-well microtitre plate with Vero cells. Control wells, which refer to wells containing cells not inoculated with toxin, were included on each plate for untoxicated cell background. After incubation at 37°C in a 5% CO_2 atmosphere for 48 h, detached cells, medium and toxin were removed by vigorous shaking. Remaining Vero cells were fixed with 2% formalin in 0.067 M phosphate-buffered saline (pH 7.2) for 1 min, the fixative was removed and the plate was stained with 0.13% crystal violet in 5% ethanol for 30 min. Excess stain was removed by rinsing, and the plates were air-dried. For quantification, stain was eluted with 200 µl of 50% ethanol, and color intensity of each well was measured with an Elx800

microplate reader (Bio-Tek Instruments, Winooski, VT) at 600 nm wavelength. The color intensity was proportional to the number of viable, attached cells in the well. The absorbance values were then plotted against of the log of the toxin dilution. For determining the toxin dilution resulting in 50% cell detachment (CD_{50}), the zero detachment dye absorbance value was obtained from control wells with untoxicated cell on each plate, and then CD_{50} value was determined by extrapolating one-half of this value to the log scale of toxin dilution. *E. coli* EDL933 and *E. coli* K-12 were used as positive and negative control, respectively. All assays were conducted in triplicate and independently repeated three times.

***eae* and EHEC-*hlyA* genes.** The STEC isolates were further tested using a multiplex PCR (assay 4; Table 1) for the presence of *eae* and EHEC-*hlyA* genes. PCR conditions used were the same as described above.

Genomic DNA fingerprinting using pulse-field gel electrophoresis (PFGE). Genomic DNA fingerprints of the STEC isolates were further determined using PFGE according to a standard protocol developed by PulseNet for *E. coli* O157:H7 (19). Briefly, agarose-embedded DNA was digested with 50 U of XbaI for 3h in a water bath at 37°C. DNA fragments were separated by electrophoresis in 0.5×Tris-borate-EDTA buffer at 14°C for 18 h on a CHEF-III Mapper electrophoresis system with pulse time of 2.2 s to 54.2 s. The gels were stained with ethidium bromide (0.5 μg /ml) and images were taken under UV transillumination. The images were analyzed with Bionumerics Software by using Dice coefficients and unweighted pair group method to achieve dendrograms with a 1.5% band position tolerance.

Identification of virulence determinants of other diarrheagenic *E. coli.*

To assess the presence of other potentially diarrheagenic *E. coli* in retail meats, 1,275 *E. coli* isolates recovered from retail meats in 2006 were chosen and examined using two multiplex PCR (assays 2 and 3; Table 1), for detecting the following virulence gene markers: *eaeA* for intimin of EPEC, *bfpA* for the bundle-forming pilus of EPEC, *elt* and *stI* for heat labile and heat stable enterotoxins of ETEC, respectively, *ial* for invasion-associated locus of EIEC, *aafII* for aggregative adherence fimbriae II in EAEC, and *daaE* for F1845 fimbriae in DAEC. Each of the two PCR assays was performed in a 25 µl reaction mixture containing 2 µl of template DNA, 2.5 µl of 10 × PCR buffer II, 2 µl of a 1.25 mM mixture of deoxynucleoside triphosphates, 4 µl of 25 mM $MgCl_2$, 0.25 µl of 5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ), and 0.5µl (25 pmol) of each oligonucleotide primer (Invitrogen, Carlsbad, CA). The thermocycling conditions were as follows: 95°C for 12 min, 94°C for 30 s, 60°C for 30 s, and 68°C for 3 min for 30 cycles, with a final 10 min extension at 72°C. PCR products (10 µl) were examined with a 2% (wt/vol) agarose gel at 120 mV for 30 min. *E. coli* strains ATCC35401 (containing *elt, stI*), ATCC43893 (*ipaH*), ATCC43887 (*eae, bfp*), 042 (*aafII*), F1845 (*daaE*) were used as positive controls, while *E. coli* K-12 served as negative control.

Serotyping. All STEC and other potentially diarrheagenic *E. coli* isolates identified in the study were sent to *E. coli* Reference Center at Pennsylvania State University to determine their O antigen and H antigen.

Nucleotide sequence accession numbers. The nucleotide sequences of the complete stx_1 operons from isolates 20177, 22813, N2688, N11354, N11355,

N13844, N15018 were submitted to GenBank and given accession numbers GQ429154 to GQ429160. The nucleotide sequences of the complete *stx*$_2$ operons from isolates N22813, 23765, N2688, N2743, N2746, N4854, N5545, N5578, N11354, N11355, N11682, N15432 and partial *stx*$_2$ operon from N5789 were also deposited in the GenBank database under accession numbers GQ429161 to GQ429173.

Statistical analysis. Differences in percentage data were analyzed by Chi-square test using SPSS software (Version 12.0, SPSS Inc. Chicago, IL). A P value of <0.05 was considered significant for all comparisons.

Results

Presence of STEC in retail meats. Among the 7,258 *E. coli* isolates recovered from retail meats collected in four states from 2002 to 2007, 17 (0.23%) *E. coli* were tested positive for the presence of *stx* gene(s) and considered as STEC (Table II-2). Almost all STEC isolates (n = 16) were from ground beef, accounting for 0.89% of 1,806 *E. coli* from this product. One STEC (0.09%) was identified among 1,167 *E. coli* from pork chop. No STEC was found in ground turkey or chicken breast. Five STEC were detected among 1,306 *E. coli* collected in 2005, followed by three STEC each in 2004, 2006 and 2007, two STEC in 2002 and one in 2003. The percentage of STEC among the *E. coli* isolates ranged from 0.08% to 0.38% annually during the six-year period, but there is no significant difference in percentage by year. Nearly 60% of the STEC (n = 10) isolates were identified among

E. coli from Maryland, where a higher percentage (0.57%) of STEC was observed compared to other states (0.05-0.22%) ($P < 0.05$)

Characterization of STEC. Serotyping results showed that H antigens were successfully typed for all 17 STEC isolates, whereas O antigen of six STEC isolates could not be determined (Table II-2). Only five serotypes were found in 11 typeable isolates. Many STEC isolates (7 out of 11) belonged to serotype O83:H8, whereas the other four typeable isolates exhibited different serotypes: O8:H16, O15:H16, O15:H27, O88:H38. Different H types were observed in six O-nontypeable isolates, among which isolates N5577 and N5578 reacted with the same H antiserum (H46).

Digestion of genomic DNA from 17 STEC isolates with *Xba*I restriction enzyme and analysis using PFGE revealed 14 distinct profiles (Figure II-1). Although isolates of the same serotypes tended to cluster together, polymorphism of genome sequence was also observed between some isolates of the same serotype according to different PFGE patterns. Three pairs of STEC (N11354 and N11355, N2743 and N2746, and N11354 and N11355) showed identical PFGE profiles and serotypes. Isolates within each pair were recovered from the same food source (*i.e.* ground beef), and the same geographic locale. Presumably they were the same clones. All other isolates had their own specific PFGE profiles, with a similarity index ranging from 67% to 84%.

STEC virulence genes and Vero cell cytotoxicity. Among the 17 STEC isolates, five contained both stx_1 and stx_2 genes, whereas two contained stx_1 only and 10 contained stx_2 only (Table II-2). None of the STEC isolates in this study carried

eae gene, while seven (41%) STEC isolates were EHEC-*hlyA* positive. Cytotoxicity of the STEC isolates was examined on Vero cells. Sixteen isolates were considered toxic to Vero cells when compared to *E. coli* K-12. Only one isolate (N5789) showed no cytotoxicity to Vero cells. For those cytotoxic STEC, their CD_{50} ranged from $10^{-1.2}$ to $10^{-3.9}$ (Figure II-2). Overall, STEC with both stx_1 and stx_2 displayed a greater toxicity ($CD_{50} < 10^{-3.2}$) than those with only one of the *stx* genes, with the exception of N2743/N2746 and N13844.

***Stx* gene sequence and phylogenetic analysis.** All stx_1 genes from the seven STEC were successfully amplified and sequenced. A portion of stx_2 from N5789 could not be amplified despite repeated efforts. In addition, chromatograms of stx_2 sequences amplified from isolates N5577 and N15018 exhibited two peaks at several positions, suggesting the presence of more than one allele of stx_2. Shiga toxin 2 gene sequences from these two isolates were not further determined in this study and were excluded from phylogenetic analysis. Two previously described stx_1 sequences (one classical stx_1 and one stx_{1c} sequences), and seven stx_1 sequences determined in this study, were aligned and used to construct a phylogenetic tree (Figure II-3a). Sequences were aligned from the start codon of stx_{1a} to the stop codon of stx_{1b}. Most (6 out of 7) of the stx_1 sequences in this study were closely related to the classical stx_1, whereas stx_1 from N15018 was very similar to stx_{1c}. Isolates N2688 and N13844 shared an identical stx_1 gene sequence. Isolates 22813, N11354 and N11355 also shared an identical stx_1 gene sequence, but different from the stx_1 gene sequence found in N2688 and N13844.

Six previously described sequences of *stx*$_2$ and its variants, and the 12 *stx*$_2$ sequences determined in this study were aligned (Figure II-3b). None of *stx*$_2$ sequences determined in this study were close to *stx*$_{2e}$ or *stx*$_{2g}$ (less than 93% similarity, data not shown).. Isolates 22813 and N11354 shared an identical *stx*$_2$ gene sequence, as did N2743, N2746 and N11682. Putative amino acid sequences analysis revealed that Stx from five isolates (N2743, N2746, N4854, N11682, N15432) possessed two amino acid substitutions Ser291 and Glu297 in Stx2 A_2 subunit compared to classical Stx2 (data not shown), which were characteristic of mucus-activatable Stx2d (Figure II-4).

Presence of virulence genes specific for other diarrheagenic *E. coli*. In addition to three STEC identified among the 1,275 *E. coli* isolates collected in the year of 2006, 11 *E. coli* isolates were *eat*$^+$/*bfp*$^-$ and were classified as atypical EPEC (Table II-2). The positive rate of atypical EPEC in *E. coli* isolates from different types of meat were 1.2% in chicken breast (5/415), 1.4% in ground beef (4/293), 1.1% in pork chop (2/180) and 0% (0/387) in ground turkey. With the exception of three atypical EPEC isolates whose O serogroups could not be determined, none of the eight typeable EPEC isolates belonged to the same serotype (Table II-2). No virulence genes specific for ETEC, EIEC, EAEC, DAEC were detected among the *E. coli* isolates.

Discussion

In this study, we analyzed 7,258 *E. coli* isolates recovered from retail meats collected by the NARMS program for the presence of *stx* genes and 1,275 *E. coli* isolates recovered in 2006 were further examined for virulence determinants of other dirreagenic *E. coli*. To our knowledge, this was the largest survey of virulence factors in *E. coli* non-selectively recovered from retail meats. Only a small number (17) of the *E. coli* were identified as STEC. No DAEC, EIEC, and ETEC were detected in the 2006 collection of the *E. coli* isolates although 11 atypical EPEC were identified.

Shiga toxin-producing *E. coli* are mostly commensal bacteria in animals with high potential for foodborne transmission to humans (9). Ruminants, primarily cattle, are the predominant reservoir of STEC and beef products serve as one of the most important sources for foodborne STEC infections (9). This consensus was supported by the result of present study that almost all STEC were recovered from ground beef. Contamination of STEC in beef has been examined by other researchers around the world. In the US, a recent study by Samadpour et al. reported STEC in 3.5% of 1, 750 retail ground beef samples collected from stores in Seattle, Washington (45). In other countries, STEC was detected in 4% of beef sample in France (43), and 3% of raw beef in Australia (11). Fantelli et al. reported STEC in 1.75% of minced beef in Switzerland (17) and Lee et al. found STEC in 1.5% of beef from Korea (32). Many factors, such as geographical locations, sampling, isolation and testing methods, make comparisons of different studies difficult. In a study using a similar protocol to ours, Lee et al. detected a higher rate of STEC in beef in Korea, possibly indicating the

influence of regional difference or different processing technologies in other countries (32). Culture confirmation is also important factor affecting the results from different studies. In some papers, samples were considered to contain STEC only based on positive PCR results in enrichment broth (43, 45), while in other studies, culture confirmation was performed to assess the real occurrence of STEC in meat samples (6, 17). Such variations could lead to a big difference in results, since isolation of STEC from *stx* positive samples was found to be relatively difficult due to low number of bacterial cells or occurrence of free *stx*-carring phages in meat samples (43, 45). In terms of isolation method, instead of picking multiple colonies randomly from one plate and testing each of them (17, 26), colony hybridization with *stx* probe would more conveniently detect any STEC from all colonies on each plate and yield more accurate results (18, 45). The level of STEC contamination (< 1%) in the NARMS ground beef appeared low compared to reports from other investigators, but caution should be exercised in interpreting this low prevalence. The NARMS retail meat program was designed to determine the prevalence of generic *E. coli,* not STEC. Moreover, only one *E. coli* isolate was picked from each *E. coli* positive meat samples in NARMS meat program. Consequently, studies specifically designed to determine the prevalence of specific pathotypes such as STEC would likely detect a much greater prevalence. Nevertheless, screening over 7,000 randomly selected *E. coli* isolates from four states over a 6-year period provides useful data on the presence of STEC in retail meats against the background of generic *E. coli* populations. Moreover, the analysis of a large number of samples enables us to estimate the extent of STEC contamination in different meats.

There is a paucity of data on the contamination of STEC in retail meats other than beef. In this study, only one STEC was found among 1,168 *E. coli* isolates from pork chop, whereas none of the *E. coli* from chicken (n = 2,181) and turkey (n = 2,106) were identified as STEC. Swine was suggested to be a potential reservoir of STEC strains (18) and presence of STEC in pork has been reported before. In a study by Samadpour et al., 9 out of 51 pork samples collected in local grocery stores in Seattle area were positive of STEC (45). In New Zealand, Brooks et al. detected one STEC from 35 pork samples (6). In contrast to swine, poultry is generally not considered to be a source of STEC. The lack of STEC in poultry meat from NARMS was consistent with the reports from Schroeder et al. who failed to detect STEC in retail chicken (51 ground chicken and 212 whole chickens) and turkey (50 ground turkey and 194 turkey breasts) samples from Washington D. C area (46). Similarly, Brooks et al. failed to isolate STEC from 36 chicken samples in New Zealand (6, 46) and Heuvelink et al. did not find O157 STEC in raw chicken (n = 744) and poultry products (n = 75) in the Netherlands (26). However, Samadpour et al. recovered 4 STEC from 33 chicken breast and 1 from 15 turkey samples (12), and Doyle and Schoeni found O157 STEC in 4 of 263 poultry products (15). These isolations were previously thought to be possibly due to exposure to infected ruminants, but recent isolation of STEC from laying hens indicated that poultry may be a source of contamination (14). As discussed before, contamination rates in different studies could be affected by many factors, and attention needs to be paid when comparing data from different studies.

Although O157:H7 is the most common STEC serotype that causes human illness in the U. S., there is growing concern over the emergence of highly virulent non-O157 STEC serotypes that are globally distributed, several of which are associated with outbreaks and/or severe human illness such as HUS and HC (2, 7). Although *E. coli* O157 was reported to be present in 0.7% of ground beef in a USDA study and 1.1% of beef in another study in Washington (38, 45), none of the STEC isolates in the present study belonged to serotype O157, which was in agreement with another study in the U. S. (50). Among the typeable STEC isolates, it is interesting to note the relatively common recovery of serotype O83:H8, which was not reported previously in food or animals. Although the seven isolates belonged to the same serotype, they did not have identical PFGE profiles and *stx* genotypes except N11354 and N11355, indicating a non-clonal spread. The fact that O83 STEC has been associated with human illness (2) raises the possibility that O83:H8 (with diverse genotypes and cytotoxicity) might transmit to humans via meat products. Additionally, isolate N15018 belonging to O15:H27, a serotype which has also been implicated in human illness (42). Failure to determine O type for several STEC isolates suggests the complexity of STEC present in the retail meats. As many STEC isolates were non-typeable by seroptying, it is important to improve the current typing scheme, and to develop new typing technologies to encompass serogroups that are untypeable at present.

Since not all STEC are equally pathogenic to humans, evaluation of virulence-associated factors is necessary to assess an individual isolate's potential to cause human illness. Our results showed none of STEC isolates carried the *eae* gene, which

is consistent with findings of other studies in which no *eae* carriage was observed among non-O157 STEC from beef (24, 43). Studies have shown that most STEC from healthy cattle do not carry *eae* gene (22, 27), and since beef is generally derived from healthy cattle, this may explain the absence of *eae* in STEC from NARMS beef samples. Although *eae* is considered important to cause attaching and effacing lesion in human intestinal epithelial cells, it may not be essential for STEC pathogenicity since *eae* negative STEC have also been reported to cause severe human infections (39). It was postulated that *eae*-negative STEC may utilize additional adherence factors such as Saa (an autoagglutinating adhesin), Iha (an adherence-conferring protein), Efa1(an EHEC factor for adherence) and LP (the long polar fimbriae protein) in the disease process (52). Interestingly, EHEC-*hlyA* gene was more frequently observed (41%, 7 out of 17) in the STEC isolates in the present study. Similar frequency (40-51%) of this gene was observed by Slanec et al. in STEC isolated from food samples (40%) and by Aidar-Ugrinovich et al. in STEC isolates from calves (51%) (1, 49). EHEC-*hlyA* is located in a large plasmid which many human pathogenic STEC strains often harbor. It may contribute to pathogenesis by acting as a pore-forming cytolysin on eukaryotic cells. The presence of this gene may enhance the virulence potential of those STEC isolates from retail meats.

Shiga toxins differ in toxicity, toxin receptor, and amino acid sequences (33). Nucleotide sequences analysis of stx_1 and stx_2 present in our STEC isolates confirmed this finding. Stx_2 gene sequences exhibited much more sequence diversity than did stx_1 sequences (Figure 3). Three stx_2 sequences were identical to a previously published stx_2 gene sequence (AY443058.1) from a human isolate, and two of them

were identical to an *stx*$_2$ allele in a bovine isolate (AY443054.1). No other *stx*$_2$ sequences were identical at the nucleotide level. All mucus-activatable *stx*$_{2d}$ sequences clustered together with two previously described activatable *stx*$_{2d}$ (*stx*$_{2d1}$ and *stx*$_{2d2}$) but with some sequence difference (Figure 3b). *Stx*$_{2c}$ and *stx*$_2$ from N5545 were also included in the cluster but they did not have the characteristic amino acid substitutions common in activatable *stx*$_{2d}$. There are two isolates (N5577, N15018) whose *stx*$_2$ chromatograms showed two peaks at multiple sites, which indicates that these two strains may carry more than one allele, an interesting phenomenon that was also observed by other researchers (3). All STEC isolates were shown to be toxic to Vero cells except one isolate (N5789). The *stx*$_2$ sequence could not be obtained for this isolate since it could not be amplified by the first pair of sequence primers. Several primers were tried to amplify a fragment which would cover the *stx2A* subunit of the gene, but part of *stx2A* could never be amplified. It is possible that a large intervening insertion may be present that prevents successful PCR amplification. It is also possible that this insertion abolishes the activity of Stx2A and consequently renders the isolate less toxic compared to other STEC.

Shiga toxin types were suggested to correlate with the clinical symptoms of STEC infection (28). Mucus-activatable Stx2d was associated with high virulence and ability to cause HUS (4, 21, 28). The particular attribute of this variant is that it could be cleaved by elastase in the intestinal mucosa causing an increase in cytotoxicity up to 1,000-fold (31). This character is attributed to two amino acid substitutions relative to the sequence of classical Stx2, Ser291 and Glu297. Based on predicted amino acid sequence analysis, 5 out of 15 *stx*$_2$-containing isolates in this

study harbored mucus-activatable *stx*$_{2d}$. The relatively high number of mucus-activatable *stx*$_{2d}$ found in STEC isolated from retail meat deserves attention. Studies found that *eae* negative, mucus-activatable Stx2d producing STEC were involved in sporadic and outbreak cases of HC and HUS (28, 40). It was also shown that although *eae* negative STEC are normally isolated from persons with no or mild disease, most *eae* negative STEC associated with severe symptoms harbor mucus-activatable *stx*$_{2d}$ as the sole *stx* gene (4). The pathogenic potential of the five isolates with mucus-activatable *stx*$_{2d}$ should not be underestimated. Currently very limited data are available concerning the presence of STEC strains harboring mucus-activatable *stx*$_{2d}$ in food or livestock source. Zheng et al. identified seven STEC carrying activatable *stx*$_{2d}$ in 153 STEC strains isolated from food, cattle and human, none of them contain *eae* gene (55). Gobius et al. investigated 311 STEC possessing *stx*$_{2}$ from food and live stock and found 12 STEC carrying activatable *stx*$_{2d}$, all of which did not have *eae* either (21). It has been suggested that the expression of an activatable toxin may compensate for the lack of intimin (34). Due to their strong association with severe clinical outcomes, more surveillance of STEC strains expressing activatable *stx*$_{2d}$ in food and human illness are warranted.

Another interesting finding of this study was the identification of several atypical EPEC. EPEC is a leading cause of infant diarrhea in developing countries and also an important cause of diarrhea in developed countries (53). Typical EPEC contain both *eae* and *bfp*, while atypical EPEC contain only *eae*. In industrialized countries, typical EPEC infections have decreased and atypical *E. coli* seems to have increased in recent years (25, 53). Unlike typical EPEC, which are found only in

humans, atypical EPEC have been isolated from a variety of animal species such as cattle, goats, sheep, chickens, pigeons and gulls (13). In addition to chicken and beef, we also identified atypical EPEC among *E. coli* isolates from pork, indicating pigs may also be potential reservoirs for the pathogen. Atypical EPEC found in this study belonged to a variety of serogroups, most of which were not found in atypical EPEC involved in human infections. However, isolate N11573 belonged to O26, a serogroup that is frequently found in classic human EPEC strains (8). Further studies are needed to determine whether atypical EPEC of animal origin could actually cause human infections when ingested.

In conclusion, retail meats, especially ground beef, were contaminated with STEC, although at a very low frequency, and some of the strains contained Shiga toxins associated with high potential to cause severe human disease. Moreover, the identification of atypical EPEC strains in retail meats is noteworthy, and the potential role of animal-derived atypical EPEC strains in causing human infections requires further investigations.

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Table II - 1. Oligonucleotide primers used in multiplex PCR and sequencing.

	Genes	Forward primer	Reverse primer	Product size (bp)	Reference
Assay 1	*stx*$_1$	GTGGCATTAATACTGAATTGTCATCA	GCGTAATCCCACGGACTCTTC	109	(29)
	stx$_2$	GGCACTGTCTGAAACTGCTCC	TCGCCAGTTATCTGACATTCTG	255	(47)
Assay 2	*stI*	TCTTTCCCCTCTTTTAGTCAGTC	CAGCACAGGCAGGATTAC	170	(36)
	elt	ACGGCGTTACTATCCTCTC	TGGTCTCGGTCAGATATGTG	274	(48)
	daaE	GAACGTTGGTTAATGTGG GGTAA	TATTCACCGGTCGGT TATCAGT	542	(54)
	aafII	CACAGGCAACTGAAA TAAGTCTGG	ATTCCCATGATGTCA AGCACTTC	378	(54)
Assay 3	*eae**	CTGAACCAGATCGTAACGGC	TGATAAGCTGCAGTCGAATCC	229	(36)
	bfpA	AACCGTTACTGCCGGTGTGA	GTTGCCGCCTCAGCAGGAGT	450	(36)
	ipaH	CTCGGCACGTTTTAA TAGTCTGG	GTGGAGAGCTGAAGTTTCTCTGC	933	(54)
Assay 4	*eae**	CTGAACCAGATCGTAACGGC	TGATAAGCTGCAGTCGAATCC	229	(36)
	EHEC-hlyA	AGCCGGAACAGTTCTCTCAG	CCAGCATAACAGCCGATGT	526	(35)

Stx1 sequencing	Paton 1	TCGCATGAGATCTGACC	AACTGACTGAATTGAGATG-	1, 470	(5)
	Paton 2	ATAAATCGCCATTCGTTGACTAC	AGAACGCCCACTGAGATCATC	180	(5)
	Gannon	ACACTGGATGATCTCAGTGG	CTGAATCCCCCTCCATTATG	603	(5)
	Vidiya	TCGCATGAGATCTGACC	AATAAGCCGTAGATTATT	448	(5)
Stx2 sequencing	Stx2-1	TTCTGAGCAATCGGTCACTG	CGGCGTCATCGTATACACAG	779	(55)
	Stx2-2	GTCACAGCAGAAGCCTTACG	ACCCACATACCACGAATCAG	714	(55)

Table II - 2. Characteristics of Shiga toxin-producing *E. coli* (STEC) and atypical enteropathogenic *E. coli* (EPEC) isolates from retail meats.[a]

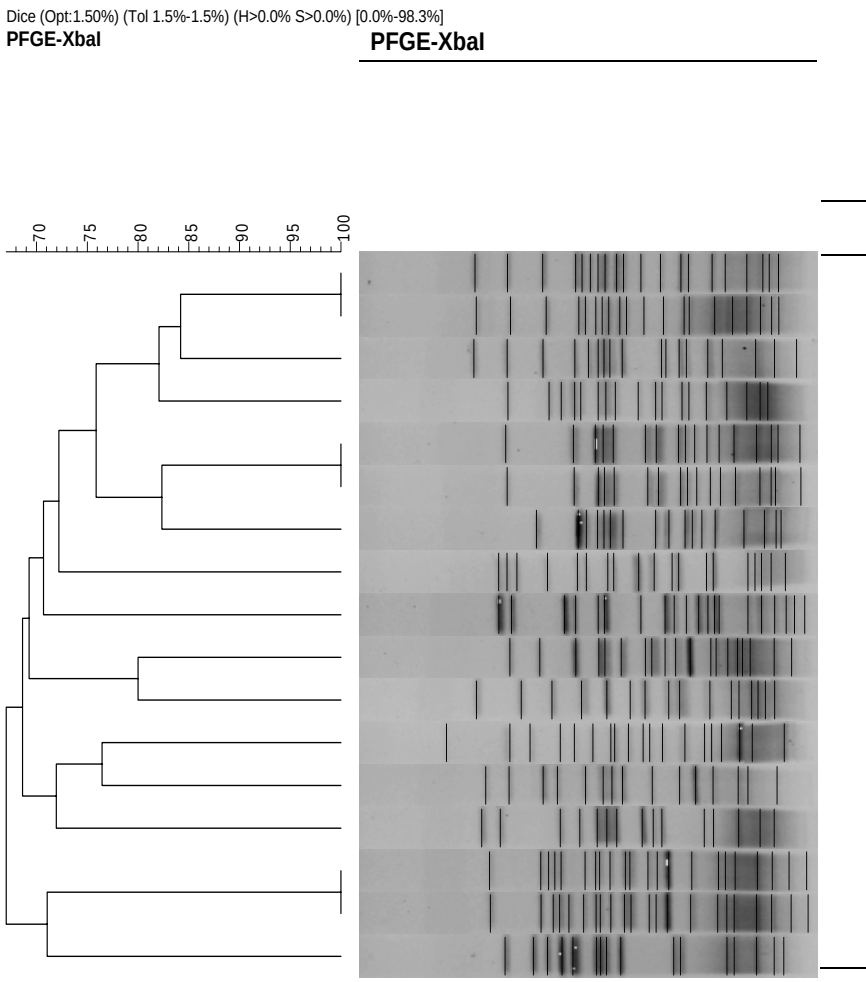
ID	Serotype	*stx*$_1$	*stx*$_2$	*eae/eaeA*	*EHEC- hlyA*	*bfp*	Source	State	Year
22813	O83:H8	+	+	-	+		Ground beef	MD	2002
N2688	O88:H38	+	+	-	+		Ground beef	MD	2004
N11354	O83:H8	+	+	-	+		Ground beef	GA	2006
N11355	O83:H8	+	+	-	+		Ground beef	GA	2006
N15018	O15:H27	+	+	-	-		Ground beef	GA	2007
20177	O8:H16	+	-	-	-		Ground beef	TN	2002
N13844	ONT:H51	+	-	-	-		Pork chop	OR	2007
N5577	ONT:H46	-	+	-	+		Ground beef	MD	2005
N5578	ONT:H46	-	+	-	+		Ground beef	MD	2005
N5789[b]	O15:H16	-	+	-	+		Ground beef	MD	2005
23765	ONT:H2	-	+	-	-		Ground beef	OR	2003
N2743	O83:H8	-	+	-	-		Ground beef	MD	2004
N2746	O83:H8	–	+	-	-		Ground beef	MD	2004
N4854	ONT:H10	–	+	-	-		Ground beef	MD	2005
N5545	ONT:H7	–	+	-	-		Ground beef	MD	2005
N11682	O83:H8	-	+	-	-		Ground beef	MD	2006
N15432	O83:H8	-	+	-	-		Ground beef	OR	2007
N11475	O154:H9			+		-	Chicken breast	GA	2006
N11537	O2:H27			+		-	Chicken breast	GA	2006

N11573	O26:H9	+	-	Chicken breast	GA	2006
N11575	O123:H51	+	-	Chicken breast	GA	2006
N12563	ONT:H7	+	-	Chicken breast	TN	2006
N11452	ONT:H8	+	-	Ground beef	GA	2006
N12148	ONT:H8	+	-	Ground beef	OR	2006
N12174	O15:H2	+	-	Ground beef	OR	2006
N12475	O10:H2	+	-	Ground beef	TN	2006
N11710	O18:H16	+	-	Pork chop	MD	2006
N12051	O81:H7	+	-	Pork chop	OR	2006

[a] Common virulence genes include *stx*$_1$ and *stx*$_2$, *eae* and *EHEC*- *hlyA* for STEC; and *eaeA* and *bfp* for EPEC.

[b] This strain was negative for vero cell cytotoxicity assay, but whether it could produce Stx or not was not tested by other assays. Here it was tentatively called STEC based on the presence of *stx*.

Figure II - 1. Dendrogram of PFGE profiles with *Xba*I for 17 STEC isolates from retail meat.
Similarities of PFGE profiles were calculated using Dice algorithm with 1.5% tolerance level.



Strains	Serotype	Source	State	Year
N11354	O83:H8	Ground beef	GA	2006
N11355	O83:H8	Ground beef	GA	2006
22813	O83:H8	Ground beef	MD	2002
N15432	O83:H8	Ground beef	OR	2007
N2743	O83:H8	Ground beef	MD	2004
N2746	O83:H8	Ground beef	MD	2004
N11682	O83:H8	Ground beef	MD	2006
N13844	ONT:H51	Pork chop	OR	2007
N5789	O15:H16	Ground beef	MD	2005
23765	ONT:H2	Ground beef	OR	2003
N15018	O15:H27	Ground beef	GA	2007
N4854	ONT:H10	Ground beef	MD	2005
N5545	ONT:H7	Ground beef	MD	2005
20177	O8:H16	Ground beef	TN	2002
N5577	ONT:H46	Ground beef	MD	2005
N5578	ONT:H46	Ground beef	MD	2005
N2688	O88:H38	Ground beef	MD	2004

Figure II - 2. Vero cell cytotoxicity of Shiga toxin producing *E. coli* isolates from retail meats.

CD_{50} is expressed here as the toxin dilution that causes 50% Vero cells detachment compared with untreated cells (control). The value on Y axis indicates the log of the reciprocal of CD_{50}. The data shown are the average of three independent assays.

*Strains EDL933 and K-12 serve as a positive and negative control, respectively.

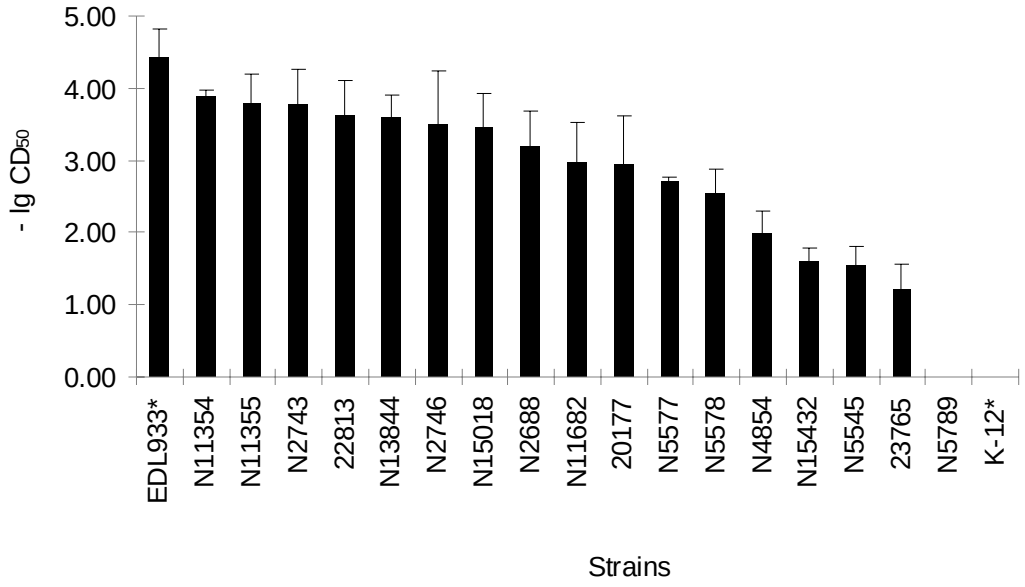
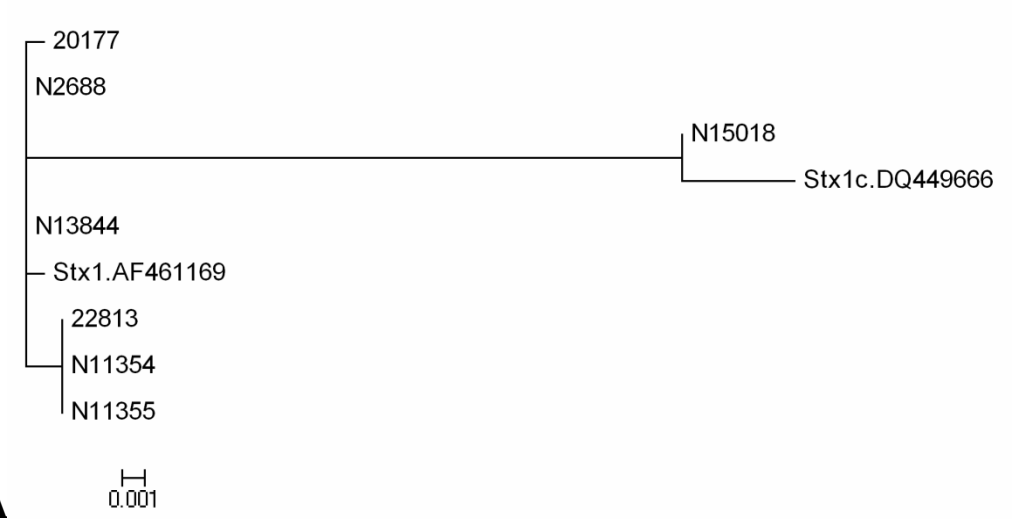
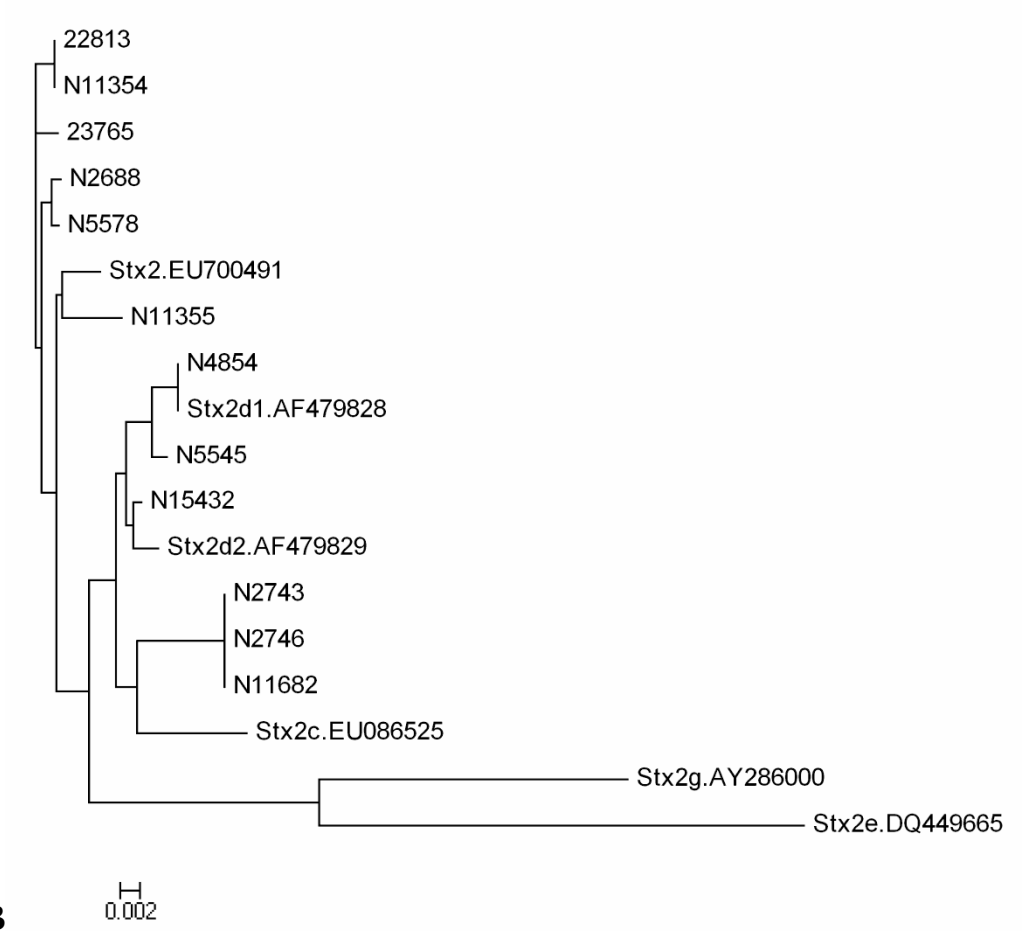


Figure II - 3. Phylogenetic trees of *stx*$_1$ sequences (A) and *stx*$_2$ sequences (B) determined in this study and sequences of previously described *stx* genes and their variants. *Stx*$_{1d}$ and *stx2f* were not included due to their considerable sequence divergence from classical *stx*$_1$ and *stx*$_2$, respectively. The horizontal bar indicates 0.001 (in A) and 0.002 (in B) nucleotide substitutions per site. Reference DNA sequences for *stx* genes were obtained from GenBank and identified in the phylogenetic trees by their accession numbers.


20177
N2688
N15018
Stx1c.DQ449666
N13844
Stx1.AF461169
22813
N11354
N11355
0.001

A


22813
N11354
23765
N2688
N5578
Stx2.EU700491
N11355
N4854
Stx2d1.AF479828
N5545
N15432
Stx2d2.AF479829
N2743
N2746
N11682
Stx2c.EU086525
Stx2g.AY286000
Stx2e.DQ449665
0.002

B

Figure II - 4. Multiple sequence alignment of predicted amino acid sequences of A subunit from five Shiga toxin 2 (Stx2) variants determined in this study with the previously published Stx2d1and Stx2d2 (both activatable) and classical Stx2.

Residues Ser313 and Glu319, which allow activation by mucus elastase, are underlined.

```
Stx2d1    1 MKCILFKWVLCLLLGFSSVSYSREFTIDFSTQQSYVSSLNTIRTEISTPLEHISQGTTSV
N4854     1 ............................................................
Stx2d2    1 ...................................S........................
N15432    1 ...................................S........................
N11682    1 .....L.............................S........................
N2743     1 .....L.............................S........................
N2746     1 .....L.............................S........................
Stx2      1 ...................................S........................

Stx2d1   61 SVINHTPPGSYFAVDIRGLDVYQARFDHLRLIIEQNNLYVAGFVNTATNTFYRFSDFTHI
N4854    61 ............................................................
Stx2d2   61 ............................................................
N15432   61 ............................................................
N11682   61 ............................................................
N2743    61 ............................................................
N2746    61 ............................................................
Stx2     61 ............................................................

Stx2d1  121 SVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEFSGNTMTRDASRAV
N4854   121 ............................................................
Stx2d2  121 ............................................................
N15432  121 ............................................................
N11682  121 ............................................................
N2743   121 ............................................................
N2746   121 ............................................................
Stx2    121 ............................................................

Stx2d1  181 LRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTLNWGRISNVLPEYRGEDGV
N4854   181 ............................................................
Stx2d2  181 ............................................................
N15432  181 ............................................................
N11682  181 ............................................................
N2743   181 ............................................................
N2746   181 ............................................................
Stx2    181 ............................................................

Stx2d1  241 RVGRISFNNISAILGTVAVILNCHHQGARSVRAVNEESQPECQITGDRPVIKINNTLWES
N4854   241 ............................................................
Stx2d2  241 ............................................................
N15432  241 ............................................................
N11682  241 ............................................................
N2743   241 ............................................................
N2746   241 ............................................................
Stx2    241 ............................................................

Stx2d1  301 NTAAAFLNRKSQSLYTTGE
N4854   301 .................
Stx2d2  301 .................
N15432  301 .................
N11682  301 .................
N2743   301 .................
N2746   301 .................
Stx2    301 ............F.....K
```

CHAPTER III. IDENTIFICATION AND ANTIMICROBIAL RESISTANCE OF EXTRAINTESTINAL PATHOGENIC *ESCHERICHIA COLI* FROM RETAIL MEAT

Abstract

Extraintestinal pathogenic *Escherichia coli* (ExPEC) cause a variety of infections outside the gastrointestinal tract. Retail meats are frequently contaminated with *E. coli* strains and might serve as a vehicle for transmitting ExPEC. A total of 1,275 *E. coli* isolates recovered from ground beef, ground turkey, chicken breast and pork chop obtained in Georgia, Maryland, Oregon and Tennessee of the United States in 2006 were investigated for the presence of ExPEC using multiplex PCR. Identified ExPEC isolates were assigned to serogroups and phylogenetic groups, and analyzed for antimicrobial susceptibility. Approximately 16% (200/1,275) of the *E. coli* isolates were identified as ExPEC based on defined genetic criteria. The occurrence of ExPEC was the highest in *E. coli* isolated from ground turkey (23.5%) and chicken breast (20.2%), but less in *E. coli* from pork chop (8.3%) and ground beef (3.4%). Phylogenetic grouping revealed that most ExPEC isolates (66.5%) fell into the same phylogenetic groups (B2 and D) as virulent human ExPEC strains. Among the 15 antimicrobial agents tested, resistance to tetracycline (67.0%), sulfisoxazole (59.5%) and streptomycin (46.0%) was most frequent. Most ExPEC isolates (n = 163, 81.5%) were resistant to at least one antimicrobial, and more than half (n = 114, 57%) exhibited resistance to at least three drugs. Our findings showed that ExPEC, including antimicrobial resistant strains, were widely distributed in

retail meats, especially in chicken and turkey products. These findings indicate a need to better understand the potential role of foods as a source of human ExPEC infection.

Introduction

Escherichia coli are prominent for their versatility in causing disease. In addition to gastrointestinal infections typically presented as diarrhea, it also causes a variety of diseases outside of the intestinal tract of humans and animals (22). Typical extraintestinal infections caused by *E. coli* include urinary tract infections (UTI), meningitis, sepsis, abdominal infections, osteomyelitis, cellulitis, and wound infections (17). *E. coli* pathotypes that cause extraintestinal infections are collectively called extraintestinal pathogenic *E. coli* (ExPEC) (32). Although ExPEC are not as well-known as diarrheagenic *E. coli*, they cause about 6.7-8.6 million cases of human infections with estimated annual medical costs of $1.5-2.3 billion dollars in the United States (31).

ExPEC are phylogenetically and epidemiologically distinct from commensal and diarrheagenic *E. coli*. In contrast to commensal strains, which mainly derive from phylogenetic groups A and B1 and lack virulence traits, most ExPEC belong to phylogenetic groups B2 and D and harbor various virulence-associated factors that allow them to induce diseases in both healthy and compromised hosts (18). Typical virulence factors in ExPEC include diverse adhesins, siderophores, capsules, toxins, proteases, invasins, and serum resistance proteins (18). Most of these virulence factors are distinct from those found in diarrheagenic *E. coli*. These determinants may be arrayed in different combinations that collectively contribute to extraintestinal infections.

In spite of the fact that ExPEC impose a great economical and medical burden on society, they have long been underappreciated. This is partly due to the sporadic nature of ExPEC infections compared to diarrheagenic *E. coli*, which are often involved in common source outbreaks (17). Many aspects of ExPEC infections, such as source and transmission pathways, remain largely undetermined. Animal-derived foods are commonly contaminated with *E. coli* and have long been known as an important vehicle for transmitting diarrheagenic *E. coli* (8, 27), but their role in spreading ExPEC has not been extensively explored. Recent studies indicated that retail meats were contaminated with ExPEC strains that share characteristics with clinical ExPEC (7, 10, 12). In addition, it was suggested that meat consumption was epidemiologically associated with antimicrobial-resistant *E. coli* causing UTI (23, 29). These findings indicate that meat may represent a possible reservoir and/or transmission vehicle of ExPEC. With the aim to gain a better understanding of extraintestinal pathogenic potential of meat-derived *E. coli*, we investigated 1,275 *E. coli* strains isolated from retail meats acquired over a year of testing (2006) in four states to determine the prevalence of *E. coli* strains carrying virulence genes associated with human ExPEC infections, and to investigate those potentially pathogenic strains for serotype, phylogenetic group and antimicrobial susceptibility profiles.

Materials and methods

Retail meat sampling and bacterial strains. *E. coli* were recovered from meat samples collected by the National Antimicrobial Resistance Monitoring Systems (NARMS) retail

meat program in local grocery stores in Georgia, Maryland, Oregon and Tennessee from January to December 2006. Detailed information on sampling, isolation, and identification can be found at http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/default.htm . NARMS testing recovered a total of 1, 275 *E. coli* isolates: 180 from pork chop, 387 from ground turkey, 293 from ground beef and 415 from chicken breast that were used in this study. Two clinical ExPEC strains, *E. coli* V27 and 2H16, kindly provided by Dr. James R. Johnson (University of Minnesota, Minneapolis, MN), were used as control organisms. All bacteria were kept at –80 °C in brain heart infusion broth with 20% glycerol until use. ExPEC isolates were sent to *E. coli* Reference Center at Pennsylvania State University to determine O antigens employing all available O (O1 to O185) antisera.

Antimicrobial susceptibility testing. Antimicrobial minimum inhibition concentrations (MICs) were determined by FDA-CVM using the Sensititre automated antimicrobial susceptibility system (Trek Diagnostic Systems, Cleveland, Ohio) and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for broth microdilution methods (2, 3). The antimicrobials tested, and the resistance breakpoint used, were as followed: amikacin (≥64 μg/ml), amoxicillin-clavulanic acid (≥32/16 μg /ml), ampicillin (≥32 μg/ml), cefoxitin (≥32 μg/ml), ceftiofur (≥8 μg/ml), ceftriaxone (≥4 μg/ml), chloramphenicol (≥32 μg/ml), ciprofloxacin (≥4 μg/ml), gentamicin (≥16 μg/ml), kanamycin (≥64 μg/ml), nalidixic acid (≥32 μg/ml), streptomycin (≥64 μg/ml), sulfisoxazole (≥512 μg/ml), tetracycline (≥16 μg/ml), and trimethoprim-sulfamethoxazole (≥4/76 μg/ml). *E. coli* ATCC 25922 and

35218, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, and *Pseudomonas aeruginosa* ATCC 27853 were used as controls in antimicrobial MIC determinations.

ExPEC virulence genotyping. The *E. coli* isolates were tested using a multiplex PCR for the following virulence associated markers: *papA* (P fimbriae structural subunit) and/or *papC* (P fimbriae assembly), *sfa/foc* (S and F1C fimbriae subunits), *afa/dra* (Dr-antigen-binding adhesins), *kpsMT* II (group 2 capsular polysaccharide units), and *iutA* (aerobactin receptor) (Table III-1). ExPEC was defined as *E. coli* containing two or more of the five virulence genes (*papA* and *papC* were analyzed collectively) (16). DNA of *E. coli* isolates were extracted using boiling methods (33). PCR was performed in a 25 μl reaction mixture containing 2 μl of template DNA, 2.5 μl of 10 × PCR buffer II, 2 μl of a 1.25 mM mixture of deoxynucleoside triphosphates, 4 μl of 25 mM $MgCl_2$, 0.25 μl of 5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Foster City, CA), and a 0.6 μM concentration of each primer (Invitrogen, Carlsbad, CA). The thermocycling conditions were as follows: 95°C for 12 min, 94°C for 30 s, 60°C for 30 s, and 68°C for 3 min for 30 cycles, with a final 10 min extension at 72°C. PCR products (10 μl) were evaluated with a 2 % (wt/vol) agarose gel at 120 mV for 30 min. A molecular marker (100 bp DNA ladder; New England BioLabs, Ipswich, MA) was run concurrently. The DNA mixture of two clinical strains (V27 and 2H16,) which together harbored all of the virulence genes tested, was used as a positive control, whereas the DNA of *E. coli* K-12 served as a negative control.

Phylogenetic grouping. ExPEC isolates were subjected to phylogenetic grouping using a PCR-based method (4). Briefly, three markers, gene *chuA*, gene *yjaA*,

and DNA fragment TspE4.C2, were amplified using primers in Table III-1. The PCR was performed under the following conditions: 94°C for 4 min, 30 cycles of 94°C for 5 s, 59°C for 10 s, and a final 5 min extension at 72°C. The results of amplification were analyzed to assign the isolates to one of four phylogenetic groups according to the following criteria: *E. coli* negative of both *chuA* and TspE4.C2 were assigned to group A; *E. coli* negative of *chuA* but positive of TspE4.C2 to group B1; *E. coli* positive of both *chuA* and *yjaA* to group B2; *E. coli* positive of *chuA* but negative of *yjaA* to group D (4).

Statistical analyses. Comparisons of proportions for a characteristic in different groups were tested by Chi-square test. The threshold for significance was $P < 0.01$. All statistical analyses were performed using Statistical Package for the Social Sciences (version 13.0).

Results

Presence of ExPEC and virulence markers in *E. coli* isolates from retail meats. ExPEC was defined by the presence of at least two of the five virulence markers tested. Two hundred (15.7%) *E. coli* isolates met the criteria and were considered as ExPEC. The occurrence of ExPEC varied in *E. coli* isolates from different meat types, with the highest rate in *E. coli* from ground turkey (23.5%) and chicken breast (20.2%), followed by those from pork chop (8.3%), and ground beef (3.4%) (Table III-2).

Among the 1,275 *E. coli* isolates, *iutA* was most frequently present and detected in 63.1 % of *E. coli* isolated from chicken, and 53%, 18.3% and 8.5% of *E. coli* from ground turkey, pork chop and ground beef, respectively. The *kpsMT* II locus occurred in 3.1-22.2% of *E. coli* from different meats, *sfa/foc* in 2.8% and 2.6% of *E. coli*

from pork and turkey, respectively, but not in *E. coli* from beef and chicken. *papA* and *papC* were in 0.7-2.8% and 2.4-13.7% of *E. coli* from different meats, respectively. None of the *E. coli* isolates carried *afa/dra* gene (Table III-2).

Serotyping. Determining O-antigen for the 200 ExPEC isolates revealed that they belonged to a variety of serogroups. One hundred and sixty-seven isolates were O-antigen typeable, including six isolates that reacted with two antisera. The remaining 33 ExPEC isolates were non-typeable using currently available antisera (Table III-3). There were 42 different serogroups among 167 typeable ExPEC isolates. The most common serogroup was O2 (20.5 %), followed by O25 (9.5 %) and O11 (5 %). The remaining *E. coli* isolates were scattered among 39 other serogroups, with less than 10 isolates per serogroup. More than half (55%) of all typeable isolates fell into serogroups commonly associated with *E. coli* causing urinary tract infection (O1, O2, O4, O6, O7, O8, O16, O18, O25, O75). Serogroup O25 (17.9%) was most common for isolates from chicken, whereas O2 (36.2%) was most frequently identified in ExPEC from turkey (Table III-3).

Phylogenetic grouping. *E. coli* can be classified into four major phylogenetic groups: A, B1, B2 and D. In general, most human ExPEC clinical isolates belong to group B2 or D. PCR-based phylogenetic analysis showed that 133/200 (66.5%) of the ExPEC isolates from retail meat fell into groups B2 (42.0%) or D (24.5%), followed by group A (22.5%) and group B1 (11.0%) (Table III-4). ExPEC from chicken breast were relatively evenly distributed in groups B2/D (55.9% of isolates) and in group A/B1 (44.1%). A large proportion of isolates from ground turkey (79.1%) and ground beef (90%) were assigned to groups B2 and D. More than half of ExPEC (66.7 %) from ground pork belonged to groups A and B1 (Table III-4).

Combining data for serogrouping revealed that 87.8% of O2 strains and all of O25 strains fell into phylogenetic B2 group, while 60% of O11 isolates belonged to group D. Non-typeable strains and the remaining uncommon isolates were evenly distributed in different phylogenetic groups (data not shown).

Distribution of virulence genes among strain subtypes. Among all virulence associated factors tested, *papC* was significantly positively associated with phylogenetic group B1 and negatively associated with B2, while *kpsMT* II was negatively associated with group B1 and A and positively associated with group B2 (Table III-5). Others virulence genes were widely dispersed and were not significantly associated with any phylogenetic group. According to resistance status, *papC* occurred more frequently in resistant isolates than susceptible isolates, while other genes were equally distributed in resistant and susceptible isolates (Table III-5).

Antimicrobial resistance. Analysis of antimicrobial resistance data from FDA-CVM revealed that most ExPEC isolates (163/200) exhibited resistance to at least one of the 15 antimicrobials tested and considered as resistant isolates (Figure III-1). The highest resistance was to tetracycline (67%), and followed by resistance to sulfisoxazole (59.5%) and streptomycin (46%). A lower level of resistance was found to antimicrobials such as gentamicin (31.5%), kanamycin (28%), ampicillin (22.5%) and nalidixic acid (10.5%), (Figure III-1). All ExPEC isolates were susceptible to ciprofloxacin, with the exception of one ExPEC from turkey, which was resistant to ciprofloxacin and 11 additional antimicrobials. Ten ExPEC isolates, including 7 (8.3%) from chicken, 2 (20%) from beef and 1(1.1%) from turkey, exhibited resistance to ceftiofur and ceftriaxone

(using the new CLSI resistance breakpoint; see ref. 3). All isolates were susceptible to amikacin.

More than half (114/200) of the ExPEC isolates were resistant to at least three antimicrobials, and nine isolates showed resistance to more than eight antimicrobials (data not shown). Three ExPEC isolates from chicken breast, two of which belonged to the same serogroup and phylogenetic group, shared the same multi-drug resistance (MDR) pattern for nine antimicrobials: amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, kanamycin, streptomycin, sulfisoxazole and tetracycline. Two isolates, one from ground turkey and one from ground beef were resistant to 12 antimicrobials. The turkey isolate was only susceptible to amikacin, ceftiofur and ceftriaxone, while the beef isolate was susceptible to amikacin, chloramphenicol and ciprofloxacin. There were no significant associations between antimicrobial resistance and the presence of specific O antigens or phylogenetic groups (data not shown).

Discussion

Foods are a main vehicle for transmitting enteric zoonotic pathogens, most notably *E. coli* O157:H7, *Campylobacter* and *Salmonella* (5, 24). More recent data imply a potential role of foods, particularly raw retail meats, as vehicles for transmitting fecal pathogens capable of causing extraintestinal infections (10, 12, 16, 23, 36). In this study, we analyzed 1,275 *E. coli* isolates recovered from four different meat products from January to December 2006 for their ExPEC virulence markers, phylogenetic background, serogroups and antimicrobial resistance. It is important to point out that the isolates for

testing in this study were obtained by selecting a single colony from the culture plate. We made no attempt to enrich for Shiga toxin-producing *E. coli* strains, nor to capture the diversity of *E. coli* within a given meat sample. With this approach, 200/1,275 retail meat strains were classified as ExPEC based on molecular criteria. Moreover, many of these meat-derived ExPEC belonged to phylogenetic clusters and serogroups commonly associated with strains causing human clinical illness. In addition, antimicrobial resistance was common among isolates carrying virulence determinants. These findings support the hypothesis that food animals may be a reservoir, and retail meats may be a vehicle, for transmitting human pathogenic *E. coli* causing extraintestinal infections, including strains that are resistant to antimicrobials.

ExPEC were much more common in poultry (20.2-23.5%) than ground beef (8.3%) and pork chop (3.4%), which is consistent with previous studies (10, 12) where more than one CFU/sample was selected for testing. A similar prevalence (20-22%) of ExPEC was reported in *E. coli* isolated from poultry products in Minnesota and Wisconsin, and among subsets of NARMS meat isolates (14, 16, 19). These comparable data observed in studies investigating foods from different areas and time periods supports a poultry meat baseline level of 18-25% for strains defined as ExPEC by these specific criteria. Poultry has long been considered as a source of *E. coli* strains with zoonotic potential (6, 21, 25, 30). Certain avian pathogenic *E. coli* (APEC), which can cause lethality in mice and exhibit pathogenic traits *in vivo* (9, 34), also share many virulence traits with human ExPEC (6, 21, 25, 30) . Bonnet et al. reported that 75% of ExPEC from poultry in Canada (defined using the same criteria as this study) also could be classified as APEC (1). Johnson et al. showed that more than 60% of *E. coli* from

retail poultry meat contained virulence genes that typify APEC (20). While we did not attempt to determine the carriage of APEC-specific factors in this study, our observation that ExPEC are common in poultry products is consistent with these findings in supporting retail meats as a source of pathogenic *E. coli* capable of infecting both birds and humans.

The strains of ExPEC found in retail meats shared other characteristics with human clinical ExPEC isolates. A considerable proportion of meat-derived ExPEC were from O serogroups commonly associated with *E. coli* causing UTI in humans (37), such as serogroups O1, O2, O4, O6, O7, O8, O18, O25. Notably, O2 was the most common O-antigen found in ExPEC isolated from retail meat. All isolates in this serogroup belonged to virulent phylogenetic groups, either group B2 (88%) or group D (12%), which may explain frequent occurrence of this serogroup in strains encountered in animal and human infections (6, 25). The second most common serogroup, O25, appeared exclusively among phylogenetic group B2. O25 has also been associated with strains causing disease in humans and animals (15, 26). Several ExPEC strains from retail meat were O11 and O73, which belong to a cluster or serogroups (typically from O11/O17/O73/O77), which were responsible for clusters of UTIs in the U.S. and other countries (13, 29). Thus, in addition to ExPEC genotypes, serogroups also overlapped with those associated with human extraintestinal infections.

An epidemiological link between frequent chicken or pork consumption and community-acquired urinary tract infections has been demonstrated, which implicated a foodborne route for strains causing UTI (23). A recent study by Vincent et al. further lent support to this hypothesis by finding isolates in retail chicken indistinguishable from

human UTI strains (36). Our finding of many meat-source ExPEC strains from UTI-associated serogroups may indicate their uropathogenic potential and also support possible food reservoir of human UTI-associated strains. In addition, a large proportion (86%) of isolates from UTI-associated serogroups were derived from chicken or turkey, suggesting that a poultry foodborne pathway is more likely in transmitting UTI-causing *E. coli* compared to pork and beef.

The virulence potential of meat-source ExPEC was further supported by phylogenetic analysis of these strains. It has been suggested that the virulence of a strain could be better inferred from its phylogenetic background than from its commensal or pathogenic origin (28). Studies demonstrated that virulent extraintestinal *E. coli* strains came mostly from phylogenetic group B2, and to a lesser extent from group D (18). Our analysis of 200 ExPEC from retail meats revealed that many ExPEC isolates belonged to groups B2 and D, with a higher proportion in turkey ExPEC strains (79%) than chicken strains (55%) despite a similar frequency of ExPEC overall. This implies that turkey may deserve greater attention than chicken with respect to the possibility of transmitting ExPEC.

Our data showed that more than 80% of the meat-derived ExPEC were resistant to at least one of the tested antimicrobials, many of which are commonly used in clinical settings. For example, 10-20% of ExPEC were resistant to trimethoprim/sulfamethoxazole, amoxicillin/clavulanic acid, ampicillin and nalidixic acid, all of which are frequently prescribed antimicrobial classes in the treatment of UTIs (35). Resistance to two important antimicrobials, ceftriaxone and ciprofloxacin, was also detected. Ceftriaxone resistance levels measured in this study are higher than previously

reported because the resistance breakpoint was revised down by the CLSI from ≥64 μg/ml to ≥4 μg/ml in January 2010 (3). Previous research suggested that resistant *E. coli* in food animals or meat may primarily derive from susceptible isolates in their animal host (11, 14, 19), the fact that resistant and susceptible ExPEC isolates in this study were similar with respect to most virulence gene carriage (except *papC*) and phylogenetic group confirmed this hypothesis.

A strength of this study is that the *E. coli* isolates were recovered from retail meats obtained in four states over a year of testing. To our knowledge, this is the first study of this extent examining randomly selected *E. coli* from foods over time, and may be considered a good preliminary assessment of ExPEC in retail meat across the U. S. There also are several limitations of this study. In the NARMS retail meat program, only one *E. coli* isolate is selected from each positive meat sample. Consequently, the true prevalence of ExPEC in retail meat was probably underestimated. Moreover, the study could be more compelling if there were temporally and spatially corresponding human clinical ExPEC strains available for comparison. Finally, ExPEC was defined based on the molecular detection of more than two of the five virulence markers, yet their pathogenic potential was not confirmed with experimental evidence. Further investigations are underway in our laboratory to determine the potential of certain meat ExPEC in causing UTI by a more extensive virulence gene analysis and an examination of pathogenicity using human cell cultures.

In summary, our results revealed that retail meats, especially poultry products, and to a lesser extent ground beef and pork chop, were contaminated with ExPEC that resembled clinical ExPEC in serogrouping and phylogenetic backgrounds. In addition,

most ExPEC from retail meats were resistant to at least one antimicrobial agent. These findings indicate the potential of retail meat as a vehicle for transmitting antimicrobial-resistant ExPEC to consumers. Because this study relied on a limited number of markers to evaluate strains, further studies with more comprehensive genetic tools are needed to more fully elucidate the role foods may play in the epidemiology of ExPEC.

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Table III - 1. Primers used in ExPEC virulence genes detection and phylogenetic grouping.

Targets	Primer name	Primer sequences (5'-3')	Size of product (bp)	Reference
papA	PapA f	ATGGCAGTGGTGTCTTTTGGTG	717	(16)
	PapA r	CGTCCCACCATACGTGCTCTTC		
papC	PapC f	GTGGCAGTATGAGTAATGACCGTTA	205	(16)
	PapC r	ATATCCTTTCTGCAGGGATGCAATA		
sfa/foc	Sfa1	CTCCGGAGAACTGGGTGCATCTTAC	410	(16)
	Sfa2	CGGAGGAGTAATTACAAACCTGGCA		
afa/dra	Afa f	GGCAGAGGGCCGGCAACAGGC	592	(16)
	Afa r	CCCGTAACGCGCCAGCATCTC		
iutA	AerJ f	GGCTGGACATCATGGGAACTGG	302	(16)
	AerJ r	CGTCGGGAACGGGTAGAATCG		
kpsMT II	kpsII f	GCGCATTTGCTGATACTGTTG	272	(16)
	kpsII r	CATCCAGACGATAAGCATGAGCA		
chuA	ChuA.1	GACGAACCAACGGTCAGGAT	279	(4)
	ChuA.2	TGCCGCCAGTACCAAAGACA		
yjaA	YjaA.1	TGAAGTGTCAGGAGACGCTG	211	(4)
	YjaA.2	ATGGAGAATGCGTTCCTCAAC		
TspE4.C2	TspE4.C2.1	GAGTAATGTCGGGGCATTCA	152	(4)
	TspE4.C2.2	CGCGCCAACAAAGTATTACG		

Table III - 2. Prevalence of ExPEC and ExPEC-defining virulence markers in *E. coli* isolated from retail meats.

Virulence factors/ExPEC[a]	No (%[b]) of strains positive for the indicated virulence factors / ExPEC status			
	Pork chop (n=180)	Ground turkey (n=387)	Ground beef (n=293)	Chicken breast (n=415)
papA	5 (2.8)	10 (2.6)	2 (0.7)	4 (1.0)
papC	13 (7.2)	53 (13.7)*	7 (2.4)*	44 (10.6)
afa/dra	0 (0)	0 (0)	0 (0)	0 (0)
sfa/foc	5 (2.8)	10 (2.6)	0 (0)	0 (0)
iutA	33 (18.3)*	207 (53.2)*	25 (8.5)*	262 (63.1)*
kps MT II	10 (5.6)*	86 (22.2)*	9 (3.1)*	61 (14.7)
ExPEC	15 (8.3)*	91 (23.5)*	10 (3.4)*	84 (20.2)*

[a] ExPEC are defined molecularly as those isolates containing two or more of the following genes: *papA and/or papC* (analyzed collectively), *afa/dra, sfa/foc, iutA, kps MT* II.

[b] Percentage of positive isolates among all *E. coli* isolates within each meat types.

* $P < 0.01$ for comparison of indicated group with all other groups.

Table III - 3. Serogroups of ExPEC isolated from retail meats.

O type	Number of ExPEC in each serogroup				
	Chicken Breast (n = 84)	Ground turkey (n = 91)	Ground beef (n = 10)	Pork chop (n = 15)	Total (%[a]) (n = 200)
ONT	16	12	3	2	33 (16.5)
1	3	2	1	0	6 (3)
2	7	33	1	0	41 (20.5)
4	0	3	0	0	3 (1.5)
5	3	0	0	2	5 (2.5)
6	3	1	0	0	4 (2)
7	1	1	2	0	4 (2)
8	2	6	0	1	9 (4.5)
9	0	1	0	0	1 (0.5)
11	0	9	0	1	10 (5)
13	1	0	0	0	1 (0.5)
15	2	1	0	0	3 (1.5)
18	0	0	0	2	2 (1)
19	2	4	0	0	6 (3)
20	2	0	0	0	2 (1)
21	3	0	0	0	3(1.5)
25	15	4	0	0	19 (9.5)
33	0	0	0	1	1 (0.5)
36	1	0	0	0	1 (0.5)
53	1	0	0	0	1 (0.5)
55	1	0	0	0	1 (0.5)
73	4	1	0	1	6 (3)
78	3	1	0	0	4 (2)
83	0	2	0	0	2 (1)
84	1	1	0	2	4 (2)
88	3	0	0	0	3 (1.5)
101	0	1	0	0	1 (0.5)
102	0	0	1	0	1 (0.5)
103	0	1	0	0	1 (0.5)
114	1	0	0	0	1 (0.5)
115	1	0	0	0	1 (0.5)
119	0	2	0	2	4 (2)
120	2	0	0	0	2 (1)
131	0	1	0	0	1 (0.5)

138	1	0	0	0	1 (0.5)
145	0	1	0	0	1 (0.5)
148	0	1	0	0	1 (0.5)
153	0	1	0	0	1 (0.5)
159	1	1	0	0	2 (1)
168	0	0	0	1	1 (0.5)
17,73	0	0	1	0	1 (0.5)
19,86	3	0	1	0	4 (2)
106, 116	1	0	0	0	1 (0.5)

[a] Percentage of isolates of each serogroup among 200 ExPEC isolates.

Table III - 4. Distribution of phylogenetic groups among ExPEC from retail meats.

Source of ExPEC	Total no. of ExPEC	Phylogenetic group, no. (%[a])			
		A	B1	B2	D
Chicken breast	84	24 (28.6)	13 (15.5)	28 (33.3)	19 (22.6)
Ground beef	10	1 (10.0)	0 (0)	2 (20.0)	7 (70.0)*
Ground turkey	91	13 (14.3)	6 (6.6)	53 (58.2)*	19 (20.9)
Pork chop	15	7 (46.7)	3 (20.0)	1 (6.7)*	4 (26.7)
Total	200	45 (22.5)	22 (11.0)	84 (42.0)	49 (24.5)

[a] Percentage of isolate among all ExPEC isolates from each type of meats.
* $P < 0.01$ for comparison of indicated group with all other groups.

Table III - 5. Distribution of virulence factors according to phylogenetic group and resistance status.

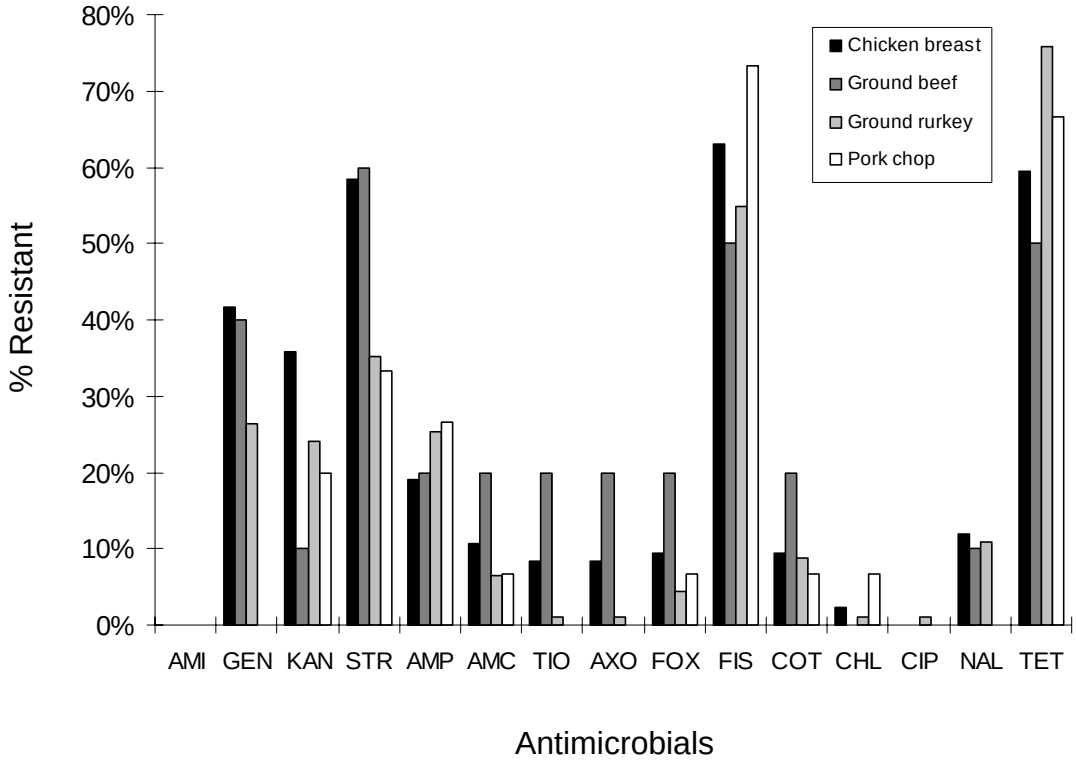
Virulence factors	Total no. of isolates with the gene (%)	Prevalence of virulence factors, no. (%[a])					
		within phylogenetic group				Resistance status	
		A n=45	B1 n=22	B2 n=84	D n=49	Resistant (n=163)	Susceptible (n=37)
papA	20 (10.0)	6 (13.3)	2 (9.1)	8 (9.5)	4 (8.2)	17 (10.4)	3 (8.1)
papC	98 (49.0)	27 (60.0)	21 (95.5)*	22 (26.2)*	28 (57.1)	90 (55.2)*	8 (21.2)
afa/dra	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
sfa/foc	13 (6.5)	3 (6.7)	1 (4.5)	8 (9.5)	1 (2.0)	9 (5.5)	4 (10.8)
iutA	197 (98.5)	45 (100)	22 (100)	82 (97.6)	48 (98.0)	160 (98.2)	37 (100)
kpsMT II	123 (61.5)	18 (40.0)*	1 (4.5)*	75 (89.3)*	29 (59.1)	96 (58.9)	27 (73.0)

[a]: Percentage of isolates with the gene among all ExPEC within each group.

* $P < 0.01$ for comparison of indicated group with all other groups.

Figure III - 1. Antimicrobial resistance of 200 ExPEC isolated from retail chicken breast, ground beef, ground turkey, and pork chop in 2006.

These isolates were tested for susceptibility to: amikacin (AMI), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), ampicillin(AMP), amoxicillin/Clavulanic acid (AMC), ceftiofur (TIO), ceftriaxone (AXO), cefoxitin (FOX), sulfisoxazole (FIS), trimethoprim/sulfamethoxazole (COT), chloramphenicol (CHL), ciprofloxacin (CIP), nalidixic acid (NAL), and tetracycline (TET).



CHAPTER IV. CHARACTERIZATION OF POTENTIALLY UROPATHOGENIC *ESCHERICHIA COLI* STRAINS ISOLATED FROM RETAIL MEATS

Abstract

Although meats are generally considered as an importance source of *E. coli* strains that cause enteric infections, it was suggested recently that meats might be a reservoir for uropathogenic *E. coli* (UPEC) that cause urinary tract infections (UTIs). The objective of this study was to determine the uropathogenic potential of *E. coli* strains isolated from retail meat. Two hundred *E. coli* strains isolated from National Antimicrobial Resistance Monitoring System (NARMS) retail meat in 2006, which were previously identified as extraintestinal pathogenic *E. coli*, were investigated by multiplex PCR for the presence of 21 virulence genes that are involved in UTI pathogenesis. Twenty three *E. coli* strains were then selected and further characterized by multi-locus sequence typing (MLST) and assays for adherence to and invasion of human bladder carcinoma T-24 cells. Moreover, the ability of those strains to induce interleukin-6 secretion from T-24 cells was also examined. Of the 21 virulence genes tested, all were detected in *E. coli* isolates except for *afa/dra* and *hlyD*. Each isolate carried a number of virulence genes ranging from 2 to 13. Twenty three *E. coli* strains were selected for further analysis based on their virulence gene profiles and serotypes. Among them, twenty one strains were assigned to 12 specific sequence types (ST), while the remaining 2 strains exhibited new STs. Seventeen strains belonged to STs associated with human UPEC infections. In T-24 cell assay, nearly all strains exhibited lower level of adherence

and invasion compared to clinical UPEC strain CFT073. But like UPEC strain CFT073, all of them induced less Interleukin (IL)-6 secretion compared to *E. coli* K-12 strain. These observations suggested that a small proportion of *E. coli* isolates from retail meats resemble human UPEC in various aspects, but their potential in causing human UTI still needs further investigation.

Introduction

Urinary tract infections (UTIs), including cystitis and pyelonephritis, are the most common bacterial infections in the United States, which account for approximately seven billion infections per year and over $1 billion cost (6). It is estimated that 40% of women and 12% of men will experienced at least one episode of UTI, and vexing recurrent UTI are very common in many individuals (5). Uropathogenic *E. coli* (UPEC), which belong to a broader group of *E. coli* that cause infections outside of gastrointestinal tract (extraintestinal pathogenic *E. coli*, ExPEC), are the causative agent for 70–95% of community-acquired UTIs and about half of nosocomial UTIs (40).

UPEC strains generally belong to a limited serogroups, while they don't share similar pattern for H antigen and K antigen (27). Most of them are assigned to phylogenetic group B2 and, to a lesser extent, phylogenetic group D (34). In contrast to commensal strains which generally harbor no or few virulence factors, UPEC have evolved an array of virulence factors and mechanisms that are important in allowing them to establish UTI (19, 40). UPEC encode various adhesive structures called pili or fimbriae to help them adhere and even invade to uroepithelial cells. To survive in a low iron environment in urinary tract, UPEC express different iron acquisition systems to take

iron from host for their benefit. Various toxins that UPEC strains secrete are also involved in the UTI pathogenesis. In response to UPEC infection, human innate immune response is initiated, which is characterized by secretion of pro-inflammatory cytokines/chemokines (including IL-6 and IL-8) by urothelial cells. Production of such molecules is essential in rapid recruitment of neutrophils into the bladder and in clearing bacteria (32, 33). But it has been shown that many UPEC strains could suppress cytokine and chemokine production during the early stage of colonization and this may serve as one of the strategies that UPEC use to evade the innate immune response (3, 10).

It is generally accepted that the immediate reservoir of UPEC is within the human intestinal tract (37). Due to the proximity of urinary tract to the rectum, bacteria from the fecal flora could seek chance to get into the urinary tract. Once inside the urinary tract, UPEC could ascend through the urethra into the bladder and cause cystitis. Sometimes bacteria could go further through ureters to kidney and cause pyelonephritis (29, 32). Despite the general consensus about the aforementioned pathway, the question about where those UPEC strains come from still remains largely unresolved. In light of the occurrence of UTI outbreaks due to closely related UPEC strains and the epidemiological association between meat consumption and UTI, it has been hypothesized that foods, especially meat, could be a possible reservoir of human UPEC strains (28, 35, 39). In many research testing this hypothesis, different methods were employed to determine similarity of meat-derived *E. coli* strains to clinical UPEC strains (16, 39). But none of them examined the virulence potential of meat-derived isolates by using tissue cultures or animal models. In this study, 200 *E. coli* isolates from retail meat, which were previously identified as ExPEC, were first characterized by extensive virulence factor profiling.

Selected isolates were subtyped by multi-locus sequencing typing (MLST). Furthermore, selected isolates were investigated for their ability to adhere to and invade bladder epithelial cells, and their ability to induce IL-6 secretion by T-24 cells was also explored.

Materials and methods

Bacterial strains. Two hundred *E. coli* isolates, including 84 from chicken breast, 91 from ground turkey, 10 from ground beef, and 15 from pork chop, were selected from 1, 275 *E. coli* isolates recovered from meat samples by the National Antimicrobial Resistance Monitoring Systems retail meat program in local grocery stores in Geogia, Maryland, Oregon and Tennessee from January to December 2006. All of isolates were selected based on their ExPEC status, which was defined by possession of two of more of the following five virulence markers: *papA* or *papC*, *sfa/foc*, *afa/dra*, *kpsMT* II and *iutA.* All of those isolates have been characterized for serogoup and phylogenetic group previously, and such information was included in this study for a comprehensive view of all available characteristics of each isolate and for selection purpose. Control strains used for genotyping were clinical *E. coli* strains JJ079, Buti 1-5-1, Buti 1-7-6, V27, 2H16 (all of strains were kindly provided by Dr. James R Johnson of the University of Minnesota). A UPEC strain CFT073 and a laboratory *E. coli* strain K-12 were used as reference strains in tissue culture studies. All strains were stored at –80°C in LB broth with 20% glycerol until use.

DNA preparation. *E. coli* cells were recovered from frozen culture at – 80°C, streaked onto blood agar plate and incubated overnight at 37 °C. For virulence genotyping, DNA of each isolate was extracted using a previously described boiling

method (42). Alternatively, for MLST analysis, DNA was prepared from each of the isolates with an UltraClean Microbial DNA Isolation kit (MoBio Laboratories, Solana Beach, CA) used according to the manufacturer's instructions.

Virulence genotyping. Twenty one virulence genes associated with UPEC pathogenesis were investigated by three multiplex PCR assays. Targeted genes and their descriptions are given in Table 1 . Primers for each virulence factor were first validated individually in single PCR assays. Then primers were placed into three pools according to primer compatibility and product size: Pool 1: *hra*, *papG* allele III , *usp*, *fimH*, *iha*, *sat*; Pool 2, *papG allele* II, *pic*, *cnf1*, *ompT*, *cvaC*, *pai*; Pool 3: *papC*, *kpsMT* II, *iut*A, *sfa/foc*, *afa/dra*, *papA*, *hlyD*. Primer pools were tested by use of control strains containing all the relevant virulence genes. For pool 1, *E. coli* strain JJ079 and Buti 1-5-1; pool2, *E. coli* strain 079, Buti 1-5-1 and Buti 1-7-6, pool 3: *E. coli* strain V27 and 2H16. (all of these strains were kindly provided by Dr. James R Johnson of the University of Minnesota).

All multiplex PCR were performed in a 25 μl reaction mixture containing 2 μl of template DNA, 2.5 μl of 10 × PCR buffer, 2 μl of a 1.25 mM mixture of deoxynucleoside triphosphates, 4 μl of 25 mM $MgCl_2$, 0.25 μl of 5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ), and a 0.6 μM concentration of each primer (Invitrogen, Carlsbad, CA). The thermocycling conditions were as follows: 95°C for 12 min, 94°C for 30 s, 60°C for 30 s, and 68°C for 3 min for 30 cycles, with a final 10 min extension at 72°C (13). PCR products (10 μl) were evaluated with a 2 % (wt/vol) agarose gel at 120 mV for 30 min. A molecular marker (100 bp DNA ladder; New England BioLabs, Ipswich, MA) was run concurrently. An isolate was considered to contain specific genes if amplicons of the expected size appeared.

Multilocus sequence typing. MLST of selected *E. coli* strains were carried out by performing a sequence analysis of seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA, and recA*) using the typing scheme described before (1, 41). Fragments of seven genes were amplified using specific primers listed in Table 1. The PCR amplicons were purified and then sequenced on both strands with the PCR primer set. Sequencing reactions were performed using Big-dye terminator cycle sequencing ready reaction DNA sequencing kit and electrophoresed on AB 3730 DNA sequencer (Applied Biosystems, Foster City, CA). DNA sequence data were compiled and analyzed using DNA Sequencher 4.0 software (Gene Codes Corp., Ann Arbor, MI). Assignment of the alleles, sequence types and sequence type complexes were performed via an electronic *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli).

Adherece and invasion assays. Adherence and invasion assays were performed according to procedures described previously with some modifications (24, 30). Briefly, T-24 human bladder epithelial cells were grown into confluent epithelial cell monolayer in 24-well plates. Bacteria, grown statically in LB broth at 37 °C for 24 h, were diluted with phosphate-buffered saline (PBS) to achieve an optical density at 600nm (OD_{600}) of 0.5. Ten microliter of bacterial suspension (a multiplicity of infection (MOI) of about 10) was added to three sets of triplicate cells. The number of cells in inoculum was titered by serial dilution. After incubation at 37 °C for 2 h, the cells in one set of wells were washed five times with PBS (containing Mg^{2+} and Ca^{2+})(Gibco), and lysed in 1 ml 0.1% Triton X-100 in H_2O to calculate the number of adherent bacteria. In another set of wells, 20 µl of 5% Triton X-100 was added to lyse the cells, and the total number of bacteria was enumerated. The percent adherence frequency was determined as the number of bacteria

recovered after PBS washes divided by the total number of bacteria present in each well. To determine invasion frequency, a third set of triplicate wells was washed twice with PBS (containing Mg^{2+} and Ca^{2+}), and incubated for another 2 h in medium containing 100 μg/ml gentamicin (Sigma) to kill all extracellular bacteria. Cells were then washed with PBS (containing Mg^{2+} and Ca^{2+}) three times, and lysed in 1 ml of 0.1% Trion X-100 in water to enumerated intracellular bacteria. The percent invasion frequency was expressed as the number of the bacteria surviving the gentamicin treatment divided by the number of bacteria in the initial inoculum.

IL-6 Cytokine induction assay. IL-6 scretion assays were performed essentially as described by Hunstad et al (11). Confluent epithelial T-24 cell monolayers were prepared as described above. Confluent bladder cell monolayers were washed once with sterile phosphate-buffered saline (PBS), and 990 μl of fresh medium was added. Ten microliters of bacterial suspension (MOI of about 10) were added to corresponding wells. Uninfected wells (in which 10 μl PBS was added) were used as control. After inoculation, tissue culture plates were incubated at 37°C with 5% CO_2 for 2 h. Culture supernatants were then collected, centrifuged at 20,000 *g* for 3 min to pellet bacteria and cell debris, and stored at -80°C until IL-6 measurement by using human IL-6 ELISA kit (R & D Systems, Minneapolis, MN).

Statistical analysis. All analyses were performed by use of Statistical Package for the Social Sciences (SPSS) version 11.0. Comparisons of proportions for a virulence gene in different groups were tested by Chi-square test (2-tailed). And comparisons of the difference in the number of virulence gene between groups were tested by Kruskal-Wallis test followed by Mann-Whitney post-hoc pairwise 2-group comparison. Comparisons of

means were tested with one-way analysis of variance followed by Dunnett's multiple comparisons test. The threshold for statistical significance was $P < 0.05$.

Results

Virulence genotyping of ExPEC strains from retail meats. Of the 21 virulence genes tested, all could be detected in *E. coli* strains isolated from retail meats except *afa/dra* gene and *hlyD* gene. Other genes occurred at rates ranging from 0.5% to 97.5% (Table 2). Nearly all isolates harbored the *fimH* gene, and some adhesion genes such as *papG* allele II and *hra* were present in 38.0% and 33.5% of the isolates, while *papG* allele III and *iha* were only found in a small percentage of the isolates (2.0% and 3.5% respectively). Iron acquisition genes, *iutA* and *iro*, were very frequently present in those meat-source isolates (80.5% and 98.5%). Protectins genes, such as *cvaC* and *kpsMT* II, were detected in more than half of the isolates. Among the five toxins examined, only *vat* and *pic* were relative common, while *cnf1* and *sat* occurred in 0.5% of all isolates and no isolates contained *hlyD* gene. For miscellaneous virulence genes, *ompT* was found in 76.0% of all isolates, *malX* and *usp* were present in 43% and 36% of all isolates. Certain virulence genes, including *cvaC, kpsMT* II*, vat, ompT, malX* and *usp*, were associated with phylogenetic group B2 (Table 2). Genes *iro*, *cvaC*,*malX* and *usp* occurred in a higher percentage of turkey isolates than isolates from other three types of meats (Table 2).

The number of virulence genes detected in different strains ranged from 2 to 13 among the 21 genes tested. The average number of virulence genes in those meat-derived ExPEC isolates was 7.6. Analysis combining phylogenetic grouping results from

previous research revealed that isolates belonging to phylogenetic group B2 (8.9) and D (7.7) contained higher number of virulence genes compared to strains in group B1(6.2) and A (5.7) (data not shown). Isolates from different meat types did not differ significantly in the number of virulence genes they contained.

MLST. Twenty three *E. coli* strains were selected from 200 *E. coli* isolates for MLST analysis based on their virulence gene profile (≥8 genes) and serotypes being one of the 10 common serogroups in *E. coli* causing UTI (Table 3). Of 23 strains analyzed by MLST, twenty one strains could be assigned to 12 specific sequence types (ST), while the remaining two strains exhibited new STs that could not be assigned a type by the *E. coli* MLST website (Table 4). Two isolates belonged to ST73 which was included in ST73 complex. One isolate was identified as ST140 and two were found to be ST95, all of them were placed into ST95 complex. One isolate was identified as ST88 and fell into ST23 complex. Other STs identified among 23 isolates were ST131(3 isolates), ST998 (3 isolates), ST117 (2 isolates), ST141 (2 isolates), ST429 (2 isolates), ST88 (1 isolates), ST135 (1 isolates), ST624 (1 isolates),ST770 (1 isolates), none of them could be placed in any ST complexes in the database.

Adherence and invasion. Since adherence and invasion are considered as important steps during UPEC pathogenesis, 23 strains were further tested for adherence and invasion using T-24 bladder carcinoma cell line together with a human UTI strain CFT073 and a laboratory strain K-12. All isolates from meat could adhere to T-24 cell to some extent, with the percentage of adherence ranged from 0.125% to 1.022% (Figure 1). Both CFT073 and non-virulent strain K-12 showed strong adherence to T-24 cell, while most of the isolates from retail meat showed weaker ability to adhere. Similar to

adherence assay, meat-source isolates also exhibited various degree of invasiveness. Based on the results of non-invasive *E. coli* K-12 strain, meat-source isolates were categorized into three groups according to their invasion percentage: non-invasive (0-0.05%), potentially invasive (0.05-0.1%) and invasive (>0.1). According to this criterion, only 1 isolate was considered as invasive, six isolates as potentially invasive, and 16 isolates were put into non-invasive group.

Cytokine secretion assay. It was demonstrated that most clinical UPEC isolates could suppress pro-inflammatory cytokine secretion by bladder epithelial cells. In order to determine whether strains from retail meat could modulate urothelial IL-6 response, we infected T-24 cells with *E. coli* isolates from meat and quantified IL-6 secretion by ELISA. Clinical strain CFT073 (117.03 ± 38.73pg/ml) induced significantly less IL-6 compared with K-12 (244.12 ± 22.15 pg/ml) strain. Similar to CFT073, all meat-source isolates (43.08 to 153.26 pg/ml) also induced far less IL-6 than K-12 strain (data not shown).

Discussion

Although it is generally accepted that UPEC strains causing UTIs come from the patients' colon, the actual source of those pathogenic strains remains elusive. Recent studies suggested that UPEC may originate from contaminated food, especially animal-derived product (14, 16, 18, 39). To test this hypothesis, we investigated *E. coli* isolates from four different types of meats for an array of UPEC virulence genes. And those strains with a high number of virulence genes were chosen for MLST typing and their interactions with human bladder epithelial cells were examined. This study demonstrated

that *E. coli* isolates from retail meats contained many UPEC virulence genes, and some of them belonged to STs that were involved in human UTIs. But the lack of certain archetypal UPEC virulence gene and poor ability to adhere to and invade epithelial cells indicated that the hypothesis of animal reservoir of UPEC still needs more evidence.

The roles of most of virulence genes chosen in this study were experimentally determined in the pathogenesis of UTI (17, 29). Except for *afa/dra* and *hlyD*, all of virulence genes were detected in meat-derived isolates including certain very typical UPCE virulence genes such as P, S, G fimbriae, and the *usp* gene, indicating at least those strains may serve as a reservoir of UPEC virulence genes that could be transferred to microflora in the human intestine. On the other hand, *E. coli* from meats lacked some virulence genes commonly found in UPEC isolates such as *hlyD* and *cnf1*, which were estimated to be present in one third and half of UPEC isolates, respectively (40). And for those virulence genes present in isolates from meat, their prevalence was different from the data in studies examining UPEC isolates (23, 36). For instance, *iha, papA, papG* III, *vat* and *malX* were found to be in a higher percentage in UPEC isolates, while higher rate of *iro* and *cvaC* were observed in meat-source isolates than in UPEC isolates (23). Those findings suggest that although there is overlap with respect to virulence genes among meat-source isolates and UPEC isolates, their distributions differ. Our results are very similar to other studies examining virulence gene profiles of ExPEC isolates from meats in spite of different sampling time and locale. Johnson *et al.* examined several virulence factors in ExPEC isolates from a more geographically limited retail chicken samples, very similar levels of *afa/dra, fimH, papC, iro,iutA, cvaC, kpsMT* II,*cnf1, hlyD, ompT, malX* were detected in that study compared to the data in this study(18). This consistency

in prevalence data indicates that it is the general picture of the presence of UPEC virulence genes in *E. coli* from retail chicken.

Isolates belonging to phylogenetic groups B2 and D have higher number of virulence genes (8.9 for B2 and 7.7 for D) than isolates from group A (5.7) and B1 (6.2). This confirmed the perception that strains in those two phylogenetic groups were generally more virulent than the other two groups (34). Many studies demonstrated that most UPEC isolates fell into those two groups (15, 20). Although many researchers focused on poultry products for meat reservoir hypothesis (18, 39), our results showed that pork or beef isolates also bear certain UPEC virulence genes. And some adhesin genes occurred at higher rates in isolates from these two groups than from poultry products (such as *iha, hra, papA, papG* III *and sfa/foc*). Even though only a small percentage of *E. coli* isolates from these two types of meat met ExPEC criteria (that is why a low number of isolates were available to be included in this study), their potential as a UPEC reservoir could not be completely excluded.

It was suggested that the number of virulence genes present in an isolate could be a good predictor of its virulence (15). So we chose 23 strains that contained more than 8 virulence genes and belonged to the common ten O groups in UPEC, with the assumption that they have higher chance of being urovirulent, and they were subjected to MLST analysis. Many typing methods have been used to identify clonal groups among UPEC. One useful technique is MLST, which utilized variations of several house keeping genes for comparison and therefore, avoid the drawbacks of gel-based fingerprinting method (41). In addition, there is a large collection of UPEC in globally accessible MLST database and that makes comparison from different studies possible and easy. Analysis of

selected isolates by MLST revealed that many of them belonged to ST complexes that included clinical UPEC strains. For instance, ST95 complex and ST73 complex are two of the four major ST complexes (another two are ST14 complex and ST69 complex) that are associated with human UPEC in the U.S. (25). Other STs, such as ST88, ST117, ST131, ST141, ST770, and ST998 were also reported to be involved in UTIs in the MLST database. This overlap of ST among meat and human isolate implied that certain *E. coli* isolates from meat may have zoonotic potential.

Although several researchers have also investigated virulence gene profiles and examined genetic similarity between meat-source isolates and human clinical isolates with different typing methods, none of them explore the virulence potential of those isolates using tissue culture or animal model (12, 23, 39). Here we used T-24 cells, a commonly used human bladder carcinoma epithelial cell, to assess the interaction between meat-derived isolates and bladder cells. Adherence was the first step of UTI pathogenesis and is very important for strain to persist in bladder. In addition, UPEC could invade into epithelial cells to form intracellular communities, which may shield the bacteria from the host immune response (32). Unexpectedly, even though some meat isolates did harbor several virulence genes including various adhesion genes, they were generally not good at attaching, and most of them fell into non-invasive group, both of which may indicate that their potential to cause UTI are limited. This incongruence of genotype with phenotype may be explained by the fact that many of the virulence genes in those meat isolates may not be or are weakly expressed. Researchers has found that even though almost 90% of *E. coli* contains the *fimH* gene, which encode the tip of type 1 pili, its expression varied greatly in different strains since *fim* operon is regulated by a

switch element. Difference in *fim* switch status (on or off, also known as phase variation) was found between UPEC compared and control isolates (8, 26). Admittedly, UPEC are comprised of a very heterogeneous group of strains and poor adhesion and invasion do not necessarily equal to no potential to cause UTI (31). So even though meat isolates have low adherence and invasion ability, the possibility for them to cause human disease still exists.

Upon UPEC infection, host bladder epithelial cells respond by secreting cytokines and chemokines, leading to inflammation and clearance the bacteria. Recent studies demonstrate that during the early stage of colonization, UPEC could modulate this response and may provide themselves more time to colonize and multiply (2, 3, 10). Since this seems to be an important characteristic in UPEC, we were interested to see if *E. coli* isolates from meat could behave like UPEC. Surprsingly, like the clinical strain CFT073, all meat isolates induced less IL-6 compared with K-12 strain. This may bring into question the use of this character to predict uropathogenic potential. Billips *et al.* investigated a panel of human *E. coli* strains including diarrheagenic strains, fecal strains and ExPEC strains, and they found that most of those strains could modulate IL-6 secretion irrespective of their origins. It is possible that this trait may give those pathogenic strains additional advantage during colonization, but it could also be shared by other pathogenic or commensal strains.

In summary, we demonstrated that a small proportion of *E. coli* strains isolated from retail meats contained UPEC virulence genes and shared ST with clinical UPEC, indicating their potential to be a reservoir of UPEC virulence genes or even UPEC. But findings from interactions with bladder epithelial cells made their uropathogenic potential

undetermined. More research (such as infection study using animal models) is warranted before we could conclude with full confidence that meat reservoir exists for UPEC.

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Table IV - 1 Primers used for virulence gene detection and MLST analysis.

Gene	Description	Size (bp)	Primer sequences (5' - 3')	Reference
Adhesins				
afa/dra	Dr antigen-specific fimbrial and afimbrial adhesin	592	F: ggcagagggccggcaacaggc R: cccgtaacgcgccagcatctc	(21)
fim	Type 1 fimbriae	508	F: tcgagaacggataagccgtgg R: gcagtcacctgccctccggta	(21)
hra	Heat-resistant agglutinin	162	F: cgaatcgttgtcacgttcag R: tatttatcgccccactcgtc	(38)
iha	Iron regulated gene homolog adhesin	829	F: ctggcggaggctctgagatca R: tccttaagctcccgcggctga	(36)
papA	Major structural subunit of P pili	717	F: atggcagtggtgtcttttggtg R: cgtcccaccatacgtgctcttc	(21)
papC	Central region of P pili	205	F: gtggcagtatgagtaatgaccgtta R: atatcctttctgcagggatgcaata	(21)
papG II	Class II P pili	190	F: gggatgagcgggcctttgat R: cgggcccccaagtaactcg	(21)
papG III	Class III P pili	258	F: ggcctgcaatggatttacctgg R: ccaccaaatgaccatgccagac	(21)
sfa/foc	Central region of S fimbriae and F1C fimbriae	410	F: ctccggagaactgggtgcatcttac R: cggaggagtaattacaaacctggca	(21)
Iron acquisition				
iro	Catecholate siderophore (salmochelin) receptor	667	aagtcaaagcaggggttgcccg gacgccgacattaagacgcag	(36)
iutA	Ferric aerobactin receptor	302	F: ggctggacatcatgggaactgg R: cgtcgggaacgggtagaatcg	(21)
Protectins				
cvaC	Colicin V	679	F: cacacacaaacgggagctgtt R: cttcccgcagcatagttccat	(21)
kpsMT II	Group II capsular polysaccharide synthesis	272	F: gcgcatttgctgatactgttg R: catccagacgataagcatgagca	(21)
Toxins				
cnf1	Cytotoxic necrotizing factor 1	498	F: aagatggagtttcctatgcaggag R: cattcagagtcctgccctcattatt	(21)
hlyD	Transporter gene of the hemolysin operon	904	F: ctccggtacgtgaaaaggac R: gccctgattactgaagcctg	(36)
pic	Serin protease autotransporter	409	F: actggatcttaaggctcagg R: tggaatatcagggtgccact	(4)
sat	Secreted autotransporter toxin	937	F: gcagctaccgcaataggaggt R: cattcagagtaccggggccta	(7)
vat	Vacuolating autotransporter toxin	289	F: agagacgagactgtatttgc R: gtcaggtcagtaacgagcac	(9)

Miscellaneous				
ompT	Outer membrane protease	559	F: atctagccgaagaaggaggc R: cccgggtcatagtgttcatc	(36)
malX	Pathogenicity-associated island marker	925	F: ggacatcctgttacagcgcgca R: tcgccaccaatcacagccgaac	(21)
usp	Uropathogen specific protein	440	F: acattcacggcaagcctcag R: agcgagttcctggtgaaagc	(22)
MLST				
adk	Adenylate kinase	583	F: attctgcttggcgctccggg R: ccgtcaactttcgcgtattt	(41)
fumC	Fumarate hydratase	806	F: tcacaggtcgccagcgcttc R: gtacgcagcgaaaaagattc	(41)
gyrB	DNA gyrase	911	F: tcggcgacacggatgacggc R: atcaggccttcacgcgcatc	(41)
icd	Isocitrate/isopropylmalate dehydrogenase	799	F: ccgattatcccttacattgaag R: ggacgcagcaggatctgtt	(1)
mdh	Malate dehydrogenase	932	F: atgaaagtcgcagtcctcggcgctgctggcgg R: ttaacgaactcctgccccagagcgatatctttctt	(41)
purA	Adenylosuccinate dehydrogenase	816	F: cgcgctgatgaaagagatga R: catacggtaagccacgcaga	(41)
rec	ATP/GTP binding motif	780	F: acctttgtagctgtaccacg R: agcgtgaaggtaaaacctgtg	(41)

Table IV - 2. Prevalence of virulence associated genes in ExPEC strains isolated from retail meat

Genes/categories	Prevelance of virulence associated genes in ExPEC from meats (%)								
	Overall	Meat type				Phylogenetic group			
		Chicken n = 84	beef n = 10	turkey n = 91	Pork n = 15	A n = 45	B1 n = 22	B2 n = 84	D n = 49
Adhesins									
afa/dra	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
fimC	97.5	96.4	100.0	100.0	86.7	88.9	100.0	100.0	100.0
hra	33.5	41.7	80.0	18.7	46.7	40.0	22.7	23.8	49.0
iha	3.5	4.8	0.0	1.1	13.3	0.0	4.5	4.8	4.1
papA	10.0	4.8	20.0	9.9	33.3	13.3	9.1	9.5	8.2
papC	49.0	51.2	50.0	40.7	86.7	60.0	95.5	26.2	57.1
*papG*II	38.0	44.0	30.0	33.0	40.0	40.0	72.7	20.2	51.0
*papG*III	2.0	2.4	0.0	0.0	13.3	4.4	9.1	0.0	0.0
sfa/foc	6.5	0.0	0.0	11.0	20.0	6.7	4.5	9.5	2.0
Iron acquisition									
iro	80.5	67.9	70.0	96.7	60.0	62.2	81.8	88.1	83.7
iutA	98.5	100.0	100.0	97.8	93.3	100.0	100.0	97.6	98.0
Protectins	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
cvaC	68.5	50.0	60.0	93.4	26.7	42.2	68.2	81.0	71.4
*kps*II	61.5	57.1	70.0	69.2	33.3	40.0	4.5	89.3	59.2
Toxins									
cnf1	0.5	0.0	0.0	1.1	0.0	0.0	0.0	1.2	0.0
hlyD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pic	15.5	7.1	40.0	17.6	33.3	0.0	4.5	15.5	34.7
sat	0.5	0.0	0.0	0.0	6.7	0.0	0.0	1.2	0.0
vat	38.5	21.4	50.0	54.9	26.7	6.7	4.5	63.1	40.8
Miscellaneous									
ompT	76.0	67.9	80.0	84.6	66.7	55.6	36.4	96.4	77.6
pai	43.0	29.8	20.0	63.7	6.7	6.7	0.0	83.3	26.5
usp	36.0	23.8	10.0	54.9	6.7	6.7	0.0	79.8	4.1

Table IV - 3. Characteristics of *E. coli* isolates selected for MLST and T-24 cell assays*

Isolate	Source	Sero	Phylo	*hra*	*usp*	*fimH*	*iro*	*iha*	*sat*	*pap* GII	*vat*	*pic*	*cnf 1*	*omp T*	*cva*	*pai*	*pap A*	*pap C*	*sfa /foc*	*iut A*	*kps M*	# of VF
N11989	Turkey	1	B2	-	+	+	+	-	-	+	+	-	-	+	+	+	+	+	-	+	+	12
N12528	Chicken	1	D	+	-	+	+	-	-	-	+	+	-	+	-	+	-	-	-	+	+	9
N11444	Turkey	2	B2	+	+	+	+	-	-	-	+	+	+	+	+	+	-	-	+	+	+	13
N12522	Chicken	2	B2	+	+	+	+	-	-	+	+	-	-	+	+	+	-	+	-	+	+	12
N11698	Turkey	2	B2	-	+	+	+	-	-	-	+	+	-	+	+	+	-	-	+	+	+	11
N11726	Turkey	2	B2	+	+	+	+	-	-	-	+	+	-	+	-	+	-	-	+	+	+	11
N11816	Turkey	2	B2	-	+	+	+	-	-	-	+	+	-	+	+	+	-	-	+	+	+	11
N12564	Turkey	2	B2	-	+	+	+	-	-	+	-	-	-	+	+	+	+	+	-	+	+	11
N11911	Chicken	2	D	+	-	+	+	-	-	+	-	-	-	+	+	+	-	+	-	+	+	10
N11479	Turkey	2	B2	-	+	+	+	-	-	-	+	+	-	+	+	+	-	-	-	+	+	10
N11279	Turkey	2	B2	-	+	+	+	-	-	-	+	-	-	+	+	+	-	-	-	+	+	9
N12138	Turkey	2	B2	-	+	+	+	-	-	-	+	-	-	+	+	+	-	-	-	+	+	9
N11940	Turkey	2	B2	-	+	+	+	-	-	-	+	-	-	+	+	+	-	-	-	+	+	9
N11306	Turkey	4	B2	-	+	+	+	-	-	-	+	+	-	+	+	+	-	-	+	+	-	10
N12064	Turkey	6	B1	-	-	+	+	-	-	+	+	+	-	+	+	-	-	+	-	+	-	9
N12306	Beef	7	D	+	-	+	+	-	-	-	+	+	-	+	+	-	-	-	-	+	+	9
N12521	Chicken	8	B2	-	+	+	+	+	-	-	+	-	-	+	+	+	-	-	-	+	+	10
N11981	Chicken	8	A	+	-	+	+	-	-	+	-	-	-	+	+	-	-	+	-	+	-	8
N12050	Pork	18	B2	+	+	+	-	+	+	-	+	+	-	+	-	+	-	-	-	+	+	11
N11610	Chicken	25	B2	+	+	+	+	-	-	-	-	-	-	+	+	+	-	-	-	+	+	9
N11473	Chicken	25	B2	-	+	+	+	-	-	-	-	-	-	+	+	+	-	-	-	+	+	8
N11668	Chicken	25	B2	+	-	+	+	-	-	+	-	-	-	+	-	-	-	+	-	+	+	8
N12423	Chicken	25	B2	-	+	+	+	+	-	-	-	-	-	+	-	+	-	-	-	+	+	8

*: None of these isolates contained *papGIII*, *hlyD* and *afa/dra*.
Abbreviations: serogroup (sero), phylogenetic group (phylo), virulence factor (VF)

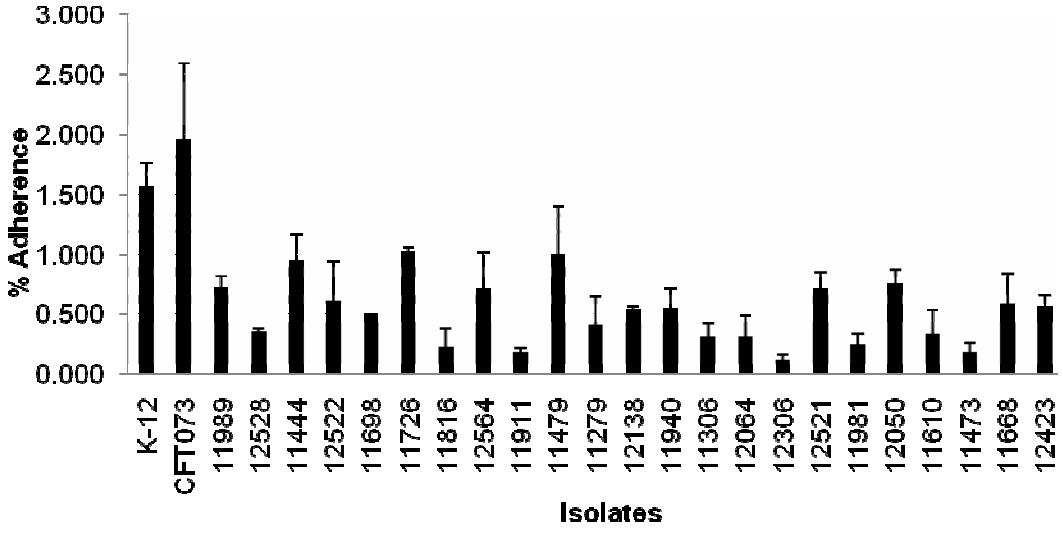
Table IV - 4. MLST of 23 *E. coli* isolates from retail meats.

Isolate	Gene allele type							ST	ST Complex
	adk	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*		
N12528	6	4	14	16	24	2	14	New	None
N12521	27	88	78	29	70	158	2	New	None
N11306	36	24	9	13	17	11	25	ST073	ST73 Cplx
N12050	36	24	9	13	17	11	25	ST073	ST73 Cplx
N11981	6	4	12	1	20	12	7	ST088	ST23 Cplx
N11989	37	38	19	37	17	11	26	ST095	ST95 Cplx
N12564	37	38	19	37	17	11	26	ST095	ST95 Cplx
N12064	20	45	41	43	5	32	2	ST117	None
N12306	20	45	41	43	5	32	2	ST117	None
N11610	53	40	47	13	36	28	29	ST131	None
N11473	53	40	47	13	36	28	29	ST131	None
N12423	53	40	47	13	36	28	29	ST131	None
N12522	13	39	50	13	16	37	25	ST135	None
N12138	55	38	19	37	17	11	26	ST140	ST95 Cplx
N11698	13	52	10	14	17	25	17	ST141	None
N11816	13	52	10	14	17	25	17	ST141	None
N11479	97	40	93	13	23	28	66	ST429	None
N11940	97	40	93	13	23	28	66	ST429	None
N11911	92	4	87	96	70	58	91	ST624	None
N11668	52	116	55	101	113	40	38	ST770	None
N11444	13	52	156	14	17	25	17	ST998	None
N11726	13	52	156	14	17	25	17	ST998	None
N11279	13	52	156	14	17	25	17	ST998	None

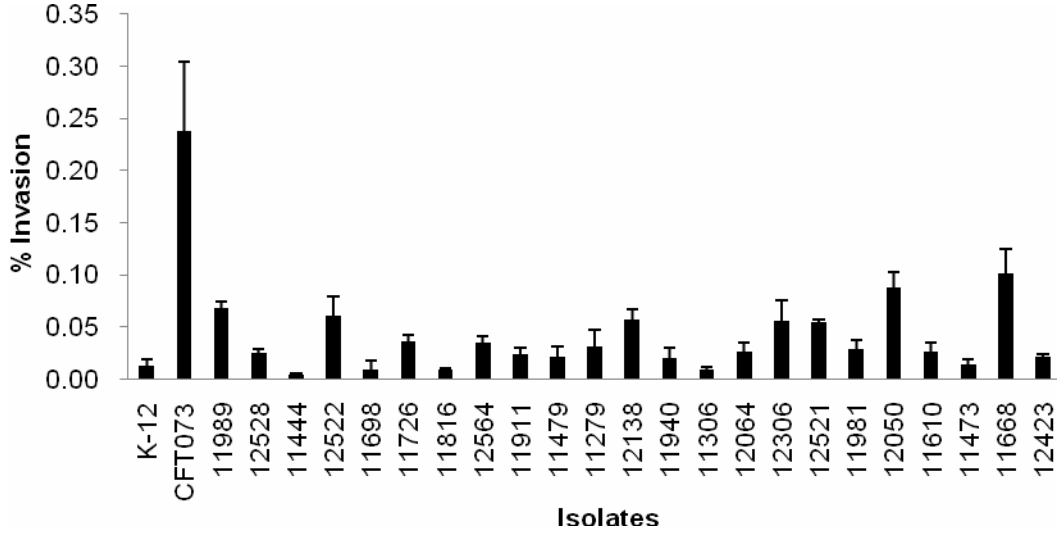
Figure IV - 1. The ability of *E. coli* isolates from retail meat to adhere to (A) and invade (B) T-24 human bladder epithelial cells.

A UPEC strain CFT073 and a laboratory strain K-12 were also included. All data represent means ± SD of three independent assays, each performed in triplicate.

A



B



CHAPTER V. SUMMARY

Food serves as a common vehicle for transmitting certain pathogens. Retail meat may be contaminated with *E. coli* during slaughtering and processing. Due to the fact that not all *E. coli* are pathogenic to human beings, differentiation between pathogenic and non-pathogenic strains becomes very important. Although *E. coli* has been isolated from retail meat and their prevalence was previously researched, few data exist about contamination of different pathogenic *E. coli* among retail meat. In this project, pathogenic *E. coli* strains, including both diarrheagenic and extraintestinal pathogenic strains, were identified from *E. coli* isolates recovered from different types of retail meats. They were further characterized genotypically and phenotypically in various aspects and their pathogenic potential were also investigated. This study would be great helpful for us to estimate the actual existence of different pathogenic *E. coli* among retail meat products in the United States and the potential they have to cause human disease. In addition, this study would also provide useful information for meat industries as well as governmental institutions controlling food safety.

In chapter 2, a total of 7,259 *Escherichia coli* isolates collected during 2002-2007 from NARMS retail meats (ground beef, ground turkey, chicken breast and pork chop) were screened for the presence of Shiga toxin genes and 1,275 *E. coli* isolates recovered in 2006 were also examined for virulence genes specific for other five groups of diarrheagenic *E. coli*. PCR assays specific for *eae* and EHEC-*hlyA* genes were employed to identify virulence factors of STEC, while serotyping, Vero cell cytotoxicity assay, *stx* gene sequence analysis and pulsed-field gel electrophoresis

(PFGE) were used to further characterize the STEC strains. The data demonstrated that diverse STEC strains were present in retail meats, especially in ground beef, and some strains contained *stx* variant that is associated with severe human infections. Retail meat may also be contaminated with atypical EPEC strains, while EIEC, ETEC and DAEC may not be a major concern in retail meat.

Although meats are generally considered as an importance source of *E. coli* strains that cause enteric infections, it was suggested recently that meats might be a reservoir for ExPEC strains. In chapter 3, the 1,275 *E. coli* isolates recovered in 2006 were investigated for the presence of five ExPEC virulence markers using multiplex PCR. Identified ExPEC isolates were characterized by serogrouping and phylogenetic grouping, and their antimicrobial susceptibility data were analyzed. Approximately 16% (200/1,275) of the *E. coli* isolates were identified as ExPEC based on defined molecular criteria. The prevalence of ExPEC was higher in the *E. coli* population isolated from poultry than *E. coli* from pork chop and ground beef. Most ExPEC isolates fell into virulent phylogenetic groups B2 and D. A large proportion of ExPEC isolates (81.5%) were resistant to at least one antimicrobial, and 57% of isolates exhibited resistance to at least three drugs. These findings showed that retail meats, especially in chicken and turkey products could be contaminated with ExPEC, including antibiotic resistant ExPEC strains. It provided the evidence for the hypothesis that retail meat may serve as a possible vehicle to transmit ExPEC strains.

Those ExPEC found in retail meat were only defined by detection of more than two of the five virulence factors, yet their pathogenic potential was still largely undetermined. An extensive virulence gene profile and their interactions with host

bladder cells could shed more light on their potential to cause human urinary tract infections. In chapter 4, 200 ExPEC strains previously identified from retail meat were investigated by multiplex PCR for the presence of an array of virulence genes that are involved in UTI pathogenesis. Those strains with a high number of virulence genes were chosen for MLST typing and their interactions with tissue cultured human bladder epithelial cells were examined. Moreover, the abilities of those strains to induce interleukin-6 secretion from T-24 cells were also examined. It was demonstrated that *E. coli* isolates from retail meats contained many UPEC virulence genes, and some of those isolates belonged to STs that were frequently involved in human UTIs. But the lack of certain archetypal UPEC virulence gene and poor ability to adhere to and invade epithelial cells indicated that the hypothesis of animal reservoir of UPEC still need more evidence.

In summary, this project demonstrated that a certain portion of *E. coli* isolates from retail meats are potentially diarrheagenic or extranintestinal pathogens based on the virulence genes they contained and their similarities to strains causing human infections with respect to some important characteristics. Due to the limitations of this project, there are still many gaps in our knowledge of meat-derived *E. coli* for future research to fill. First, although we investigated the presence of pathogenic *E. coli* against the background of *generic E. coli* populations, the prevalence of pathogenic *E. coli* in retail meats could not be determined. Future work specifically designed to isolate the specific pathotypes of *E. coli* from meats are necessary to answer the aforementioned question. Furthermore, the pathogenic potential of the *E. coli* isolates was largely predicted rather than being verified by *in vivo* study, animal

infection studies will be important to elucidate the actual ability of those *E. coli* strains to cause infections. Finally, the virulence factors chosen to define ExPEC were epidemiologically linked with ExPEC but not unique to ExPEC. Availability of whole genome sequence of more clinical ExPEC strains and bioinformatics tools may help find certain virulence markers exclusively occurring in ExPEC and contribute to elucidate the real source and transmission pathway of human ExPEC strains.

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