EXECUTIVE SUMMARY

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The Significance of Non-O157 Shiga Toxin-producing *Escherichia coli* in Food

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ABSTRACT

Although *Escherichia coli* O157:H7 has received considerable attention in the United States, other pathogenic *E. coli* strains are also known to cause foodborne and water borne illnesses. However, since these strains have not received the same level of attention, from either a scientific or a public health point of view, it is important for the food safety community to understand the biology of these bacteria. This manuscript is a review of the current state of knowledge on the taxonomy and pathogenicity of the non-O157 shiga toxin producing *E. coli* strains (non-O157 STEC), as well as their significance to human health. Of this group, six serovars have been responsible for most of the human disease outbreaks in North America: O26, O45, O103, O111, O121, and O145. The methodology used for detection of these organisms is a key issue and is also discussed in detail. Finally, current knowledge about the prevalence of non-O157 STEC in food, and possible methods of disease control and prevention, are reviewed.

"The authors of this article and editors of *Food Protection Trends* dedicate this article to Mike Grant, who contributed greatly to the successful completion of the project. Mike passed away on 28 August 2010. His active participation and love for food microbiology will be missed."

INTRODUCTION

The species *Escherichia coli* consists of a large number of serovars, many (if not most) of which are not pathogenic to humans. Those that are pathogenic are generally classified into groups based on the presence of specific virulence factors that impact the type of disease manifestation observed in infected individuals. These major groups of *E. coli* are referred to as diffuse-adhering (DAEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC) and enterohemorrhagic (EHEC) *(30)*. The most widely recognized serotype of pathogenic *E. coli* associated with foods in the United States (U.S.) is the enterohemorrhagic serotype O157:H7 or O157:NM. This serotype belongs to a group of Shiga toxin-producing *E. coli* (STEC). Well over 100 different *E. coli* serotypes in addition to O157 have been classified as STEC *(93)*, and it is this group of bacteria that is the subject of this paper.

Among the STEC, *E. coli* O157:H7 has received the most attention by the scientific and regulatory community because of its association with several large outbreaks of human illness with severe manifestations. As a result of a large outbreak associated with undercooked hamburgers in the Pacific Northwest in the fall of 1992 and spring of 1993, the United States Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) declared in 1994 that *E. coli* O157:H7 was an adulterant in ground beef; a similar pronouncement was made in 1999 regarding all non-intact raw beef products *(30)*.

In the U.S., twenty-three outbreaks of non-O157 STEC illnesses were reported between 1990 and 2007. The common modes of transmission were food (primarily), followed by person-to-person, lake water, animal contact and unknown sources *(36)*. The main foods

associated with human illnesses were salads, berries, milk, cider and punch. The U.S. Centers for Disease Control and Prevention (CDC) estalished illness caused by STEC *E. coli* as a reportable illness in 2000. As would be expected with such a diverse group of bacteria, the reported illnesses are attributable to a wide variety of serotypes. However, six serotypes accounted for 75% of the reported cases in the U.S. *(36)*: O26, O45, O103, O111, O121, and O145.

Non-O157 STEC are a challenging problem because, unlike O157, they have no unique or distinguishing physiological features or phenotypic characteristics to readily distinguish them from other *E. coli* strains. As a result, it is quite likely that our knowledge of non-O157 STEC in foods and human health is limited by our relative inability to identify the bacteria. The burden of human illness from non-O157 STEC is likely much greater than currently reported, and based on our existing knowledge, food and water are likely to be the leading modes of transmission. In 2008, USDA-FSIS announced that testing would be done for non-O157 STEC in regulatory ground beef samples taken for O157 analysis *(84)*. The intention of this action was to better characterize the prevalence of these bacteria in ground beef and to use this knowledge to inform risk-based inspection of USDA-FSIS regulated products. Given the association of non-O157 STEC with salads, it would seem reasonable to conduct surveys to understand the prevalence of these organisms in leafy green vegetables.

TAXONOMY AND PHYSIOLOGY

Escherichia coli, as a group of bacteria, are Gram negative short bacilli that belong to the family *Enterobacteriaceae*. They are generally motile, although many non-motile variants exist. As facultative anaerobes, they have both respiratory and fermentative metabolism. They appear as convex round colonies with entire edges on solid culture media and are readily cultivatable on a variety of standard nutritive media *(14, 39)*.

Physiologically, *E. coli* are capable of growing over a broad temperature range. Although the optimum temperature is usually reported as 37°C, the range of growth is considered to be between 7°C and 46°C. They are capable of growing over a wide range of pH values as well, from pH 4.5 to pH 9 *(43)*. However, the nature of the acidifying agent, the serotype and various environmental conditions are also important; for example, *E. coli* have not been reported to grow in cheese at pH values of less than 5.4.

As with other enteric bacteria, *E. coli* can be further differentiated by the unique polysaccharide antigens expressed on the outer membrane (O antigens) and by the protein antigens on their flagella (H antigens). The uniqueness of these antigens is useful both for classification and for development of diagnostic tests *(92)*. Genetic characterization is rapidly replacing traditional serotyping and may be more relevant to human illness, as these methods can allow for identification of strain-specific virulence factors *(48)*.

Shiga toxin-producing *E. coli* (STEC), by definition, produce Shiga-like toxins (Stx). There are two distinct types of these toxins, referred to as Stx 1 and Stx 2, and a given STEC strain may produce either one or both of the toxins. Unfortunately, except for the production of Stx, there are no other physiological characteristics that differentiate STEC from other non-pathogenic *E. coli*. The one exception to this is *E. coli* O157:H7, which does not ferment sorbitol to produce acidic end products within 24 hours at 37°C. Sorbitol fermentation has formed the basis of methods to detect *E. coli* O157:H7 in contaminated foods, but since other STEC do not display this characteristic, it is virtually impossible to distinguish them from non-pathogenic *E. coli* that are common co-contaminants in many foods *(36)*. This makes the detection and identification of STEC in both food and clinical samples very difficult.

The available evidence suggests that STEC are physiologically similar to other *E. coli* strains. From a food processing point of view, perhaps the most significant physiological characteristic is their tolerance to acidic environments. The pH of many foods, including fermented products and fruit juices, is in the acidic range, and although pH values below 4.4 effectively inhibit growth *(43)*, the bacteria are capable of surviving for weeks in such environments. STEC are believed to have a low infectious dose, so the mere presence of even small numbers of these bacteria in foods may be sufficient to cause human illnesses *(58)*.

MECHANISMS OF PATHOGENICITY

Pathogenicity in Shiga toxin-producing *Escherichia coli* (STEC) is linked to several factors. From an environmental perspective, the ability of the pathogen to survive in foods and in the host gut is critical *(59)*. A number of virulence factors allow the organism to attach and colonize the bowel, invade tissues, and produce toxins that contribute to disease symptoms and progression. Significant virulence factors associated with the pathogenicity of STEC have been identified by means of histopathology of tissues taken from patients with hemolytic uremic syndrome (HUS) and hemorrhagic colitis and on studies using tissue culture and animal models *(60)*. Additional factors that influence pathogenesis include the diversity of the serotypes that cause disease as well as the infective dose and the level and type of toxin produced.

Attaching and effacing

Many studies on the pathogenicity of STEC have focused on elucidating the mechanisms of adherence and colonization *(48)*. Most highly pathogenic STEC, including *E. coli* O157:H7, colonize the large intestine and produce a characteristic histopathological feature known as the attaching and effacing lesion (A/E) induced by a bacterial type 111 secretion system. This lesion is characterized by intimate attachment of the bacteria to the plasma membranes of the host epithelial cells, localized destruction of the brush border microvilli, and assembly of highly organized pedestal-like actin structures. By adhering to intestinal epithelial cells, the STEC cells subvert cytoskeletal processes.

All proteins associated with the formation of the A/E lesion identified to date are encoded on a chromosomal pathogenicity island known as the locus of enterocyte effacement (LEE). These include structural components of a type III secretion system (TTSS), intimin, and translocated intimin receptor (Tir) and other effector proteins

(37). One of these, intimin, is a 94-kDa outer membrane protein encoded by the *eae* (*E. coli* attaching and effacing) gene. The patterns of attachment and interaction of STEC with epithelial cells are different when *eae*-positive and *eae*-negative STEC are compared, with the *eae*-positive strains producing the characteristic A/E lesion. Intimin is exported via the general secretory pathway into the periplasm, where it is inserted into the outer membrane by a putative autotransport mechanism. Interaction of intimin with host cells stimulates production of microvilli-like processes.

TTSS components are associated with the virulence of many Gram-negative bacterial pathogens. The TTSS apparatus is a complex "needle and syringe" structure that is assembled from the products of approximately 20 genes in the LEE. Numerous effector proteins have been identified in STEC cells, and these are translocated into the host cell via the LEE-encoded TTSS.

Shiga toxins

STEC produce one or two Shiga toxins (Stx) *(69)*. Molecular studies of different *E. coli* strains have revealed that Stx1 is either identical to the Stx of *Shigella dysenteriae* type 1 or differs by only one amino acid. Several antigenic variants of Stx1 have been described. Unlike Stx1, toxins of the Stx2 group show significant genetic and antigenic variability; they are not neutralized by antiserum produced against Stx1 and do not cross hybridize with Stx1-specific DNA probes. At least 11 antigenic and genetic variants of Stx2 have been identified, including Stx2, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g *(60)*.

Shiga toxins act by inhibiting protein synthesis. However, the precise roles of Stx in mediating colonic disease, HUS and neurological disorders have not been fully elucidated, as there is no satisfactory animal model for hemorrhagic colitis or HUS, and the severity of disease precludes study of experimental infections in humans. The involvement of Stx in enterocolitis was demonstrated when fluid accumulation and histological damage occurred after purified Stx was injected into ligated rabbit intestinal loops. Histopathologic examination of kidney tissue from HUS patients revealed profound structural alterations in the glomeruli, the basic filtration unit of the kidney. Arteriolar damage, involving internal cell proliferation, fibrin thrombi deposition and perivascular inflammation, also occurs. Neurological symptoms in patients and experimental animals infected with STEC have also been described and may be associated with secondary neuron disturbances that result from endothelial cell damage by Stx.

Plasmid mediated factors

Many STEC possess a highly conserved plasmid, such as pO157, pSFO157 *(19, 20)*, and pO113 *(67)*. On the basis of DNA sequence analysis, pO157, initially identified in *E. coli* O157:H7, has been shown to be a 92-kb F-like plasmid composed of segments of putative virulence genes in a framework of replication and maintenance regions, with seven insertion sequence elements located largely at the boundaries of the virulence segments. There are 100 open reading frames, of which 19 have been sequenced and implicated as potential virulence genes, including those encoding a potential adhesin (ToxB), Enterohemorrhagic *E. coli* (EHEC)-hemolysin, a serine protease (EspP), a catalase and the StcE protein. ToxB shares sequence similarity with the enteropathogenic *E. coli* (EPEC) LifA and Efa-1 genes. The term EHEC-hemolysin is used to distinguish it from α-hemolysin, to which it is related but not identical. EHEC-hemolysin belongs to the repeats-in-toxin (RTX) family of exoproteins. Four gene products of the *hlyCABD* operon encode a pore-forming cytolysin and its secretion apparatus. Toxicity results from the insertion of HlyA into the cytoplasmic membrane of target mammalian cells, with resulting disruption of permeability. The EHEC catalase-peroxidase is encoded by *katP*, whose product is a bifunctional periplasmic enzyme that protects the bacterium against oxidative stress, a possible defense strategy of mammalian cells during bacterial infection.

In summary, STEC strains possess a number of factors that mediate their pathogenicity in humans. Currently there is considerable diversity in the range and types of virulence factors associated with STEC infections in humans and food animals, which makes defining single traits of virulence difficult. It is likely that virulence results from a combination of factors.

ROLE OF STEC IN HUMAN DISEASE AND THE ROLE OF HIGHLY PATHOGENIC STEC

Before *E. coli* O157:H7 was first associated with foodborne outbreaks of bloody diarrhea, post-diarrheal hemolytic uremic syndrome (HUS) was recognized as a distinct clinical entity with a higher rate of occurrence in Argentina than in the U.S. or the Netherlands *(29)*. Shortly after *E. coli* O157:H7 was associated with foodborne outbreaks of bloody diarrhea, this and several non-O157 serotypes of STEC were also causally linked to the occurrence of post-diarrheal hemolytic uremic syndrome (HUS) *(50, 53)*.

In the U.S. and several other countries, *E. coli* O157:H7 was quickly recognized as the primary STEC strain of public health importance because of its association with various foodborne, waterborne and child daycare outbreaks of bloody diarrhea and HUS *(83)*. This recognition was facilitated by the unique biochemical characteristic of *E. coli* O157:H7, i.e., the inability to ferment sorbitol, which allowed the development of highly specific selective media that could be used in clinical microbiology laboratories. However, individual cases of HUS and outbreaks of bloody diarrhea associated with non-O157 STEC serotypes O111 and O26 were recognized throughout the world, and appeared to be more common across Europe, South America and in Australia *(9)*. Unfortunately, as already stated, non-O157 STEC cannot easily be distinguished from commensal *E. coli*.

In 1999, Mead and colleagues estimated that there were 73,000 *E. coli* O157:H7 infections, with up to 3,000 cases of HUS, in the U.S. annually *(58)*. They estimated that the rate of illness associated with non-O157:H7 infections was approximately half that for *E. coli* O157. Mead's estimate for O157 was based on data collected from the first two years of active surveillance by

TABLE 1. Methodologies to detect non-O157 STEC's

Method	Limitations	References
Cultural Methods		
Enrichment	No clearly identified selective agents specific to non-O157 STEC's	*87, 88*
Immunomagnetic separation	Need for specific antibody coated beads to non-O157 serotypes	*94*
Selective/Differential Media	Lack of clearly identified selective and differential agents	*8, 42, 65*
Immunological Detection	Need for antibodies specific to strains	*30, 70*
Verotoxin Detection	Identifies presence of toxin, but not specific strain	*1, 72*
PCR	Specific for toxin genes, but not specific for serotype	*10, 28, 95*
Alternate Methods	Microarrays, conductance, flow cytometery; experimental and not commercially available	

FoodNet. However, the non-O157 estimate was derived from preliminary studies conducted outside of FoodNet *(44)*. More recently, FoodNet has implemented active surveillance for non-O157 STEC, and the diagnosed rate of non-O157:H7 infections (0.57/100,000 population) remains about half the rate of *E. coli* O157:H7 infections (1.2/100,000 population) *(22)*. Although the results of FoodNet active surveillance eliminated potential reporting bias as a cause of the different rates for *E. coli* O157:H7 and non-O157 STEC, they still reflect differences in laboratory diagnostic practices required to establish the diagnosis.

Perhaps the best perspective on the role of various STEC strains in human health can be provided by the experience of Argentina, which has an incidence rate of HUS at least twice the rate reported in the US *(77)*. In a prospective study of STEC infection among children in Argentina, 60% of culture-confirmed STEC infections were caused by O157:H7. Sixteen other STEC serotypes have been implicated in disease, the most common of which, O145:NM, accounted for 29% of the non-O157 STEC infections. However, *E. coli* O157:H7 was isolated from 85% of patients with confirmed or probable HUS, compared with 53% of patients without HUS *(77)*. These data better define the current epidemiology of STEC infections. Non-O157 STEC appear to be as common as O157:H7 strains as a cause of diarrheal illness, but most non-O157 STEC are not associated with severe clinical outcomes.

Highly pathogenic STEC

The epidemiology of human STEC infections demonstrates that *E. coli* O157:H7 is more pathogenic than most strains of non-O157 STEC and that highly pathogenic non-O157 STEC share many of the same virulence factors as *E. coli* O157:H7. However, the precise combination of factors that contributes to the pathogenicity of *E. coli* O157:H7 and other highly pathogenic STEC is not fully known *(25)*. Early analysis identified Stx2-producing, *eae*-positive STEC as more likely to be associated with HC and HUS *(16)* although some highly virulent STEC such as serotype O26:H11 produce only Stx1. Serotype appears to remain an important indicator of virulence, and Karmali et al. *(52)* recently classified STEC into five seropathotypes (A through E) based on the frequencies of their associations with outbreaks and/or severe disease. Seropathotype A (*E. coli* O157:H7/NM) has the highest frequency/severity and seropathotypes D and E the lowest, producing mild disease, if any. This may form the basis of a molecular analytic method that can be used to compare strains based on sequence analysis of pathogenicity islands. Such an approach could be used to discriminate highly pathogenic STEC strains from those posing less serious public health risks *(91)*. Clearly, establishing a functional definition of highly pathogenic STEC will be necessary as we seek to evaluate the need for regulatory control of these organisms in food or in the environment.

METHODS OF ENRICHMENT AND DETECTION

The primary limitation in the understanding of non-O157 STEC is the lack of standardized methodology to either detect or quantify the bacteria. Because of the lack of standardized, commercially available methods, much of the methodology is based on developments with *E. coli* O157:H7. However, O157:H7 is a single, specific serotype, while the non-O157 STEC are a group of different serotypes. Developing assays to detect or enumerate multiple serotypes, particularly when there are similar serotypes that are not pathogenic, is challenging. These methodological approaches and their limitations are summarized in Table 1.

Cultural methods

Procedures for detection of non-O157 STEC are hampered by a lack of known physiological characteristics that distinguish these strains from non-pathogenic *E. coli*. Difficulty in detection is also exacerbated by the ability of STEC to be internalized in foods and to survive for long periods of time *(31)*. However, because of their potential to cause serious disease, it is important to be able to detect small numbers of STEC even in complex food samples, such as those in which competitors greatly outnumber STEC. Even though awareness of the potential importance of non-O157 STEC has grown, there has been a tendency to use detection methods developed for O157:H7. Such approaches may lead to inaccurate conclusions.

Enrichment procedures pertinent to all STEC

In spite of the inherent difficulties, attempts have been made to optimize methods for selective enrichment of non-O157 STEC. Vimont et al. *(87)* reviewed enrichment protocols from a total of 380 experiments, identifying key variables that included type of broth medium, presence of antibiotics, and/or selective ingredients and incubation time/temperature. No clear conclusions or recommendations could be drawn, and more extensive work, which would include multiple serotypes and sample matrices, was suggested. In a subsequent study, Vimont et al. *(88)* examined the comparative growth of background microflora and two strains of fluorescent, ampicillin-resistant non-O157 STEC inoculated into bovine feces. They found a simple competitive interaction, with the growth of STEC terminating as soon as the background microflora reached a maximal level of 8 $\log_{10}$ CFU ml^{-1}, which was relatively constant across all enrichment protocols. Use of EC broth appeared more favorable for enrichment of the test strains than other evaluated media .

Incorporation of IMS to facilitate enrichment and isolation

Immunomagnetic separation (IMS), which provides enhanced isolation capacity through the use of paramagnetic beads coated with specific antigens *(94)*, has become particularly popular. Many studies have continued to use anti-O157 beads, and then make observations as to how this method may be pertinent to non-O157 serotypes after further characterization of isolates. Utilization of IMS is likely to expand, as beads specific for O26, O103, O111 and O145 are now available.

Evaluation of selective media applicable to non-O157 STEC

Several chromogenic culture media have been developed for the detection of *E. coli* O157, and studies have been conducted to determine whether these are also applicable to non-O157 serotypes.

Current chromogenic agars are based primarily on traits such as sorbitol fermentation, glucuronidase activity and galactosidase activity, and are largely effective for discrimination of O157:H7. However, their usefulness for differentiation of non-O157 STEC is mixed. For example, Bettelheim *(8)* found that all sorbitol negative, shiga-like toxin-producing (SLT) strains of O157:H7 and O157:H^- produced highly characteristic gray-black or steel-black colonies on Rainbow™ Agar O157, but interpretation of the results from non-O157 cultures was more complex. Several toxigenic strains of O111, O26 and O128 produced deep blue-black colonies, which would allow their discrimination; however, other serotypes previously associated with HUS yielded red, pink or mauve colonies indistinguishable from SLT-negative generic *E. coli*. Additional studies utilizing Rainbow agar include those of Müller et al. *(65)* and Ingram et al. *(42)* as applied to water and clinical samples, respectively.

Attempts have therefore been made to utilize other characteristics for differentiating non-O157 STEC using media. Enterohemolysin agar was developed based on the observation by Beutin et al. *(13)* that many non-O157 and most O157:H7 strains produce narrow turbid zones of hemolysis on blood agar supplemented with sheep RBC and Ca^{+2} ions, evident after 18–24 h of incubation. This medium is commercially available and effective, with some caveats: enterohemolysin-positive colonies must be screened for Stx production, some non-O157 STEC and sorbitol-fermenting O157 do not produce enterohemolysins, and large enteric populations or non-STEC α-hemolytic colonies can interfere *(49)* with interpretations.

Enumeration/quantification

Few studies have attempted to quantify non-O157 strains or to establish a correlation with standard indicators, such as *E. coli* enumerated by the most probable number (MPN) method. However, quantitative methods used for O157 STEC are likely applicable to non-O157 STEC's. Jordan et al. *(47)* monitored *E. coli* O157:H7 levels in spiked cheese and milk and could detect 1 CFU/g and 4 CFU/10 ml, respectively, by using CT-O157:H7 ID medium, which was more sensitive than TCSMAC by approximately two logs. Mora et al. *(62)* studied STEC in minced beef, comparing *E. coli* MPN and Petrifilm. The largest percentage of non-O157 STEC (28%) was found in samples with > 999 MPN/g. Fremaux et al. *(35)* utilized a MPN procedure using 96-well plates and testing each well for Stx. Fox et al. *(34)* determined the sensitivity and specificity of O157 MPN methods and observed that the most useful approach was pre-enrichment direct streaking of 1:10-diluted feces in triplicate. Similar approaches may be applicable to non-O157 STEC's.

Use of immunologically-based methods for the detection of non-O157 from clinical and food samples

Most immunological detection methods for O157 and non-O157 strains rely on the detection the Shiga toxins or other cellular antigens. Originally, some of these tests were designed for the detection of O157 strains only but also have application for non-O157 strains. These assays are based on immunocapture, sometimes in the lateral flow kit format. Detection from contaminated foods must be preceded with a primary enrichment phase to increase the population of the organism and its associated antigen to a detectable level, usually lasting between 8 to 24 hours. In

a recent white paper, Eblen *(30)* commented that although there is a need for test kits specifically designed to rapidly identify pathogenic non-O157 *E. coli* strain types, there appears to be little impetus for diagnostic companies to develop them because of a limited market. Perelle et al. *(70)* noted that the five main serogroups most often associated with foodborne illness are O26, O103, O111, O145, and O157:H7, so future assays may focus on these strains.

DETECTION OF NON-O157 STEC IN FOOD-STUFFS

Research on detection of non-O157 VTEC in foodstuffs is limited. Although research has been focused largely on the detection of O157 type strains, those methods do have potential application to non-O157 strains.

Aldus et al. *(1)* designed and developed a novel dipstick and lateral flow immunological method for the detection of one or both of the Stx 1 and Stx 2 toxins simultaneously. The test was, however, limited by lack of antibody that could be used to detect strains producing VT 2 solely, and combinations of VT 1 and 2 were necessary for positive results.

Pontell et al. *(72)* used immunoassays to evaluate O157 and non-O157 strains recovered from 352 food samples collected in the Lombardy region of Italy. Samples tested included raw milk, fresh meat (beef, veal), dairy foods, and processed and salted meats. The two immunoassays used were the Premier EHEC test, designed to identify toxin presence in foods, and the Vidas ECO analyzer, an automated immunoassay, designed to specifically identify *E. coli* O157:H7. The study detected non-O157 STEC's in two samples, with five additional raw milk and cheese curd samples displaying weakly positive reactions and subsequently yielding non-typable *E. coli* isolates. The researchers noted that the Premier EHEC gave better detection rates for VTEC-producing strains than the ECO VIDAS, which detected serotype O157 exclusively.

Detection of VTEC using verocell assays

Moreira et al. *(63)* investigated the direct isolation of STEC from cattle feces and toxin detection using Vero cells. Of 1127 *E. coli* isolates recovered from cattle, 243 were tested for verotoxins; overall, 49% of the animals tested were positive for verotoxin-producing *E. coli* strains. Pathogenic isolates represented serogroups O157, O91, O125, O119, O112 and O29, some of which were associated with human disease.

PCR-based methods

Because of the lack of distinguishing physiological characteristics and their serological diversity, the only broadly applicable targets for non-O157 STEC strains are direct detection of the verotoxins or detection of the *stx1/stx2* genes encoding these toxins *(10)*. A very large number of PCR-based methods have been used in this regard, some of which are quite rapid and amenable to multiplexing, although these are primarily research tools and not commercially available as kits. Certain caveats apply when using DNA-based detection methods, including concerns about amplification of DNA from non-viable cells and inhibition by components of certain samples *(28)*, but these are increasingly addressed by inclusion of internal controls and other means by which to assure detection of viable cells *(95)*.

Efforts to expand the number of isolates detected in a sample

Colony lift hybridization methods have been applied in an effort to increase isolation of non-O157 STEC strains, particularly when these are present at low concentrations relative to background microflora. In this regard, Nielsen and Anderson *(68)* isolated STEC from cattle feces by enrichment followed by analysis for *stx1/stx2* genes. Positive samples were screened by colony hybridization, which permitted simultaneous probing of colonies on 1600-cell membranes. This approach facilitated characterization of many more isolates than in previous studies and was highly efficient, allowing isolation of a single STEC colony from approximately 500 background coliform organisms. The same method was used by Cobbold and Desmarchelier *(23)* in a year-long survey of bovine fecal and dairy environmental samples. Enrichments that were positive for *stx* genes by use of PCR were filtered through a HGMF filter and subjected to DNA hybridization targeting the stx genes. Subsequent testing for four serogroups permitted evaluation of multiple STEC strains over time and by cattle group.

Alternative strategies for the detection of non-O157 STEC

Alternative strategies for the detection of non-O157 strains can theoretically follow the typical approaches used in the detection of O157 type strains, including microarrays, conductance, sensors, immunomagnetic separation and flow cytometry.

PREVALENCE OF NON-O157 STEC IN ANIMAL FOOD PRODUCTS

Soon after initial reports that O157 and non-O157 STEC were the cause of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans, STEC were identified as zoonotic foodborne/waterborne pathogens harbored in the intestinal tract of healthy cattle and other ruminants *(12, 50)*. Shedding of STEC in manure at levels up to 10^6 CFU/g can result in hide contamination and dissemination into the immediate environment and local water supplies *(12)*. In light of this ecology, meats, milk, water and fresh produce are the most frequently implicated vehicles of STEC infection *(12, 21, 31, 36)*. STEC strains belonging to over 400 serotypes have been isolated from humans, animals and food-related sources *(9)*, (http://www.microbionet.com.au/vtectable.htm, http://www.usc.es/ecoli/index.html). However, many non-O157 serotypes from food animals and foods have not been linked to human disease, or have lower outbreak potential and cause less serious disease than O157 STEC *(44, 51)*.

Concern about non-O157 STEC has increased primarily because of several outbreaks, most of which have been foodborne *(36, 80)*. However, outbreaks account for less than 20% of reported STEC O157 infections *(89)*, and

the same appears to hold true for non-O157 infections *(44)*. Because they are less likely than O157 STEC to cause outbreaks or severe disease and because they are more challenging diagnostically, many non-O157 infections may not be investigated fully and their sources may thus remain undetermined. Consequently the true burden of illness due to non-O157 STEC infections, many of which are likely to be foodborne, is probably underestimated.

Reports of O157 and non-O157 STEC in foods from numerous countries reveal wide variation in prevalence estimates *(31, 33, 40, 41)*. While differences in food production practices and geographic, seasonal and climatic factors influence prevalence, much of the variability results from differences in study design and in the methods used for detection and isolation of all STEC. The most reliable estimates of the true prevalence of any STEC in foods are derived by testing food enrichment cultures for *stx* genes or Stx production. Given the challenges of isolation of non-O157 STEC, prevalence estimates of these strains are probably relatively unreliable unless hundreds or thousands of individual colonies are tested for Stx production or the presence of *stx* genes. These methodological challenges deserve consideration in interpretation of prevalence estimates.

Meats

Meats become contaminated on carcass surfaces, primarily at slaughter, during hide removal and bung preparation *(31, 40)*; contamination may also occur during subsequent carcass washing and dressing, during post-dressing processing such as grinding, and by cross-contamination during packaging, handling and food preparation. Ground (minced) meats pose a particular risk, because grinding results in comingling (spreading) and internalization of the bacteria, meaning that product must be cooked thoroughly for adequate bacterial inactivation. Fermented sausages and other meat products that are not heated to lethal cooking temperatures also appear to carry a particularly high risk.

Beef

Non-O157 STEC are more prevalent than O157 STEC in beef products. Data from numerous countries indicate that while STEC O157 is rarely present in more than 1% of raw beef products, the prevalence of non-O157 or "STEC-positive" *E. coli* strains in raw beef ranges from 2.4 to 49.6% *(31, 40)*. Also, STEC were present in 63% of ground veal samples *(78)*. Overall, a range of 5–40% is consistent with early estimates of all STEC in raw beef in Canada, the U.S. and the United Kingdom, where reliable methods for detection of Stx were used *(45)*. In the U.S., *stx* genes were detected by PCR in enrichment cultures of 5.7–26.2% of samples of domestic beef and 1.8–15.6% of samples of imported beef *(54)*. Similar methods revealed STEC prevalence ranging from 15.5 to 33.3% in beef in Canada *(4)*, Australia *(7)*, France *(6)* and Sweden *(57)*. Interestingly, only 1.1% of domestic Swedish beef had evidence of STEC contamination, and a similarly low prevalence of contamination (0–5.9%, including STEC O157) was reported by eight member states of the EU *(33)*. Although caution is recommended in interpreting these EU data, both O157 and non-O157 STEC appear to be less prevalent in cattle meat products in several European countries than in other countries.

Sheep and other ruminant meats

Since STEC are also prevalent in sheep *(12)*, sheep meat products are likely to be contaminated with STEC. The fleece of sheep is frequently STEC-positive *(54)*, suggesting that contamination occurs by pathways similar to those described for beef meats. The prevalence of *stx* genes in cultures of lamb meats in the U.S. and Australia ranged from 47.6 to 75% *(7, 78)* and were lower in Belgium (6%) *(71)* and in the EU (0%) in 2005 *(33)*. Meats from goats and wild or exotic ruminants (deer, antelope, etc.) can also contain STEC, as suggested by a small study demonstrating that STEC strains were present in 22–100% of meats from several of these species *(71)*.

Meats from other species

The reported prevalence of STEC in pork products is relatively low, including the absence of the organism in Belgian pork *(71)*, and prevalence of 0–6.2% in the EU *(33)*, 15.3% in Canada *(74)* and 18% in the U.S. *(78)*. Although STEC O157 and several non-O157 STEC implicated in human disease have been isolated from pigs and/or pig meat, most STEC from pigs are those associated with porcine edema disease and are rarely isolated from humans *(38)*. Also, the presence of STEC in pigs may be due to contaminated feed and associated with farms having multiple production species, and may therefore be due to transmission from ruminants. Without on-farm exposure or the potential for post-production contamination, pork meats appear to pose a lower risk than ruminant meats as a source of human exposure to STEC.

Poultry products also appear to carry little risk; the prevalence rates of STEC in these products are generally less than 1% *(71, 74)*. At retail locations in the U.S., however, 6.7% of turkey samples and 12.1% of poultry samples were STEC-positive *(78)*, possibly due to post-production cross-contamination during handling and packaging. STEC may also be found in fish or shellfish *(31, 78)* but appear to pose little risk to humans. However, since some seafoods are consumed with little or no cooking, there may be a potential for transmission to humans through consumption of these foods.

Dairy products

Milk and dairy products from cattle and other milk-producing ruminants are also potential vehicles of STEC and have been linked to outbreaks of both O157 and non-O157 STEC infections *(33)*. Milk becomes contaminated from skin, hides and the dairy environment during milking. Since both O157 and non-O157 STEC are sensitive to pasteurization, raw milk and raw milk products are the main public health risks, which are greater in countries where raw milk and raw milk cheeses are consumed more frequently. Estimates of the prevalence of STEC in raw milk products from cattle, sheep and goats vary considerably, ranging

from 0–16.7% *(31, 33, 41)*. The wide variation in these estimates again reflects differences in methodology as well as regional and cultural differences in agri-food practices.

Significance of STEC in animal food products

Among the many factors influencing the health risks from STEC associated with the consumption of animal food products, the most significant are the levels of contamination, the survival of the organism during processing and preparation, and the virulence of the contaminating strains. Limited data suggest that levels of STEC O157 and probably non-O157 STEC in meats and milk are usually low (less than 10 CFU/g or ml) and that without further inactivation, they can survive for weeks or months in milk and meat products *(31)*. At these relatively low levels of contamination, the potential risks associated with residual STEC in foods appear to be influenced strongly by their virulence.

Of the hundreds of STEC serotypes isolated from foods of animal origin, fewer than 20 O-serogroups have been associated with outbreaks, and over 30 others have been linked repeatedly to serious human disease *(9, 36, 44, 51, 80)*. Consequently, not all STEC constitute the same health risks, which makes it very difficult to evaluate risk. Although many virulent STEC are among the more common serotypes isolated from ruminants and their products *(40, 41, 45)*, serotype alone cannot be relied upon as a clear indicator of virulence. STEC virulence is strongly associated with the type and subtype of Stx and with mobile genetic elements such as the LEE and other pathogenicity islands, presence of all of which can be variable within serogroups and serotypes *(51, 80)*. Moreover, new serotypes of virulent STEC will likely emerge as the result of transfer of these mobile virulence-associated genetic elements *(12, 51)*.

Control of STEC in animal food products

Interventions during slaughter, dressing and processing can reduce carcass contamination and hence the levels of STEC and other pathogens in meat products. Similarly, STEC contamination of milk products can be effectively reduced by processing interventions including pasteurization and exposure to acidic conditions. Further reductions in meat contamination are likely to be achieved with the introduction of recently developed on-farm and pre-slaughter interventions commonly employed to reduce the incidence of *E. coli* O157.

CONTROL OF *ESCHERICHIA COLI* NON-O157:H7 IN ANIMALS, PLANTS AND WATER

Microbial contamination originating from animal fecal waste can serve as the source of STEC contamination to soil, or to water used for irrigation or washing of plant crops. Thus, *E. coli* O157 and non-O157 STEC strains are found in meat products such as ground beef, in water and in produce. However, most published studies on various aspects of STEC in foods deal with serotype O157, which has been implicated in documented foodborne illness outbreaks, especially through consumption of undercooked ground beef, in which it has been declared an adulterant. Thus, there is a need to control serotype O157 and all other pathogenic STEC, as well as all pathogens in foods where they are documented to persist and cause human health problems.

Animals

Methods to control *E. coli* O157:H7 in animals should be equally effective in controlling the non-O157 STEC, and this has been confirmed by Cutter and Rivera-Betancourt *(26),* who demonstrated that decontamination interventions used by the beef industry during slaughter and dressing (i.e., water, hot water, organic acids and trisodium phosphate) were similarly effective against *E. coli* O157:H7, other STEC serotypes, and *Salmonella.*

Plants

Most studies of pathogenic *E. coli* in produce have focused on O157:H7. Because non-O157 STEC share many physiological characteristics with O157:H7, it is likely that the same control measures will work for both *E. coli* groups.

The importance of effective control measures is illustrated by the growing number of outbreaks and cases of foodborne illnesses linked to fresh produce. A CDC survey of U.S. outbreaks between 1973 and 1997 revealed non-O157 STEC outbreaks associated with consumption of carrots and pineapple *(81)*. An additional 13 outbreaks were linked to *E. coli* O157:H7, and it is likely that others occurred, although EHEC infections were not reportable during much of this period. Another analysis of produce-related outbreaks during the period 1990–2004 concluded that 27 out of 84 were due to pathogenic *E. coli (17)*. Between 1990 and 2007, there were eleven documented foodborne disease outbreaks with non-O157 STEC's in the U.S. *(36)*. Of these, the source was never identified in 5 outbreaks, while the sources of the remaining 6 fell into no particular category but included a salad bar, salad, berries, milk and ice. The largest *E. coli* O157:H7 outbreak ever recorded was due to consumption of contaminated radish sprouts in Japan in 1996 *(61)*.

Overall, the incidence of O157:H7 in produce has been reported to be very low. Arthur et al. *(3)* found no verotoxigenic *E. coli* in 1,183 samples of Ontario-grown produce. Surveys of fresh produce in the U.S. screened in 2000–2002 and 2003–2004 returned no positive results from 398 and 2,029 samples, respectively *(46, 64)*. However, in spite of these generally encouraging results, there are reasons for concern *(2)*. The low infective dose of STEC makes detection of small numbers of the organism critical, and detection may be compromised by large numbers of non-pathogenic competitors *(27)*. Furthermore, STEC may be internalized in plants and are capable of long-term survival in the environment *(31)*.

Produce washing

One or more post-harvest washing steps are common to most produce items. Washes may involve only water, or water supplemented with disinfecting agents. Regardless of the wash used, conditions such as flow velocity, agita-

tion rate and contact time impact the degree of reduction of pathogen populations on produce during washing *(90)*. Commercially available sanitizers have been evaluated for their potential to effect a 5 log reduction of *E. coli* O157:H7 in artificially inoculated fresh produce of various types. For example, treatment of apples, tomatoes, and oranges with 1.5% lactic acid and 1.5% hydrogen peroxide at 40°C resulted in pathogen reduction of > 5 log CFU per fruit, while a simple deionized water wash resulted in only a 2 log reduction *(85)*. However, results in commercial settings may vary widely.

Inclusion of hypochlorite or hypochlorous acid in produce wash solutions, typically at 50 – 200 ppm, is perhaps the most common approach to microbial load reduction (*55*). However, chlorine may also be less effective when pathogens gain access to the interior of the produce *(32)*. A common approach to enhancing the effectiveness of chlorine is to use it in combination with acids or other bactericidal treatments.

Microbial antagonism

Reduction of *E. coli* O157:H7 and other pathogens through microbial antagonism has been investigated as an alternative to more traditional chemical and physical sanitizers. The potential for reduction of *E. coli* O157:H7 on pre-harvest produce was illustrated by the study of Cooley et al. *(24)*, who demonstrated that the intentional addition of *Enterobacter asburiae* decreased *E. coli* O157:H7 survival on lettuce 20–30 fold.

Physical controls

Inactivation of STEC by ionizing irradiation has been examined in several recent studies. Three strains of *E. coli* O157 in clarified apple juice demonstrated D_{10} values ranging from 0.12 to 0.21 kGy *(11)*. After acid adaptation, these values increased to 0.22–0.31 KGy. The authors concluded that a 1.8 kGy dose should provide 5 log inactivation.

Water

Reports concerning STEC control measures in water deal primarily with *E. coli* O157:H7. These studies are described here on the assumption that other *E. coli* serotypes respond in a broadly similar fashion to control measures such as chlorination, filtration, predation, etc.

STEC strains transmitted by waterborne routes have been the cause of several outbreaks. Between 1989 and 2004, 23 outbreaks occurred, including 12 cases in Japan linked to O26:H11 and 11 cases in Connecticut linked to O121:H19 *(66)*. Illnesses have been associated with water used for a variety of purposes, including recreational water, drinking water and irrigation water. However, the three largest outbreaks between 1989 and 2004 were due to drinking water contaminated with O157:H7 in Ontario, New York, and Missouri *(66)*.

Wastewater

Control of pathogens in human wastewater has been practiced for decades, including such standard techniques as filtration, chlorination, and UV and ozone treatment *(76)*. Long experience with these methods should provide information on effective control. However, the ability of STEC to survive in wastewater is notable and can serve as the source of contamination of groundwater, irrigation water or water used in livestock production. A study of five STEC strains in dairy lagoon microcosms was conducted to evaluate the effect of circulating aerators *(73)*. The importance of appropriate treatment of sludge and manure was also emphasized in an extensive study with 752 samples, including bovine and porcine feces *(86)*. Twenty-four percent contained Stx-positive isolates, representing 21 VTEC strains.

The potential for pathogen dissemination in gray water (waste water from residential use which does not include sewage) has also been evaluated *(15)*. Consistently high levels of indicator organisms indicated the need for treatment if the water is to be used for recycling. However, *E. coli* O157:H7, and by implication the non-O157 STEC's, was not detected in any sample.

Surface water

Surface waters represent an intermediate potential for STEC contamination, with wastewater having the highest enteric bacterial populations and ground waters (because they are typically purified by natural filtration processes), having the lowest. In 1999, 36 people were infected with *E. coli* O157:H7 as a result of swimming in a lake in Washington state *(79)*. PFGE patterns of isolates from duck feces and water samples were identical to those of patient isolates. Five other outbreaks associated with recreational waters had been reported prior to the Washington outbreak.

Drinking water

Little work has been done with non-O157 STEC strains, but *E. coli* O157:H7 is not unusually resistant to chlorination practices commonly used to purify water. An EPA report noted that inactivation rates of *E. coli* O157:H7 and wild-type *E. coli* were similar *(75)*. Water utilities in the U.S. maintain a median chlorine residual of 1.1 mg/L, with a 45 minute lapse before the first point of use in the distribution system. *E. coli* O157:H7 is therefore unlikely to survive conventional water treatment practices. However, the possibility of acquiring STEC infection from drinking water remains. For example, not all municipal water utilities use chlorine, and adverse conditions can greatly diminish chlorine levels *(75)*. Proper maintenance of wells is also crucial and sometimes overlooked. Weather conditions may also degrade drinking water quality. The largest waterborne O157:H7 outbreak on record, 2,000 cases in Ontario, Canada, was apparently caused by municipal water contaminated by a heavy rainfall event immediately prior to the outbreak *(5)*.

Livestock drinking water

If water provided to livestock is contaminated with fecal material or otherwise of poor microbiological quality, it may serve as a source of transmission of STEC. In a study of 473 cattle water troughs, *E. coli* O157 was found in 1.3% *(56)*. By additional use of experimentally inoculated

indicator microcosms into water troughs, the authors concluded that these troughs are a major source of exposure to enteric bacteria. It does appear that contamination may be controlled by manipulating factors such as exposure to sunlight, cleaning frequency, trough design, and protozoan predation. Trough water with turbidity scores above 4 were correlated with total *E. coli* counts > 5,800 CFU/100 ml and greater likelihood of *E. coli* O157:H7 *(82)*.

CONCLUSIONS

The role of non-O157 STEC's in foods are best understood from known outbreaks, as there has been little monitoring or surveillance in the food supply. This is largely attributable to the lack of standardized methods for the detection or enumeration of these bacteria in food matrices, and perhaps also to the lack of agreement on which serotypes are most important. Non-O157 STEC's present a methodological challenge similar to that of salmonellae, where the methods have to be equally sensitive as applied to multiple strains. Unlike salmonellae, however, there are many serovars of *E. coli* which are not human pathogens, and the challenge then becomes to separate the pathogenic strains from the non pathogenic strains. The current regulatory interest in non-O157 STEC's will undoubtedly spur further developments in methodologies, as it did with *E. coli* O157:H7. Until then, our knowledge of non-O157 STEC's in foods will likely be limited to specific outbreak investigations and may perhaps be best described as "things we do not know we don't know."

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