Development of a rapid PCR method for direct *Escherichia coli* O157:H7 detection in meat

by

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GENERAL INTRODUCTION

Demand for specific *Escherichia coli* O157:H7 detection methods

Enterohemorrhagic *Escherichia coli* O157:H7 is a human pathogen belonging to the group of Shiga-like toxin-producing *E. coli* (STEC) (Marques *et al.*, 1986, Tarr, 1995). After its identification in 1982, this organism was placed in a group of major foodborne pathogens because of the severe and potentially life threatening illnesses it causes and the steadily increasing number of food-borne outbreaks associated with it (Griffin and Tauxe, 1991). The illnesses include hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), the latter of which may result in life long complications or even death (Griffin *et al.*, 1988). Numerous epidemiological studies indicate that cattle are a major reservoir for *E. coli* O157:H7 (Cray and Moon, 1995; Wells *et al.*, 1991; Whipp *et al.*, 1994); however, any food product or water that comes into contact with contaminated meat or bovine feces becomes infectious.

Rapid and accurate detection methods are important tools for fast identification of contaminated foods or environments and thus for protection of consumers against STEC associated illnesses. Currently, diagnosis in most clinical laboratories is based on culture utilizing the organism's unique phenotypic characteristics which allow the differentiation of O157:H7 from other *E. coli* serotypes (Boyce *et al.*, 1995a; Smith and Scotland, 1993). Although these procedures are reliable and easily adaptable in bacteriology laboratories, they are not fast nor can they detect phenotipically changed variants of *E. coli* O157:H7 (Acheson and Keusch, 1996; Feng, 1995).

The production of Shiga-like toxin 1 and/or 2 (Stx1 and Stx2) by *E. coli* O157:H7 is considered to be a major virulence attribute, but knowledge of the organism's pathogenesis is incomplete (Griffin and Tauxe, 1991; O'Brien *et al.*, 1992; Brotman *et al.*, 1995). More than 100 Stx-producing *E. coli* serotypes are known to exist and a few of them have been associated with foodborne HC and HUS, although not nearly to the extent of O157:H7 (Feng and Lampel, 1994; Robson *et al.*, 1993). The question remains as to why the O157:H7

serotype is the predominant one involved in HC and HUS cases. Until this is answered, and while other STECs continue to be a potential threats to humans, there is a need for diagnostic methods to detect *E. coli* O157:H7 specifically, along with the ability to identify the presence of other Stx-producing bacteria.

Many advanced methods such as toxin detection, ELISA, immunofluorescence, and DNA probes have been employed extensively and described in the literature, but none of them solve all the problems mentioned above (Padhey and Doyle, 1992; Smith and Scotland, 1993; Su and Brandt, 1995). The currently widely used polymerase chain reaction (PCR) procedure could meet these demands as it can target detection of several genetic markers of bacteria simultaneously and can be applied to bacterial cultures as well as clinical, environmental and food samples. Additionally, PCR has the potential for increased specificity and sensitivity as compared to biochemical tests.

Numerous alternative and improved PCR methods for STEC and *E. coli* O157:H7 detection have been generated in the last decade. A few procedures have been described to detect STEC in meat (Begum and Jackson, 1995; Gannon *et al.*, 1992; Read *et al.*, 1992); however, none have been aimed at detection of *E. coli* O157:H7 and Stx sequences simultaneously. Furthermore, the previously described PCR methods, applied directly to meat, involve the labor intensive sample preparation steps necessary for removal of PCR inhibitors in meat. Thus, development of a rapid and specific PCR-based method for detection of *E. coli* O157:H7 and other STEC directly from meat would be an important step in satisfying the increasing demand for safer meat products.

Objectives of the study

The ultimate goal of this research was to develop a sensitive, one-day PCR technique that would allow specific detection of *E. coli* O157:H7 and other Stx-producing bacteria from ground meat -- the most common vehicle implicated in STEC associated food-borne illnesses. Other goals include:

1) development of a simplified meat sample processing technique which avoids time consuming DNA extraction procedures and thus would be easily adaptable for use in other laboratories;

2) determination of the influence of ground beef fat content on PCR test results;

3) determination of sensitivity and specificity of the PCR procedure in comparison to bacteriologic culturing.

Thesis organization

This thesis contains a manuscript which will be submitted to the journal *Applied and Environmental Microbiology*. The master's candidate, Ilze Matise, is the research investigator and senior author of the paper. The manuscript is preceded by a literature review and followed by a general summary and an appendix to the study described in the manuscript. References and acknowledgments appear after the appendix.

LITERATURE REVIEW

Significance of *E. coli* O157:H7 as a foodborne pathogen

The Centers for Disease Control and Prevention (CDC) estimate that 10,000-20,000 cases of *E. coli* O157:H7 infection occur in the USA each year (CDC, 1993) with a calculated incidence of 4-8 infections per 100,000 persons per year. This is an approximation of the real number of *E. coli* O157:H7 cases and the true incidence is probably much higher due to illnesses not reported. It might appear that with this low incidence *E. coli* O157:H7 would not be such an important foodborne pathogen. However, the potentially life threatening diseases associated with it, namely hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), are the cause of public awareness, especially after an *E. coli* O157:H7 associated outbreak in a fast-food restaurant chain in 1993 which caused over 500 illnesses and 4 deaths (Bell *et al.*, 1994; Griffin and Tauxe, 1991). After that outbreak, *E. coli* O157:H7 was included in the group of major foodborne pathogens and remains in this group due to steadily increasing numbers of O157:H7-associated cases and foodborne outbreaks (Brotman *et al.*, 1994).

Unfortunately, *E. coli* O157:H7 is not the only *E. coli* serotype implicated in HC and HUS cases. The pathogenicity of this organism is attributed to the production of Shiga-like toxins 1 and 2 (Stx1 and Stx2), but the exact mechanisms involved in disease are not clear (Griffin and Tauxe, 1991, O'Brien *et al.*, 1992). Other *E. coli* strains are known to produce the same toxins and some sporadic cases of bloody diarrhea and HUS have been associated with these Stx-producing *E. coli* (STEC) strains (Robson *et al.*, 1993). The report of a 1994 outbreak of acute gastroenteritis characterized by bloody diarrhea was attributed to *E. coli* O104:H21 strain producing Stx2 (U. S. Department of Health and Human Services, 1995), indicating that public health workers should be aware of other *E. coli* organisms which are similar to O157:H7.

The food source implicated in most of the *E. coli* O157:H7 outbreaks has been undercooked ground beef, but unpasteurized milk and apple cider as well as salad, raw potatoes, fermented sausage, mayonnaise, and water have also been associated with O157:H7 illness (Centers for Epidemiology and Animal Health, 1994a). The information collected from numerous O157:H7 cases involving food of bovine origin and the continuous presence of *E. coli* O157:H7 in national cattle herd surveys indicate that cattle are a major reservoir of O157:H7 (Wells *et al.*, 1991; Whipp *et al.*, 1994). Experimental infection of cattle doesn't produce signs of the disease, however, O157:H7 colonizes the intestinal tract (Cray and Moon, 1995). When shed in environment, *E. coli* O157:H7 survives well in normal or low temperatures but is destroyed by heat. Human infection with O157:H7 occurs primarily through ingestion of raw or undercooked food contaminated with bovine fecal material.

It is not yet known what specific factors contribute to increased colonization and shedding of *E. coli* O157:H7, what the transmission patterns within cattle are, if any, and whether or not other animal reservoirs exist. Therefore all prevention measures have been focused on the post-harvest control of *E. coli* O157:H7 to ensure that bovine origin products are well cooked and that no product crosscontamination occurs during food preparation.

Disease surveillance has been heightened in the U.S. to record *E. coli* O157:H7 cases and outbreaks. Hazard Analysis Critical Control Point plans which recognize critical points for *E. coli* O157:H7 control are being implemented in food processing and production establishments. Efforts to educate food producers and consumers have been employed frequently; nevertheless, the measures taken to prevent *E. coli* O157:H7 infection are still inadequate. Currently, the consumers' protection is in their own hands -- they can protect themselves from O157:H7 and other foodborne pathogens through proper food handling and cooking. Meanwhile, industry, together with agencies of the USDA and researchers, are exploring O157:H7 control means at all levels: farm, slaughter plants, meat processing plants, distributors, retailers and restaurants. Much knowledge has been gained in the past 15 years regarding the nature of O157:H7 and other STEC and the diseases they cause; yet more knowledge about the epidemiology and ecology of these organisms is necessary to assure safe food for consumers.

E. coli O157:H7 within a group of pathogenic *E. coli*

Most strains of *E. coli* are harmless commensals and necessary components of intestinal flora, but others are virulent pathogens which affect the intestine or extraintestinal sites (Brenner, 1984). In recent years human pathogenic *E. coli* serotypes have been divided into 5 groups, depending on their virulence mechanisms and the disease produced (Levine, 1987):

1) enterotoxigenic (ETEC); if they are non-invasive and produce secretory toxins that do not damage the host's mucosal epithelium. ETEC cause infantile diarrhea and traveler's diarrhea;

2) enteroinvasive (EIEC); if they penetrate and disrupt the host's epithelial cells. EIEC produces bacillary dysentery;

3) enteropathogenic (EPEC); if they adhere to epithelial cells in localized microcolonies and cause attaching and effacing lesions. EPEC cause enteric disease, which is, particularly in small children, characterized by watery diarrhea with mucus but not blood;

4) enterohemorrhagic (EHEC); if the strains are associated with bloody diarrhea;

5) enteroaggregative *E. coli* (EaggEC); if they adhere to epithelial cells in a pattern resembling brickwork. EAggEC has been associated with chronic diarrhea.

E. coli O157:H7 is included in the group of EHEC due to its frequent association with bloody diarrhea cases. However, clinical studies and human stool screening indicate that *E. coli* O157:H7 is often present in the stools of patients with non-bloody diarrhea. Furthermore, the classification leaves out other Stx-producing *E. coli* (STEC) strains which also are capable of causing diarrhea ranging from mild to severe, with or without the presence of blood (Tarr, 1995).

Genetic relationship between the O157:H7 serotype and other *E. coli*

A study by Whittam *et al* (1988) examined 100 Stx-producing *E. coli* strains by multilocus enzyme electrophoresis. The electrophoretic-typing is based on genetic variation in loci that encode a variety of enzymes. Proteins in extracts from bacterial lysates are separated by horizontal starch gel electrophoresis and stained for specific enzymes. Based on different mobilities of variants of an enzyme, distinctive patterns can be identified and used to group and to relate isolates (Sealander *et al.*, 1986). In Whittman's study the 100 strains, including 75 of serotype O157:H7, represented isolates from North America and Great Britain recovered mostly from patients with HC and HUS. The results established that isolates of serotype O157:H7 have descended from a clone that is widely distributed in North America and, as determined by a genetic comparison with isolates of other *E. coli* serotypes, was only distantly related to other Stx-producing *E. coli*. Furthermore, these researchers demonstrated that O157:H7 strains are only distantly related to other strains of the O157 group associated with enteric infections in animals (Whittman and Wilson, 1988). In a later study Whittman *et al* (1993) examined 1,300 isolates of pathogenic *E. coli* serotypes from EHEC, ETEC, and EPEC groups. Comparisons of the multilocus enzyme profiles revealed that, genetically, the O157:H7 clone is most closely related to a clone of the O55:H7 strain which belongs to the group of EPEC and has long been associated with worldwide outbreaks of infantile diarrhea. They proposed that *E. coli* O157:H7 emerged when an O55:H7-like progenitor, already possessing a mechanism for adherence to the intestinal cell, acquired secondary virulence factors such as bacteriophage-encoded Shiga-like cytotoxins and plasmid-encoded adhesins. It is not known when this change occurred or what ecological conditions stimulated emergence of this *E. coli* O157:H7 clone.

Historically, O157:H7 has been a serotype rarely isolated from humans or animals. The CDC detected only a single strain of O157:H7 in more than 3,000 serotyped in the period from 1973 to 1982 and the *E. coli* Reference Center at Pennsylvania State University reported no serotype O157:H7 in more than 20,000 serotyped cultures isolated from animals (Riley *et al.*, 1983).

E. coli O157:H7 biochemical, growth and survival characteristics

As a typical *E. coli*, the O157:H7 is a gram negative, rod-shaped, bacterium which ferments lactose and produces characteristic colonies on differential media such as MacConkey's agar (Farmer, 1995). However, this organism has a number of properties which distinguish it from the majority of *E. coli*. For example, more than 90% of O157:H7 isolates fail to ferment sorbitol within 24 hours when grown on agar containing sorbitol such as Sorbitol MacConkey (SMAC) (Farmer and Davis, 1985). Thus, this media, in which O157:H7 forms colorless colonies while sorbitol fermenting bacteria appear red, is extensively used for cultural detection of O157:H7. Another distinguishing characteristic of O157:H7 is the lack of β-glucuronidase activity which by contrast is present in 93% of *E. coli* of other serotypes (Feng and Hartman, 1982). The production of β-glucuronidase by bacteria is the basis for rapid fluorogenic assay; glucuronide, incorporated in media is hydrolyzed to a fluorogenic end product by the enzyme. *E. coli* O157:H7 produces inactive β-glucuronidase; therefore no fluorescence is observed in the assay. This feature, in addition to the utilization of SMAC, is also employed in cultural detection of *E. coli* O157:H7.

E. coli O157:H7 grows poorly at temperatures used for culturing coliforms from foods, i.e., 44°C-45°C; instead it grows the best at 30°C-42°C, with 37°C being optimal (Doyle and Shoeni, 1984). The organism has no unusual heat resistance as determined in experiments with contaminated milk and ground beef. No *E. coli* O157:H7 were found in milk after heating to 63°C (D'Aoust *et al.*, 1988), nor were any O157:H7 found in contaminated ground beef heated at 64.3°C for 9.6 sec (Doyle and Shoeni, 1984). Survival studies of *E. coli* O157:H7 in frozen meat showed that in ground beef frozen at -80°C and held at -20°C it can survive for up to 9 months without a major change in numbers (Doyle and Shoeni, 1984). Jackson *et al.* (1996) studied the influence of storage and holding temperatures on *E. coli* O157:H7 survival in ground beef patties. Patties were contaminated with the bacteria and stored at 3°C or 15°C for 9 hours or at -18°C for 8 days and then held at 21°C or 30°C for 0 or 4 hours. For all grilling temperatures (54.4°C-68.3°C), *E. coli* O157:H7 was most heat resistant in frozen patties, while the bacterium in patties stored at

15°C were the most heat sensitive. Holding patties at 21°C or 30°C prior to grilling resulted in increased sensitivity of the organisms to heat. The authors proposed that the greater survival of *E. coli* O157:H7 during the grilling of frozen meat patties could be a result of a slower rate of heating during cooking or of physiological changes within the bacterial cells as a result of freezing.

The influence of pH on survival or growth of *E. coli* O157:H7 has been determined in enrichment media such as Trypticase soy broth (TSB) and in fermented, dry sausage during its production and storage. The organism grew in TSB at a pH 4.6 to 9.0 and survived but didn't grow in fermented sausage at pH 4.8 when stored at 4°C. A two log reduction was achieved after storing the contaminated sausage for two months (Glass *et al.*, 1992). When *E. coli* O157:H7 cells were adapted to acidic conditions, it was found that these cells had increased resistance to lactic acid and survived better than nonadapted cells in dry salami (pH 5.0) and apple cider (pH 3.4) (Leyer *et al.*, 1995). Results of *E. coli* O157:H7 survival studies in apple cider revealed that the bacteria were capable of survival in fresh apple cider at 20°C for more than 10 days, while alcoholic fermentation of fresh cider was an effective means of destroying this pathogen (Semanchek and Golden, 1996). The pH of fermenting cider was not statistically different from that of nonfermenting cider throughout the study period. Inactivation of *E. coli* O157:H7 in fermenting cider was attributed to the combined effects of pH and ethanol.

Another aspect of bacterial acid resistance is the ability to survive acidic environments while passing through the gastrointestinal tract to cause disease. Recent studies of acid resistance mechanisms of *E. coli* O157:H7 have revealed that several systems potentially exist, such as an acid-induced oxidative system, an acid-induced arginine-dependent system, and a glutamate-dependent system; all of which were able to protect *E. coli* O157:H7 against pH 2.5 (Lin *et al.*, 1996). Of importance, was the finding that once induced, the acid resistance systems remained active for prolonged periods (more that 28 days) of cold storage at 4°C.

Virulence attributes of *E. coli* O157:H7

Several factors, such as the production of Shiga-like toxins, hemolysin, and heat-stable enterotoxin as well as the ability to colonize the intestinal tract, are believed to determine the pathogenicity of *E. coli* O157:H7. The relative importance of these factors is not clear.

Shiga-like toxins

Shiga-like toxin 1 and 2 (Stx1 and Stx2), also called verocytotoxins due to their toxicity to Vero cells, are produced by all *E. coli* O157:H7 isolated from patients with diarrhea. The majority of *E. coli* O157:H7 strains express both toxins, but strains exist which express only one of them. Stx1 is completely neutralized by antibodies to Shiga toxin produced by *Shigella dysenteriae* and DNA sequence analysis has revealed 99% homology in the genes encoding for the toxin production. Stx2 is only partially neutralized by antibodies to Shiga toxin and shares approximately 66% DNA homology (O'Brien and Holmes, 1987; O'Brien *et al.*, 1992). Thus, Stx2 is genetically related to but antigenically distinct from Stx1. Also the sequence and antigenic variability within Stx2 is greater than that of Stx1, and a growing number of closely related Stx have been identified that belong to the Stx2 family (Gyles, 1992). Genes for both Stx1 and Stx2 toxins are encoded by bacteriophages while the Shiga toxin gene of *S. dysenteriae* is found on chromosomal DNA.

Structurally, Shiga toxin and Shiga-like toxins consist of one A subunit and 5 B subunits. The A subunit contains an enzymatically active molecule and the B subunit binds to glycolipid receptors on the cells (Gb3). After internalization, the A subunit is cleaved to a smaller A1 fragment which targets 60S ribosomes and causes inhibition of protein synthesis and cell death (O'Brien and Holmes, 1987; O'Brien *et al.*, 1992). Histopathologic examinations of human and animal tissues suggest that the target cells for toxin action are vascular endothelial cells (Obrig *et al.*, 1988). In a recent report Ramegowda and Tesh (1996) proposed that proinflammatory cytokines, such as tumor necrosis factor alpha and interleukin 1β, could sensitize the target cells to the toxic action of Stxs by upregulating Gb3 expression on endothelial cells.

It is not known whether Stx1 action differs from that of Stx2 since strains producing either toxin or both have been implicated in hemorrhagic colitis and HUS cases. Interestingly, *in vitro* experiments comparing Stx effects on cultured human umbilical vein and human renal microvascular endothelial cells demonstrated that Stx2 was 1000 times more potent as a cytotoxic agent than Stx1 toward renal endothelial cells, despite the fact that these cells could bind 10 times more Stx1 than Stx2. The preferential action of Stx2 was specific for renal endothelial cells, as human umbilical vein endothelial cells were almost equally affected by Stx1 and Stx2 (Louise and Obrig, 1995). In HUS animal model studies, Tesh *et al.* (1993) demonstrated that Stx2 was more lethal for mice than Stx1. These observations of a greater toxicity of Stx2 *in vitro* and animal model studies are supported by epidemiological data which suggest that Stx2 may also be more toxic *in vivo*, as STEC strains producing Stx2 only are more commonly associated with HUS than those producing Stx1 alone or Stx1 and Stx2 (Ostroff *et al.*, 1989b).

Adherence and attachment

While the structure and function of Stx genes and their products have been extensively studied, the mechanisms of bacterial adherence and colonization of the intestinal tract are less clear, but may include an initial contact step followed by more intimate attachment. The initial attachment is thought to be mediated by a 60-megadalton plasmid which encodes structural and regulatory genes for fimbriae (Karch *et al.*, 1987). However, the presence or absence of this plasmid in *E. coli* O157:H7 strain 933 did not appear to be important in mediating bacterial adherence in the cecum and colon of gnotobiotic piglets (Tzipori *et al.*, 1987). Waldolkowski *et al.* (1990) utilized a murine model of EHEC infection to study the importance of plasmid-encoded adherence factors in the development of hemorrhagic colitis. When streptomycin treated mice were individually fed either *E. coli* O157:H7 strain 933 or its plasmid-cured derivative, both strains colonized the mice. However, when both strains were simultaneously fed to the same type of mice, the plasmid-cured derivative was usually unable to co-colonize the intestinal tract, suggesting that the plasmid may encode factors important in establishing colonization.

The attachment factor, intimin protein, which provides closer adherence of EHEC to intestinal cells is encoded by the chromosomal *eae* gene and produces the attaching and effacing lesion. This adhesion pattern was first observed in association with EPEC isolates (Jerse *et al.*, 1990). The attaching and effacing lesion as caused by EPEC is characterized by a loss of intestinal epithelial cell microvilli and the intimate association of bacteria with cell membranes. The same lesions have been reproduced in a gnotobiotic piglet model (Tzipori *et al.*, 1988; 1989) and in cell cultures (Knutton *et al.*, 1989) infected with *E. coli* O157:H7; however, the attaching and effacing lesion has not been demonstrated in pathologic studies of human colonic specimens (Griffin *et al.*, 1990; Kelly *et al.*, 1987; Richardson *et al,*. 1988; Ryan *et al.*, 1986). Analysis of human and animal STEC isolates have shown that the *eae* gene is present in most *E. coli* O157:H7 strains, but is not restricted to this serotype of Stx-producing *E. coli*. A study of prevalence of the *eae* gene in STEC strains from dairy cattle in Ontario (Sandhu *et al.*, 1996) determined that both *eae*-positive and *eae*-negative STEC were present in this cattle population in high frequency. A study conducted by Louie *et al.* (1994) looked at the distribution of the *eae* gene in strains of STEC isolated from cattle and humans in association with disease symptoms. They found that the majority of strains isolated from humans with bloody diarrhea or HUS and cattle with severe diarrhea were *eae* positive (82% and 83%, respectively). In contrast, 59% of STEC isolated from asymptomatic cattle were *eae* negative and of the remaining 41% that were *eae* positive, the majority were serotype O157:H7.

Recently another adherence factor, called "log jam" was found in EHEC (McKee and O'Brien, 1995) interactions with human ileocecal epithelial cells in an *in vitro* study. It was independent of the *eae* gene and was also shared by some non-pathogenic *E. coli* strains. Therefore, this ability did not appear to be associated with virulence but may represent a basic adherence mechanism that allows a variety of *E. coli* bacteria to bind to and colonize the human intestine whether or not the organism expresses additional specific adherence factors.

It is probable that not a single factor but a coordinated action of several factors determines the ability of *E. coli* O157:H7 to attach to intestinal epithelial cells. The importance of each factor in mediating adherence and colonization is not clear; however, it

has been proposed that the plasmid may be responsible for regulating chromosomally encoded genes involved in lesion formation (Tesh and O'Brien, 1992).

Heat stable enterotoxin and hemolysins

More possible virulence factors have been discovered in recent years. Some of them were first identified in other groups of pathogenic *E. coli* but later were found in EHEC such as *E. coli* O157:H7. For example, it was determined that some strains of enteroaggregative *E. coli* associated with persistent childhood diarrhea produce a low-molecular weight peptide referred to as EAggEC heat-stable enterotoxin (EAST1) (Savarino *et al.*, 1991). When the distribution and prevalence of the *EAST1* gene in selected *E. coli* groups including EHEC were determined by colony hybridization (Savarino *et al.*, 1996), the results revealed that all 75 tested *E. coli* O157:H7 hybridized with an EAST1 DNA probe and 41% of ETEC, 41% of EAggEC, and 22% of EPEC isolates also had this gene. Thus, it was shown that the *EAST1* gene is broadly distributed among diarrheogenic *E. coli* and may represent an additional determinant in the pathogenesis.

The production of phage-encoded enterohemolysins (Ehly1 and Ehly2) and the possession of a large plasmid encoding for a pore-forming type of hemolysin have been associated with human pathogenic STEC strains; however, no definitive role of these factors as necessary and sufficient virulence markers has been demonstrated (Bauer and Wech, 1996; 1994; Beutin *et al.*, 1993; Schmidt *et al*). When STEC strains (total of 208) originating from healthy cattle, sheep, goats, pigs, cats, and dogs were investigated for these properties, it was found that 61.5% were carrying DNA sequences specific for the plasmid-encoded hemolysin but only 14.4% had bacteriophage-associated enterohemolysin-specific sequences (Beutin *et al.*, 1995).

Pathogenesis of *E. coli* O157:H7 infections

In 1983 Karmali and colleagues proposed that Stx is the direct etiologic agent in the pathogenesis of both HC and HUS. Numerous toxin studies in animal models and *in vitro* cell cultures indeed support this hypothesis. Rabbits injected with Stx1 developed thrombotic microangiopathic lesions similar to those seen in humans with HUS (Richardson *et al.*, 1988, 1992) supporting *in vitro* observations of a direct cytotoxic effect of Stx1 in vascular endothelial cells (Obrig *et al.*, 1987). When partially purified Stx from culture filtrates of *E. coli* O157:H7 or an Stx-producing strain of *E. coli* O157:H7 were intragastrically administered to infant rabbits, all rabbits produced the same histopathologic lesions in mid- and distal colon, providing evidence that a toxin plays a role in pathogenesis (Pai *et al.*, 1986). Marques *et al.* (1986) studied numerous *E. coli* strains for their cell-associated cytotoxicity by testing culture supernatants for cytotoxic effects on HeLa cells. They classified strains according to the level of cell-associated toxin produced. Of 418 strains analyzed, 10 strains produced moderate levels of cell-associated cytotoxin and 39 strains made high levels of cytotoxin. All but one of 49 strains were associated with diarrhea, hemorrhagic colitis, or HUS, and all of them made only Stx1 or Stx2, or both. This finding indicated that such elevated levels of cytotoxin may play a role in the pathogenesis of the HC and HUS.

Current thinking is that *E. coli* O157:H7 releases Stx in the intestinal tract which is then absorbed into the circulation producing vascular endothelial damage with subsequent intravascular coagulation and fibrin deposition and ultimately resulting in various clinical features of *E. coli* O157:H7 infection (Su and Brandt, 1995). It has been proposed that microvascular thrombi form through a direct cytotoxic effect on vascular endothelium or a direct effect on platelet aggregation (Morrison *et al.*, 1986). Ischemic changes precipitated by platelet-fibrin thrombi in the colonic microvasculature result in hemorrhagic colitis but their more disseminated distribution and location in kidneys result in HUS. Hemolytic anemia as it is observed in HUS results from the passage of red blood cells through damaged blood vessels, leading to fragmentation of RBCs as they impinge on intraluminal strands of fibrin. The damaged cells are then removed by the reticuloendothelial system.

Clinical manifestations of *E. coli* O157:H7 in humans

The spectrum of clinical manifestations of *E. coli* O157:H7 infection is very broad and can range from asymptomatic and self-limiting to painful and severe bloody diarrhea with life threatening complications -- hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). The syndromes are well described, but the factors determining the nature of infection are not fully understood. It is most likely that an interaction of host and bacterial factors is responsible.

Nonbloody diarrhea and asymptomatic infections

The frequency with which *E. coli* O157:H7 causes nonbloody diarrhea is uncertain because of the tendency of patients with nonbloody diarrhea not to seek medical care and physicians' tendency to only perform stool cultures on more severely affected patients. In community outbreaks, nonbloody diarrhea is suspected to occur in a larger percentage of patients than is verified in the laboratory because severe cases are more likely to be identified. In reported outbreaks, the frequency of nonbloody diarrhea has ranged from 10 to 65% (Bell *et al.*, 1994; Swerdlow *et al.*, 1992). However, data from laboratories where all stools submitted for bacterial culture were screened for *E. coli* O157:H7 suggested that more than 90% of the cases of diarrhea caused by this organism were indeed bloody (MacDonald *et al.*, 1988). Patients infected with *E. coli* O157:H7 whose stools do not become bloody have less severe illness; in one study, the duration of diarrhea, number of stools per day, and proportion of patients with abdominal cramps, vomiting, and fever were all lower in patients with nonbloody diarrhea (Spika *et al.*, 1986).

Cases of asymptomatic *E. coli* O157:H7 infections have occasionally been detected in outbreaks. In a study of *E. coli* O157:H7 infections in a kindergarten group who drank raw milk at a dairy, *E. coli* O157:H7 was isolated from the stool of an asymptomatic child (Ontario Disease Surveillance Report, 1986). Asymptomatic *E. coli* O157:H7 infection was also reported in a nurse who cared for ill residents of a nursing home (Canadian Disease

Weekly Report, 1983) and in a resident who ate hamburger implicated in another *E. coli* O157:H7 nursing home outbreak (Ryan *et al.*, 1986).

Hemorrhagic colitis

Hemorrhagic colitis is the most often observed syndrome caused by *E. coli* O157:H7. It is characterized by severe abdominal cramps, bloody stools, little or no fever, and evidence of colonic mucosal edema, erosion, or hemorrhage in the absence of conventional enteric pathogens in the stools (Riley *et al.*, 1983). The incubation period is 3-9 days, after which the first symptoms noticed are abdominal cramps and nonbloody diarrhea. Bloody stools appear on the second or third day of illness and continue for 2-4 days. Vomiting occurs in about half of the patients, and low grade fever is reported to occur in fewer than one-third of the patients. In most cases, the disease is self-limiting and resolves over a few days without specific treatment. About 18% of hemorrhagic colitis cases are severe enough to require hospitalization because of dehydration, poor fluid intake, or the need for observation (Marks and Roberts, 1993). The major, and most severe, complication in about 2-7% of hemorrhagic diarrhea cases is the development of HUS or TTP, both life-threatening syndromes, but the factors predisposing these complication have not been identified. Patients whose diarrhea remains resolved for 2 or 3 days with no evidence of microangiopathic hemolytic anemia are considered to have avoided the HUS/TTP complication (Tarr, 1995). Other post-infectious gastrointestinal complications are very rare but have included colonic strictures and crampy abdominal pain for extended periods after the initial *E. coli* O157:H7 infection (Tarr, 1995).

Fecal shedding of *E. coli* O157:H7 by infected humans usually lasts about 4-7 days (Church *et al.*, 1995; Griffin *et al.*, 1988; Tarr *et al.*, 1990). Pai *et al.* (1984) reported a longer carriage in children than in adults. Their observation was supported by Belongia and colleagues (1993) investigation of transmission of *E. coli* O157:H7 in Minnesota child day-care facilities. The researchers reported the median duration of shedding 17 days (range 2-62 days) when 24 symptomatic, culture-positive children were subjected to follow-up study. The duration of shedding was similar in children with HUS or bloody diarrhea compared with those with non-bloody diarrhea. Intermittent fecal shedding of *E. coli* O157:H7 was found in

three of 24 children. Although these data show that *E. coli* O157:H7 is present in fecal specimens more than 2 weeks after the first symptoms, the larger studies indicate that an interval of no more than 7 days between the onset of symptoms and the collection of stool for *E. coli* O157:H7 culture appears critical for recovery of this pathogen (Church *et al.*, 1995; Griffin *et al.*, 1988; Tarr *et al.*, 1990).

No specific therapy has proven effective in patients with *E. coli* O157:H7 infection. Although the organism is found to be uniformly susceptible to most antimicrobial drugs effective against gram-negative bacteria, there are several reasons why antibiotic treatment is not recommended. Antibiotic treatment initiated early in *E. coli* O157:H7 infection would kill the bacteria but could also render the released toxin more available for systemic absorption (Walterspiel, 1992). In a prospective study in which trimetoprim-sulfamethoxazole was administered late after onset of symptoms, neither benefit nor harm was apparent in the treated group (Proulx *et al.*, 1992). Moreover, the results of one retrospective study indicated that patients who were treated with antibiotics during *E. coli* O157:H7 diarrhea faced the same or greater risk of developing HUS in comparison with those who received no antimicrobial agents (Ostroff *et al.*, 1989a; Pavia *et al.*, 1990). Antibiotic use prior to the onset of disease has been identified as a risk factor for poor outcome of *E. coli* O157:H7 infection (HUS complication) (Carter *et al.*, 1987) as was the administration of anti-motility agents during the course of the disease (Cimolai *et al.*, 1990).

Hemolytic uremic syndrome and thrombotic thrombocytopenic purpura

Hemolytic uremic syndrome and thrombotic thrombocytopenic purpura are sequelae of bloody or nonbloody diarrhea in approximately 2-7% of *E. coli* O157:H7 infections. HUS is characterized by hemolytic anemia, thrombocytopenia, and renal failure whereas thrombotic thrombocytopenic purpura has all the clinical features of HUS but with more prominent neurologic involvement and less severe renal injury (Ruggenenti and Remuzzi, 1990). Thrombi are found in small blood vessels throughout the body in both HUS and TTP. The proposed distinction between HUS and TTP involves the observation of more frequent and severe renal failure in HUS cases and more frequent and severe neurologic involvement in

TTP cases; however, these symptoms do not clearly separate the syndromes (Remuzzi and Garella, 1987). Therefore, to avoid confusion, TTP following severe diarrhea and HUS with neurological signs should not be differentiated but should be recognized as HUS cases (Griffin and Tauxe, 1991; Tarr, 1995).

HUS develops an average of one week after the onset of diarrhea with symptoms of pallor, oligouria or anuria, edema, and, in rare instances, seizures or a generalized hemorrhagic diathesis (Tarr, 1995). The syndrome is most likely to occur in young children and the elderly (Griffin and Tauxe, 1991), and is now considered to be the leading cause of acute renal failure in children. Approximately 10% of *E. coli* O157:H7 diarrhea cases in children under age 10 progress to HUS compared to a 2-7% rate of infection in the populations of all ages. In addition to age, risk factors favoring the development of the HUS include bloody diarrhea, fever, an elevated leukocyte count, and treatment with antimotility agents (Cimolai *et al.*, 1990); however, all of these factors are also directly associated with the more severe cases of hemorrhagic colitis. It is possible, but not proven, that the severity of disease depends on the amount of Stx produced and/or the host's susceptibility to Stx.

It is not possible to predict which *E. coli* O157:H7 diarrhea illnesses will result in HUS. Therefore, during the week after the onset of diarrhea, patients with documented infection should be monitored for the signs of pallor and oliguria which are indicative for HUS. During this period, blood cell counts and urinalysis should be used to monitor patients at high risk such as children less than five years old and the elderly over 65 (Boyce *et al.*, 1995b). The management of HUS is supportive and includes meticulous attention to fluid and electrolyte balance (Tarr, 1995). Approximately half the patients with HUS require dialysis, and three-quarters require transfusions of erythrocytes and/or platelets (Tarr *et al.*, 1989a). The mortality rate is 3-5% (Siegler *et al.*, 1994; Martin *et al.*, 1990; Tarr *et al.*, 1989a) and about 5% of patients who survive may have severe sequelae, such as end-stage renal disease or permanent neurologic injury (Siegler *et al.*, 1994).

Clinical illnesses associated with STEC serotypes other than *E. coli* O157:H7

Numerous reports of human illnesses associated with STEC strains other than *E. coli* O157:H7 indicate that these strains may cause diseases similar to or less severe than those caused by *E. coli* O157:H7. The severity of diseases caused by non-O157:H7 STEC vary in the same range as O157:H7 infections from mild to bloody diarrhea with occasional HUS complications; however, this severe manifestation is seldom observed. The reported incidence of STEC clinical illnesses is much lower than that for *E. coli* O157:H7 infections. Analysis of stool specimens from 5,415 patients in a two-year prospective study in Alberta (Canada) indicated that 130 patients were infected with *E. coli* O157:H7 strain but another 29 were infected with STEC non-O157:H7 strains, including O26:H11, O103:H2, O91:H-, O145:H-, O111:H-, O38:H21, O6:H-, O5:H- and O?:H21. Severity of disease varied from one strain to another, but only 42% of those individuals infected with the non-O157:H7 strains had bloody diarrhea compared with 98% of those infected with O157:H7. According to another prospective study conducted in Seattle, Washington, 13 of 445 patients with diarrhea had *E. coli* O157:H7 infections, whereas 5 were infected with various STEC other than O157:H7 strains, including O26:H-, O153:H2, O68:H-, and O-:H11. Similar to the results from other studies, the non-O157:H7 strains were associated with bloody diarrhea less frequently than were the O157:H7 strains (Bokete *et al.*, 1993). A recent report indicates that non-O157:H7 strains may be involved in outbreaks. Eleven confirmed and 7 suspected cases of bloody diarrhea were attributed to Stx-producing *E. coli* serotype O104:H21 in Helena, Montana (U. S. Department of Health and Human Services, 1995).

Prospective and retrospective studies of association of STEC with HUS show that although *E. coli* O157:H7 is a major cause of this syndrome, some other STEC serotypes are involved. A prospective study of exposure to STEC among Canadian children with HUS (Rowe *et al.*, 1993) studied 34 patients. *E. coli* O157:H7 was isolated from the stools of 26 along with 2 STEC other than O157:H7, namely O55:H7 and O111:NM. The latter strain was also involved in a family infection which caused diarrhea and led to one case of HUS. Additionally, in the last five years sporadic cases of diarrhea and HUS associated with *E. coli*

strains O101 and an O9ab:H-producing a variant of Stx2 (called Stx2e and known to be produced by porcine edema *E. coli* strains but not implicated in human disease) have been reported (Pierard *et al.*, 1991; Thomas *et al.*, 1994). These findings suggest a need for evaluation of the frequency of Stx2e-producing *E. coli* in human infections and investigation of the pathogenic mechanisms of these strains.

Immune response to STEC infections

Patients with *E. coli* O157:H7 infection show an increase in Stx1 and Stx2 neutralizing antibody titers (Karmali *et al.*, 1983, 1985b). This serologic finding supports the idea that Shiga-like toxins are important in *E. coli* O157:H7 infection and suggests that antibodies to Stx may play a role in the pathogenesis of the disease. Questions addressing the protective nature of these antibodies and duration of immunity in human patients need to be answered. Several HUS studies have measured the level of IgM and IgG antibodies against Stx1 and Stx2. The antibody titers ranged from 4 to 80 in acute serum specimens collected between days 4 and 18 after the onset of illness; they ranged from 32 to 1280 in convalescent serum specimens collected between days 13 and 43 (Karmali *et al.*, 1983, 1985b), demonstrating a 4-fold rise in convalescent sera. In contrast to these studies, a multicenter study of HUS in a pediatric population in Europe conducted by Bitzan *et al.* (1993) revealed that a 4-fold titer rise against Stx1 was observed in only 6% of patients. Interestingly, the comparison of Stx2 titers in HUS patients and controls showed that the sera from both these groups neutralized Stx2. The Stx2 neutralizing activity of normal human sera was retained even after selective removal of major immunoglobulin classes. Therefore, further studies are needed to define the Stx2 neutralizing principle in patients with HUS and in normal sera as well as its functional role in STEC infections.

Animal studies have demonstrated the presence of Stx antibodies in serum of animals infected with *E. coli* O157:H7. The life span of these antibodies is unknown (Pirro *et al.*, 1995). In experimentally challenged calves previous infection did not prevent reinfection by

the same strain of *E. coli* O157:H7 (Cray and Moon, 1995) when calves were reinfected 13-14 weeks after the detection of the last positive fecal sample. However, the calf model may not be an appropriate model to draw conclusions from regarding the immunogenic properties of *E. coli* O157:H7 because only occasionally is this bacteria associated with clinical disease in the bovine species.

Additional information about the immunologic nature of Stx comes from studies in weaning pigs of edema disease caused by *E. coli* strains producing a variant of Stx2. It has been shown that the clinical manifestations of edema disease, such as palpebral edema and neurologic dysfunction, result from microangiopathy and vascular necrosis which are attributed to Stx2e action (Gannon *et al.*, 1989; Marques *et al.*, 1987). Recently, Bosworth *et al.* (1996) reported the prevention of clinical edema disease by vaccination of pigs with genetically modified Stx2e toxin; thus demonstrating the protective role of antitoxin immunity. On the basis of these observations, it can be proposed that Stx antibodies may have a defensive role in human *E. coli* O157:H7 infections if they are present in the body prior to the toxins produced by the bacteria reaching their receptors.

Epidemiology of *E. coli* O157:H7 and other STEC infections

E. coli O157:H7 outbreaks

The history of *E. coli* O157:H7 began with the 1982 outbreaks of hemorrhagic diarrhea in Michigan and Oregon linked to consumption of fast-food hamburgers contaminated with, as it was then called, a rare type of *E. coli*. (Riley *et al.*, 1983). Forty-seven people became ill with HC, but none progressed to HUS. Analysis of *E. coli* O157:H7 isolated from the stools of the affected persons matched that of the *E. coli* O157:H7 strains isolated from hamburgers, indicating that undercooked hamburgers were the source of infection. During the mid-1980s, *E. coli* O157:H7 outbreaks were identified not only in the United States but also in Canada and the United Kingdom, causing awareness of this worldwide emerging pathogen which led to measures of increased surveillance. From 1982

till 1992, a total of 18 O157:H7 outbreaks were documented in the U.S.; an outbreak being defined by CDC as an incident in which two or more persons experienced similar illness and epidemiological analysis implicated a common exposure. During this time, an average of 2-3 outbreaks per year of *E. coli* O157:H7 infections were investigated, and *E. coli* O157:H7 became a well-recognized human pathogen involved in hemorrhagic colitis and HUS cases. The majority of the outbreaks occurred in communities (6) and were linked to a common source such as contaminated bovine products (3), apple cider (1), unchlorinated water (1), and swimming in a lake (1). Nine outbreaks reported from institutional settings -- a nursing home, 4 day-care centers, 3 schools and a mental institution -- revealed that secondary person-to-person transmission is an important means for spread of infection. Several conclusions were drawn from these outbreaks. The high proportion of outbreaks in institutional settings indicate that children and older people may be more prone to *E. coli* O157:H7 infection upon exposure. The evidence of person-to-person transmission indicates that the infectious dose is probably low. Nine outbreaks (50%) were directly linked to the consumption of bovine-origin products. Cross-contamination with cattle feces was suspected in outbreaks associated with apple cider, vegetables, municipal water and lake water.

A major event that drew wide public attention was a multi-state outbreak in 1992-1993 (CDC, 1993; O'Brien *et al.*, 1993; Dorn, 1993). According to the reports, 583 persons were affected across four states, with 477 cases in Washington state. Most patients had eaten at a restaurant, which is a part of a chain of hamburger restaurants, or had close contact with a confirmed case. In all, 171 patients required hospitalization, 41 developed HUS and four children died. An extensive microbiological study was conducted on isolates of *E. coli* O157:H7 from the multistate outbreak to compare human isolates with isolates from meat as well as to compare outbreak *E. coli* O157:H7 isolates with those from sporadic cases in Washington state occurring at the same time as the outbreak. All isolates were examined by pulsed-field gel electrophoresis and phage typed. All 26 isolates from patients associated with the multistate outbreak and all 27 isolates from incriminated lots of hamburger meat had the same electrophoretic pattern and belonged to the same phage type. Of the sporadic case

isolates obtained during the outbreak, a variety of phage and electrophoretic types were found.

In 1993 and 1994, 46 outbreaks of *E. coli* O157:H7 infection were reported to the CDC (CDC, 1995). This dramatic increase was partially influenced by measures taken to heighten surveillance, i.e. increased reporting of and screening for *E. coli* O157:H7 infection by laboratories. In June 1993, the Association of State and Territorial Public Health Laboratory Directors, in a joint resolution with the Council of State and Territorial Epidemiologists, recommended that clinical laboratories screen at least all bloody stool specimens for *E. coli* O157:H7 with sorbitol-MacConkey medium (Council of the State and Territorial Epidemiologists, 1993). As a result, more small outbreaks were identified; for example, of 16 outbreaks reported in 1993, 92% involved 11 or fewer confirmed cases. At the end of 1993, 18 states required reporting of O157:H7 cases; by January 1995, *E. coli* O157:H7 infection was a reportable disease in 33 states (CDC, 1995). In 1996, this number reached 41. However, not all clinical laboratories screen submitted stool samples for *E. coli* O157:H7. A nationwide survey of 230 randomly selected clinical laboratories determined that only 54% of laboratories were screening bloody stools for *E. coli* O157:H7 (Boyce *et al.*, 1995a).

Beef and bovine-origin products are the major source of *E. coli* O157:H7 infection; however, fast food restaurant involvement in outbreaks is not prevalent. More frequent are reports of unpasteurized apple cider as a source and a few recalls have been made (CDC, 1996). Outside the U.S. outbreaks have been documented in Canada, Europe, Australia, South Africa, Japan, Argentina, and Chile (Albert and Bettelheim, 1989; Browning *et al.*, 1990; Caprioli *et al.*, 1992; Cordovez *et al.*, 1992; Hamano *et al.*, 1993; Lopez *et al.*, 1989; Sramkova *et al.*, 1990). The most recent reports are of *E. coli* O157:H7 outbreaks in Japan -- source still unknown but more than 9,000 illnesses (Swinbanks, 1996); and in Scotland -- linked to the consumption of contaminated cooked meats. The total number of cases in Scotland was 409 with 17 deaths and no new cases were reported after the incriminated meat supplier was closed (IFST, 1997). To the author's knowledge, the two latest *E. coli* O157:H7 outbreaks in the U.S. were in the Fall of 1996, both associated with unpasteurized

commercial apple juice. In the western states these caused illness in 66 persons and one death, and in the northeastern U.S. 14 cases were identified (CDC, 1996).

Non-O157:H7 STEC involvement in outbreaks

In the U.S. there is only one reported outbreak attributed to non-O157:H7 STEC (U.S. Department of Health and Human Services, 1995). Eleven confirmed and 7 suspected cases with gastrointestinal illness symptoms of abdominal cramps, vomiting, diarrhea and bloody stools were identified. *E. coli* O104:H21 was isolated from the stools of patients in the absence of other gastrointestinal pathogens -- *Salmonella, Shigella, Campylobacter* and *E. coli* O157:H7. The isolated serotype O104:H21 produced Stx2. An epidemiological analysis indicated that drinking brand A milk 7 days prior to illness was associated with an increased risk. Although the organism was not isolated from the milk, investigation documented post-pasteurization contamination of milk products with fecal coliforms.

E. coli O157:H7 is the predominant strain implicated in outbreaks in the USA, Canada and the United Kingdom, but other STEC serotypes are more prevalent in Australia and some European countries (Italy, Germany). For instance, an outbreak due to an O111:NM strain led to 23 cases of HUS and one death in Australia (Bettelheim, 1996). The same serotype has been detected in association with sporadic cases in Italy since 1988 and in 1992 it was involved in a STEC outbreak (Caprioli *et al*, 1994).

Incidence of E. coli O157:H7 infections

The true incidence of human *E. coli* O157:H7 infections can only be estimated. The most reliable data come from prospective studies. These studies are limited to the defined populations which were investigated but may not represent the general population. On the other hand, data collected by the surveillance system provide a nationwide summary but are biased for several reasons: only persons who seek medical attention are screened, screening tests differ among clinical laboratories, and laboratories differ on screening; some screen all stool specimens, some only bloody stools.

The number of HUS cases could be used as an indicator of *E. coli* O157:H7 incidence because *E. coli* O157:H7 is the predominant enteric pathogen in sporadic HUS cases in the U.S. (Neill, 1989), with positive O157:H7 isolation ranging from 58 to 65%. This can be used to determine the possible incidence of *E. coli* O157:H7 infections prior to 1982. In Minnesota, the incidence of HUS in children less than 18 years old increased from 0.5 to 2.0 per 100,000 from 1979 through 1988 (Martin *et al.*, 1990). A retrospective study in Washington state indicated that HUS incidence may have been 2.5 times higher among children in 1980 than in 1976 (Tarr *et al.*, 1989a). These studies present evidence that *E. coli* O157:H7 was indeed a rare serotype before 1980 and that *E. coli* O157:H7 incidence has increased since then. However, a retrospective study of childhood HUS cases in Utah between 1971 and 1990 by Siegler *et al.* (1994) showed that periods of high HUS incidence had occurred, but there was no overall sustained increase in incidence. The mean annual incidence in Utah during this period was 1.42/100,000 children with a range between 0.2 and 3.4 per 100,000 children per year.

In a population-based study conducted in Washington state during 1985-1986 all submitted stool specimens were screened for *E. coli* O157:H7. Results revealed that the O157:H7 incidence rate was 8 per 100,000 per year (MacDonald *et al.*, 1988). Among the 4,539 patients who submitted stool specimens, *E. coli* O157:H7 was isolated in 25 cases (0.6%) and was the third most common cause of bacterial diarrhea after *Campylobacter* and *Salmonella.* In a state-wide study in Washington state in 1987, the incidence was only 2.1 cases per 100,000 persons per year, but many laboratories were not screening for the organism (Ostroff *et al.*, 1989a). Currently, the CDC estimates that from 10,000 to 20,000 cases of *E. coli* O157:H7 occur in the U.S. every year. This number is based on the prospective studies and the assumption that the true incidence is underestimated due to mild *E. coli* O157:H7 diarrhea cases not being diagnosed.

To determine the fluctuations in incidence of *E. coli* O157:H7 infections under conditions of steady surveillance, five year studies have been performed in Alberta, Canada and in Scotland. The enhanced surveillance for *E. coli* O157:H7 in these regions has been in place since 1987 due to high rates of STEC infections (Waters *et al.*, 1994). The mean annual

rates of *E. coli* O157:H7 infections were 12.1/100,000 and 2.0/100,000 for Alberta and Scotland, respectively. Over the five years fluctuations in the number of cases were seen in both areas. In Alberta, the number of cases was relatively constant during 1987-1989; thereafter, however, rates fell dramatically -- from 16.0/100,000 in 1989 to 10.0/100,000 and 8.4 in 1990 and 1991, respectively. The next year (1992) the rate was again up, to around 14.0/100,000 followed by a sharp drop in 1993 to 6.1 cases per 100,000. In Scotland, a slow increase in the incidence rate from 1987 until 1991 was seen, climbing from 0.2 to 4.0/100,000. In 1992-1993, by which time more laboratories had expanded their criteria for screening but no specific intervention measures had been taken, rates of *E. coli* O157:H7 had fallen to around half the 1991 figure (2.3 and 2.4, respectively, vs. 4.0). This study showed the incidence of *E. coli* O157:H7 infections varied between years with some periods of decline. The reason for a decrease in *E. coli* O157:H7 infection cases could be attributed to greater public awareness, with resulting improvements in food handling procedures. Other conditions independent of human factors may be a consideration; however, it is possible that no specific reason is responsible.

In the state of Iowa, *E. coli* O157:H7 cases have been reportable since 1989. During the period from 1989 until 1996, the State Hygienic laboratory identified 191 cultures of *E. coli* O157:H7 from cases throughout Iowa, with the largest numbers occurring in counties with greater populations (Moyer, 1996). A marked increase was seen in cultures identified during 1993-1996, but this is attributed to heightened awareness and improvements in laboratory techniques rather than changes in the epidemiology of the organism.

Outbreaks and sporadic cases of *E. coli* O157:H7 infection occur all year around; however, a considerable peak during the warmest months of the year is a consistent finding in the USA and other countries. Thus, *E. coli* O157:H7 incidence studies in Washington state in 1987 revealed that the peak incidence of cases occurred from June though August (Ostroff *et al.*, 1989a). In the five year review period from 1987 to 1991, the incidence of this infection in Alberta, Canada, as well as in Scotland, was consistently highest in the summer: 64-81% of cases occurred between May and September (Waters *et al.*, 1994). Also, analysis of *E. coli* O157:H7 cases in Iowa from 1993 through 1996 show that the number of hemorrhagic colitis

cases peaks between June and September (Moyer, 1996). This seasonal variation may reflect the ecology of the organism; but it may also be associated with greater human exposure to ground beef or other O157:H7-contaminated foods during the "cook-out" months and/or more improper handling such as temperature abuse or incomplete cooking of products such as ground beef during warmer months (Boyce *et al.*, 1995b; Centers for Epidemiology and Animal Health, 1994a).

Transmission of E. coli O157:H7

The most important means of *E. coli* O157:H7 infection is as a foodborne transmission. More than 50% of *E. coli* O157:H7 outbreaks have been associated with consumption of contaminated, undercooked beef products or unpasteurized milk. However, a wide variety of other foods, i.e., salad vegetables, cantaloupe, potatoes, salad dressing, mayonnaise, hot dogs, lunch meat, dry-cured salami, and apple cider account for the remainder of the outbreaks. Each outbreak has taught an additional lesson about *E. coli* O157:H7. Thus, frequent association of outbreaks with bovine-origin products and later animal studies suggested that cattle may be a reservoir of this organism. It was also recognized that most of the outbreaks were associated with food mishandling, such as insufficient cooking time or temperature, or cross-contamination of fresh and cooked products (Griffin and Tauxe, 1991). Cattle were suspected to be a primary cause of *E. coli* O157:H7 outbreaks linked to salad vegetables; in most cases it was determined that vegetables were cross-contaminated with fecal material or meat products containing *E. coli* O157:H7. Recently, unpasteurized apple cider has become a common source of the infection. The epidemiological analyses of the outbreaks have revealed that the apple cider was partially made from drop apples (apples picked from the ground) possibly exposed to cattle manure.

Person-to-person transmission is another well documented route of *E. coli* O157:H7 infection. It occurs after exposure to the primary case under conditions of improper hygiene, especially in day care centers and nursing homes. In these settings, primary cases usually are residents who have consumed contaminated food and have developed hemorrhagic diarrhea and then transmitted infectious organisms through close contact with other residents. Person-

to person transmission, as well as cross-contamination of vegetables from meat, indicate that the infectious dose is probably low. The exact amount of an infectious dose is not known. As few as 40 CFU/g have been found in implicated meat patties, and it has been suggested that 10-100 organisms would be a large enough inoculum to produce disease (Griffin and Tauxe, 1991; Roberts *et al.*, 1995).

A few large waterborne outbreaks attributed to *E. coli* O157:H7 have been reported (Dev *et al.*, 1991). A community outbreak in 1989 was due to a contaminated water supply (Swerlow *et al.*, 1992), and a few other waterborne outbreaks have been linked to swimming in contaminated lakes or pools (Brewster *et al.*, 1994; Hornsby-Lewis, 1995; Keene *et al.*, 1994).

Risk factors for human E. coli O157:H7 infections

Several factors may be associated with increased risk of human *E. coli* O157:H7 infections. The consumption of bovine products is currently the most important risk factor; however, despite the perception that *E. coli* O157:H7 infection is most likely to be contracted by eating hamburgers at fast-food restaurants and that home-cooked food is safer, the consumption of hamburger at home (in a noncommercial establishment) was identified in one study as a risk factor (LeSaux *et al.*, 1993). Although *E. coli* O157:H7 is found in very few samples of retail ground beef, consumers in general should be aware that food cannot be assumed safe unless it is cooked properly. The consumption of rare ground beef, defined as red or pink meat, certainly carries a risk of *E. coli* O157:H7 infection (Tarr, 1995).

Age is another significant determinant of *E. coli* O157:H7 infection and the outcome; the very young people and the elderly are at increased risk. The age-specific prevalence is highest in children under 5 years of age, gradually decreases with age, then increases again in persons age 65 or greater (Pai *et al.*, 1988). People at extreme ages are also most susceptible to the sequelae of *E. coli* O157:H7 infection, i.e., HUS and death from disease-associated complications.

If age is a risk factor, the residents of institutions such as daycare centers and nursing homes present a high risk population. Indeed, up to 30% of U.S. outbreaks have been related

to daycare centers, schools, mental health facilities, or nursing homes indicating that institutional settings may be an additional risk factor. However, age and institutional settings may be correlated factors as people associated with such institutions are likely to have similar dietary intakes; therefore, if *E. coli* O157:H7 is present in a food item, a potentially large number of people may be exposed. Person-to person transmission in daycare centers poses a particular risk to young children. In these settings, *E. coli* O157:H7 may be readily transmitted from person-to-person through improper diaper changing practices, inadequate toilet hygiene, and failure to exclude ill children.

E. coli O157:H7 and other STEC in foods

Direct evidence of food as a major vehicle for *E. coli* O157:H7 transmission comes from outbreaks where an identical *E. coli* O157:H7 has been recovered from both humans with the illness and a suspected food source. For example, the same strain of *E. coli* O157:H7 was isolated from ground beef and humans involved in the first O157:H7 outbreak; and the multistate outbreak analysis found the same O157:H7 isolate in humans and in ground beef. However, it is not always possible to obtain this evidence; therefore, thorough epidemiological analysis is the best tool for source identification.

A few local and national surveys of retail meats have been conducted. Doyle and Schoeni in 1987 surveyed retail, raw meats and poultry in Wisconsin and Alberta. The results revealed *E. coli* O157:H7 in 3.7% (6/164) of ground beef, 1.5% (4/264) of pork, 1.5% (4/263) of poultry and 2% (4/205) of lamb retail samples. The proportion of positive ground beef samples from Alberta, an area with a high incidence of *E. coli* O157:H7 infections and HUS, was particularly high -- 29% (5/17). In a national survey of raw meats obtained between 1987 and 1989, *E. coli* O157:H7 was isolated from 0.5% (7/1,478) bob veal kidneys whereas only 0.06% (2/3,475) fancy veal kidneys were positive (Griffin and Tauxe, 1991). Bob veal refers to male calves from dairy herds while fancy veal refers to milk-fed calves raised for veal; however, the difference (7 positive samples versus 2) is questionable. In this study, 0.12% (2/1,668) of raw beef samples were found to contain *E. coli* O157:H7.

In contrast to a very low isolation rate of *E. coli* O157:H7 from meat samples, the isolation rate for all STEC in retail meats is much higher as indicated by several studies. Of the 294 fresh meat, poultry and seafood samples purchased from Seattle area grocery stores, 17% were STEC positive (Samadpour *et al.*, 1994b). An earlier study of STEC in foods in Ontario surveyed beef and pork samples (Read *et al.*, 1990). It was found that 36.4% of beef and 10.6% of pork samples were positive; however, no STEC of serotype O157:H7 were isolated.

Recently (1995-1996), a nationwide pork microbiological data collection was completed in market hogs and no *E. coli* O157:H7 was recovered from any of the 2,112 carcasses (FSIS, 1996). A nationwide microbiological testing program of raw ground beef is underway (American Meat Institute, 1997).

Animal reservoirs of E. coli O157:H7 and other STEC

Zoonotic transmission of O157:H7 is thought to occur because of epidemiological associations of HC and HUS with consumption of ground beef and unpasteurized milk. Studies of cattle as reservoirs of STEC pathogenic for humans have included general surveys as well as investigations of cattle herds implicated as sources of human infections. In some of these studies, investigators have tested only for O157:H7, whereas others have looked for STEC in general. STEC studies reveal that cattle are colonized with a number of STEC serotypes (Chapman *et al.*, 1993; Hankock *et al.*, 1994; Wells *et al.*, 1991; Whipp *et al.*, 1994); however, most often they differ from human STEC isolates (Chapman and Siddons, 1994; Rietra *et al.*, 1989). The same conclusion was reached when STEC isolated from sheep, goats, pigs, dogs, and cats were compared with human STEC (Beutin *et al.*, 1995; Wray *et al.*, 1993). Surveys of *E. coli* O157:H7 in other animal species have not been extensive.

E. coli O157:H7 does not appear to be pathogenic in cattle and clinical disease associated with natural *E. coli* O157:H7 infection in cattle has not been documented (Wells *et al.*, 1991; Zhao *et al.*, 1995). Experimentally infected calves occasionally develop transient mild diarrhea shortly after inoculation; however, no significant gross or histological lesions

have been reported in these calves (Brown *et al.*, 1997; Cray and Moon, 1995). *E. coli* O157:H7 is capable of colonizing the intestinal tract, but the factors favoring it are not known. The prevalence of *E. coli* O157:H7 in individual cattle appears to be low, although the herd prevalence can vary significantly. Studies estimating the prevalence of herds with *E. coli* O157:H7 reported a range from 1.8 to 100%; however, some high herd prevalences were attributed to the fact that these premises were tracebacks associated with human cases. For example, of 11 dairies which were traced back and sampled in the Wells *et al.* study (1991), 63.6% (7) were found to be positive for O157:H7. Of 1,139 nontraceback dairies sampled in the same study, only 2.4% were positive. In a National Animal Health Monitoring System survey the prevalence of O157:H7 in preweaned dairy calves was analyzed by testing fecal samples collected from approximately 7,000 calves from over 1,000 operations in 28 states (Centers for Epidemiology and Animal Health, 1994b). Samples from a total of 25 calves from 19 farms tested positive for the organism; thus the individual animal prevalence of *E. coli* O157:H7 was 0.36% and herd prevalence was 1.8%. Repeated sampling of *E. coli* O157:H7 positive and negative herds revealed that the herd O157:H7 status is variable -- negative herds may have some positive animals identified the next time they are sampled or vice versa. Several studies have identified a greater prevalence of *E. coli* O157:H7 in weaned calves than in adult cattle. Garber *et al.* (1995) compared *E. coli* O157:H7 prevalence in preweaned and weaned dairy calves and found that calves were 3 times more likely to shed the bacteria after weaning than before weaning. Sample prevalence of *E. coli* O157:H7 in calves less than 8 weeks old was 1.4% and in calves 8 weeks or older was 4.8%. The cause of these prevalence differences are not clear. Several explanations have been proposed, such as increased stress just prior to weaning, dietary changes, and physiologic changes in the calf gastrointestinal system.

Studies of the duration of fecal shedding are particularly relevant to the epidemiology of *E. coli* O157:H7. Preliminary evidence suggests that cattle transiently shed O157:H7 in their feces and that the excretion period ranges from hours to weeks (Wells, 1991). In the experimental study done by Cray and Moon (1995), the pattern (magnitude and duration) of shedding of *E. coli* O157:H7 was evaluated in experimentally inoculated cattle. Fecal

shedding varied widely among animals of the same age group but persisted longer in calves than in adult animals. For example, 8 of 8 calves but only 2 of 9 adult steers had positive fecal cultures 7 weeks post inoculation. Most calves shed more than 10^6 CFU/g of feces, and some adults maintained this peak magnitude of shedding for several days during the first week post inoculation. In the same study, the various inocula were tested for establishing infection in adult cattle. *E. coli* O157:H7 was recovered from none of five steers infected with 10^4 CFU and from only 2 of the 5 adults inoculated with 10^7 CFU. The magnitude of shedding by the two infected animals was low (<5.0 CFU/g). Whether the same shedding patterns occur on farms is not known. But if the shedding pattern is similar to the one observed in the study, then heavy excretion of organisms may be required to maintain the infection within a herd. This may also occur if more susceptible animals or more virulent bacterial strains are present in a herd (Cray and Moon, 1995).

Several factors that could increase colonization and fecal shedding of *E. coli* O157:H7 have been proposed and studies conducted. It is well known that stressors such as weaning, dietary changes, shipping, fasting, and changes in immunological status and disease, predispose animals to shifts in indigenous microflora of the gastrointestinal tract (Tannock, 1983). Starvation or abrupt changes in the diet of adult animals have been associated with increases in all *E. coli* numbers (Brownlie and Grau, 1967). This process is thought to be regulated largely by volatile fatty acids produced by the anaerobic members of the large intestinal flora, which inhibit the growth of coliforms (Lee and Gemmel, 1972; Schaedler *et al.*, 1965). Dietary stress which alters the microbial flora of the intestinal tract presumably influences the anaerobe population and this decrease in anaerobes may allow rise of coliform numbers. Recent work has shown that O157:H7 grows poorly in the ruminal environment of a well-fed animal; however, the rumen contents of cattle held off feed for 48 hours did not inhibit the growth of O157:H7 (Rasmussen *et al.*, 1993). Thus, there is evidence that dietary stress may indeed influence the development, and presumably shedding, of O157:H7 in cattle.

Cattle starvation prior to slaughter and transportation are well defined stress factors; therefore, it has been suggested that animals at slaughter may shed increased numbers of *E. coli* O157:H7. Most cattle surveys have been done at the farm level and have found a very

low prevalence (Hancock *et al.*, 1994; Wells *et al.*, 1991; Whipp *et al.*, 1994; Wilson *et al.*, 1992; Zhao *et al.*, 1995). By comparison, in the few studies of abattoirs, a higher incidence of *E. coli* O157:H7 in cattle was observed (Chapman *et al.*, 1993). The effect of animal fasting was studied in experimentally infected calves (Brown *et al.*, 1997). A clear association between fasting and increased fecal shedding of *E. coli* O157:H7 was not evident in this study; however, it was observed that calves which shed low numbers of the bacteria prior to fasting had the most consistent increase in shedding of *E. coli* O157:H7 after fasting. Additionally, the researchers addressed questions about the sites of colonization of the organism. As previously reported, *E. coli* O157:H7 was confined to the gastrointestinal tract in inoculated calves. Within the gastrointestinal tract, the forstomachs were a primary site of *E. coli* O157:H7 localization and proliferation. Moreover, in two-culture negative calves, necropsied 21 and 25 days post inoculation, *E. coli* O157:H7 was found only in the rumen and omasum. These findings indicate that the primary sites of localization of *E. coli* O157:H7 in calves shedding low numbers of bacteria or shedding intermittently appear to be the rumen and omasum. Factors which influence the proliferation of *E. coli* O157:H7 in the forstomachs may ultimately determine the magnitude of fecal shedding (Rasmussen *et al.*, 1993)

Slaughter plants are the first critical control point because here *E. coli* O157:H7 enters the food chain. The exact mechanism of the contamination of ground beef is still unclear, but most likely it occurs through contamination of carcasses with fecal contents of slaughtered animals. Although O157:H7 is a surface contaminant on beef, the grinding process mixes surface material throughout a ground beef product. Cooking the surface of beef cuts is likely to kill O157:H7, but ground beef must be cooked to a higher internal temperature because of the possibility of O157:H7 contamination in the interior.

E. coli O157:H7 detection methods

Numerous methods have been developed for the detection and isolation of *E. coli* O157:H7 in foods and clinical samples. These methods can be divided into the following

categories: isolation of *E. coli* O157:H7; demonstration of Stx; presence of antibodies to Stx and/or O157 lipopolysaccharide, and demonstration of the presence of *E. coli* O157:H7 genetic material in specimens.

Routine methods used in clinical laboratories rely on biochemical features of serotype O157:H7 such as its inability to ferment sorbitol within 24 hours in sorbitol-MacConkey agar and the absence of active β-glucuronidase in MUG media (Smith and Scotland, 1993). Suspected isolates are tested with a latex agglutination test to confirm O157 and H7 antigens. However, use of biochemical characteristics as a base for diagnostic methods can lead to false positive or false negative results. This is very likely as there are some bacteria other than *E. coli* O157:H7 (e.g. *E. hermanii* and *E. coli* O157:H16) which can also be sorbitol negative, and there are some atypical isolates of O157:H7 which are found to be able to ferment sorbitol as well as produce β-glucuronidase. An additional limitation of using bacteriology for *E. coli* O157:H7 detection in clinical samples such as stools is that the rate of isolation decreases with delays in collection of stool samples; cultures obtained more than 6 days after the onset of illness or after administration of antibiotics often produce negative results.

Many modified bacteriological culture methods have been described in the literature. These procedures vary in the sensitivity, specificity, complexity and time necessary to complete them (Table 1). Generally the methods applied to specimens directly are not as sensitive as those applied after enrichment or immunomagnetic separation. The latter two procedures increase the sensitivity 10-100 times and allow the detection of contamination as low as 0.5 cells per g of food or feces.

The presence of *E. coli* O157:H7 in foods and fecal samples can be detected by colony immunoblotting and ELISAs utilizing specific polyclonal and monoclonal antibodies against O157:H7. These methods are faster than bacteriologic culturing, as they do not require further serologic or biochemical characterization of positive colonies; however, false-positive results may occur at higher rates. A summary of immunoblotting and ELISA methods for *E. coli* O157:H7 detection is presented in Table 2.

Shiga-like toxin detection in *E. coli* culture broth and in specimens such as feces and foods by cytotoxin assays and ELISAs are alternative methods of diagnosing *E. coli* O157:H7

TABLE 1. Bacteriological culture methods for *E. coli* O157:H7 detection

Specimen	Reference	Description	Time to complete	Comments
Bacterial isolates	Farmer and Davis, 1985	Single-tube semisolid medium containing sorbitol and H7 antiserum; strains grow in a characteristic pattern.	18-24 h	Not highly specific for *E. coli* O157:H7.
Food	Szabo *et al.*, 1986	Food homogenates plated onto medium composed of tryptone, sorbitol, bile salts, MUG, and bromscresol blue; observe characteristic colonies, need serological confirmation.	18-24 h	Detects ground beef samples inoculated with 10^3 to 10^4 CFU/g.
Ground beef	Okrend *et al.*, 1990a	Enrichment in *E. coli* broth supplemented with novobiocin, for 6-24 h, followed by plating on SMAC, and transfering suspect colonies to MUG media followed by biochemical or serological confirmation.	>72 h	Detects 5 CFU/g. Time consuming.
Ground beef	Okrend *et al.*, 1990b	Enrichment in modified *E. coli* broth, inoculation onto 3M Petrifilm™ *E. coli* count plates, immunoblot assay of colonies. Positive sample confirmation by isolating organism.	26-28 h	Detects 0.6 CFU/g 0% false-negatives and 2% false-positive results.
Ground beef	Okrend *et al.*, 1992	Enrichment, capture of *E. coli* O157:H7 by using O157 antibody coated magnetic beads, plating on SMAC. Serological identification of suspect colonies.	48 h	Detects 0.6-0.9 CFU/g.

TABLE 1. (continued)

Specimen	Reference	Description	Time to complete	Comments
Meat, milk and feces	Wright *et al.*, 1994; Chapman *et al.*, 1994	Enrichment (6 h) in buffered peptone water supplemented with vancomycin, cefsulodin and cefixime, subculture in cefixime tellurite SMAC directly and after immunomagnetic separation (IMS); serological confirmation of suspect colonies.	34 h	Detects 200 CFU/g without IMS and 2 CFU/g with IMS in meat and 100-1,000 CFU/ml of feces with IMS.
Stools	Chapman *et al.*, 1991	Direct plating of fecal samples onto SMAC or SMAC supplemented with rhamnose and cefixime (CR-SMAC), serological confirmation of suspect colonies.	24 h	Smaller numbers of sorbitol-negative non-*E. coli* O157:H7 colonies grew on CR-SMAC compared to SMAC (171 vs 404).
Feces	Sanderson *et al.*, 1995	Comparison of 3 enrichment (none; TSB+cefixime +vancomycin; TSB+C+V+ tellurite) and 3 plating protocols (SMAC; SMAC+ cefixime; SMAC+C+ tellurite); further identification of suspect colonies.	48 h	All broth enrichment protocols superior to direct plating. SMAC+CT -- the most sensitive plating medium.

TABLE 2. Immunoblotting and ELISA methods for *E. coli* O157:H7 detection

Specimen	Reference	Description	Time to complete	Comments
Food	Todd *et al.*, 1988	Food is filtrated through hydrophobic grid membrane (HGM) and membranes incubated on agar containing tryptone, bile salts, sorbitol, MUG, and bromcresol purple. HGM is immunostained with O157:H7 monoclonal antibodies (O-antigen specific).	24 h	Detects 10^1 - 10^3 CFU/g of food.
Food, feces	Szabo *et al.*, 1990	Same as above (Todd *et al.*, 1988) with an enrichment step in TSB prior to filtering.	40 h	Detects 1 CFU/g.
Food	Padhey and Doyle, 1991	Enrichment of food sample in selective media for 16-18 h followed by application of culture to a sandwich-ELISA. This procedure uses polyclonal antibodies specific for *E. coli* O157:H7 as the capture antibodies and the monoclonal antibodies for detection.	<20 h	Monoclonal antibodies reacted also with O26:H11 STEC. Detects as few as 0.2 CFU/g.
Ground beef	Kim and Doyle, 1992	Dipstic immunoassay: ELISA type assay (as described above, Padhey and Doyle, 1991) on a hydrophobic polyvinylidine difluoride-based membrane.	16 h	Detects 0.1 - 1.3 CFU/g, with a false-positive rate of 2%.
Bacterial culture	Toth *et al.*, 1991	Direct ELISA utilizing antiserum specific for 60-MDa plasmid of *E. coli* O157:H7.	3-4 h	In addition to *E. coli* O157:H7 also detects other STEC strains.

TABLE 2. (continued)

Specimen	Reference	Description	Time to complete	Comments
Ground beef and milk	Acheson *et al.*, 1996	Stx detection by commercial enzyme immunoassay after food sample overnight enrichment.	20 h	Detects both Stx simultaneously without differentiation; not specific for *E. coli* O157:H7; detects 1-4 CFU/g (ml).

and other STEC caused infections (Maniar *et al.*, 1990; Smith and Scotland, 1993; Weeratna and Doyle, 1991). Stx present in specimens can be detected directly by their cytotoxic effect on Vero cells or, alternatively, by testing Shiga-like toxin activity of *E. coli* strains isolated from the specimens. Early cytopathic effects (rounding and detaching of Vero cells) can be observed after one day of incubation; however, final readings are done after plates have been incubated for 3 or 4 days. Increased sensitivity of Shiga-like toxin assays can be achieved by adding polymyxin to the tested cell pellet (Karmali *et al.*, 1985a). Stx detection is not specific for *E. coli* O157:H7 unless it is followed by culturing. Moreover, after demonstrating the cytotoxic effect of the specimen or culture, it is necessary to confirm that these effects are indeed due to the presence of Stx. For that purpose, neutralization tests using antisera against Stx1 and Stx2 are used (Smith and Scotand, 1993). The advantage of Stx detection methods is their ability to identify any STEC; however, the disadvantage is the major time and labor requirement.

Serologic testing is based on increases in serum Stx-neutralizing or O157 lipopolyssacharide antibody titers during *E. coli* O157:H7 infections. A rise in Stx1 antibodies during the convalescent period is a good indication of recent exposure to STEC-

producing Stx1 and can be used as an additional diagnostic method (Karmali *et al.*, 1983, 1985b). However, the presence of Stx2 neutralizing antibodies in human serum has been detected in healthy individuals without known exposure to STEC, suggesting that human serum contains nonspecific Stx2 in vitro neutralizing substances (Bitzan *et al.*, 1993); therefore, the value of Stx2 antibody measuring is questionable. Serologic response to O157 lipopolysaccharides of *E. coli* O157:H7 has been reported and can be a useful adjunct for diagnosing *E. coli* O157:H7 infection (Bitzan *et al.*, 1991; Chart *et al.*, 1989, 1991; Yamada *et al.*, 1993). Serologic tests detecting antibodies to O157 are not highly specific to *E. coli* O157:H7 because the O157 antigen cross reacts with a few other organisms and also detects nontoxigenic and non-H7 strains of *E. coli* O157.

DNA probes and PCR-based techniques for detection of *E. coli* O157:H7 and other STEC are targeted to identify specific nucleotide sequences characteristic to Stx genes or other regions of STEC. They are generally more sensitive and more specific than culturing and require less time; but laboratory effort is greater. DNA hybridization assays (probes) utilize a short sequence of nucleotide bases that will bind to a single strand of DNA from the specimen or culture fixed on a nitrocellulose filter (Wolkott, 1992). Depending on the homology between the DNA of the bound bacteria and the probe, hybridization will produce double stranded DNA which will vary in stability. A perfect match in the sequence of nucleotides produces very stable double stranded DNA, whereas one or more base mismatches impart increasing instability that can lead to weak hybridization of strands. The event of hybridization is detected due to a label fixed to the probe. It can be labeled with radioactive isotopes such as ^{32}P, ^{35}S, or ^{125}I, enzymes such as alkaline phosphatase or radish peroxidase, or with biotin incorporated into the probe. Most of the DNA probes that have been developed target detection of different regions of *stx* genes in bacterial cultures or cultured specimens (Brown *et al.*, 1989; Karch and Meyer, 1989a; Newland and Neill, 1988; Samadpour *et al.*, 1990, 1994a; Scotland *et al.*, 1988; Thomas *et al.*, 1991; Willshaw *et al.*, 1987); therefore, they do not detect *E. coli* O157:H7 specifically. Samadpour *et al.* (1990) evaluated the application of DNA probes to detect STEC in food and fecal samples. The dot blot hybridization assay detected 1.3 CFU of STEC per g of meat after sample overnight

enrichment. The total time required to complete the procedure was 48 hours. Instead of *stx* genes, Levine *et al.* (1987) and Gicquelais *et al.* (1990) targeted their DNA probe to detect the 60-megadalton plasmid typically carried by *E. coli* O157:H7 and other enterohemorrhagic *E. coli*. This DNA probe detected 99% of all *E. coli* O157:H7 tested, but it also hybridized with other EHEC and therefore could not be used for specific STEC or *E. coli* O157:H7 detection.

Automation and wide application of PCR to bacterial cultures and clinical specimens stimulated development of numerous PCR procedures (Table 3) for *E. coli* O157:H7 and other STEC detection. Like DNA probes, most of the PCR methods have been targeted to amplify various segments of conserved sequences within *stx* genes. Some additional targets such as the *eae* gene (Deng and Fratamico, 1996) and the 60-megadalton plasmid encoding for fimbrial adhesion have been used to identify pathogenic STEC (Fratamico *et al.*, 1995). Recently, a unique sequence in *E. coli* O157:H7 was identified in the *uidA* gene which encodes for β-glucuronidase (Feng, 1993). Although *E. coli* O157:H7 is characterized by a lack of this enzyme, the bacterium has the *uidA* gene and produces an inactive β-glucuronidase. It was found that the conserved sequence of the *uidA* gene in *E. coli* O157:H7 was highly specific to this serotype but not to other *E. coli*. The sequence differences did not determine the activity or inactivity of the β-glucuronidase (Feng and Lampel, 1994). The scientists developed a PCR assay in which they utilized three target genes -- one for each *stx* and a third for the *uidA* specific and conserved sequence (Cebula *et al.*, 1995).

The entire PCR procedure consists of DNA template preparation, PCR amplification, and PCR product detection. PCR procedures described in the literature vary widely in the target sequences, DNA template preparation procedures, PCR conditions, time required to complete the assay, and the lowest detectable limit. These are presented in Table 3. Advantages of PCR are: high specificity due to detection of specific sequences on DNA, ability to target several genetic loci simultaneously, and ability to detect low amounts of DNA material present in the specimens. On the other hand, the high sensitivity of PCR creates a contamination problem -- the slightest carryover of PCR end-product into PCR reagents can

TABLE 3. PCR methods for detection of *E. coli* O157:H7 and other STEC

Specimen	Reference	Description: DNA template preparation (1), target genes (2), and PCR product detection technique (3)	Time to complete	Sensitivity and other comments
Bacterial culture	Pollard *et al.*, 1990	1. Nucleic acid extracted from bacterial culture with phenol-chloroform and precipitated with ethanol. 2. Simultaneous identification and differentiation of stx_1 and stx_2. 3. Agarose gel electrophoresis.	8 h	Sensitivity 1 ng of total nucleic acid. Procedure also identified Shiga toxin gene of *Shigella dysenterae*.
Bacterial culture	Karch and Meyer, 1989b	1. Nucleic acid extraction and heat denaturation of bacterial culture. 2. Both *stx* genes detected by single pair of primers. 3. Agarose gel electrophoresis.	8 h	Sensitivity 100 CFU/ PCR reaction.
Bacterial colonies	Fratamico *et al.*, 1995	1. Heat lysis of a bacterial colony. 2. Simultaneuos identification and differentiation of 3 targets: *eaeA*, both *stx* and 60-MDa plasmid. 3. Agarose gel electrophoresis.	8 h	Sensitivity: 1.2 CFU for plasmid, 100 CFU for *eaeA* gene and 1000 CFU for *stx* genes. Positive signal for all 3 targets only in toxigenic O157 strains.
Foods, feces	Read *et al.*, 1992	1. Heat lysis of enrichment cultures of specimens. 2. Common sequence for both *stx*. 3. Agarose gel elctrophoresis.	36 h	17 CFU/g.

TABLE 3. (continued)

Specimen	Reference	Description: DNA template preparation (1), target genes (2), and PCR product detection technique (3)	Time to complete	Sensitivity and other comments
Water	Lang *et al.*, 1994	1. Enrichment and isolation of bacterial colonies from water prior to DNA extraction. DNA prepared by lysozyme digestion followed by lysis with proteinase K. 2. Simultaneous identification and differentiation of stx_1, stx_2, and heat labile toxin gene *lt*. 3. Dot blot analysis by internal oligonucleotide probes to stx_1, stx_2, and *lt* and agarose gel electrophoresis.	48 - 72 h	Sensitivity not determined.
Foods (ground beef and milk)	Deng *et al.*, 1996	1. Suspect colony from enriched food cultured on MAC lysed by heat. 2. Three target sequences: *eae*, *stx*, and 60-MDa plasmid. 3. Digoxigenin labeled oligonucleotide probe.	48 h	4 - 9 CFU/g or ml.
Feces	Rahn *et al.*, 1996	1. Overnight enrichment of fecal sample (2x), use of broth extracts after several centrifugations and heat lysis for DNA template. 2. Conserved sequences of stx_1 and stx_2. 3. Agarose gel electrophoresis.	72 h	Sensitivity not determined.
Bacterial colonies	Cebula *et al.*, 1995	1. Crude cell lysis by heat. 2. Three target genes: two *stx* and *uidA* with specific sequence in *E. coli* O157:H7. 3. Agarose gel electrophoresis.	6 h	Sensitivity not determined.

TABLE 3. (continued)

Specimen	Reference	Description: DNA template preparation (1), target genes (2), and PCR product detection technique (3)	Time to complete	Sensitivity and other comments
Ground beef	Gannon *et al.*, 1992	1. Six hour meat sample enrichment followed by DNA lysis with lysozyme and proteinase K and lysate precipitation with ethanol. 2. Two targets - each for one of *stx*. 3. Agarose gel electrophoresis.	9 h	1 CFU/g.
Foods	Jinneman *et al.*, 1995	1. Six hour enrichment followed by 1 ml or 10 ml size aliquot centrifugation with or without immunomagnetic separation with O157 beads. DNA released by boiling. 2. Two targets - each for one of *stx*. 3. Agarose gel electrophoresis.	12 h	10 ml analysis more sensitive than 1 ml. IMS + 10 ml analysis detected 43% samples with 0.1 CFU/g, 77% with 1 CFU/g and 93% with 10 CFU/g.
Ground beef	Witham *et al.*, 1996	1. Template preparation by boiling of meat samples after enrichment. 2. Shiga-like toxin 1. 3. Fluorogenic probe.	36 h	5-15 CFU/g after 8 h enrichment; 0.5 CFU/g after 12 h enrichment.
Ground beef	Begum and Jackson, 1995	1. Direct heat lysis of ground beef slurry or the same after 4 h enrichment. 2. Shiga-like toxin 2. 3. Agarose gel electrophoresis and dig-labelled DNA probe.	8-12 h	10^6 CFU/g without enrichment; 30 CFU/g in most of samples after enrichment.

cause false positive results. Tedious laboratory procedures as well as careful monitoring to identify contamination are necessary in laboratories performing PCR. Another deficiency of PCR is the inability to differentiate between viable and non-viable cells as PCR identifies any DNA material present in a specimen. In the case when PCR is used for simultaneous detection of several target genes in a pool of cells, a shortcoming is the inability to determine whether these targets are derived from a single cell population or several.

Other diagnostic methods have been used in epidemiological studies to help distinguish *E. coli* O157:H7 outbreak isolates from those of sporadic and unrelated cases. These methods include Stx genotyping (Paros *et al.*, 1993; Tarr *et al.*, 1989b; Thomas *et al.*, 1993), plasmid DNA profiling (Ahmed *et al.*, 1987; Paros *et al.*, 1993; Wells *et al.*, 1983), bacteriophage typing (Ahmed *et al.*, 1987; Khakhria *et al.*, 1990; Paros *et al.*, 1993; Tarr *et al.*, 1989b), restriction fragment length polymorphism (Paros *et al.*, 1993; Samadpour *et al.*, 1993), pulsed-field gel electrophoresis (Bohm and Karch, 1992), multilocus enzyme electrophoretic typing (Whittham *et al.*, 1988), and patterns of antibiotic susceptibility (Swerdlow *et al.*, 1992).

Control and prevention of *E. coli* O157:H7 infections

Modern meat processing systems must focus on the protection of public health through the reduction of hazards such as *E. coli* O157:H7. However, there are currently no preharvest interventions known to control *E. coli* O157:H7, and there is no evidence to suggest that O157:H7 can be eliminated at the preharvest level in the immediate future. Beef and dairy cattle are a principal source of *E. coli* O157:H7. Because of the low prevalence, sporadic shedding and absence of clinical disease in cattle, it is difficult, if not impossible, to identify the organism on the farm. Despite that, the hazard posed by *E. coli* O157:H7 can be reduced at multiple points along the way from slaughter plants to consumer. The Hazard Analysis Critical Control Point (HACCP) system is an approach that shifts the primary emphasis for food safety away from end-product testing. In the case of *E. coli* O157:H7, end-

product testing, based on a statistically derived number of samples, would be neither an effective nor a feasible means of assuring food safety due to the heterogeneous distribution and low prevalence of *E. coli* O157:H7 contamination. HACCP instead focuses on identification and monitoring of critical points in the production, slaughter, processing, storage, distribution, and final food preparation stages which could allow unacceptable levels of contamination, cross-contamination, survival, or growth of *E. coli* O157:H7. Use of good manufacturing practices through the production line from slaughter to table should include avoidance of cross-contamination, cleaning of equipment, proper cooking, cooling, hot holding, reheating and personal hygiene.

At present, the only other method, aside from cooking, that can eliminate *E. coli* O157:H7 from food is irradiation. It is approved for pork and poultry and experiments with beef have been successful as well. However, two major problems are associated with food irradiation. The first is a question of acceptance; second is the question of cost-effectiveness. While acceptance largely depends on consumer education, the physical ability to irradiate all beef would require a major financial investment. It may be worth considering the irradiation of beef that is served to high risk populations in institutional settings.

The importance of consumer education cannot be overstressed. Consumers can protect themselves by following "good manufacturing practices" at home -- they should be urged to adopt the food handling practices advocated on current meat labels: keep refrigerated or frozen; thaw in refrigerator or microwave; keep raw meat separate from other foods; wash working surfaces, utensils, and hands after touching raw meat; cook thoroughly; keep hot foods hot; and refrigerate leftovers immediately or discard them.

A COMPARISON OF TRIPLEX PCR AND MICROBIOLOGICAL CULTURING FOR THE DETECTION OF ESCHERICHIA COLI O157:H7 IN GROUND MEAT

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Abstract

A rapid triplex polymerase chain reaction (PCR) procedure was developed for detection of *Escherichia coli* O157:H7 and other Shiga-like toxin producing *E. coli* (STEC) from ground beef and ground pork at contamination levels of 0.14, 1.4 and 14 CFU per g of meat. It was simultaneously compared for sensitivity to bacteriologic culture technique. The PCR amplified three target genes: *uidA* which is specific for *E. coli* O157:H7, and stx_1 and stx_2, the genes for Shiga-like toxins 1 and 2. The entire PCR procedure, which consisted of a simplified sample preparation step, PCR amplification, and agarose gel electrophoresis was 7 hours after overnight sample enrichment. Bacteriologic culture required 52 hours for detection of the target. Detection of the *uidA* gene by triplex PCR was 91% sensitive in beef and 55% sensitive in pork, and 90% and 71% sensitive for *stx* gene detection in beef and pork, respectively. Detection of *E. coli* O157:H7 by culture was 53% sensitive in beef and 11% sensitive in pork. The greater sensitivity of the PCR procedure was statistically significant regardless of type of meat. There was also a statistically significant difference in sensitivity for beef and pork by both detection methods. Fat content in beef did not statistically influence PCR results.

Introduction

Enterohemorrhagic Shiga-like toxin-producing *Escherichia coli* O157:H7 (STEC) is a foodborne pathogen which may cause life-threatening diseases, namely hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Griffin *et al.*, 1988; Tarr, 1995). The microorganism was first identified in 1982 and since then the number of recognized STEC cases and foodborne outbreaks have increased in each succeeding year (Boyce *et al.*, 1995b; Brotman *et al.*, 1995). Most of the outbreaks associated with *E. coli* O157:H7 infection have been traced to the consumption of undercooked beef products, and epidemiologic analyses indicate that cattle are a reservoir for *E. coli* O157:H7 (Cray and Moon, 1995; Wells *et al.*, 1991; Whipp *et al.*, 1994). Information from outbreaks suggests that the infectious dose for *E. coli* O157:H7 is low (Griffin and Tauxe, 1991; Roberts *et al.*, 1995).

The pathogenicity of *E. coli* O157:H7 is attributed to the production of Shiga-like toxins 1 and 2 (Stx1 and Stx2) (Griffin and Tauxe, 1991; O'Brien *et al.*, 1992), but knowledge of pathogenesis is incomplete. It is unclear why *E. coli* O157:H7 is the predominant serotype associated with HC and HUS when more than 100 STEC strains are known. Importantly, O157:H7 is not the only STEC serotype known to be involved in HC and HUS (Robson *et al.*, 1993). In recent years, the incidence of HC and HUS associated with STEC strains other than O157:H7 has been reported at increasing rates outside the USA (Acheson and Keusch, 1996). Within the USA, sporadic cases of HC and/or HUS and one foodborne outbreak associated with STEC other than O157:H7 have been documented (Acheson and Keusch, 1996; U.S. Department of Health and Human Services, 1995).

Currently, laboratory diagnosis of *E. coli* O157:H7 HC and HUS cases is accomplished by isolation of *E. coli* O157:H7 from specimens by bacteriologic culture followed by O157 antigen detection, toxin detection, or biochemical characterization. Culture techniques are based on the unique biochemical and physiological properties of O157:H7 which distinguish it from other *E. coli* strains; i.e., O157:H7 does not ferment sorbitol and is negative in glucuronidase assay (Boyce *et al.*, 1995a; Padhey and Doyle, 1992; Smith and Scotland, 1993). Although bacteriological identification techniques are reliable and easily

performed standard procedures, major shortcomings have been recognized (Acheson and Keusch, 1996; Feng, 1995). For example, the inability to detect STEC strains that do not share the same biochemical characteristics as O157:H7 and the inability to detect *E. coli* O157:H7 strains with unusual phenotypes, such as strains with sorbitol fermenting ability or glucuronidase activity (Gannon *et al.*, 1992; Hayes *et al.*, 1995), limit the effectiveness of methods to ensure reliable bacteriological identification of pathogenic *E. coli*.

Numerous alternative and improved methods for STEC and O157:H7 serotype specific detection have been generated in the last decade (Chapman *et al.*, 1994; Kim and Doyle, 1992; Law *et al.*, 1994; McCleary and Rowe, 1995; Okrend *et al.*, 1990ab, 1992; Padhey and Doyle, 1991; Szabo *et al.*, 1990; Wrigth *et al.*, 1994; Yamada and Kudoh, 1994). The PCR technique has enabled the detection of genetic markers rather than the use of biochemical properties to identify bacteria in cultures and clinical specimens. Currently, PCR is being widely explored for detection of STEC in meat (Begum and Jackson, 1995; Gannon *et al.*, 1992; Read *et al.*, 1992), and feces (Paton *et al.*, 1993; Read *et al.*, 1992). However, none of the PCR procedures reported in the literature have been targeted to specifically detect *E. coli* O157:H7 and Stx1 and Stx2 sequences in contaminated sources.

With the limitations in detection of *E. coli* O157:H7 in mind, the purpose of our research was threefold: (i) to develop a rapid PCR-based procedure that detects specifically *E. coli* O157:H7 and other Stx-producing bacteria in ground meat by avoiding time consuming DNA extraction procedures, (ii) to determine sensitivity and specificity of the PCR procedure in comparison to bacteriologic culturing methods, and (iii) to determine whether the fat content of ground beef influences PCR test results.

Materials and Methods

Bacterial strains and media

Fifteen *E. coli* strains with known *stx* gene data were provided by Dr. T. Casey (National Animal Disease Center) (Table 4). These and another eighteen STEC strains, all *E. coli* O157:H7 with unknown *stx* genotypes, provided by Dr. J. Dickson (Iowa State University), were analyzed by multiplex PCR in our laboratory. *E. coli* strains used as positive and negative controls were ATCC *E. coli* O157:H7 strain 35150 producing both Stx1 and Stx2 and a laboratory *E. coli* strain lacking *stx* genes, respectively.

TABLE 4. Serotypes and *stx* gene probe data of STEC strains subjected to PCR analysis

Strain ID	Serotype	stx_1	stx_2	*uidA*
2162	O139:H(+)	-	+	-
2228[a]	O139 NM	-	+	-
2482[a]	unknown	-	+	-
2597	O139:H1	-	+	-
2602	unknown	-	+	-
3081[a]	O157:H7	+	+	+
3094	O111	+	-	-
3108[a]	O26	+	-	-
3128	O113	+	+	-
3239[a]	O165	-	+	-
3244	O119:H16	-	+	-
4718	O157:H7	+	+	+
4719	O157:H7	+	+	+
4720	O157:H7	-	+	+
4721	O157:H7	+	-	+
35150[ab]	O157:H7	+	+	+

[a] Strains used in the first experiment.
[b] ATCC strain, positive control.

Total aerobic and coliform counts of meat were determined by plating meat homogenates on plate count agar (PCA, Difco) and violet red bile agar (VRBA, Difco), respectively. Trypticase soy broth (TSB, Difco) was used to enumerate bacteria in cultures and meat. For the cultural detection of *E. coli* O157:H7, enriched meat samples were plated on sorbitol-MacConkey agar (SMAC, Difco), followed by transfer of suspect colonies to MacConkey agar (MAC, Difco), and media containing 0.08% p-nitrophenyl-β-D-glucuronide (Sigma).

Meat sample bacteriology

(i) Total aerobic plate and coliform plate counts. Ground beef (single batches of 70%, 80%, and 93% lean) and ground pork (two batches of similar fat content) were purchased from local retail stores. For each meat batch, total aerobic and coliform plate counts were performed twice and results averaged. Twenty-five g of meat were blended with 225 ml phosphate buffer solution (PBS) for 2 min in a Stomacher 80 to yield a 1:10 dilution. One hundred µl of 10-fold dilutions made in PBS were plated on PCA and VRBA. Plates were incubated at 37°C for 24 (VRBA) or 48 hours (PCA), after which colony counts were determined.

(ii) *E. coli* O157:H7 culturing. The protocol for cultural detection of *E. coli* O157:H7 was adapted from the National Animal Disease Center Bacteriology Laboratory procedure (Dargatz *et al.*, 1997) and modified to *E. coli* O157:H7 isolation from meat. Ten g of control and seeded ground meat samples were blended with 35 ml of TSB for 2 min in a Stomacher 80. Samples were incubated overnight at 37°C. The next day, 10-fold dilutions in PBS were made from sample homogenates. From each sample, 100 µl of dilutions 10^{-4}, 10^{-5}, and 10^{-6} were plated on SMAC and incubated for 24 hours. A plate with a count between 30-300 clear colonies was chosen from each sample following incubation and the total number of sorbitol negative colonies determined. Eight sorbitol negative (clear) *E. coli* O157:H7 characteristic colonies were inoculated to media containing 0.08% β-D-glucuronide and streaked on MAC. After overnight incubation, lactose positive (red on MAC) and β-D-glucuronidase negative (no fluorescence) colonies were tested for indole production by liquid

indole reagent (Bactidrop Spot Indole; Remel). Indole positive (blue) *E. coli* O157:H7 suspect colonies were tested with the latex O157 antigen (ECOLEX ^{TM}; Orion Diagnostica) test.

Inoculation of STEC onto meat

STEC isolates were analyzed by triplex PCR prior to inoculating the meat, as described below. To adjust inoculum, STEC strains were grown overnight in TSB and centrifuged (15 min; 5125 x g) in an IEC clinical centrifuge. The supernatant fluid was removed and the pellet was resuspended in PBS to give 50% light transmittance at 400 nm in a Bausch and Lomb® Spectronic 20. Decimal dilutions up to 10^{-8} were made in PBS. To determine the inoculum for seeding meat samples, 100 µl of the 10^{-5} and 10^{-6} dilutions were plated in duplicate on SMAC. Plates were incubated overnight and colony counts obtained the following day. Negative control samples were inoculated with corresponding dilutions of either the laboratory strain of non-Stx producing *E. coli* or 100 µl PBS.

Meat negative for *E. coli* O157:H7 as detected by culturing was used in the experiments. After inoculation, all contaminated and control meat samples were left on the counter for one hour. Thirty-five ml of TSB were then added to each sample and blended in a Stomacher 80. Prepared meat samples were incubated at 37°C overnight.

(i) Preliminary experiment. This experiment was performed to determine whether the meat sample processing technique described below could serve for detection of low inoculation levels by PCR. Seven 8 g samples of ground beef (93% lean) were inoculated with a 10^{-5} dilution of 6 selected STEC (Table 4) and with negative control *E. coli* strain. Each sample received a different STEC strain and the dose varied from 0.5 to 15 CFU/g. Samples were subjected to triplex PCR analysis.

(ii) Primary experiment. This experiment was conducted to determine the sensitivity and specificity of the PCR and compare it to the sensitivity and specificity of culturing technique. Four types of ground meat were used: ground pork, 70% lean ground beef, 80% lean ground beef, and 93% lean ground beef. Ten g meat samples were inoculated with randomly selected STEC strains from a pool of 30 strains. The thirty STEC strains

included 22 *E. coli* O157:H7 and 8 STEC other than O157:H7. The experiment was conducted in 4 blocks, each block employing one kind of meat. For each block, 100 µl of decimal dilutions 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} of 8 randomly chosen STEC strains were used to inoculate 32 meat samples. The dilutions delivered approximately 14, 1.4, 1.4 x 10^{-1}, and 1.4 x 10^{-2} CFU STEC per g of meat, respectively. Eight control meat samples, each receiving 100 µl PBS, were included in the block. The experimental design provided use of 40 samples in a block and a total of 160 samples in the experiment. All samples were analyzed by PCR and bacteriology.

Meat processing prior to PCR

After overnight incubation, meat samples were centrifuged (15 min; 110 x g) in an IEC, model UV, centrifuge. The supernatant was separated from meat particles and subjected to low speed centrifugation again (15 min; 110 x g). For the first experiment, only one low speed centrifugation was performed. After either the first or second centrifugation, supernatant fluids were collected and centrifuged at high speed (15 min; 1000 x g). The supernatant fluids were discarded and the pelleted cells were resuspended in 30 ml PBS. High speed centrifugation (15 min; 1000 x g) was repeated and the supernatant fluids were removed. The resulting pellets were resuspended in 1 ml water. Each sample was divided in half: 500 µl of each suspension were frozen for later analysis, and the other 500 µl were used immediately in a PCR reaction, as described below.

Triplex PCR procedure

A triplex PCR was developed to simultaneously identify *E. coli* O157:H7 and the two Shiga-like toxin genes (stx_1 and stx_2) encoded on bacteriophages. Sequences for the three pairs of primers employed in triplex PCR were specific to conserved regions of each *stx* gene (13), and the *uidA* gene (7) as reported previously (stx_1: 5'-ACA CTG GAT GAT CTC AGT GG-3' and 5'-CTG AAT CCC CCT CCA TTA TG-3'; stx_2: 5'-CCA TGA CAA CGG ACA GCA GTT-3' and 5'-CCT GTC AAC TGA GCA CTT TG-3'; *uidA*: 5'-GCG AAA ACT

GTG GAA TTG GG-3' and 5'-TGA TGC TCC ATA ACT TCC TG-3'). Oligonucleotides were synthesized at the Iowa State University Nucleic Acid Facility.

The 50 µl PCR reaction solutions contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl; 0.2 mM (each) dATP, dGTP, dCTP, and dTTP (Perkin and Elmer); 1 µM of each primer; 2.5 units of Taq DNA polymerase (Perkin and Elmer); and 10 µl of template. DNA from bacterial colonies was prepared by resuspending a single colony in 500 µl water containing 4 mM NaOH. To target the DNA liberation from the meat samples, 10 µl of 1 N NaOH solution was added to 500 µl cell suspensions. All samples were boiled for 10 min, cooled on ice for 10 min, centrifuged (2 min; 16,000 x g) in an Eppendorf centrifuge 5415 C, and the supernatant subjected to PCR. Amplifications were performed in a DNA thermal cycler (Gene Amp PCR System 2400, Perkin and Elmer) for 40 cycles with 4 min initiation at 94°C followed by cycles of 1 min at 94°C, 1 min at 58°C, 1.5 min at 72°C, and a final extension at 72°C for 7 minutes. After PCR amplification, DNA samples were analyzed by agarose gel electrophoresis, visualized by UV light, and photographed. A molecular size marker, 1kb DNA ladder (Life technologies, Inc.), was included in each gel.

Statistical analyses

Statistical analyses were applied to the pooled results of the primary experiment. Chi squared and general linear model (SAS program) were used to determine the influence of meat type and to compare PCR to bacteriological culturing for 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} inocula levels separately. Values 1 and 0, respectively, were assigned to positive and negative samples. Test sensitivity and specificity were calculated for both PCR and bacteriology by pooling results from all samples inoculated with doses varying from 1.4×10^{-1} to 14 CFU/g. The 1.4×10^{-2} CFU/g inoculum was considered too low to deliver a single cell per 10 g meat sample and therefore was not used in calculation of the sensitivity of the technique.

Results

Shiga-like toxin and uidA gene detection by triplex PCR in STEC strains

DNA from colonies of 6 *E. coli* O157:H7 and 10 other STEC strains with known stx_1 and stx_2 gene profiles (Table 4) were amplified by triplex PCR to determine whether the PCR procedure could correctly identify the target genes. Expected *stx* profiles were generated from all STEC strains and the 6 *E. coli* O157:H7 strains were correctly distinguished from 10 other STEC strains by the PCR procedure. Eighteen *E. coli* O157:H7 strains with unknown *stx* types were also analyzed by the triplex PCR. Electrophoretic analysis revealed the *uidA* band and distinct *stx* band patterns in all strains. Eleven strains carried both *stx* genes, whereas 7 strains had only the stx_2 sequence.

Preliminary experiment

Six ground beef samples inoculated with selected STEC strains (Table 4) all yielded PCR products of expected size, as determined by Stx genotype data and PCR analysis of bacterial cultures (Figure 1). No PCR products were detected when non-Stx *E. coli* contaminated meat was analyzed.

Primary experiment

A total of 160 meat samples were analyzed simultaneously by PCR and bacterial culture. The strains for meat inoculation were selected at random from a pool of 22 *E. coli* O157:H7 strains and 8 strains of other STEC serotypes. In total, 16 *E. coli* O157:H7 strains and 5 STEC strains other than O157:H7 were analyzed. The total aerobic and coliform plate counts for all meat batches are given in Table 5.

(i) *uidA* gene detection by PCR. The analysis of meat samples seeded with a dose of 14 CFU of *E. coli* O157:H7 per g of meat showed that this procedure was 100% and 83% effective in detection of the *uidA* sequence in ground beef and ground pork samples, respectively (Figure 2). Positive sample detection at an inoculum level of 1.4 CFU/g

FIGURE 1. Agarose gel electrophoresic analysis results of STEC seeded meat samples amplified by triplex PCR. Lanes A, B, C in each gel show 1 kb DNA ladder and positive and negative control samples, respectively. The highest molecular weight band in a positive control sample (lane B) corresponds to stx_2 fragment (779 bp), the next lower corresponds to stx_1 fragment (614 bp), and the lowest molecular weight band is *uidA* (252 bp). Lanes 1-5 are meat samples inoculated with selected STEC strains (Table 4). Lane 1 - #2482; 2 - #3081; 3 - #3239; 4 - #2228; and 5 - #3108. Lane 6 - meat sample seeded with ATCC *E. coli* O157:H7 strain 35150, lane 7 - meat sample inoculated with laboratory strain *E. coli* (no Stx).

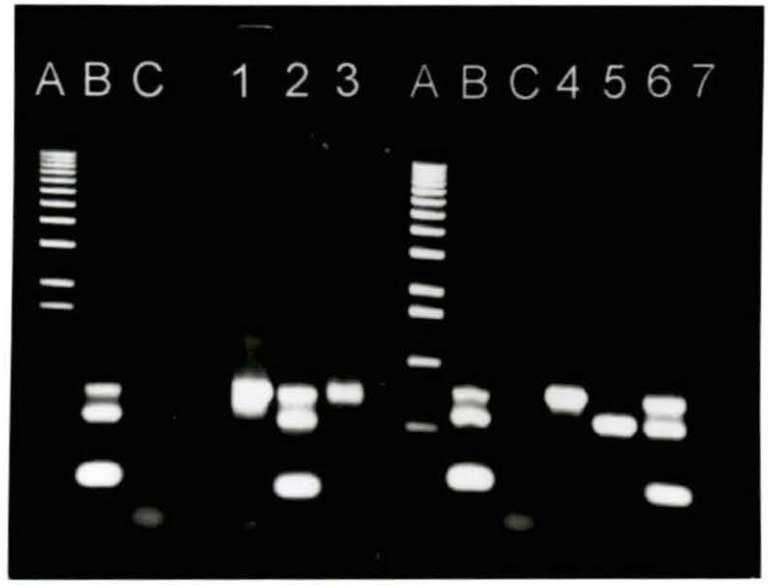
A B C 1 2 3 A B C 4 5 6 7

TABLE 5. Ground pork and ground beef total aerobic and coliform plate counts

Meat	Plate counts CFU/g	
	Aerobic	Coliforms
Ground pork[a]	3.1×10^5 1.5×10^6	1.8×10^4 5.0×10^2
Ground beef[b] 70% lean	6.7×10^5	6.0×10^2
Ground beef 80% lean	2.3×10^5	1.8×10^2
Ground beef 93% lean	5.2×10^4	1.0×10^3

[a] Two separately purchased ground pork batches were used.
[b] All ground beef samples were purchased from single lots.

diminished to 84% and 67%, respectively, for ground beef and pork. The difference due to meat type at these inocula levels was not significant ($p = 0.07$ and $p = 0.4$ for inocula of 14 and 1.4 CFU/g, respectively). Triplex PCR detected 89% of the positive beef samples and 17% of the pork samples contaminated with a dose of 0.14 CFU/g; the difference due to meat type was statistically significant ($p = 0.0001$). Furthermore, the triplex PCR procedure detected 21% of ground beef samples seeded with 10-fold fewer cells, 1.4×10^{-2} CFU/g, but none of the seeded pork samples. When the *E. coli* O157:H7 detection levels were compared in beef samples of differing fat content, no statistically significant difference was found at any of the inocula levels ($p > 0.6$). In summary, the overall sensitivity of the triplex PCR for meat sample detection seeded with 0.14 - 14 CFU/g was 91% in ground beef and 55% in ground pork (Table 6), and the specificity of the PCR procedure was 100% regardless of meat type.

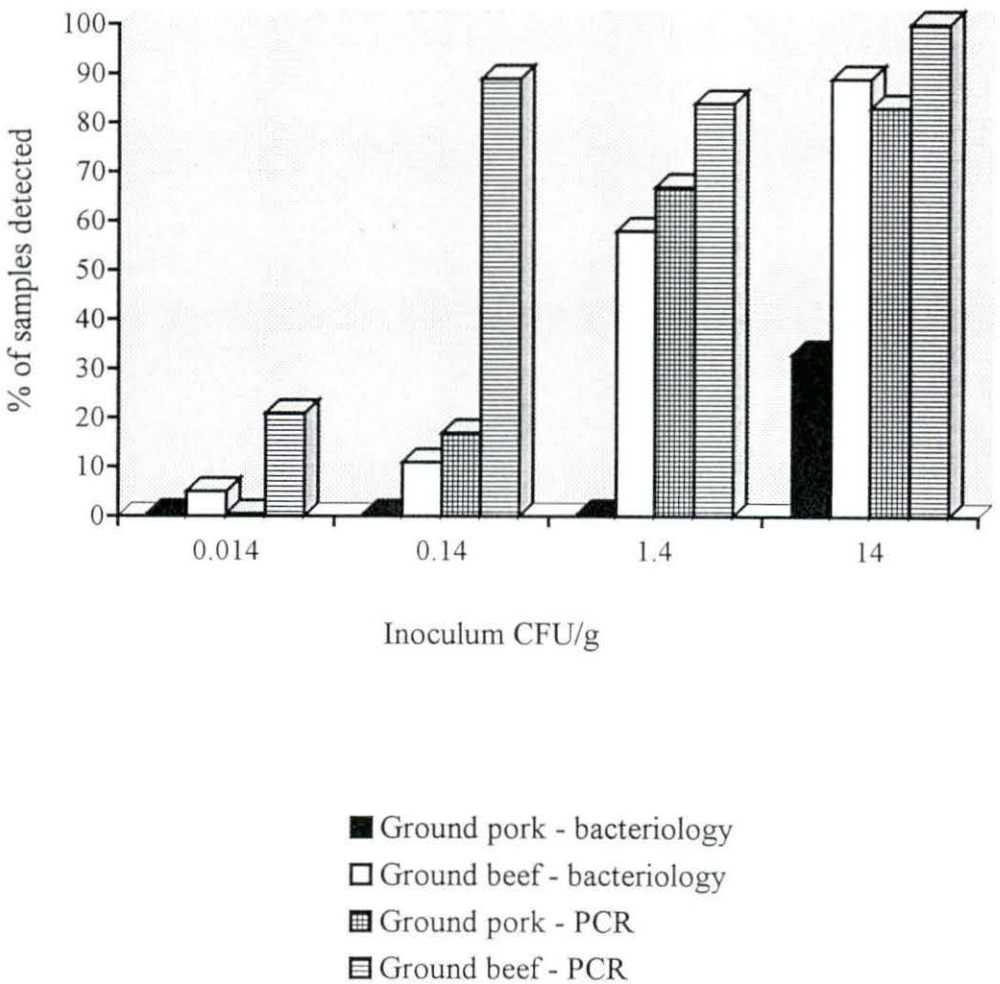

FIGURE 2. *E. coli* O157:H7 detection by PCR and bacteriology in ground meat

(ii) *Stx* gene detection by PCR. The presence of the *stx* sequences was correctly identified in 88% of ground pork samples and 100% of ground beef samples seeded with 14CFU/g (data not shown). When meat samples were seeded with 10-fold fewer cells (1.4 CFU/g), *stx* genes were identified in 88% of contaminated beef and pork samples. The identification rate dropped to 38% in ground pork samples and to 83% in ground beef samples inoculated with 0.14 CFU per g (data not shown). As presented in Table 6, the overall sensitivity of Stx marker detection was 71% in ground pork and 90% in ground beef. Shiga-like toxin genes were found in two ground beef negative control samples (80% lean) which showed bands corresponding to stx_1 and stx_2 (one band/sample). Additionally, PCR products of both *stx* genes were found in 5% of meat samples known to be inoculated with STEC strain carrying only a single *stx* gene.

(iii) *E. coli* O157:H7 detection by culture. As shown in Figure 2, the bacteriologic culturing procedure identified 89% of the ground beef samples seeded with 14 CFU/g of meat and 33% of ground pork samples inoculated with the same dose. This trend was also observed at an inoculum level of 1.4 CFU/g; however, at a considerably lower detection level for both beef and pork with only 58% of seeded ground beef samples and none of the seeded pork samples being identified by cultural isolation procedures. The ability to detect *E. coli* O157:H7 contaminated samples at these two inocula levels differed significantly depending on meat type, i.e., beef versus pork ($p = 0.005$). With the decrease of inoculum to 0.14 CFU/g, bacteriologic culture techniques identified only 11% of the inoculated beef samples and none of the pork samples. This difference was not dependent on meat type ($p = 0.43$). In summary, the overall sensitivity of culturing for detection of meat samples inoculated with 0.14-14 CFU/g was 53% in ground beef and 11% in ground pork (Table 6). The specificity of bacteriology was 100% as none of the control meat samples were positive for *E. coli* O157:H7. The fat content of beef, however, was not a significant factor influencing *E. coli* O157:H7 recovery ($p > 0.4$).

TABLE 6. Comparison of the sensitivity[a] of culturing and triplex PCR methods for *E. coli* O157:H7 and *stx* gene detection

Meat type[b]	Detection method[c]	Detection target	No. of samples pos/total (%)
Ground pork	Bacteriology	*E. coli* O157:H7	2/18 (11)
	PCR	*uidA*	10/18 (55)
	PCR	*stx* genes	17/24 (71)
Ground beef (average)	Bacteriology	*E. coli* O157:H7	30/57 (53)
	PCR	*uidA*	52/57 (91)
	PCR	*stx* genes	65/72 (90)
Ground beef 70% lean	Bacteriology	*E. coli* O157:H7	11/24 (46)
	PCR	*uidA*	21/24 (88)
	PCR	*stx* genes	21/24 (88)
Ground beef 80% lean	Bacteriology	*E. coli* O157:H7	8/12 (67)
	PCR	*uidA*	12/12 (100)
	PCR	*stx* genes	23/24 (96)
Ground beef 93% lean	Bacteriology	*E. coli* O157:H7	11/21 (52)
	PCR	*uidA*	19/21 (90)
	PCR	*stx* genes	21/24 (88)

[a] Sensitivity calculated for pool of contaminated meat samples consisting of an equal number of samples inoculated with 14 , 1.4 and 0.14 CFU/g.
[b] Significant differences between pork and beef samples within each detection method.
[c] Significant difference between PCR and bacteriology for *E. coli* O157:H7 detection regardless of meat type.

Discussion

In this paper we present a rapid and specific PCR procedure for *E. coli* O157:H7 and *stx* gene detection in ground beef and pork which was faster, more sensitive and detected lower contamination levels than bacteriological culturing. The greater sensitivity of PCR was

statistically significant at all inoculum levels regardless of meat type. The time required to complete the entire procedure consisting of meat sample preparation, PCR amplification, and agarose gel electrophoresis was 7 hours, after overnight enrichment, compared to 52 hours for detection by bacterial culturing.

The triplex PCR procedure was established by choosing three target genes: *uidA*, stx_1, and stx_2. The *uidA* gene encodes β-glucuronidase in *E. coli* and although *E. coli* O157:H7 strains do not exhibit glucuronidase activity, the *uidA* gene is present on the chromosome. This gene has some conserved sequences that are unique to the *E. coli* O157:H7 serotype (Cebula *et al.*, 1994; Feng and Lampel, 1994) and can be used to specify this serotype. However, it is not the serotype which determines the pathogenicity of the bacteria but the production of Stx. Since HUS cases associated with STEC other than O157:H7 have been reported at an increasing rate (Robson *et al.*, 1993), the simultaneous targeting of stx_1 and stx_2 seqences was important in detection of foods contaminated with STEC. Additionally, some studies indicate that Stx2 is a more potent toxin than Stx1 (Russman *et al.*, 1994); thus a method characterizing *stx* genotype, such as triplex PCR, would be of value to study the association between the *stx* type of the source of infection and the severity of disease.

The development of a triplex PCR procedure for STEC detection in meat involved three stages. In the beginning, conditions of PCR were adjusted to optimize the synthesis of target sequences. The sensitivity, e.g. ability to detect true positive samples as positives, and specificity, e.g., ability to detect true negative samples as negatives, were tested by subjecting STEC strains with known serotype and Stx status to PCR analysis. In the second step, a new, simplified, meat processing technique was developed and applied to seeded meat samples to determine whether it served for detection of low contamination levels. Finally, a larger series of experiments was conducted to determine the sensitivity of PCR at low STEC contamination levels in comparison with standard bacteriology techniques in seeded meat samples. Like most of the standard cultural methods for detection of *E. coli* O157:H7 (Smith and Scotland, 1993), our culturing protocol was based on the inability of *E. coli* O157:H7 to ferment sorbitol in SMAC media. We performed bacteriologic culturing on enriched meat and analyzed 8 sorbitol negative colonies.

The specificity of PCR procedure in detection of *uidA* sequences was 100%; that is, it correctly identified the *uidA* sequence in all *E. coli* O157:H7 strains. Moreover, it correctly distinguished between *E. coli* O157:H7 and other STEC contaminated meat samples, emphasizing the specificity of the *uidA* primers used in this procedure for *E. coli* O157:H7 detection. The triplex PCR procedure did not detect the *uidA* sequence in any of the non-contaminated samples, but found *stx* genes in two of them. Two possible explanations are that (a) these were true findings, meat samples contained bacteria producing Stx, or (b) these were false positive samples.

The sensitivity of the PCR procedure was determined by analysis of STEC-contaminated samples. The bacteriological culturing was performed on the same samples to compare the two methods. Meat was inoculated with a fairly low inoculum (0.14 to 14 CFU/g) to simulate a natural low infectious dose which is believed to be as low as a few cells (Griffin and Tauxe, 1991; Roberts *et al.*, 1995). The PCR analysis determined that the overall sensitivity of this method was high for both *uidA* and *stx* gene detection in beef (91% and 90%, respectively) but not as high in pork (55% and 71%, respectively). In comparison, the overall sensitivity of culturing was only about 50% in ground beef and 10% in ground pork, and the difference between sensitivities of the two methods was statistically significant.

Statistical analysis of separate inocula levels revealed that the sensitivity at each inoculum level was greater in beef than in pork for both methods. We explored possible explanations. As regards PCR, fat content as a PCR inhibitor was one of our considerations. We excluded this possibility for two reasons: (i) ground pork was on average 80% lean (fat content of 20%) which was the same as one of ground beef batches, yet PCR sensitivity for *uidA* gene detection in pork was 33% compared to 100% in ground beef 80% lean, and (ii) PCR analysis of beef samples differing in fat content (70%, 80%, and 93% lean) determined that fat content was not a significant factor influencing PCR outcome.

As regards bacteriology, several factors were investigated. Inconsistency in technique was excluded since bacteriology on pork and beef samples was done by the same person. Further, we compared total APC and coliform counts of ground beef and ground pork to determine whether the number of microorganisms in meat interfered with the cultural recovery

of *E. coli* O157:H7. We found that one of two ground pork batches had a 10- to 100-fold higher APC and the other had 10- to 100-fold higher coliform count compared to the rest of the meat batches (Table 5). The comparison of bacteriology results of seeded ground pork samples revealed consistently low O157:H7 detection ability (1 positive of 9 seeded) in both ground pork batches, indicating that factors other than the total number of aerobic bacteria or coliforms account for the sensitivity differences in beef and pork by culture.

We analyzed the number of sorbitol negative colonies (data not shown) in non-inoculated meat samples and found that the total number was higher in ground pork compared to ground beef. As we were testing 8 sorbitol-negative colonies according to our culturing protocol, the larger total number of sorbitol negative colonies in ground pork samples could have contributed to a greater chance to miss the true *E. coli* O157:H7 colonies. To determine whether there was a difference in *E. coli* O157:H7 growth rates in beef and pork, we compared the number of sorbitol negative colonies in seeded and corresponding control samples. The results revealed that after overnight enrichment there was a larger increase in the number of sorbitol negative colonies in ground beef samples than in ground pork (35% increase in beef versus 25% increase in pork). Although we don't know the actual increase in *E. coli* O157:H7 numbers, the last observation supports the hypothesis that ground pork is not as favorable a medium for *E. coli* O157:H7 growth as ground beef. It also validates the sensitivity differences between pork and beef detected by PCR because the overnight enrichment determined the number of bacteria subjected to PCR analysis. More extensive research is needed on this subject, especially since there are no other studies comparing *E. coli* O157:H7 growth in ground beef and ground pork.

In summary, we have described a new PCR procedure which was superior to bacterial culture in *E. coli* O157:H7 detection in meat. Because PCR cannot determine whether the detected genes derive from a single STEC strain, the primary use of the reported PCR technique is to identify meat samples containing STEC. Use of overnight sample enrichment prior to PCR allowed detection of low levels of bacterial contamination after a simple preparation procedure, while eliminating detection of DNA derived from non-viable bacterial cells. This technique can be applied to PCR detection of other meat borne organisms. Several

improvements of the PCR protocol are under consideration for enhanced sensitivity. These include use of higher quality Taq polymerase and development of an automated PCR product detection system such as an ELISA which would exceed electrophoretic product analysis in speed and precision.

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GENERAL SUMMARY

Escherichia coli O157:H7 is a common human foodborne pathogen that has a wide spectrum of involvment, ranging from the asymptomatic carrier state to hemorrhagic colitis, and complications such as the hemolytic uremic syndrome. The increasing frequency of recognized cases may be due in part to a heightened awareness of the organism and increased surveillance measures, but may also reflect a true increase in incidence of O157:H7. Cattle are a major reservoir of *E. coli* O157:H7 and, although O157:H7 prevalence is low at the farm level, it may be considerably higher at slaughter facilities. Currently, bovine-origin products are defined as high risk products for O157:H7 infection, but a wide variety of other *E. coli* O157:H7 infection sources have been recognized. Importantly, infectious dose is low, which facilitates person-to-person transmission of the organism.

Shiga-like toxin production is believed to be the primary etiologic event in *E. coli* O157:H7 infection, but detailed pathogenic sequences still need to be characterized. Several Stx-producing *E. coli*, other than those with O157:H7 serotype, have been associated with human disease and may become increasingly important. The potentially high morbidity and mortality associated with *E. coli* O157:H7 and other STECs warrant development and implementation of effective preventive actions. Rapid and accurate detection methods to identify sources of infection as well as surveys of food products are important in the enhancement of food safety.

The development of a triplex PCR technique which allowed detection of *E. coli* O157:H7 and other STEC contamination lower than 1 CFU/g of ground meat is described in a manuscript to be submitted for publication. The time required to complete the entire PCR procedure consisting of a simplified sample preparation step, PCR amplification, and agarose gel electrophoresis was 7 hours after overnight sample enrichment. In comparison with standard bacteriologic culturing, triplex PCR was faster, more sensitive, and detected lower contamination levels. Thus, this can be an alternative method for *E. coli* O157:H7 and other STEC detection in laboratories utilizing PCR as a diagnostic tool.

APPENDIX

Objectives

The STEC gene encoding for Stx1 is 99% homologous to the Shiga toxin gene of *Shigella dysenteriae*; therefore, several *Shigellae* strains needed to be assayed by the triplex PCR. Additionally, a report of a β-D-glucuronidase positive variant of *E. coli* associated with a case of bloody diarrhea warranted testing this isolate by the triplex PCR (Hayes *et al.*, 1995). As one of the targets in the triplex PCR is the *uidA* gene, we were interested determining the ability of *uidA* primers to detect *E. coli* O157:H7 producing active β-D-glucuronidase. Determination of the specificity of the triplex PCR when applied to *Shigella* strains and β-D-glucuronidase positive *E. coli* O157:H7 strains was our first goal.

Our second goal was to determine the repeatability of triplex PCR analysis of contaminated and control meat samples.

Materials and Methods

Bacterial strains

Six *Shigella* strains provided by the Iowa Environmental and Public Health Laboratory included 3 *S. sonnei*, 2 *S. flexneri*, and one *S. dysenteriae* isolates cultured from human stool samples. Another *Shigella* strain, *S. dysenteriae* type 1, was obtained from ATCC (ATCC #9361). The *E. coli* O157:H7 strain producing active β-D-glucuronidase was kindly provided by Dr. Peggy Hayes (CDC). Strains used as positive and negative controls were ATCC *E. coli* O157:H7 35150 producing both Stx1 and Stx2, and laboratory *E. coli* lacking *stx* genes, respectively.

Strains were grown on brain heart infusion agar and single colonies were used for PCR analysis. Prior to PCR, the colonies were resuspended in 500 µl water containing 4 mM

NaOH. Further preparation, the PCR procedure, and PCR product analysis were completed as described in the manuscript (Materials and Methods; Triplex PCR procedure).

Repeated PCR on meat samples

As described in the manuscript, suspensions of contaminated and control meat samples prepared by repeated slow and fast centrifugation were divided in half. Five hundred µl were used for immediate PCR and the other 500 µl were frozen for later analysis. Samples from the frozen sets were prepared for PCR by adding 10 µl of 1 N NaOH and the entire PCR procedure repeated as described in the Materials and Methods part of the manuscript.

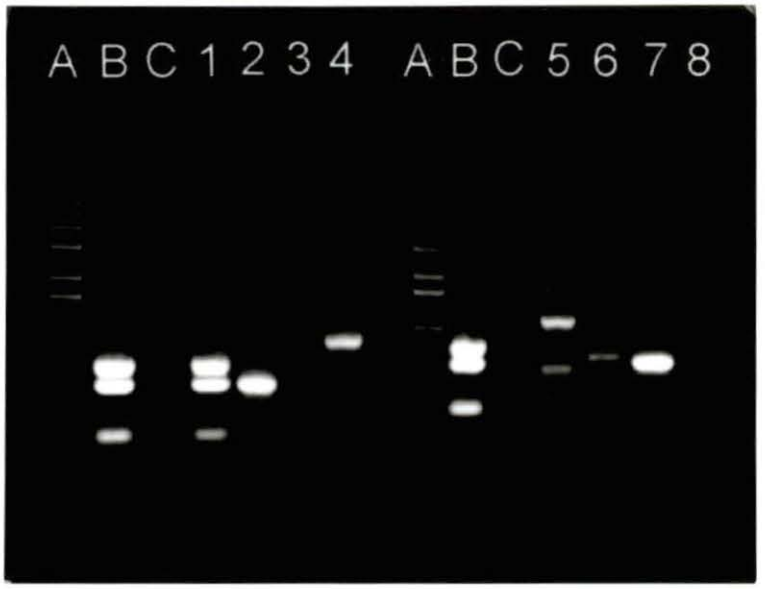
Results and Discussion

Triplex PCR analysis of* Shigellae *strains and β-D-glucuronidase positive* E. coli *O157:H7

As shown in Figure 3, triplex PCR amplified *stx*$_1$ gene corresponding sequences in both *S. dysenteriae* isolates. The other 5 *Shigella* isolates (3 *S. sonnei*, 2 *S. flexneri*) displayed several bands but none of them corresponded to the targets of triplex PCR. The detection of *stx*$_1$ gene sequences in two *S. dysenteriae* isolates was not surprising because the Shiga toxin gene of *S. dysenteriae* is known to be 99% homologous to that of *stx*$_1$ in *E. coli*. These findings are important in interpretation of triplex PCR results in that identification of *stx*$_1$ sequences in meat samples in the absence of the *uidA* marker serves as an indication of the presence of *S. dysenteriae* or STEC other than the O157:H7 serotype.

The triplex PCR analysis identified the β-D-glucuronidase producing-*E. coli* O157:H7 strain as having the *uidA* band and both *stx* markers (Figure 3), showing that *uidA* primers are specific to *E. coli* O157:H7 strains carrying an atypical β-D-glucuronidase phenotype. Although expected, this observation emphasized the fact that the specific sequences targeted in the *E. coli* O157:H7 *uidA* gene are unique to this serotype, but do not determine the active or inactive state of the encoded enzyme.

FIGURE 3. Agarose gel electrophoresic analysis results of bacterial isolates amplified by triplex PCR. Lanes A, B, C in each gel show 1 kb DNA ladder and positive, and negative control samples, respectively. The highest molecular weight band in a positive control sample corresponds to the stx_2 fragment (779 bp), the next lowest corresponds to the stx_1 fragment (614 bp), and the lowest molecular weight band is *uidA* (252 bp). Lanes 1-8 are PCR amplification results of the following strains of bacteria: 1 - β-D-glucuronidase positive *E. coli* O157:H7; 2 - *Shigella dysenteriae* type 1 (ATCC strain #9361); 3 - *S. sonnei*; 4 - *S. flexneri*; 5 - *S. flexneri*; 6 - *S. sonnei*; 7 - *S. dysenteriae*; 8 - *S. sonnei*. Bacterial strains in lanes 3-8 were provided by the Iowa Environmental and Public Health Laboratory.


A B C 1 2 3 4 A B C 5 6 7 8

Repeated PCR analysis of contaminated and control meat samples

The second set of 160 meat samples was prepared 45-120 days prior to testing and stored frozen until analysis by triplex PCR. This procedure identified *uidA* sequences in 100% of the ground beef and ground pork samples seeded with a dose of 14 CFU *E. coli* O157:H7 per g of meat (Table 7). At a 10-fold lower inoculum, i.e., 1.4 CFU/g, 89% of ground beef samples and 100% of ground pork were detected. With another 10-fold decrease in inoculum

TABLE 7. Sensitivity of the triplex PCR for *uidA* and *stx* gene detection[a] at various inocula

Meat type	Detection target	Samples detected pos/total (%) seeded with inoculum 1.4 CFU/g			
		Dilution			
		x 10	x 1	x 10^{-1}	x 10^{-2}
Ground pork	*uidA*	6/6 (100)	6/6 (100)	1/6 (17)	0/6 (0)
	stx	8/8 (100)	8/8 (100)	2/8 (25)	0/8 (0)
Ground beef (average)	*uidA*	19/19 (100)	17/19 (89)	12/19 (63)	2/19 (10)
	stx	24/24 (100)	22/24 (92)	16/24 (67)	5/24 (21)
Ground beef 70% lean	*uidA*	8/8 (100)	7/8 (87)	4/8 (50)	0/8 (0)
	stx	8/8 (100)	7/8 (87)	6/8 (75)	2/8 (25)
Ground beef 80% lean	*uidA*	4/4 (100)	4/4 (100)	4/4 (100)	1/4 (25)
	stx	8/8 (100)	8/8 (100)	6/8 (75)	1/8 (12)
Ground beef 93% lean	*uidA*	7/7 (100)	6/7 (86)	4/7 (57)	2/7 (29)
	stx	8/8 (100)	7/8 (87)	4/8 (50)	2/8 (25)

[a] The second analysis of samples. Results of the first PCR analysis are presented in the manuscript.

(0.14 CFU/g), detection of positive samples by PCR dropped to 63% in ground beef and 17% in ground pork. Furthermore, the triplex PCR procedure detected 10% of ground beef samples seeded with 10-fold fewer cells, 0.014 CFU/g, but none of the seeded pork samples. The overall sensitivity of the PCR procedure for *uidA* gene detection for samples inoculated with 0.14 to 14 CFU/g was 88% in ground beef and 72% in ground pork. In comparison, the overall sensitivity of PCR in the first set of samples was 91% and 55% in ground beef and ground pork, respectively. The specificity of PCR in the second set of samples was the same as in the first, i.e., 100% regardless of type of meat. None of the control meat samples and no meat samples contaminated with STEC other than O157:H7 displayed the *uidA* band.

The presence of the *stx* sequence was correctly identified in 100% and 92% of ground beef samples seeded with 14 and 1.4 CFU/g, respectively, and in 100% of ground pork samples seeded with the same doses (Table 7). For the next two inocula levels, i.e., 0.14 and 0.014 CFU/g, triplex PCR correctly detected *stx* genes in 67% and 21% ground beef samples and in 25% and 0% ground pork samples, respectively. The overall sensitivity of triplex PCR for *stx* sequence detection in samples seeded with 0.14 to 14 CFU/g was 86% in ground beef and 75% in ground pork compared to 90% and 71% sensitivity in the first set of samples of beef and pork, respectively. In four samples, *stx* genes were identified in non-seeded meat samples: two in ground beef 80% lean and one each in ground pork and 93% lean ground beef. Additionally, PCR products of both stx genes were found in 3% of meat samples known to be inoculated with a STEC strain carrying a single *stx* gene.

The comparison of the triplex PCR results in the two sets of samples (immediate PCR and PCR on samples stored frozen) is displayed in Figure 4. The sensitivities calculated for each separate inoculation level varied insignificantly between the first and second set of samples. An improvement in PCR sensitivity for *uidA* and *stx* gene detection was observed in second set of ground pork samples seeded with 1.4-14 CFU/g. The sensitivity was the same in ground beef seeded with 14 CFU/g and slightly higher in the second set of ground beef samples seeded with 1.4 CFU/g. Meanwhile, a small decrease was detected in *uidA* and *stx* gene identification in both meats at the two lowest inocula (0.14 and 0.014 CFU/g). The individual agreement between the PCR results of the first and second samples varied

considerably, especially at inoculum levels 1.4 and 0.14 CFU/g (Table 8). In total, 83% of samples subjected to PCR analysis produced identical target band profiles in both amplifications.

Repetition of PCR analysis of samples saved from the first series of experiments revealed comparable sensitivity. While the individual results varied between the first and second samples, the overall sensitivity did not differ significantly.

TABLE 8. Discrepancy between the results of the first[a] and the second[b] triplex PCR.

Meat type	Detection target	No. of samples with disagreement/total (%) in meat seeded with mean inoculum 1.4 CFU/g			
		Dilution			
		x 10	x 1	x 10^{-1}	x 10^{-2}
Ground pork	*uidA*	1/6 (17)	2/6 (33)	2/6 (33)	0/6 (0)
	stx	1/8 (13)	1/8 (13)	3/8 (38)	0/8 (0)
Ground beef (average)	*uidA*	0/19 (0)	5/19 (26)	5/19 (26)	2/19 (11)
	stx	0/24 (0)	5/24 (21)	8/24 (33)	4/24 (17)
Ground beef 70% lean	*uidA*	0/8 (0)	3/8 (38)	3/8 (38)	0/8 (0)
	stx	0/8 (0)	3/8 (38)	2/8 (25)	2/8 (25)
Ground beef 80% lean	*uidA*	0/4 (0)	0/4 (0)	0/4 (0)	1/4 (25)
	stx	0/8 (0)	0/8 (0)	2/8 (25)	1/8 (13)
Ground beef 93% lean	*uidA*	0/7 (0)	2/7 (29)	2/7 (29)	1/7 (14)
	stx	0/8 (0)	2/8 (25)	4/8 (50)	1/8 (13)

[a] The first sets of meat samples were analyzed by triplex PCR immediately after preparation. The results are described in the manuscript.
[b] The second sets of samples were stored frozen until later analysis to determine the repeatability of the PCR results.

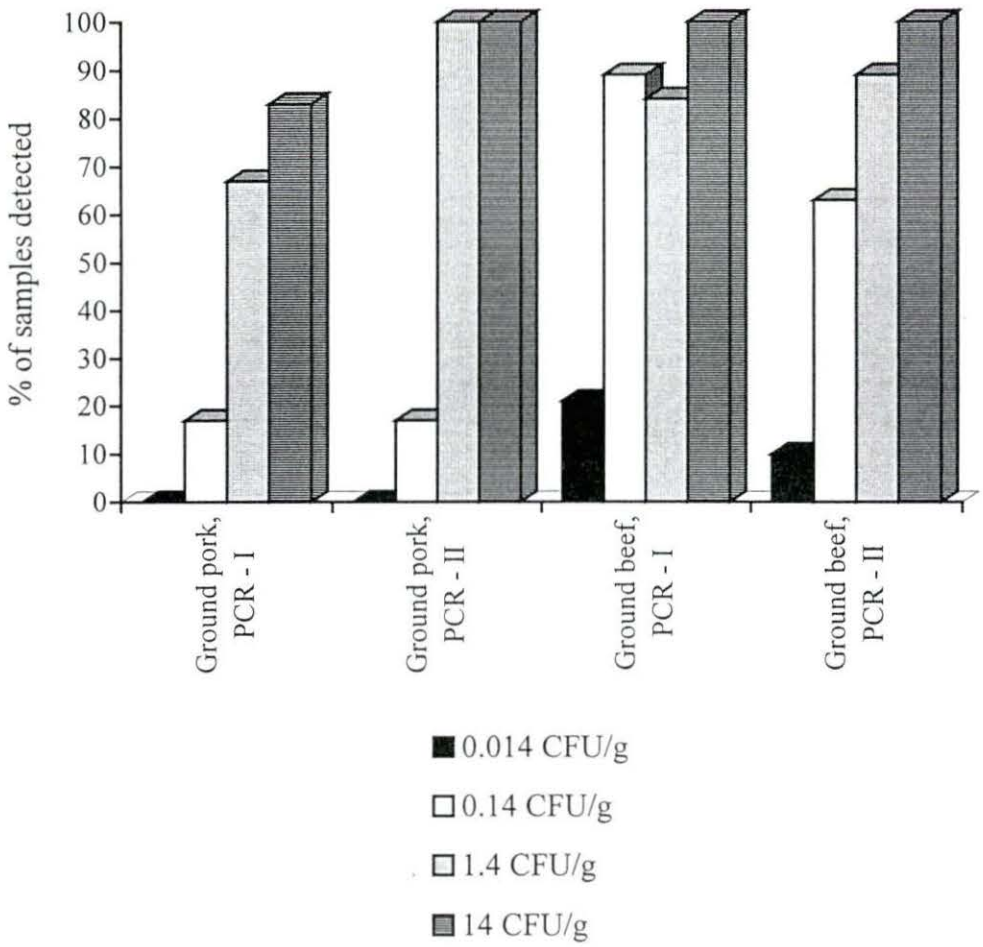


FIGURE 4. Comparison of PCR sensitivity in the first and second set of meat samples

There was a pattern in discrepancies at various inocula levels: at the highest and the lowest inocula levels (14 and 0.014 CFU/g) only few results disagreed (8 of 114 samples), while at the medium inocula (1.4 and 0.14 CFU/g) this disagreement was more frequent (31 of 114 samples). A possible explanation is that in high and low inocula seeded samples the amount of DNA template is constantly low or high and therefore PCR amplification results do not vary widely. Meat samples seeded with inocula 1.4 and 0.14 appear to contain marginal amounts of DNA which are detected only if PCR conditions are optimal. Although PCR reactions contain the same PCR reagents, the condition of the PCR may vary in each tube after addition of the DNA template. This accounts for the greater variability between results of the first and second PCR analysis of meat samples seeded with the medium size inoculum. If the PCR method described here was to be adopted for routine use, it might be of value to perform several PCR amplifications of the same meat sample preparation. The identification of *stx* or *uidA* corresponding markers in one of the PCR reactions of the sample would be sufficient evidence of contamination.

REFERENCES CITED

Acheson, D. W. K., L. L. Lincicome, S. De Breucher, and G. T. Keusch. 1996. Detection of Shiga-like toxin-producing *Escherichia coli* in ground beef and milk by commercial enzyme immunoassay. J. Food Protect. **59:**344-349.

Acheson, D. W. K., and G. T. Keusch. 1996. Which Shiga toxin-producing types of *E. coli* are important? ASM News **62:**302-306.

Ahmed, R., C. Bopp, A. Borczyk, and S. Kasatiya. 1987. Phage-typing scheme for *Escherichia coli* O157:H7. J. Infect. Dis. **155:**806-809.

Albert, M. G. and K. A. Bettelheim. 1989. Enterohemorrhagic *E. coli* O157:H7 in central Australia (Letter). J. Diarrhoeal Dis. Res. **7:**96-97.

American Meat Institute. 1997. Issue: microbiological testing program for *Escherichia coli* O157:H7 in raw ground beef. <http://ifse.tamu.edu/ifse/ECOLI/amiinfo1.htm> (Accessed 28 January, 1997)

Bauer, M. E. and R. A. Welch. 1996. Characterization of an RTX toxin from enterohemorrhagic *Escherichia coli* O157:H7. Infect. Immun. **64:**167-175.

Begum, D. and M. P. Jackson. 1995. Direct detection of Shiga-like toxin-producing *Escherichia coli* in ground beef using the polymerase chain reaction. Mol. Cell. Probes **9:**259-264.

Bell, B. P., M. Goldoft, P. M. Griffith, M. A. Davis, D. C. Gordon, P. I. Tarr, C. A. Bartleson, J. H. Lewis, T. J. Barrett, and J. G. Wells 1994. A multistate outbreak of *Escherichia coli* O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers: the Washington experience. JAVMA **272:**1349-1353.

Belongia, E. A., M. T. Osterholm, J. T. Soler, D. A. Ammend, J. E. Braun, and K. L. MacDonald. 1993. Transmission of *Escherichia coli* O157:H7 infection in Minnesota child day-care facilities. JAMA. **269:**883-888.

Beutin, L., D. Geier, S. Zimmermann, and H. Karch. 1995. Virulence markers of Shiga-like toxin-producing *Escherichia coli* strains originating from healthy domestic animals of different species. J. Clin. Microbiol. **33:**631-635.

Beutin, L., U. H. Stroeher, and P A. Manning. 1993. Isolation of enterohemolysin (Ehly2)-associated sequences encoded on temparate phages of *Escherichia coli*. Gene. **132:**95-99.

Bettelheim, K. A. 1996. Enterohemorrhagic *Escherichia coli*: a new problem, an old group of organisms. Austr. Vet. J. **73:**20-26.

Bitzan, M., K. Ludwig, M. Klemt, H. Konig, J. Buren, and D. E. Muller-Wiefel. 1993. The role of *Esherichia coli* O157 infections in the classical (enteropathic) Haemolytic uraemic syndrome: Results of a Central European, multicentre study. Epidemiol. Infect. **110:**183-196.

Bitzan, M., E. Moebius, K. Ludwig, D. E. Muller-Wiefel, J. Heesemann, and H. Karch. 1991. High incidence of serum antibodies to *Escherichia coli* O157 lipopoly saccharide in children with hemolytic-uremic syndrome. J. Pediatr. **119:**380-385.

Bokete, T. N., C. M. O'Callahan, C. R. Clausen, N. M. Tang, N. Tran, S. L. Moseley, T. R. Fritsche, and P. I. Tarr. 1993. Shiga-like toxin-producing *Escherichia coli* in Seattle children: a prospective study. Gastroenterology **105:**1724-1731.

Bosworth, B. T., J. E. Samuel, H. W. Moon, A. D. O'Brien, V. M. Gordon, and S. C. Whipp. 1996. Vaccination with genetically modified Shiga-like toxin IIe prevents edema disease in swine. Infect. Immun. **64:**55-60.

Boyce, T. G., A. G. Pemberton, J. G. Wells, and P. M. Griffin. 1995a. Screening for *Escherichia coli* O157:H7 - a nationwide survey of clinical laboratories. J. Clin. Microbiol. **33:**3275-3277.

Boyce, T. G., D. L. Swerdlow, and P. M. Griffin. 1995b. *Escherichia coli* O157:H7 and the hemolytic-uremic syndrome. N. Engl. J. Med. **333:**364-368.

Brenner, D. J. 1984. Family *Enterobacteriaceae*, p. 408-420. *In* N. R. Krieg and J. G. Holt (ed.), Manual of Systematic Bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.

Brewster, D. H., M. I. Brown, D. Robertson, G. L. Houghton, J. Bimson, and J. C. Sharp. 1994. An outbreak of *Escherichia coli* O157 associated with children's paddling pool. Epidemiol. Infect. **112:**441-447.

Brian, M. J., M. Frososlono, B. E. Murray, A. Miranda, E. L. Lopez, H. F. Gomez, and T. G. Cleary. 1992. Polymerase chain reaction for diagnosis of enterohemorrhagic *Escherichia coli* infection and hemolytic uremic syndrome. J. Clin. Microbiol. **30:**1801-1806.

Brotman, M., R. A. Giannella, C. P. F. Alm, H. Bauman, A. R. Bennet, R. E. Black, C. M. Bruhn, M. B. Cohen, S. L. Gorbach, J. B. Kaper, M. R. Roberts, J. L. Staneck, S. Taylor, and H. F. Troutt. 1995. Consensus conference statement:

Escherichia coli O157:H7 infections - an emerging national health crisis, July 11-13, 1994. Gastroenterology **108:**1923-1934.

Brown, C. A., B. G. Harmon, T. Zhao, and M. P. Doyle. 1997. Experimental *Escherichia coli* O157:H7 carriage in calves. Appl. Environ. Microbiol. **63:**27-32.

Brown, J. E., O. Sethabutr, M. P. Jackson, S. Lolekha, and P. Echeverria. 1989. Hybridization of *Escherichia coli* producing Shiga-like toxin I, Shiga-like toxin II, and variant of Shiga-like toxin II with synthetic oligonucleotide probes. Infect. Immun. **57:**2811-2814.

Browning, N. G., J. R. Botha, H. Sacho, and P. J. Moore. 1990. *Escherichia coli* O157:H7 haemorrhagic colitis. Report of the first South Africa case. S. Afr. J. Surg. **28:**28-29.

Brownlie, L. E. and F. H. Grau. 1967. Effect of food intake on growth and survival of salmonellas and *Escherichia coli* in the bovine rumen. J. Gen. Microbiol. **46:**125-134.

Canadian Disease Weekly Report. 1983. Hemorrhagic colitis in a home for the aged - Ontario. **9-8:**29-32.

Caprioli, A., I. Luzzi, F. Rosmini, P. Pasquini, R. Cirrincione, A. Gianviti, M. C. Matteucci, and G. Rizzoni. 1992. Hemolytic-uremic syndrome and Vero cytotoxin-producing *Escherichia coli* infection in Italy. The HUS Italian Study Group. J.Infect. Dis. **166:**154-158.

Caprioli, A., I. Luzzi, F. Rosmini, C. Resti, A. Edefonti, F. Perfumo, C. Farina, A. Goglio, A. Gianviti, and G. Rizzoni. 1994. Communitywide outbreak of hemolytic-uremic syndrome associated with with non-O157 verocytotoxin-producing *Escherichia coli*. J. Infect. Dis. **169:**208-213.

Carter, A. O., A. A. Borczyk, J. A. Carlson, B. Harvey, J. C. Hockin, M. A. Karmali, C. Krishnan, D. A. Korn, and H. Lior. 1987. A severe outbreak of *Escherichia coli* O157:H7-associated hemorrhagic colitis in a nursing home. N. Engl. J. Med. **317:**1496-1500.

Cebula, T. A., W. L. Payne, and P. Feng. 1995. Simultaneous identification of strains of *Escherichia coli* serotype O157:H7 and their Shiga-like toxin type by mismatch amplification mutation assay-multiplex PCR. J. Clin. Microbiol. **33:**248-250.

Center for Disease Control and Prevention. 1993. Update: multistate outbreak of *Escherichia coli* O157:H7 infections from hamburgers - Western States, 1992-1993. MMWR **42:**258-262.

Center for Disease Control and Prevention. 1996. Outbreaks of *Escherichia coli* O157:H7 infection and cryptosporidiosis associated with drinking unpasteurized apple cider - Connecticut and New York, October 1996. MMWR. **46:**4-8.

Centers for Epidemiology and Animal Health. 1994a. *Escherichia coli* O157:H7 - Issues and Ramifications. USDA:APHIS:VS; Fort Collins, Colorado.

Centers for Epidemiology and Animal Health. 1994b. *Escherichia coli* O157:H7 in U.S. dairy calves. USDA:APHIS:VS; Fort Collins, Colorado.

Chapman, P. A. and C. A. Siddons. 1994. A comparison of strains of *Escherichia coli* O157 from humans and cattle in Sheffield, United Kingdom. J. Infect. Dis. **170:**251-252.

Chapman, P. A., C. A. Siddons, D. J. Wright, P. Norman, J. Fox, and E. Crick. 1993. Cattle as a possible source of verocytotoxin-producing *Escherichia coli* O157 infection in man. Epidemiol. Infect. **111:**439-447.

Chapman, P. A., C. A. Siddons, P. M. Zadic, and L. Jewes. 1991. An improved selective medium for the isolation of *Escherichia coli* O157:H7. J. Med. Microbiol. **35:**107-110.

Chapman, P. A., D. J. Wright, and C. A. Siddons. 1994. A comparison of immunomagnetic separation and direct culture for the isolation of verocytotoxin-producing *Escherichia coli* from bovine faeces. J. Med. Microbiol. **40:** 424-427.

Chart, H., S. M. Scotland, H. R. Smith, and B. Rowe. 1989. Antibodies to *Escherichia coli* O157 in patients with hemorrhagic colitis and haemolytic uraemic syndrome. J. Clin. Pathol. **42:**973-976.

Chart, H., H. R. Smith, S. M. Scotland, B. Rowe, D. V. Milford, and C. M. Taylor. 1991. Serological identification of *Escherichia coli* O157:H7 infection in haemolytic uraemic syndrome. Lancet. **337:**138-140.

Church, D. L., G. Cadrain, A. Kabani, T. Javadji, and C. Trevenen. 1995. Practice guidelines for ordering stool cultures in a pediatric population. Am. J. Clin. Pathol. **103:**149-153.

Cimolai, N., J. E. Carter, B. J. Morrison, and J. D. Anderson. 1990. Risk factors for the progression of *Escherichia coli* O157:H7 enteritis to hemolytic-uremic syndrome. J. Pediatr. **116:**589-592.

Cordovez, A., V. Prado, L. Maggi, J. Cordero, J. Martinez, A. Misraji, R. Rios, G. Soza, A. Ojeda, and M. M. Levine. 1992. Enterohemorrhagic *Escherichia coli*

associated with hemolytic-uremic syndrome in Chilean children. J. Clin. Micrbiol. **30:**2153-2157.

Cray, W. C. Jr. and H. W. Moon. 1995. Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. Appl. Environ. Microbiol. **61:**1568-1590.

Dargatz, D. A., S. J. Wells, L. A. Thomas, D. D. Hancock, and L. P. Garber. J. Food. Prot., in press.

Deng, M. Y. and P. M. Fratamico. 1996. A multiplex PCR for rapid identification of Shiga-like toxin-producing *Escherichia coli* O157:H7 isolated from foods. J. Food. Prot. **59:**570-576.

Dev, V. J., M. Main, and I. Gould. 1991. Waterborne outbreak of *Escherichia coli* O157. Lancet (letter) **337:**1412.

Doyle, M. P. and J. L. Schoeni. 1987. Isolation of *Escherichia coli* O157:H7 from retail fresh meats and poultry. Appl. Environ. Micrbiol. **53:**2394-2396.

Farmer, J. J. 3rd. 1995. *Enterobacteriaceae*: introduction and identification, p. 438-449. *In* P. R. Murray, E. J. Baron, M. A. Phaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of Clinical Microbiology, 6th ed. American Society for Microbiology, Washington, D. C.

Farmer, J. J. and B. R. Davis. 1985. H7 antiserum-sorbitol fermentation medium: a single tube screening medium for detecting *Escherichia coli* O157:H7 associated with hemorrhagic colitis. J. Clin. Microbiol. **22:**620-625.

Farmer, J. J. 3rd, M. E. Potter, L. W. Riley, T. J. Barrett, P. A. Blake, and C. A. Bopp. 1983. Animal models to study *Esherichia coli* O157:H7 isolated from patients with haemorrhagic colitis. Lancet. **1:**702-703.

Feng, P. 1993. Identification of *Escherichia coli* serotype O157:H7 by DNA probe specific for an allele of *uidA* gene. Mol. Cell. Probes. **7:**151-154.

Feng, P. 1995. *Escherichia coli* serotype O157:H7: novel vehicles of infection and emergence of phenotypic variants. Emerg. Infect. Dis. **1:**47-52.

Feng, P. and K. A. Lampel. 1994. Genetical analysis of *uidA* expression in enterohemorrhagic *Escherichia* coli serotype O157:H7. Microbiology **140:**2101-2107.

Food Safety and Inspection Service. 1996. Nationwide pork microbiological baseline data collection program: market hogs. U. S. Department of Agriculture, Washington, DC.

Francis, D. H., J. E. Collins, and J. R. Duimstra. 1986. Infection of gnotobiotic pigs with an *Escherichia coli* O157:H7 strain associated with an outbreak of hemorrhagic colitis. Infect. Immun. **51:**953-956.

Fratamico, P. M., R. L. Buchanan, and P. H. Cooke. 1993. Virulence of an *Escherichia coli* O157:H7 sorbitol-positive mutant. Appl. Environ. Microbiol. **59:**4245-4252.

Fratamico, P. M., S. K. Sackitey, M. Weidmann, and M. Y. Deng. 1995. Detection of *Escherichia coli* O157:H7 by multiplex PCR. J. Clin. Microbiol. **33:**2188-2191.

Gannon, V. P. J., C. L. Gyles, and B. P. Wilcock. 1989. Effects of *Escherichia coli* Shiga-like toxins (verotoxins) in pigs. Can. J. Vet. Res. **53:**306-312.

Gannon, V. P. J., R. K. King, J. Y. Kim, and E. J. G. Thomas. 1992. Rapid and sensitive method for detection of Shiga-like toxin-producing *Escherichia coli* in ground beef using the polymerase chain reaction. Appl. Environ. Microbiol. **58:**3809-3815.

Garber, L. P., S. J. Wells, D. D. Hancock, M. P. Doyle, J. Tuttle, J. A. Shere, and T. Zhao. 1995. Risk factors for fecal shedding of *Escherichia coli* O157:H7 in dairy calves. JAVMA. **207:**46-49.

Gicquelais, K. G., M. M. Baldini, J. Martinez, L. Maggi, W. C. Martin, V. Prado, J. B. Kaper, and M. M. Levine. 1990. Practical and economical method for using biotinylated DNA probes with bacterial colony blots to identify diarrhea-causing *Escherichia coli*. J. Clin. Microbiol. **28:**2485-2490.

Griffin, P. M., L. C. Olmstead, and R. E. Petras. 1990. *Escherichia coli* O157:H7-associated colitis. A clinical and histological study of 11 cases. Gastroenterology. **99:**142-149.

Griffin, P. M., S. M. Ostroff, R. V. Tauxe, K. D. Greene, J. G. Wells, J. H. Lewis, and P. A. Blake. 1988. Illnesses associated with *Escherichia coli* O157:H7 infections. Ann. Intern. Med. **109:**705-712.

Griffin, P. M. and R. V. Tauxe. 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. Epidemiol. Rev. **13:**60-98.

Hamano, S., Y. Nakanishi, T. Nara, T. Seki, T. Ohtani, T. Oishi, K. Joh, T. Oikawa, Y. Muramatsu, and Y. Ogawa. 1993. Neurologic manifestations of hemorrhagic colitis in the outbreak of *Escherichia coli* O157:H7 infection in Japan. Acta Paediatr. **82:**454-458.

Hancock, D. D., T. E. Besser, M. L. Kinsel, P. I. Tarr, D. H. Rice, and M. G. Paros. 1994. The prevalence of *Escherichia coli* in dairy and beef cattle in Washington state. Epidemiol. Infect. **113:**199-207.

Hayes, P. S., K. Blom, P. Feng, J. Lewis, N. A. Strockbine, and B. Swaminathan. 1995. Isolation and characterization of a β-D-glucuronidase-producing strain of *Escherichia coli* serotype O157:H7 in the United States. J. Clin. Microbiol. **33:**3347-3348.

Hornsby-Lewis, L. 1995. A newly recognized source of *Escherichia coli* O157:H7 infections. Gastroenterology **108:**1597-1602.

Institute of Food Science and Technology. 1997. VTEC: Editorial footnote, updated to 17 January 1997. Serious outbreak in Scotland. <http://www.easynet.co.uk./ifst/vtecb.htm#top> (Accessed 28 January 1997).

Jackson, T. C., M. D. Hardin, and G. R. Acuff. 1996. Heat resistance of *Escherichia coli* O157:H7 in a nutrient medium and in ground beef patties as influenced by storage and holding temperatures. J. Food Protect. **59:**230-237.

Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kapler. 1990. A genetic loci of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions in tissue culture cells. Proc. Natl. Acad. Sci. USA. **87:**7839-7843.

Jinneman, K. C., P. A. Trost, W. E. Hill, S. D. Weagant, J. L. Bryant, C. A. Kaysner, and M. M. Wekell. 1995. Comparison of template preparation methods from foods for amplification of *Escherichia coli* O157 Shiga-like toxins type I and II by multiplex polymerase chain reaction. J. Food Prot. **58:**722-726.

Karch, H., J. Heeseman, R. Laufs, A. D. O'Brien, C. O. Tacket, and M. M. Levine. 1987. A plasmid of enterohemorrhagic *Escherichia coli* O157:H7 is required for expression of a new fimbrial antigen and for adhesion to epithelial cells. Infect. Immun. **55:**455-461.

Karch, H. and T. Meyer. 1989a. Evaluation of oligonucleotide probes for identification of Shiga-like toxin-producing *Escherichia coli*. J. Clin. Microbiol. **27:**1180-1186.

Karch, H. and T. Meyer. 1989b. Single primer pair for amplifying segments of distinct Shiga-like-toxin genes by polymerase chain reaction. J. Clin. Microbiol. **27:**2751-2757.

Karmali, M. A., M. Petric, C. Lim, R. Cheung, and G. S. Arbus. 1985a. Sensitive method for detecting low numbers of Verotoxin-producing *Escherichia coli* in mixed culture by use of colony sweeps and polymyxin extraction of verotoxin. J. Clin. Microbiol. **22:**614-619.

Karmali, M. A., M. Petric, C. Lim, P. C. Fleming, G. S. Arbus, and H. Lior. 1985b. The association between idiopathic hemolytic-uremic syndrome and infection by verotoxin-producing *Escherichia coli*. J. Infect. Dis. **151:**775-782.

Karmali, M. A., B. T. Steel, M. Petric, and C. Lim. 1983. Sporadic cases of haemolytic-uremic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. Lancet. **1:**619-620.

Kelly, J. K., C. H. Pai, I. H. Jadusingh, M. L. Macinnis, E. A. Shaffer, and N. B. Hershfield. 1986. The histopathology of rectosigmoid biopsies from adults with bloody diarrhea due to verotoxin-producing *Escherichia coli*. Am. J. Clin. Pathol. **88:**78-82.

Khakhria, R., D. Duck, and H. Lior. 1990. Extended phage-typing scheme for *Escherichia coli* O157:H7. Epidemiol. Infect. **105:**511-520.

Kim, M. S. and M. P. Doyle. 1992. Dipstick immunoassay to detect enterohemorrhagic *Escherichia coli* O157:H7 in retail ground beef. Appl. Environ. Microbiol. **58:**1764-1767.

Lang A. L., Y. L. Tsai, C. L. Mayer, K. C. Patton, and C. J. Palmer. 1994. Multiplex PCR for detection of the heat-labile toxin gene and Shiga-like toxin I and II genes in *Escherichia coli* isolated from natural waters. Appl. Environ. Microbiol. **60:**3145-3149.

Law, D., A. A. Hamour, D. W. K. Acheson, H. Panigrahi, L. A. Ganguli, and D. W. Denning. 1994. Diagnosis of infections with Shiga-like toxin-producing *Escherichia coli* by use of enzyme-linked immunosorbent assays for Shiga-like toxins on cultured stool samples. J. Med. Microbiol. **40:**241-245.

Lee, A. and E. Gemmel. 1972. Changes in the mouse intestinal microflora during weaning: role of volatile acids. Infect. Immun. **5:**1-7.

LeSaux, N., J. S. Spika, and B. Friesen. 1993. Ground beef consumption in noncommercial settings as a risk factor for sporadic *Escherichia coli* O157:H7 infection in Canada. J. Infect. Dis. **167:**500-502.

Levine, M. M. 1987. *Escherichia coli* that cause diarhhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. **155:**377-389.

Levine, M. M., J. Xu, J. B. Kaper, H. Lior, V. Prado, B. Tall, J. Nataro, H. Karch, and K. Wachmuth. 1987. A DNA probe to identify enterohemorrhagic *Escherichia coli*

of O157:H7 and other serotypes that cause hemorrhagic colitis and hemolytic uremic syndrome. J. Infect.Dis. **156:**175-182.

Leyer, G. J., L. L. Wang, and E. A. Johnson. 1995. Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. Appl. Environm. Microbiol. **61:**3752-3755.

Lin, J., M. P. Smith, K. C. Chapin, H. S. Baik, G. N. Bennet, and J. W. Foster. 1996. Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. Appl. Environ. Microbiol. **62:**3094-3100.

Lopez, E. L., M. Diaz, S. Grinstein, S. Devoto, F. Mendilaharzu, B. E. Murray, S. Ashkenazi, E. Rubeglio, M. Woloj, and M. Vasquez. 1989. Hemolytic uremic syndrome and diarrhea in Argentine children: the role of Shiga-like toxins. J. Infect. Dis. **160:**469-475.

Louie, M., J. De Azavedo, R. Clarke, A. Borczyk, H. Lior, M. Richter, and J. Brunton. 1994. Sequence heterogeneity of the *eae* gene and detection of verotoxin producing *Escherichia coli* using serotype-specific primers. Epidemiol. Infect. **112:**449-461.

Louise, C. B. and T. G. Obrig. 1995. Specific interaction of *Escherichia coli* O157:H7-derived Shiga-like toxin II with human renal endothelial cells. J. Infect. Dis. **172:**1397-1401.

MacDonald, K. L., M. J. O'Leary, and M. L. Cohen. 1988. *Escherichia coli* O157:H7, an emerging gastrointestinal pathogen. Results of a one-year, prospective, population based study. JAVMA. **259:**3567-3570.

Maniar, A. C., T. Williams, C. M. Anand, and G. W. Hammond. 1990. Detection of Verotoxin in stool specimens. J. Clin. Microbiol. **28:**134-135.

Marks, S. and T. Roberts. 1993. *E. coli* O157:H7 ranks as the fourth most costly foodborne disease. Food Rev. **16:**51-59.

Marques, L. R. M., M. A. Moore, J. G. Wells, I. K. Wachsmuth, and A. D. O'Brien. 1986. Production of Shiga-like toxin by *Escherichia coli*. J. Infect. Dis. **154:**338-341.

Marques, L. R. M., J. S. M. Petris, S. J. Cryz, and A. D. O'Brien. 1987. *Escherichia coli* strains isolated from pigs with edema disease produce a variant of Shiga-like toxin II. FEMS Microbiol. Lett. **44:**33-38.

Martin, D. L., K. L. MacDonald, K. E. White, J. T. Soler, and M. T. Osterholm. 1990. The epidemiology and clinical aspects of the hemolytic uremic syndrome in Minnesota. N. Engl. J. Med. **323:**1161-1167.

McCleary, D. R. and M. T. Rowe. 1995. Development of a selective plating technique for the recovery of *Escherichia coli* O157:H7 after heat stress. Lett. Appl. Microbiol. **21:**252-256.

McKee, M. L. and A. D. O'Brien. 1995. Investigation of enterohemorrhagic *Escherichia coli* O157:H7 adherence characteristics and invasion potential reveals a new attachment pattern shared by intestinal *E. coli*. Infect. Immun. **63:**2070-2074.

Morrison, D. M., D. L. Tyrell, and L. D. Jewell. 1986. Colonic biopsy in verotoxin-induced hemorrhagic colitis and thrombotic thrombocytopenic purpura (TTP). Am. J. Clin. Pathol. **86:**108-112.

Moyer, N. P. 1996. *E. coli* O157:H7 infection in Iowa: 1989 to 1996. <http://www.uhl.uiowa.edu/pubs/hotline/9608/e_coli.html> (Accessed 4 December, 1996).

Neill, M. A. 1989. *E. coli* O157:H7 -- current concepts and future prospects. J. Food Safety. **10:**99-106.

Newland, J. W. and R. J. Neill. 1988. DNA probes for Shiga-like toxins I and II and for toxin-converting bacteriophages. J. Clin. Microbiol. **26:**1292-1297.

O'Brien, A. D., A. R. Melton, C. K. Schmitt, M. L. McKee, M. L. Batts, and D. E. Griffin. 1993. Profile of *Escherichia coli* O157:H7 pathogen responsible for hamburger-borne outbreak of hemorrhagic colitis and hemolytic uremic syndrome in Washington. J. Clin. Microbiol. **31:**2799

O'Brien, A. D. and R. K. Holmes. 1987. Shiga and Shiga-like toxins. Microbiol. Rev. **51:**206-220.

O'Brien A., V. L. Tesh, A. Donohue-Rolfe, M. P. Jackson, S. Olsnes, K. Sandvig, A. A. Lindberg, and G. T. Keusch. 1992. Shiga toxin: biochemistry, genetics, mode of action, and role in pathogenesis. Curr. Top. Microbiol. Immunol. **180:**65-94.

Obrig, T. G., P. J. DelVecchio, J. E. Brown, T. P. Morgan, B. M. Rowland, T. K. Judge, and S. W. Rothman. 1988. Direct cytotoxic action of Shiga toxin on human vascular endothelial cells. Infect. Immun. **56:**2373-2378.

Obrig, T. G., P. J. DelVecchio, M. A. Karmali, M. Petric, T. P. Moran, and T. K. Judge. 1987. Pathogenesis of haemolytic uraemic syndrome. Lancet. **2:**687.

Okrend, A. J. G., B. E. Rose, and B. Bennett. 1990a. A screening method for the isolation of *Escherichia coli* O157:H7 from ground beef. J. Food Prot. **53:**249-252.

Okrend, A. J. G., B. E. Rose, and C. P. Lattuada. 1992. Isolation of *Escherichia coli* O157:H7 using O157 specific antibody coated magnetic beads. J. Food Prot. **55:**214-217.

Okrend, A. J. G., B. E. Rose, and R. Matner. 1990b. An improved screening method for the detection and isolation of *Escherichia coli* O157:H7 from meat, incorporating the 3M Petrifilm™ test kit - HEC - for hemorrhagic *Escherichia coli* O157:H7. J. Food Prot. **53:**936-940.

Ontario Disease Surveillance Report. 1986. Outbreak of gastrointestinal disease in Sarnia, Ontario. **7:**604-611.

Ostroff, S. M., J. M. Kobayashi, and J. H. Lewis. 1989a. Infections with *Escherichia coli* O157:H7 in Washington State. The first year of statewide disease surveillance. JAVMA. **262:**355-359.

Ostroff, S. M., P I. Tarr, M. A. Neill, J. H. Lewis, N. Hargrett-Bean, and J. M. Kobayashi. 1989b. Toxin genotypes and plasmid profiles as determinants of systemic sequelae in *Escherichia coli* O157:H7 infections. J. Infect. Dis. **160:**994-999.

Padhey, N. V. and M. P. Doyle. 1992. *Escherichia coli* O157:H7: epidemiology, pathogenesis, and methods for detection in food. J. Food Prot. **55:**555-565.

Padhey, N. V. and M. P. Doyle. 1991. Rapid procedure for detecting enterohemorrhagic *Escherichia coli* O157:H7 in food. Appl. Environ. Microbiol. **57:**2693-2698.

Pai, C. H., N. Ahmed., and H. Lior. 1988. Epidemiology of sporadic diarrhea due to verocytotoxin-producing *Escherichia coli*: a two year prospective study. J. Infect. Dis. **157:**1054-1057.

Pai, C. H., R. Gordon, H. V. Sims, and L. E. Bryan. 1984. Sporadic cases of hemorrhagic colitis associated with *Escherichia coli* O157:H7: clinical, epidemiological, and bacteriological features. Ann. Intern. Med. **101:**738-742.

Pai, C. H., J. K. Kelly, and G. L. Meyers. 1986. Experimental infection of infant rabbits with verotoxin-producing *Escherichia coli*. Infect. Immun. **51:**16-23.

Paros, M., P. I. Tarr, H. Kim, T. E. Besser, and D. D. Hancock. 1993. A comparison of human and bovine *Esherichia coli* O157:H7 isolates by toxin genotype, plasmid profile, and bacteriophage lambda-restriction fragment length polymorphism profile. J. Infect. Dis. **168:**1300-1303.

Paton, A. W., J. C. Paton, P. N. Goldwater, and P. A. Manning. 1993. Direct detection of *Escherichia coli* Shiga-like toxin gene in primary fecal cultures by polymerase chain reaction. J. Clin. Microbiol. **31:**3063-3067.

Pavia, A. T., C. R. Nichols, D. P. Green, R. V. Tauxe, S. Mottice, K. D. Greene, T. G. Wells, R. L. Siegler, E. D. Brewer, and D. Hannon. 1990. Hemolytic uremic syndrome during an outbreak of *Escherichia coli* O157:H7 infections in institutions for mentally retarded persons: clinical and epidemiologic observations. J. Pediatr. **116:**544-551.

Pierard, D., L. Huyghens, S. Lauwers, and H. Lior. 1991. Diarrhea associated with *Escherichia coli* producing porcine oedema disease verotoxin. Lancet. **338:**762.

Pirro, F., L. H. Wieler, K. Failing, R. Bauerfeind, and G. Baljer. 1995. Neutralizing antibodies against Shiga-like toxins from *Escherichia coli* in colostra and sera of cattle. Vet. Microbiol. **43:**131-141.

Pollard, D. R., W. M. Johnson, H. Lior, S. D. Tyler, and K. R. Rozee. 1990. Rapid and specific detection of Verotoxin genes in *Escherichia coli* by the polymerase chain reaction. J. Clin. Microbiol. **28:**540-545.

Proulx, F., J. P. Turgeon, G. Delage, L. Lafleur, and L. Chicoine. 1992. Randomized, controlled trial of antibiotic therapy for *Escherichia coli* O157:H7 enteritis. J. Pediatr. **121:**299-303.

Rahn, K., J. B. Wilson, K. A. McFadden, S. C. Read, A. G. Ellis, S. A. Renwick, R. C. Clarke, and R. P. Johnson. 1996. Comparison of Vero cell assay and PCR as indicators of the presence of Verocytotoxigenic *Escherichia coli* in bovine and human fecal samples. Appl. Environ. Microbiol. **62:**4314-4317.

Ramegoda, B. and V. L. Tesh. 1996. Differentiation-associated toxin receptor modulation, cytokine production, and sensitivity to Shiga-like toxins in human monocytes and monocytic cell lines. Infect. Immun. **64:**1173-1180.

Rasmussen, M. A., W. C. Cray, Jr., T. A. Casey, and S. C. Whipp. 1993. Rumen contents as a reservoir of enterohemorrhagic *Escherichia coli*. FEMS Microbiol. Lett. **114:**79-84.

Read, S. C., R. C. Clarke, A. Martin, S. A. De Grandis, J. Hii, S. McEwen, and C. L. Gyles. 1992. Polymerase chain reaction for detection of verocytotoxigenic *Escherichia coli* isolated from animal and food sources. Mol. Cell. Probes **6:**153-161.

Read, S. C., C. L. Gyles, R. C. Clarke, H. Lior, and S. McEwen. 1990. Prevalence of verotoxigenic *Escherichia coli* in ground beef, pork, and chicken in southwest Ontario. Epidemiol. Infect. **105:**11-20.

Richardson, S. E., M. A. Karmali, L. E. Becker, and C. R. Smith. 1988. The histopathology of the hemolytic-uremic syndrome associated with verocytotoxin-producing *Escherichia coli* infections. Hum. Pathol. **19:**1102-1108.

Richardson, S. E., T. A. Rotman, V. Jay, C. R. Smith, L. E. Becker, M. Petric, N. F. Olivieri, and M. A. Karmali. 1992. Experimental verocytotoximia in rabbits. Infect. Immun. **60:**4154-4167.

Rietra, P. G. M., G. A. Willshaw, H. R. Smith, A. M. Field, S. M. Scotland, and B. Rowe. 1989. Comparison of Vero-cytotoxin-producing phages from *Escherichia coli* of human and bovine origin. J. Gen. Microbiol. **135:**2307-2318.

Riley, L. W., R. S. Remis, S. D. Remison, H. B. McGee, J. G. Wells, B. R. Davis, R. J. Herbert, E. S. Olcott, L. M. Johnson, N. T. Hargrett, P. A. Blake, and M. L. Cohen. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N. Engl. J. Med. **308:**681-685.

Roberts, C. L., P. A. Mshar, M. L. Carrter, J. L. Hadler, D. M. Sosin, P. S. Hayes, and T. J. Barret. 1995. The role of heightened surveillance in an outbreak of *Escherichia coli* O157:H7. Epidemiol. Infect. **115:**447-454.

Robson, W. L., A. K. Leung, and B. S. Caplan. 1993. Hemolytic-uremic syndrome. Curr. Probl. Pediatr. **23:**16-33.

Rowe, P. C., E. Orrbine, H. Lior, G. A. Wells, and P. N. McLaine. 1993. A prospective study of exposure to vertoxin-producing *Escherichia coli* among Canadian children with hemolytic uraemic syndrome. Epidemiol. Infect. **110:**1-7.

Ruggeneti, P. and G. Remuzzi. 1990. Thrombotic thrombocytopenic purpura and related disorders. Hematol. Oncol. Clin. North Am. **4:**219-241.

Russmann, H., H. Schmidt, J. Heesemann, A. Caprioli, and H. Karch. 1994. Variants of Shiga-like toxin II constitute a major toxin component in *Escherichia coli* O157 strains from patients with hemolytic uraemic syndrome. J. Med. Microbiol. **40:**338-343.

Ryan, C. A., R. V. Tauxe, G. W. Hosek, J. G. Wells, D. A. Stoez, H. W. McFadden, Jr., P. W. Smith, G. E. Wright, and P. A. Blake. 1986. *Escherichia coli* O157:H7 diarrhea in a nursing home: clinical, epidemiological, and pathological findings. J. Infect. Dis. **154:**631-638.

Samadpour, M., L. M. Grimm, B. Desai, D. Alfi, J. E. Ongerth, and P. I. Tarr. 1993. Molecular epidemiology of *Escherichia coli* O157:H7 strains by bacteriophage lambda restriction fragment length polymorphism analysis: application to a multistate food-borne outbreak and a day-care center cluster. J. Clin. Microbiol. **31:**3179-3183.

Samadpour, M., J. Liston, J. E. Ongerth, and P. I. Tarr. 1990. Evaluation of DNA probes for detection of Shiga-like toxin-producing *Escherichia coli* in food and calf fecal samples. Appl. Environ. Microbiol. **56:**1212-1215.

Samadpour, M., J. E. Ongerth, and J. Liston. 1994a. Development and evaluation of oligonucleotide DNA probes for detection and genotyping of Shiga-like toxin producing *Escherichia coli*. J. Food. Protect. **57:**399-402.

Samadpour, M., J. E. Ongerth, J. Liston, N. Tran, D. Nguyen, T. S. Whittam, R. A. Wilson, and P. I. Tarr. 1994b. Occurrence of Shiga-like toxin-producing *Escherichia coli* in retail fresh seafood, beef, lamb, pork, and poultry from grocery stores in Seattle, Washington. Appl. Environ. Microbiol. **60:**1038-1040.

Sanderson, M. W., J. M. Gay, D. D. Hancock, C. C. Gay, L. K. Fox, and T. E. Besser. 1995. Sensitivity of bacteriologic culture for detection of *Escherichia coli* O157:H7 in bovine feces. J. Clin. Microbiol. **33:**2616-2619.

Sandhu, K. S., R. C. Clarke, K. McFadden, A. Brouwer, M. Louie, J. Wilson, H. Lior, and C. L. Gyles. 1996. Prevalence of the *eae*A gene in verotoxigenic *Escherichia coli* strains from dairy cattle in Southwest Ontario. Epidemiol. Infect. **116:**1-7.

Savarino, S. J., A. Fasano, D. C. Robertson, and M. M. Levine. 1991. Enteroaggregative *Escherichia coli* elaborate a heat-stable enterotoxin demonstrable in an *in vitro* rabbit intestinal model. J. Clin. Invest. **87:**1450-1455.

Savarino, S. J., A. McVeigh, J. Watson, A. Cravioto, J. Molina, P. Echeverria, M. K. Bhan, M. M. Levine, and A. Fasano. 1996. Enteroaggregative *Escherichia coli* heat-stable enterotoxin is not restricted to Enteroaggregative *E. coli*. J. Infect. Dis. **173:**1019-1022.

Schaedler, R. W., R. Dubos, and R. Costello. 1965. The development of the bacterial flora in the gastrointestinal tract of mice. J. Exp. Med. **122:**59-66.

Schmidt, H., H. Karch, and L. Beutin. 1994. The large-sized plasmids of enterohemorrhagic *Escherichia coli* O157 strains encode hemolysins which are presumably members of the *E. coli* α-hemolysin family. FEMS Microbiol. Lett. **117:**189-196.

Scotland, S. M., B. Rowe, H. R. Smith, G. A. Willshaw, and R. J. Gross. 1988. Vero cytotoxin-producing strains of *Escherichia coli* from children with haemolytic uraemic syndrome and their detection by specific DNA probes. J. Med. Microbiol. **25:**237-243.

Sealander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. H. Gilmour, and T. S. Whittam. 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. **51:**873-884.

Semanchek, J. J. and D. A. Golden. 1996. Survival of *Escherichia coli* O157:H7 during fermentation of apple cider. J. Food. Protect. **59:**1256-1259.

Siegler, R. L., A. T. Pavia, R. D. Christofferson, and M. K. Milligan. 1994. A 20-year population-based study of postdiarrheal hemolytic uremic syndrome in Utah. Pediatrics. **94:**35-40.

Smith, H. R. and S. M. Scotland. 1993. Isolation and identification methods for *Escherichia coli* O157 and other Vero cytotoxin producing strains. J. Clin. Pathol. **46:**10-17.

Spika, J. S., J. E. Parsons, and D. Nordenberg. 1986. Hemolytic uremic syndrome and diarrhea associated with *Escherichia coli* O157:H7 in a day care center. J. Pediatr. **109:**287-291.

Sramkova, L., M. Bielaszewska, J. Jauda, K. Blahova, and O. Hausner. 1990. Vero cytotoxin-producing strains of *Escherichia coli* in children with haemolytic uraemic syndrome and diarrhoea in Czechoslovakia. Infection. **18:**204-209.

Su, C. and L. J. Brandt. 1995. *Escherichia coli* O157:H7 infections in humans. Ann. Intern. Med. **123:**689-714.

Swerdlow, D. L., B. A. Woodruff, R. C. Brady, P. M. Griffin, S. Tippen, H. D. Donnell, Jr., E. Geldreich, B. J. Payne, A. Meyer, Jr., and J. G. Wells. 1992. A waterborne outbreak in Missouri of *Escherichia coli* O157:H7 associated with bloody diarrhea and death. Ann. Intern. Med. **117:**812-819.

Swinbanks, D. 1996. Outbreak of *E. coli* infection in Japan renews concerns. Nature **382:**290.

Szabo, R. A., E. C. D. Todd, and A. Jean. 1986. Method to isolate *Escherichia coli* O157:H7 from food. J. Food Prot. **49:**768-772.

Szabo, R., E. Todd, J. MacKenzie, L. Parrington, and A. Armstrong. 1990. Increased sensitivity of the rapid hydrophobic grid membrane filter enzyme-labeled antibody

procedure for *Escherichia coli* O157 detection in foods and bovine feces. Appl. Environ Microbiol. **56:**3546-3549.

Tannock, G. W. 1983. Effect of dietary and environmental stress on the gastrointestinal microbiota, p 517-539. *In* D. J. Hentges (ed.), Human intestinal microflora in health and disease. Academic press, Inc. New York, NY.

Tarr, P. I. 1995. *Escherichia coli* O157:H7: clinical, diagnostic, and epidemiological aspects of human infection. Clin. Infect. Dis. **20:**1-10.

Tarr, P. I., M. A. Neill, J. Allen, C. J. Siccardi, S. L. Watkins, and R. O. Hickman. 1989a. The increased incidence of hemolytic-uremic syndrome in King County Washigton: lack of evidence for ascertainment bias. Am. J. Epidemiol. **129:**582-586.

Tarr, P. I., M. A. Neill, C. R. Clausen, J. W. Newland, R. J. Neill, and S. L. Moseley. 1989b. Genotypic variation in pathogenic *Escherichia coli* O157:H7 isolated from patients in Washington, 1984-1987. J. Infect. Dis. **159:**344-347.

Tarr, P. I., M. A.Neill, C. R. Clausen, S. L. Watkins, D. L. Christie, and R. O. Hickman. 1990. *Escherichia coli* O157:H7 and the hemolytic uremic syndrome: importance of early cultures in establishing the etiology. J. infect. Dis. **162:**553-556.

Tesh, V. L. and A. D. O'Brien. 1992. Adherence and colonization mechanisms of enteropathogenic and enterohemorrhagic *Escherichia coli*. Microbial pathogenesis. **12:**245-254.

Tesh, V. L., J. A. Burris, J. W. Owens, V. M. Gordon, E. A. Wadolkowski, A. D. O'Brien, and J. E. Samuel. 1993. Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. Infect. Immun. **61:**3392-3402.

Thomas, A., T. Cheasty, H. Chart, and B. Rowe. 1994. Isolation of Verocytotoxin-producing *Escherichia coli* serotypes O9ab:H- carrying VT2 variant gene sequences from a patient with hemolytic uraemic syndrome. Eur. J. Microbiol. Infect. Dis. **13:**1074-1076.

Thomas, A., H. R. Smith, and B. Rowe. 1993. Use of digoxigenic-labelled oligonucleotide DNA probes for VT2 and VT2 human variant genes to differentiate Vero Cytotoxin-producing *Escherichia coli* strains of serogroup O157. J. Clin. Microbiol. **31:**1700-1703.

Thomas, S., H. R. Smith, G. A. Willshaw, and B. Rowe. 1991. Non-radioactively labeled polynucleotide and oligonucleotide DNA probes, for selectively detecting *Escherichia coli* strains producing verocytotoxins VT1, VT2, and VT2 variant. Mol. Cell. Probes. **5:**129-135.

Todd, E. C. D., R. A. Szabo, P. Peterkin, A. N. Sharpe, L. Parrington, D. Bundle, M. A. J. Gidney, and M. B. Perry. 1988. Rapid hydrophobic grid membrane filter-enzyme-labeled antibody procedure for identification and enumeration of *Escherichia coli* O157 in foods. Appl. Environ. Microbiol. **54:**2536-2540.

Toth, I., T. J. Barrett, M. L. Cohen, H. S. Rumschlag, J. H. Green, and I. K. Wachsmuth. 1991. Enzyme-linked immunosorbent assay for products of the 60-megadalton plasmid of *Escherichia coli* serotype O157:H7. J. Clin. Microbiol. **29:**1016-1019.

Tzipori, S., H. Karch, and I. K. Wachsmuth. 1987. Role of 60-megadalton plasmid and Shiga-like toxins in the pathogenesis of infection caused by enterohemorrhagic *Escherichia coli* O157:H7 in gnotobiotic piglets. Infect. Immun. **55:**3117-3125.

U. S. Department of Health and Human Services. 1995. Outbreak of acute gastroenteritis attributable to *Esherichia coli* serotype O104:H21 - Helena, Montana, 1994. MMWR **44:**501-503.

Voelker, R. 1994. New strategies aimed at *E. coli* O157:H7. JAVMA. **272:**503.

Waldolkowski, E. A., J. A., Burris, and A. D. O'Brien. 1990. Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7. Infect. Immun. **58:**2438-2445.

Walterspiel, J. N., S. Ashkenazi, A. L. Morrow, and T. G. Cleary. 1992. Effect of subinhibitory concentrations of antibiotics on extracellular Shiga-like toxin I. Infection. **20:**25-29.

Waters, J. R., J. C. M. Sharp, and V. J. Dev. 1994. Infection caused by *Escherichia coli* O157:H7 in Alberta, Canada, and in Scotland: a five-year review, 1987-1991. Clin. Infect. Dis. **19:**834-843.

Weeratna, R. D. and M. P. Doyle. 1991. Detection and production of Verotoxin 1 of *Escherichia coli* O157:H7 in food. Appl. Environ. Microbiol. **57:**2951-2955.

Wells, J. G., B. R. Davis, I. K. Wachsmuth, L. W. Riley, R. S. Remis, R. Sokolov, and G. K. Morris. 1983. Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Esherichia coli* serotype. J. Clin. Microbiol. **18:**512-520.

Wells, J. G., L. D. Shipman, K. D. Greene, E. G. Sowers, J. H. Green, D. N. Cameron, F. P. Downes, M. L. Martin, P. M. Griffin, S. M. Ostroff, M. E. Potter, R. V. Tauxe, and I. K. Wachsmuth. 1991. Isolation of *Escherichia coli* serotype

O157:H7 and other Shiga-like toxin-producing *E. coli* from dairy cattle. J. Clin. Microbiol. **29:**985-989.

Whipp, S. C., M. A. Rasmussen, and W. C. Cray, Jr. 1994. Animals as a source of *Escherichia coli* pathogenic for human beings. JAVMA **204:**1168-1175.

Whittham, T. S., I. K. Wachmuth, and R. A. Wilson. 1988. Genetic evidence of clonal descent of *Escherichia coli* O157:H7 associated with hemorrhagic colitis and hemolityc uremic syndrome. J. Infect. Dis. **157:**1124-1133.

Whittham, T. S. and R. A. Wilson. 1988. Genetic relationships among pathogenic *Escherichia coli* of serogroup O157. Infect. Immun. **56:**2467-2473.

Willshaw, G. A., H. R. Smith, S. M. Scotland, A. M. Field, and B. Rowe. 1987. Heterogeneity of *Escherichia coli* phages encoding Vero cytotoxins: comparison of cloned sequences determining VT1 and VT2 and development of specific gene probes. J. Gen. Microbiol. **133:**1309-1317.

Wilson, J. B., S. A. McEwen, R. C. Clarke, K. E. Leslie, R. A. Wilson, D. Waltner-Toews, and C. L. Gyles. 1992. Distribution and characteristics of verocytotoxigenic *Escherichia coli* isolated from Ontario dairy cattle. Epidemiol. Infect. **108:**423-439.

Witham, P. K., C. T. Yamashiro, K. J. Livak, and C. A. Batt. 1996. A PCR-based assay for the detection of *Escherichia coli* Shiga-like toxin genes in ground beef. Appl. Environ. Microbiol. **62:**1347-1353.

Wolcott, M. J. 1992. Advances in nucleic acid-based detection methods. Clin. Microbiol. Rev. **5:**370-386.

Wray, C., I. M. McLaren, and P. J. Carroll. 1993. *Escherichia coli* isolated from farm animals in England and Wales between 1986 and 1991. Vet. Record. **133:**439-442.

Wright, D. J., P. A. Chapman, and C. A. Siddons. 1994. Immunomagnetic separation as a sensitive method for isolating *Escherichia coli* O157 from food samples. Epidemiol. Infect. **113:**31-39.

Yamada, S., S. Matsushita, A. Kai, M. Sasaki, A. Tsuji, and T. Kanemitsu. 1993. Detection of verocytotoxin from stool and serological testing of patients with diarrhea caused by *Escherichia coli* O157:H7. Microbiol. Immun. **37:**111-118.

Zhao, T., M. P. Doyle, J. Shere, and L. Garber. 1995. Prevalence of enterohemorrhagic *Escherichia coli* O157:H7 in a survey of dairy herds. Appl. Environ. Microbiol. **61:**1290-1293.

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