National Advisory Committee on Microbiological Criteria for Foods (NACMCF) Subcommittee on Virulence Factors and Attributes that Define Foodborne Shiga Toxin-producing *Escherichia coli* (STEC) as Severe Human Pathogens

EXECUTIVE SUMMARY

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF or Committee) was asked to report on: 1) what is currently known about virulence and pathogenicity of Shiga toxin-producing *Escherichia coli* (STEC) and how they cause illness in humans; 2) what methods are available to detect STEC and their specific virulence factors; and most importantly, 3) how to rapidly identify foodborne STEC that are most likely to cause serious human disease. Individual working groups were developed to address the charge questions, as well as to identify gaps and give recommendations for additional data or research needs. A complete list of Committee recommendations is in Chapter 4.

STEC infections cause illnesses that range in severity from diarrhea to diarrhea with grossly bloody stools, called hemorrhagic colitis, HC, to the life-threatening sequela of infection, the hemolytic uremic syndrome (HUS). STEC are ingested in contaminated food or water or through direct contact with infected animals or people. Of all STEC that cause disease in the U.S., *E. coli* O157:H7 (O157) causes the most outbreaks and the largest number of cases of serious illness (as assessed by the number of patients hospitalized or with HUS). The infectious dose fifty percent (ID_{50}) of O157 is low (estimated to be between 10-100 bacteria). As determined in animal models, these bacteria bind to enterocytes in the large intestine through the intimin outer membrane protein (gene for intimin is *eae*), attach and efface the mucosa, and elaborate Shiga toxin (Stx) that passes from the intestine through the bloodstream to sites in the

kidney. Certain Stx subtypes are more commonly associated with severe STEC human illness, e.g. Stx2a, Stx2c, and Stx2d. The serogroups (O antigen type only) linked to most cases of illness in the U.S. are O157, O26, O103, O111, O121, O45, and O145 in order of decreasing incidence. STEC disease is linked most often to foods of bovine origin and fresh produce; disease burden attributed to beef and dairy products is broadly similar in numbers to that attributed to fresh produce.

Stx production, a phage-encoded trait, and intimin, but not the O antigen type, are major drivers of pathogenicity. Thus, predictions of the pathogenic potential of STEC can be made based on Stx subtype and the potential of the bacteria to attach in the intestine. The combination of virulence genes in *E. coli* that has led to the most severe disease is stx_{2a} with *aggR* [a genetic marker for enteroaggregative *E. coli* (EAEC)]. The second highest risk group are those O157 STEC that have stx_{2a} and *eae*, followed by that same combination in O26, O103, O111, O121, O45, or O145. The combination of stx_{1a} and stx_{2a}, or stx_{2a} and stx_{2c}, or stx_{2d} with *eae* is also of particular concern. The lack of *eae* suggests a reduced potential for human disease except when *aggR* or stx_{2d} is present. There have been a few exceptions to this hierarchy such as O103 that produce only Stx1 and O113 that is *eae*-negative.

The protocols currently used by the FDA, USDA FSIS, clinical/public health laboratories and the food industry include enrichment, culture, multiplex RT-PCR, toxin immunoassays, biochemical characterization, DNA-based serotyping, DNA microarray, and whole genome sequencing (WGS). The advantages and limitations of each method are summarized in this report. New and developing high throughput methods are discussed and include metagenomics, digital PCR, biosensors, and microarray.

STEC disease prevention has been and will continue to be driven by improvement in outbreak detection, investigation, and food industry practices. Highlights of Committee recommendations include the following:

- Develop a new universal enrichment culture medium that can be broadly used for all STEC in any food.
- Explore high-throughput methods that can detect STEC virulence factor genes directly from enrichment medium and develop/improve methods that can ascertain that all critical STEC markers found in the enrichment broth are within the same cell to eliminate the need to isolate the organism.
- Expand systematic sampling of food, animals, and water for STEC.
- Explore ways for industry to share test data, anonymously.
- Fund academic research on: 1) the regulation of toxin expression and the phages that encode toxin; 2) mechanisms of attachment by *eae*-negative STEC; 3) oral-infection animal models or cell culture models that are more reflective of human disease; and, 4) human host factors that influence the outcome of STEC infection.
- Link standardized epidemiological, clinical, and STEC WGS data to monitor trends in recognized and emerging virulence attributes such as Stx type and phage profiles.
- Further develop WGS methods to: 1) predict toxin levels produced by an STEC; and, 2) generate a classification scheme based on genomic clusters.

The Committee agrees that a combination of genetic characteristics (attributes) exist that signal potentially high risk STEC and that they will eventually be identifiable using high throughput techniques that analyze gene profiles. Thus, to rapidly identify foodborne STEC that are most likely to cause serious human disease, the Committee recommends that STEC analyses move

toward using virulence markers rather than serogroup or serotype to identify pathogens. The Committee concurs that as ease-of-use increases and costs decrease, culture-independent diagnostic tests (CIDT) based on genomic clusters or lineages will be more broadly used to predict whether an STEC isolate is likely to cause serious human disease.

Executive summary of the charge:

Shiga toxin-producing *Escherichia coli* (STEC) are a large, diverse group of bacteria that are characterized by the production of Shiga toxin (Stx). There are two main Stx types, designated Stx1 and Stx2 and within each are many subtypes. Currently, there are three known Stx1 (Stx1a, Stx1c and Stx1d) and seven known Stx2 (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f and Stx2g) subtypes, but some of these are produced mostly by environmental- or animal-associated strains. Thus far, Stx1a, Stx2a, Stx2c and Stx2d are the subtypes most frequently implicated in human illness. There are estimated to be >400 known STEC serotypes that can produce any of the Stx types, subtypes or combination of subtypes. However, only a subset of these STEC serotypes have been associated with human illness. Furthermore, the production of Stx alone without other virulence factors, such as intimin, has been deemed to be insufficient to cause severe human illness.

Background:

Many STEC serotypes have been isolated from various foods, including ground meats, fresh produce and dairy products. Of the >400 known STEC serotypes, ~100 serotypes have reportedly caused the most human illnesses. Some of these, such as various serotypes in the serogroups O26, O111, O103, O121, O145 and O45 that also include the adherence factor intimin, which are commonly referred to as the "big 6", are well recognized pathogens and are of human health concern. The virulence potential of other STEC strains is more difficult to determine due to the lack of a clear understanding of STEC pathogenesis. In addition to the previously mentioned adherence factor, there may be additional virulence determinants required for a particular STEC strain to be fully virulent. Recent FDA investigations on STEC in fresh

produce showed that multiple STEC serotypes, including members of the "big 6", can be found in many types of fresh produce. Conversely, a majority of the produce-derived STEC strains lacked at least one virulence factor and were of serotypes not associated with human illness. Furthermore, the methods that are used to isolate STEC from foods are inefficient and too time consuming. The confirmatory tests used for assessing the risk potential of STEC strains are limited and lastly, these algorithms are unable to provide timely health risk information, especially for products like fresh produce that has an average shelf-life of 2 weeks.

The Food and Drug Administration (FDA), the Centers for Disease Control and Prevention (CDC), The Food Safety and Inspection Service (FSIS), the National Marine Fisheries Service (NMFS), and the Department of Defense Veterinary Service Activity (DoDVSA) believe that enhancing the scientific information available on STECs and improved detection and identification methodology will assist in reducing illness from STEC. For this reason they provided the following charge questions to NACMCF.

Charge questions for the subcommittee:

1. What is currently known about the virulence and pathogenicity of STECs and how they cause illness in humans? Address data generated within and outside of the U.S.

   Addressed in Chapters 1 & 2

   a) What defines or differentiates an STEC as a human pathogen from other STEC that are underrepresented in severe illnesses? **Addressed in Chapters 1, 3 and Figure 7**

   b) Please discuss all combinations of virulence attributes that contribute to human illness and the probable severity associated with certain combinations. Are there specific

attributes that can be identified as associated with STEC virulence in humans and the colonization and persistence on fresh produce, in lieu of colonization of environmental or host animal niches? **Addressed in Chapters 1, 2, & 4**

c) In terms of pathogenicity and virulence, please discuss what is known empirically and what has been clearly defined. **Addressed in Chapter 2**

2. What methods are available to detect STEC and their specific virulence factors, either separately or in combination? **Addressed in Chapter 3**

a) What data gaps exist and what research is required to improve the effectiveness of these methods? For example, please discuss the strengths and weaknesses of using molecular subtyping/genotyping approaches for characterization any genetic markers that contribute to STEC virulence, including specific toxin gene subtypes (i.e., *stx*$_1$, *stx*$_{1c}$, *stx*$_{1d}$ and *stx*$_2$, *stx*$_{2b}$, *stx*$_{2c}$, *stx*$_{2d}$, etc.). **Addressed in Chapter 4**

3. What are the principal attributes that can be exploited to rapidly detect STEC that are a high probability of causing severe human illness? **Addressed in Chapter 2 & Figure 7**

a) If such attributes exists, can they be implemented in a high throughput tool to ensure public health and help industry rapidly decide to hold or release product? **Addressed in Chapter 4**

b) What data gaps exist and what research is required to determine an accepted set of attributes for virulence and pathogenicity determination? **Addressed in Chapter 4**

c) What are the limitations to establishing such a rapid, high-throughput method for this determination? **Addressed in Chapter 3**

d) Are there a collection of SNPs, or other molecular identifiers that can be used in these methods (i.e. virulent lineages)? **Addressed in Chapter 4**

4. If the attributes critical for differentiating pathogenic STEC from non-pathogenic STEC can be identified, what concerns and confounding issues do you foresee in the need to determine whether those attributes are expressed or not? **Addressed in Chapters 2, 3, & 4**

a) What data gaps exist and what research is required to support methods development in relation to gene expression? **Addressed in Chapter 4.** For example, is there a need for a national research effort to establish a STEC full "transcriptome" multi-lab collaborative project on a strain-to-strain basis? **Addressed in Chapter 3**

5. What data gaps exist and what research is required to characterize and estimate the probability that a particular STEC isolate is highly virulent to humans? **Addressed in Chapters 1, 2, & 4**

a) What can be learned by assessing collections of virulence genes present in the genome or SNPs of virulent lineages? **Addressed in Chapters 3 & 4**

b) What degree of uncertainty is associated with such an assessment? **Addressed in Chapter 4**

*A few changes were made to the wording of the charge.

- A hyphen was added after environmental and animal in the first paragraph of the charge.
- Numbers less than 10 were spelled out.
- The number of STEC serotypes was changed from 300-400 to >400.
- The words “STEC serotypes” was inserted in place of “types” in the executive summary.
- The words “the most” were inserted between “caused” and “human” in the background section.
- The sentence “These STEC seem to be more prevalent in spinach and cilantro” was removed.
- The Stx subtypes were spelled out and the subtype subscripted as per convention in charge question 2a.
- The sub-questions underneath the charge questions were designated with letters rather than bullet points.
- Charge question 3, changed “detected” to “detect”.

Introduction

A. Overview

E. coli O157:H7 (hereafter referred to as O157 and includes both motile and non-motile variants) was first described as a human pathogen after a multistate outbreak of diarrhea and grossly bloody diarrhea (called HC) was linked to ground beef in 1982 (see case studies in Appendix 1) *(255, 328)*. The association between Stx (also known as Vero toxin) and the severe complication called the HUS was first noted in 1985 *(142)*. However, HUS was initially described in 1955 in rural Switzerland *(102, 142)*. The bovine reservoir for O157 was identified in 1985 *(186, 329)*. While O157 was the first serogroup of STEC to be recognized and is the most common type isolated from humans in the U.S. and other developed countries, strains from other serogroups of STEC that cause similar illnesses (diarrhea, HC, HUS) have been described. Currently, an estimated 265,000 STEC illnesses occur annually in the U.S. *(271)*. Diagnosis of STEC infection from stool samples depends on isolation of *E. coli* that produce Stx or are positive for the gene (*stx*) that encodes the toxin. In some HUS cases, presumptive STEC infection is confirmed by detection of a serologic response to the O157 or other common STEC O antigens.

STEC can produce the non-cross-neutralizable prototype toxins Stx1a, Stx2a, or variants thereof [reviewed by *(201)*], and a single STEC isolate can produce one or more of these toxins *(201)*. The toxin genes are carried on phages that are integrated into the bacterial genome. Exposure to some antibiotics can lead to induction of the phage as viral particles that can infect other *E. coli*, along with an increase in toxin production *(122, 223, 281, 298, 343)*. The term STEC includes a subset of organisms called enterohemorrhagic *E. coli* (EHEC) that are defined as those STEC that can cause HC and HUS, harbor a large ~90Kb plasmid (called pO157 for

O157), and attach closely to the mucosal surface of the bowel with subsequent effacement of the microvilli *(162)*. This close association of the organisms to intestinal cells reflects the expression of the *eae* gene for intimin, an outer membrane protein first described on enteropathogenic *E. coli* (EPEC) *(134)* and later on the prototype EHEC O157 *(340)*. Although the most common attachment mechanism for pathogenic STEC is intimin-mediated, there are STEC that are *eae*-negative. For example, STEC of serotypes O91:H21 and O113:H21 have caused HUS but do not produce intimin. In addition, an Stx2a-producing strain of *E. coli* O104:H4 that was *eae*-negative caused a large outbreak in Germany in 2011 with hundreds of cases of HUS *(86)*, see case studies in Appendix 1. While this strain was *eae*-negative, it had typical adherence genes of EAEC.

B. Steps in STEC pathogenesis

The steps in the pathogenesis of O157 infection and subsequent illness are better understood than for other types of STEC (see Fig. 1). For this reason we use O157 as an illustrative example, although the same sequence can follow infection with many other STEC. First, infection typically occurs after ingestion of O157-contaminated food or water or through direct contact with infected people or animals. The ID_{50} of O157 for humans, as estimated from a few foodborne outbreaks, appears to be very low [e.g., < 50 bacteria in dry fermented salami *(304)*, and <700 organisms in hamburger *(309)*]. Second, O157 transit through the stomach and small intestine and then establishes itself in (colonizes) the colon, as inferred from radiologic findings on O157-infected individuals *(255)*. This colonization step in animal models requires the expression of the locus of enterocyte effacement (LEE) pathogenicity island (PAI)-encoded proteins intimin and its translocated intimin receptor, Tir *(60, 66, 136, 192, 256)* to form attach and efface (A/E) lesions. Third, infected individuals develop watery diarrhea after about three

days (range 2-12 days) followed by bloody diarrhea or HC in 80-90% of O157-infected people *(231)*. The gross blood evident in the stools of people with O157-evoked diarrhea appears to be directly linked to production of Stx by the infecting organism since infection with EPEC that also produce intimin, but not Stx, does not lead to HC. Blood in the stools of O157-infected individuals is likely a result of Stx damage to small vessel endothelial cells in the colon *(131)* rather than bacterial penetration of intestinal cells. Indeed, O157 are not considered enteroinvasive organisms *(193)*. Fourth, HUS, the potentially life-threatening sequela of O157 infection that occurs in 10-15% of patients in the U.S. *(231)* fully depends on the production of Stx(s). How the Stxs exit the gut to target the kidney is not completely understood, but patients with Stx-mediated HUS exhibit acute renal injury *(231)* in addition to thrombocytopenia and hemolytic anemia.

C. Prevention, detection, and surveillance

Preventing STEC infections is of particular importance as there is no treatment other than careful fluid management and supportive care, and further, antimicrobial therapy is not usually recommended due to the potential for increased risk of HUS *(59, 210)*. Prevention entails interrupting transmission through contaminated food or water, or from animals to children, or from person to person. Most STEC have a ruminant animal reservoir though they are rarely associated with illness in the animals themselves. Meat can be contaminated during slaughter, while produce and drinking water can be contaminated by feces and runoff in the fields. Decreasing the incidence of STEC disease thus depends on reducing contamination of food and water, the use of hygienic measures in food processing and preparation areas, petting zoos, and childcare centers, and on prompt detection and control of outbreaks. While substantial success in

controlling contamination of ground beef has been achieved with measures focused on specific serogroups *(216),* STEC remain an important public health problem.

Public health surveillance for O157 began in the late 1980s after clinical laboratories started to use sorbitol MacConkey agar [SMAC *(181)*] plates routinely for diarrheal disease diagnosis, to confirm strains at public health labs, and to report infections. SMAC is an efficient way to screen for O157 as most do not rapidly ferment sorbitol. Colonies are off-white on SMAC, unlike the vast majority of *E. coli* which are pink. The state of Washington made O157 infections reportable in 1987 *(228)*. Many other states followed suit after the large West Coast outbreak in 1993 (see case studies in Appendix 1) *(44)*, when the Council of State and Territorial Epidemiologists (CSTE) recommended O157 infection be made nationally reportable *(54)*. The detection of non-O157 STEC infections became feasible when routine diagnostic methods were developed based on enzyme immune-assay (EIA) for detection of toxin in stool enrichment broths. Later, PCR-based diagnostic tools were developed for screening stool enrichment broths for *stx*. Routine screening with these diagnostic tools followed by isolation means that both non-O157 and O157 infections can be identified equally. In 2000, the CSTE recommended that all STEC infections be nationally notifiable *(55)*.

Improving the ability to identify clusters of persons infected with closely related STEC strains has been critical to detecting and controlling more outbreaks and guiding prevention efforts *(300)*. Routine molecular subtyping using pulsed-field electrophoresis (PFGE) was applied to O157 through the PulseNet network starting in 1996, and this technique has contributed greatly to the detection and investigation of outbreaks that would otherwise have been missed *(253)*. PulseNet extended PFGE to molecular subtyping of non-O157 STEC in 2009. State public health laboratories continue to perform PFGE on STEC isolates and an

increasing number of these laboratories are also performing WGS. By the end of 2018, all state public health laboratories are expected to sequence STEC isolates *(40)*. First applied systematically to *Listeria monocytogenes* in 2013, WGS methods led to a three-fold increase in the number of outbreaks detected and solved, and similar effectiveness may be obtained with STEC *(132)*.

To confirm STEC detected by non-culture methods and to allow subtyping for outbreak detection, CDC issued guidelines to clinical labs to send either the suspect *E. coli* isolate or the enrichment broth in which the Stx protein or gene was detected for final identification and characterization to the Public Health Laboratory (PHL) *(6, 106)*. Recently, STEC has been included in multiplex diagnostic panels that are increasingly used in clinical laboratories. Even with such platforms, it remains important that the PHL receive the clinical specimen or enrichment broth that yielded the STEC-specific signal, so as to obtain an isolate that can be further characterized. Such information will facilitate detection of outbreaks and will increase the understanding of key virulence determinants of STEC that lead to human disease.

Chapter 1: Clinical and epidemiologic features of STEC

A. Descriptive epidemiology of STEC infections

A.1 Surveillance of human infections in the U.S. Several surveillance systems are used to provide information about the occurrence of STEC infections in the U.S. Foodborne outbreaks of STEC infection are reported to the national Foodborne Disease Outbreak Surveillance System (FDOSS), with a summary of investigative findings (https://www.cdc.gov/foodsafety/fdoss/data/food.html). STEC surveillance data on individual cases are collected by state and territorial public health departments through passive, laboratory-based surveillance. To overcome differences in reporting requirements and completeness and to

297 provide reliable estimates of the frequency of STEC and other pathogens commonly transmitted
298 through food, the Foodborne Disease Active Surveillance Network (FoodNet) was established
299 *(117)*. FoodNet conducts active, population-based surveillance for laboratory-confirmed human
300 infections with STEC and other pathogens causing enteric diseases. FoodNet surveillance is
301 conducted in 10 sites across the U.S. and includes ~48 million persons (15% of the U.S.
302 population). FoodNet staff actively seek reports of STEC identified in clinical laboratories,
303 verify that all STEC are reported, and track the diagnostic methods used
304 (https://www.cdc.gov/foodnet/reports/index.html). A case of STEC infection is defined as the
305 identification in a clinical specimen of an *E. coli* that produces Stx or has a gene that encodes for
306 Stx production from a person in the FoodNet surveillance catchment. FoodNet tracks changes in
307 the incidence of STEC to assess the effectiveness of measures aimed at preventing these
308 illnesses.

309 **A.2 Incidence and serogroups causing STEC infections.** During 2008–2014, 6,824
310 cases of STEC infection were reported to FoodNet surveillance. Serogroup O157 caused half of
311 all STEC infections and together with the six most commonly isolated non-O157 STEC
312 serogroups (O26, O103, O111, O121, O45, and O145, referred to as the big 6) accounted for
313 86% of all STEC infections (see Table 1). Other serogroups each accounted for 1% or fewer of
314 STEC infections. The average annual incidence of STEC infection from 2008-2014 varied by
315 serogroup and year. Incidence of O157 infections remained relatively constant over the seven
316 year period, while incidence of all non-O157 STEC infections increased from 0.53 per 100,000
317 persons in 2008 to 1.4 per 100,000 persons in 2014, concurrent with the increased use of CIDTs
318 in clinical laboratories (Fig. 2).

A.3 Characteristics of patients with STEC infection and selected exposures by serogroup. The highest incidence of infection identified in the FoodNet surveillance from 2008-2014 was in children one to four years of age for all STEC serogroups and lowest among adults aged 18–64 years and older than 65 years (Fig. 3). In all age groups, the incidence of O157 infection was higher than for any other STEC serogroup. Compared to patients with infection caused by O157, patients with infections caused by O26, O103, and O111 STEC were more likely to be of Hispanic ethnicity and to have traveled internationally during the seven days before illness (Table 2).

A.4 Clinical features and complications of STEC infection by serogroup. Clinical manifestations and disease severity vary by STEC serogroup. In general, patients with an O157 infection have more severe illness compared with patients with an STEC infection caused by other serogroups. In U.S. FoodNet surveillance, a larger proportion of patients with O157 infection have bloody diarrhea (84%), require hospitalization (40%), and develop HUS (15%) compared with patients with non-O157 STEC infections; however, infections by non-O157 serogroups can also cause severe illness (Table 2). Strains that produce Stx2 are more likely to cause HUS than those that make Stx1 alone *(273)*, and stx_2 and *eae* have been associated with increased risk of bloody diarrhea and hospitalization *(31, 297)*. In an unusual outbreak caused by *E. coli* O104:H4 in Europe, 25% of patients developed HUS *(86)*.

Summary. STEC infections range in severity from subclinical infections to severe diarrhea to life-threatening HUS. In the U.S., 86% of STEC infections in humans are caused by O157 and the six most commonly isolated non-O157 STEC serogroups (O26, O103, O111, O121, O45, and O145). Reporting of non-O157 STEC infections has increased due to the improvements in clinical diagnostics and to increased surveillance. The clinical and

epidemiological features vary among these serogroups. In general, non-O157 STEC cause less severe infection than O157, and fewer non-O157 STEC infections have been associated with an identified foodborne outbreak.

B. Burden of illness

B.1 Health and economic burden of STEC illnesses in the U.S. Estimates of the overall number of illnesses caused by STEC are important to assess health burden and can be used to direct food safety policy and interventions. It is important to remember that the cases of STEC illness that are diagnosed, confirmed by microbiological testing, and reported to public health agencies represent only a small proportion of the actual number of illnesses that occur. Some of the reasons for the under identification of STEC infections are that some ill persons do not seek medical care, a clinical specimen for testing may not have been obtained, or a laboratory may not have performed appropriate diagnostic tests. The numbers of illnesses, hospitalizations, and deaths caused by STEC that occur each year in the U.S. have been estimated based on FoodNet data with statistical adjustments to account for under-diagnosis. For every reported O157 infection it has been estimated that 26 infections were not reported; for every non-O157 STEC infection reported, 107 infections were not reported *(271)*. Thus, STEC caused approximately 265,000 illnesses each year in the U.S. Of these, O157 STEC caused 96,000 illnesses, 3,300 hospitalizations, and 31 deaths and non-O157 STEC caused an estimated 169,000 illnesses and 400 hospitalizations each year. After excluding infections related to foreign travel or non-food exposures, an estimated 63,000 O157and 113,000 non-O157 STEC illnesses are caused each year by contaminated food eaten in the U.S. *(271)*.

The same estimates have been used to describe the economic burden of these infections, including medical costs of illness and productivity loss. Hoffman et al. estimated that the annual

cost of domestically-acquired foodborne STEC illness is $279 million; the majority of cost ($255 million) is associated with O157 infection while $24 million is associated with non-O157 infection *(121)*. In a different manner, Scharff et al. estimated that the cost per case of STEC foodborne illness is approximately $10,500 ($9,600 per case of O157 and $900 per case of non-O157 STEC), and the annual economic cost approaches $800 million ($635 million for O157 and $154 million for non-O157 STEC) *(272)*.

B.2 International burden of STEC illness. The recent World Health Organization (WHO) Foodborne Disease Burden Epidemiology Research Group (FERG) report is a systematic review and meta-analysis of 16 publications and notifiable disease databases from 21 nations *(332)*. STEC infection incidence rates were estimated in WHO-designated sub-regions based on a known incidence rate from a country within that sub-region if available, or extrapolated from a neighboring sub-region. A multiplier of 36 was used to account for likely underreporting of illness to health departments and applied to the best available surveillance data. It was assumed that the proportion of cases in which the infected person develops HUS or end-stage renal disease (ESRD) is the same across the globe *(179)*. Researchers thus estimated that each year STEC causes 2,801,000 acute illnesses worldwide (95% CI: 1,710,000; 5,227,000), which leads to 3,890 cases of HUS (95% CI: 2,400; 6,700), 270 cases of ESRD (95% CI: 20; 800), and 230 deaths (95% CI: 130; 420) *(179)*.

The estimated proportion of all STEC infections caused by O157 ranged from 0 (countries largely found in the Middle East) to 36% in European and Organization for Economic Co-operation and Development (OECD) nations, approximations which may indicate real variation in prevalence of O157. STEC infections may be a particular hazard in the youngest children in less developed nations. Researchers also estimated that based on age distributions of

the sampled nations, STEC causes 809,000 cases in those under 4 years of age, 554,000 in those 5–15 years, 974,000 in those 16–59 years, and 464,000 in those greater than or equal to 60 years of age *(179)*. A prospective three year multi-center study of causes of enteric infection measured the impact of STEC and other infections in children less than 5 years old in South Asia and Sub-Saharan Africa. Researchers found that children 0-11 months old with STEC were almost twice as likely to die as were healthy control children, as the adjusted elevated risk of death for that group was 1.9 (95% CI: 0.99-3.5); it was not significantly elevated for children older than 11 months *(149)*.

The global estimate of STEC illness is complicated by the variation in methods used to estimate the burden of illness from one country to another *(112)*. More systematic and harmonized estimates from more countries around the world would be useful to improve the global estimates.

C. Trends in incidence and attribution to food sources

C.1 Change in incidence can be due to surveillance artifacts. The number of STEC infections that are reported depends on which diagnostic methods are used, the clinical circumstances in which diagnosis is sought, reporting requirements, and the actual incidence of disease. Thus, national trends in reported cases need to be interpreted in the light of changes in diagnosis and reporting. If non-O157 infections are not diagnosed, O157 may be the only STEC reported. When methods that identify non-O157 strains are adopted, non-O157 STEC cases increase and may exceed O157 cases. In the U.S., reports of O157 infections increased through the 1990s as more states required them to be reported. More recently, reports of non-O157 STEC

increased as Stx screening was adopted for routine diagnosis and ultimately exceeded reported O157 infections (Fig. 4).

Similar trends can be observed in other countries. For example, the incidence of O157 infections in Norway and Japan has been relatively stable since 2000, after diagnostic testing and reporting for this STEC serogroup became routine (Fig. 5). In both countries, as in the U.S., reported non-O157 STEC infections have increased and now exceed O157 cases, as diagnostic methods that screened for all STEC began to be used *(29, 214)*. In Canada, reporting of non-O157 is variable across the provinces, and these STEC remain relatively under-diagnosed and under-reported compared to O157 (2016 *E. coli* fact sheet, PHAC Canada, www.phac-aspc.gc.ca/fs-sa/fs-fi/ecoli-eng.php).

Diagnostic practices can also affect the apparent likelihood of severe infections. For example, in the United Kingdom, O157 are sought routinely by clinical microbiologists in cases of diarrheal illness, while non-O157 STEC diagnosis is typically only pursued in the event of hospitalization or HUS. Because of the bias in testing, hospitalization and HUS appear to be far more frequent among non-O157 than O157 infections *(38)*.

C.2 Change in incidence due to improved food safety measures. STEC incidence can also change when changes in food safety regulations and practices that prevent STEC infection reduce transmission, and documenting these impacts can guide further improvements. For example, in 1994 O157 was declared an adulterant in ground beef and in 1996 the rule on Pathogen Reduction; Hazard Analysis Critical Control Point System (PR/HACCP) was published by the USDA. Despite efforts to control O157 contamination of beef, O157 infections had not decreased substantially by 2002, large outbreaks related to ground beef continued to occur, and approximately 0.8% of ground beef samples had O157 STEC (Fig. 6). In 2002, the

USDA-Food Safety and Inspection Service (FSIS) asked the beef industry to reassess their Hazard Analysis Critical Control Point (HACCP) plans to specifically address O157, and industry began testing beef trim lots used to make ground beef *(148, 215)*. The following year, contamination of ground beef dropped substantially and human O157 infections decreased by 30%, likely as a result of reduced contamination of ground beef *(215)*. In Canada, reported STEC infections due to O157 in the last 15 years have decreased steadily since 2000, also likely due to progressive improvement in meat safety measures *(248)*.

The impact of changes in food safety practices can also be seen through serial measurements of risk. For example, in the U.S. a nationwide case-control study of O157 infections conducted from 1990-1992 found the main risk factor was eating undercooked hamburger and eating in a fast-food restaurant was significantly associated with infection in a univariate analysis (60% of cases exposed, compared to 45% of controls) *(289)*. Several years later, in 1996-1997, after the large fast food hamburger-related outbreak in 1993 and promulgation of federal guidelines for cooking temperatures for burgers *(83, 313)*, a second case-control study of O157 infections found that illness was still associated with eating ground beef but not in a fast-food restaurant *(145)*.

In Japan, after several large outbreaks of STEC infections related to raw beef and liver, the Japanese Food Safety Law was revised in 2011 to prohibit the serving of raw ground beef in restaurants *(337)*. Case-control studies of O157 infections done before and after that regulatory change documented that raw ground beef and raw liver served in restaurants accounted for 47% of the O157 STEC cases before the regulatory change, and none of the risk afterwards *(338)*.

C.3 Attribution of health burden to food sources. As with individual cases, the number of outbreaks detected and reported varies depending on the methods used to detect them.

The deployment of molecular subtyping methods as a routine part of public health surveillance for O157 has greatly contributed to the detection and investigation of dispersed outbreaks *(300)*. Such systems have been adopted systematically in Japan, Canada, the United Kingdom, France, and Scandinavia. The absence of such systems in much of the rest of Europe may explain the rarity of reported STEC outbreaks in many European countries *(74)*.

Attribution of the health burden to different food commodity groups can be based on reported foodborne outbreaks, sporadic case-control studies, and comparison of collections of subtyped isolates *(113)*. Recent outbreak summaries provide the most comprehensive information for building attribution models. Painter et al. attributed illnesses to food commodities based on data from foodborne outbreaks associated with both simple and complex foods reported to the CDC from 1998–2008 *(233)*. Among the 17 food commodities, based on 186 outbreaks of O157 STEC infections, 39% of foodborne illness was attributed to beef, 27% to leafy greens, 21% to fruits and nuts, and 8% to dairy. Based on six outbreaks of non-O157 STEC infection, 62% of those infections were attributed to fruits and nuts, 30% to beef, and 8% to leafy greens *(233)*.

A review of 255 foodborne outbreaks of O157 infections in the U.S. from 2003–2012 revealed that beef and leafy vegetables were the most common food commodities associated with O157 infections. Among outbreaks with a single food commodity vehicle reported, beef accounted for 55% of the outbreaks and 48% of outbreak-associated illnesses, leafy greens accounted for 21% of the outbreaks and 38% of illnesses, and dairy products (all unpasteurized) accounted for 11% of outbreaks and 6% of illnesses *(116)*.

To harmonize source attribution based on outbreak data, the Interagency Food Safety Analytics Collaboration (IFSAC) estimated attribution for O157 by single food category,

478 including a 90% confidence interval (90% CI) *(127)*. Data on outbreaks reported in 1998-2012
479 with an implicated food category were analyzed with a Bayesian model that included pathogen,
480 food category, venue of food preparation, and a variable identifying multistate outbreaks. The
481 model gave less weight to data from 1998 through 2007 than to the data from the more recent
482 years (2008–2012). For O157-related illnesses, out of 17 food categories identified, likely
483 contaminated products were as follows: 46% were attributed to beef (90%CI 36-55%); 36% to
484 vegetables and row crops (90%CI 26-46%); 9% to unpasteurized dairy (90%CI 5-14%), and, 7%
485 to fruits (90%CI 3-12%) *(127)*.

486 In the European Union (EU), among 57 foodborne outbreaks caused by pathogenic *E.*
487 *coli* (predominantly STEC) that were reported by member states in 2007–2009, a food vehicle
488 was identified in 40 outbreaks. The implicated food vehicle was meat (mainly bovine) in 16
489 outbreaks, dairy products in nine outbreaks and "other" or "mixed food" in 15 outbreaks. Quite
490 different from what is observed in North America, no outbreak was associated with fruits or
491 vegetables *(74)*.

492 Outbreaks due to non-O157 STEC are less frequent and their sources less well-
493 established. In the U.S., among 38 single-etiology non-O157 STEC outbreaks reported from
494 1990 through 2010, 17 (45%) were foodborne, and, of those, five were caused by STEC O111
495 and five by STEC O26. The food vehicles most often implicated were dairy products, leafy
496 greens, game meat, and fruits/nuts *(176)*. Of the 38 outbreaks in the published series, eight
497 resulted in HUS. Strains from seven of these eight outbreaks had stx_2, compared to only 11
498 (37%) of the 30 outbreaks that did not result in HUS. Since that summary, 24 non-O157 STEC
499 foodborne outbreaks were reported to CDC from 2011 through 2014 (CDC unpublished data
500 from FDOSS). Among those, eight were caused by STEC O26 and five by STEC O111, and the

food vehicles most commonly implicated were leafy greens, sprouts, raw milk, ground beef, and salsa. There are no apparent correlations between serogroups and specific food vehicles.

C.4 Attribution by case-control studies. Source attribution can also use case-control studies of risk factors for sporadic infections. In the U.S., case-control studies of O157 infections have identified consumption of pink or undercooked ground beef, farm visits, drinking untreated surface water, and contact with cattle as risk factors for infection *(145, 319).* A few small case-control studies have been undertaken in other countries in which exposures were compared between non-O157 STEC-infected patients and population controls *(93, 194, 258)*. These studies have identified risk factors including consumption of sliced delicatessen meat, infant formula, catered meals, beef, raw spreadable sausage, and infants teething on undercooked beef. Other non-food-based exposures associated with non-O157 STEC infection included having a family member with occupational exposure to animals, contact with young children, wearing diapers, camping, living in an overcrowded setting, and contact with animals *(94, 194, 258)*. To examine sources of sporadic cases of non-O157 STEC, FoodNet has begun a large, population-based case-control study of risk factors for sporadic non-O157 infection. Interim analyses of data from the ongoing study has suggested consumption of fast-food hamburger (perhaps undercooked), travel, taking acid-reducing medication, and many animal and environmental exposures as risk factors for infection *(182)*. No elevated risk was associated with consumption of produce items *(182)*.

Summary. Ongoing national surveillance may show increasing STEC incidence as diagnostic and reporting practices change, while decreases can reflect improvements in food safety. In the U.S., the attribution of the health burden of illness to specific food commodities has been estimated from foodborne outbreaks and from case-control studies. Such estimates

are reasonably robust for O157. The burden attributed to beef and dairy products has been broadly similar in numbers to that attributed to fresh produce, each accounting for approximately half of illnesses, though the contribution of beef is declining. Fewer data are available for non-O157 STEC attribution and suggest a broad spectrum of food commodities are sources. Plant-derived foods predominate as identified vehicles in outbreaks, though beef and wild game are also involved. Case-control studies of sporadic cases typically identify beef and direct animal contact as important sources of sporadic infections but rarely identify produce as a risk factor.

D. Prevalence of STEC in cattle

While many ruminants serve as reservoirs for STEC, cattle are overwhelmingly associated with human illness and food-product contamination *(82)*. As such, STEC from cattle have been extensively studied for prevalence, diversity, and virulence factors. In a review by Hussein and Sakuma, it was estimated that worldwide prevalence of O157 and non-O157 STEC in dairy cattle ranged from 0.2 to 48.8% and 0.4 to 74.0%, respectively *(125)*. There were 193 STEC serotypes from cattle listed in that report, of which 24 were previously linked to HUS cases. In Spain, the overall prevalence of STEC in cattle was 27% and in calves 37% *(21)*. There were 112 STEC serotypes identified in that publication, and 22 of those had previously been associated with human illness *(21)*. The most common serotypes found in cattle in Spain were O20:H19, O22:H8, O26:H11, O77:H41, O105:H18, O113:H21, O157:H7, and O171:H2 *(21)*.

D.1 Virulence and putative virulence genes in STEC found in cattle. In general, among hundreds of STEC serotypes found in cattle, a smaller subset has been reported to be associated with human illness. Some studies have evaluated serotypes found in cattle for the presence of virulence factor genes including *stx*$_1$, *stx*$_2$, *eae*, and the enterohemolysin gene, *ehxA*,

a marker for many STEC. For example, in Spain, Blanco et al. evaluated 432 strains from cattle and found *stx*$_1$ (in 23% of strains), *stx*$_2$ (54%), both *stx*$_1$ and *stx*$_2$ (23%), *ehxA* (56%) and *eae* (17%) *(21)*. Blanco et al. also evaluated STEC isolates from cattle and beef products in Argentina and found *stx*$_1$ (in 14% of strains), *stx*$_2$ (74%), both *stx*$_1$ and *stx*$_2$ (12%), *eae* (24%), *ehxA* (46%), and a gene for a protein associated with adherence, *saa* (22%) *(22)*. Of the 153 strains characterized among 112 serotypes, 84% of the isolates belonged to serotypes previously associated with human illness. There is generally more diversity of non-O157 STEC in food animals than in ill humans, a finding that suggests that some bovine STEC may be less virulent than others (see chapter **2** section **A.** below).

E. Prevalence of STEC in foods

The prevalence of STEC in foods varies widely. Moreover, many STEC found in food belong to serotypes that have not caused recognized illness. Indeed, there is little association of specific serotypes with particular food commodities other than the well-recognized link between O157 and beef. Interpretation of the data from many surveys is complicated by the great genetic and serological diversity within STEC, the report of Stx gene testing without other virulence markers, limited serotyping of isolates, and varied sampling, isolation, and analytical methodologies. These issues make comparisons among studies and across years of surveillance difficult.

E.1 Prevalence in produce. The prevalence of non-O157 STEC in vegetables and fruits has been reported by the member states of the EU for years 2004–2009 *(74)*. During this period, member states reported results from a total of 5,910 samples of fruits and vegetables tested. Only 11 (0.19%) of these samples yielded STEC, and, of these, eight were O157 and

three were non-O157 STEC. Furthermore, none of 211 foodborne outbreaks of STEC infection reported from 2007-2009 was linked to fruits or vegetables.

Similar surveys of fresh produce were conducted annually by the Microbiological Data Program (MDP) of the Agricultural Marketing Service (AMS) of the USDA between 2001 and 2011 (https://www.ams.usda.gov/sites/default/files/media/2011MDPDatabase.zip). Of the ~2,200 samples per produce type tested each year, the prevalence of STEC was 0.5 to 0.6% in spinach, 0.3 to 0.5% in cilantro and 0.04 to 0.18% in lettuce. Combining the 112 AMS isolates with those from other produce surveys, 132 STEC isolates were further characterized. Among the 132 strains, *eae* was found in 9% (mainly O157:H7 and O26:H11) and *ehxA* in 61%; none had *aggR* (EAEC gene that encodes a positive regulator of aggregative adherence genes). Among the *eae*-negative strains, *saa* was found in 35%, and the operon for subtilase toxin, *subAB,* in 32%. The presence of more than one *stx* subtype was common; 23% of strains had two different *stx* subtypes and 2% had three. The most common *stx* subtypes were stx_{2a} (56% of the strains), stx_{2d} (28%, most often found in combination with stx_{2a} or stx_{1a}), and stx_{1a} (22%). Less common *stx* subtypes were stx_{2c} (7.5%), stx_{2e} (3%) and stx_{2g} (2%). Serotyping was incomplete for 42% of the strains. Of the 58% of strains that were completely serotyped, a number of the reported serotypes (O157:H7, O26:H11, O121:H19, O113:H21, O165:H25, and O91:H21) had previously been associated with human illness *(81).*

Samadpour et al. reported the prevalence of both non-O157 and O157 in various retail food samples using PCR that targeted stx_1 or stx_2 and *eae*, as well as specific O157 markers on enriched culture broths; positive broths were cultured only for O157 *(268)*. Non-O157 STEC with *eae* and at least one *stx* were identified in 6% of 200 sprout samples and 4% of 100 mushroom samples. A single sprout sample yielded O157 on culture *(268).*

E.2 Prevalence of STEC in beef and dairy products. In a study by Hussein et al., 162 STEC serotypes were found on beef products in the U.S., of which 43 were previously linked to the HUS and 36 with gastrointestinal illness, while 83 serotypes were not known to be associated with human illness at the time of publication *(124)*. Mathus et al. reviewed the literature for studies assessing the prevalence of STEC in foods *(189)*. The authors reported on studies that found STEC in various foods including beef, veal, pork, chicken, turkey, lamb, fish, shellfish, raw milk, minced meat, and cheese. The percentage of positive samples for STEC ranged from 1% in cheese samples to 63% in veal *(189)*. However, most of these studies relied on PCR tests that targeted *stx* and had limited cultural or serological confirmation.

Farrokh et al. reviewed numerous studies worldwide focused on the prevalence of STEC in raw milk and other dairy products made from raw milk *(76)*. They concluded that the prevalence of STEC in raw milk was relatively stable for the last 10 years at 0-2%. The frequency of virulence gene detection was greater than the frequency of culturable isolates *(76)*. A separate study in the U.S. reported the presence of virulence genes (stx_1 and stx_2) in 21% of 531 raw milk samples by PCR, but only 3.2% of samples were confirmed by culture methods *(51)*.

A Swiss study of the prevalence of STEC in raw milk cheeses collected from 2006-2008 found 86 (5.7%) of 1,502 samples positive for STEC by PCR after enrichment *(348)*. Through use of colony hybridization, 29 STEC strains were isolated. Among the 29 strains, 27 had at least one stx_2 gene and two had stx_1 only; nine (31%) had *ehxA* and none had *eae*. Of the 24 serotyped strains, none belonged to STEC serogroups O26, O103, O111, O145, or O157, though 13 (59%) were other STEC serotypes previously associated with human illness.

Overall chapter summary:

Since first identified as a pathogen in 1982, STEC have emerged as major cause of enteric illness around the world, sometimes complicated by HUS, neurologic sequelae, and death. Among the many *E. coli* strains that produce Stx, some cause illness more frequently and are more likely to lead to severe complications than other STEC. The frequency of reported infections depends greatly on the diagnostic strategies used in clinical laboratories; reporting of non-O157 STEC has increased with improvements in clinical diagnostics. In the U.S., the non-O157 STEC as a group are now more frequently identified in patients than O157 STEC, and a short list of seven serogroups, including O157, cause 86% of infections. Severe sequelae are more common in O157 infections than in non-O157 STEC infections. However, the risk of severe sequelae varies among different strains of O157, and some non-O157 STEC outbreaks, like those caused by O104:H4 in Germany *(86)* and by O111:H8 in Japan *(337)*, had unusually high complication rates. Solved outbreaks and case-control studies indicate that STEC can be transmitted via a variety of foods, including beef, dairy products, leafy greens, and fruits.

Chapter 2: Virulence profile and pathogenesis of STEC

A. Serotypes associated with human disease

Our current understanding suggests that any serotype (defined by serological type of O antigen, H or flagellar antigen, and where applicable, capsular or K antigen) of *E. coli* can be the cause of human disease if that organism makes Stx and has a mechanism by which it can adhere to the human colon. Of particular relevance are those STEC that make intimin and Stx1a (usually only when co-expressed with Stx2a), Stx2a, Stx2c, or Stx2d *(206, 273)*, Fig. **7**. Nevertheless, to date, only certain serotypes are linked to outbreaks. As reviewed in chapter 1, the serogroups (O antigen type only) associated with most cases of illness in the US are O157, O26, O111, O103, O121, O145 and O45, in order of decreasing incidence, Table 1 and Fig. 3. Thus, serogroup is a marker to readily identify these STEC, but the O antigen does not, in and of itself, contribute to pathogenesis.

The population structure of O157 has been defined by a variety of methods [e.g., single nucleotide polymorphisms (SNPs) and octamer-based scanning] *(26, 50, 155, 180)*. One conclusion from these systematic categorization approaches is that bacteriophage are a major contributor to the genetic plasticity seen among members of O157 *(5, 150, 224)*. Certain genetic lineages are more often associated with human disease *(4)*, while other lineages are more often associated with carriage in cattle *(12, 146, 330, 339)*. These O157 lineages differ significantly in relative frequency across geographic regions. For example, O157 that appear to belong to clinical lineages are isolated more frequently from cattle in countries with higher incidences of HUS *(87, 158, 161, 199, 245, 331)*. Also, certain lineages associated with O157 isolated from

humans with clinical illness do not appear to be represented in cattle, an observation that suggests the existence of a non-cattle reservoir *(26)*.

B. Virulence factors

B.1. Colonization factors

B.1.1. Relevance of adhesion. Among the hundreds of STEC serotypes, very few are commonly isolated from human gastroenteritis cases *(275)*. The reason that there is only a small subset of STEC linked to human disease may, in part, be because most such organisms do not have the capacity to intimately adhere to the intestinal epithelia. The relevance of bacterial adhesion to disease can be appreciated by considering the 2011 outbreak of bloody diarrhea that began in Northern Germany (see case studies in Appendix 1) *(86)*. The atypical, Stx2a-producing EAEC O104:H4 displays augmented adherence to intestinal epithelia compared to O157 because of the capacity of EAEC to form biofilms *(25)*. This thickly layered enteroaggregative attachment pattern likely facilitated systemic absorption of Stx and caused the unusually high rate of HUS (n = 855) that occurred *(17, 25)*.

Characterization of O157 adherence to host cells evolved from studying the related enteropathogenic *E. coli* (EPEC). The hallmark of EPEC intestinal attachment is the A/E lesion, mediated by the adhesin intimin. Only a subset of STEC, including those in the O157 and the big 6 serogroups (Table 2), share with EPEC the genes to produce A/E intestinal lesions. A/E lesion histopathology is characterized by intimate bacterial adherence to the apical surface of intestinal cells, sometimes on raised pedestals, and localized microvilli effacement. Reports of such lesions are lacking in human infections but are observed in several different animal models of disease and in healthy ruminants carrying O157 *(218, 256, 311)*.

B.1.2. Intimin encoded by *eae*. The LEE PAI carries the genes necessary for the formation of A/E lesions. Intimin is a 94- to 97-kDa outer membrane adhesin produced by all EHEC strains. Intimin is required, but not sufficient, to induce A/E lesions *in vitro* and *in vivo* *(66, 135, 192)*. Intimin plays a pivotal role in persistence and pathogenesis in animal models of disease (mice, infant rabbits, neonatal calves, and lambs) and in the colonization of healthy reservoir animals (adult cattle and sheep) *(136, 256, 285, 318)*. The primary receptor for intimin, Tir (for translocated intimin receptor), is also encoded by the LEE and translocated into the host cell plasma membrane via the LEE-encoded type III secretion system (T3SS). There are numerous subtypes of intimin (designated Int-α, -β, -γ, -δ, -ε, –θ, etc.) that are thought to mediate avidity and specificity of bacterial adherence in the intestine.

B.1.3. LEE PAI. The *eae* intimin gene lies within a ~40 kb LEE PAI that contains 41 open reading frames organized in five operons *(242)*. The locus encodes genes for attachment, actin nucleation, the T3SS, effector secreted proteins (such as EspFU that rearranges host-cell actin and effaces microvilli), chaperone proteins, regulators, and open reading frames of unknown function *(190, 294)*. The LEE is conserved among EHEC and EPEC isolates and is not present in non-pathogenic strains of *E. coli*. It is likely that the LEE was acquired by horizontal gene transfer because it contains prophage remnants, has lower % G + C content compared to *E. coli* genomic DNA (38% versus 50%), and is inserted at tRNA loci.

B.1.4. How do *eae-negative* strains colonize? Strains of non-O157 STEC that lack intimin are infrequently associated with severe disease. The mechanism(s) by which they colonize are not understood. However, it is likely that they do not intimately associate with intestinal cells but rather attach and persist using various combinations of the other numerous *E. coli* adhesins (see section **B.1.5** below). In addition, adherence capacity may be acquired in some

non-O157 STEC by horizontal gene transfer of putative adherence genes from *Shigella dysenteriae* type 1 and *Salmonella* spp., as is the case for *E. coli* O117:H7 *(57)*.

B.1.5. Other adhesins. Although intimin is the primary O157 adhesin, other factors may contribute to bacterial attachment. Several fimbrial, autotransporter, and flagellar proteins also mediate bacterial adhesion to human/animal intestinal epithelia, plants, and abiotic surfaces. At least nine fimbrial proteins contribute to O157 (or other STEC as indicated) attachment [reviewed in *(195)*].

In addition to the various fimbrial proteins, a group of surface-exposed structures referred to as STEC autotransporters contribute to the interaction between STEC and host cells and are often associated with the formation and maintenance of biofilms *(75)*. Finally, a complete picture of the highly complex situation of O157 and likely other EHEC adhesion to host cells and abiotic surfaces also includes the participation of flagella, the O-antigen, other adhesin proteins and certain pO157-encoded type II secretion system effectors [reviewed by *(195)*]. Many of these latter putative effectors of adherence are found in LEE-negative STEC as well.

B.1.6. Role of Stx in adherence. A contribution by Stx2a for adherence by STEC to tissue culture and in animals is supported by some studies, whereas others have not demonstrated such a role *(256, 259)*. Stx2a has the capacity to induce nucleolin expression at the surface of cells, and nucleolin can bind to intimin *(170, 259, 288)*. Stx2a expression is associated with cattle colonization as well in some studies *(62, 175)* but not in another *(285)*. The reasons for these discrepant findings are not clear but may be related to the particular animal model, the amount of Stx2a the challenge strain of *E. coli* produces, the site in the intestine at which colonization is assessed, and the timing of that evaluation, i.e., when during the course of infection the impact of Stx2a on colonization is measured.

B.1.7. STEC colonization of produce and/or animals

B.1.7.1 Produce. Bacterial attachment is likely the first step in fresh produce contamination. Cellular appendages such as curli fimbriae, *E. coli* common pili, hemorrhagic coli type 4 pili, and flagella, as well as T3SS, extracellular matrix cellulose, and colanic acid are involved in *E. coli* attachment to and persistence on plants. Curli fibers are essential for adherence of O157 to spinach and cultivar leaf roughness contributes to that strong attachment *(157, 177, 178, 267)*. Of note, O157 can be found inside spinach leaf stomata, in intercellular spaces, and within vascular tissue (xylem and phloem), where the bacteria are protected from gentamicin, sodium hypochlorite, or ozonated water treatments. The LEE T3SS system and effector proteins increase the efficiency of bacterial internalization in the stomata *(267)*. Several studies show that flagella are responsible, in part, for O157 binding to leafy greens and that mutation of *fliC* reduces binding to produce *(213, 262, 336)*. Plant cell wall arabinans are targets for *E. coli* common pilus and meningitis-associated fimbriae *(261)*. In addition to attachment capabilities, O157 biofilm formation may contribute to produce contamination *(344)*. There are differences in the mechanisms of *E. coli* retention by sprouts compared to leaves and fruits *(188)*. *E. coli* bind to lettuce, spinach, alfalfa, bean, tomato, cress, cucumber, pepper leaves, and fruits faster than to sprouts. Further, wounding leaves and fruit, but not sprouts, increases *E. coli* binding. There is no significant difference in the retention of an O157 and a generic *E. coli* K12 strain by fruits or leaves, but sprouts retain O157 better than they retain K12 strains *(188)*.

B.1.7.2 Animals. Because many human disease outbreaks are linked to contaminated foods of bovine origin or contact with cattle or cattle farms, O157 attachment/adherence in ruminants and in their environments impacts public health. The recto-anal junction mucosa is considered the primary site of O157 colonization in cattle and sheep

(108, 217). As with colonization in the human intestine, colonization of the bovine and ovine recto-anal junction mucosa is mediated by cell-surface structures of O157 such as intimin (the primary adhesin), other outer membrane proteins, fimbriae, flagella, the O antigen, and products of the LEE such as Tir *(172, 285)*. Adherence of O157 to bovine rectal squamous epithelial cells in culture requires factors other than those encoded by the LEE *(151)*. The mechanisms of O157 adherence to hair on bovine coats, bovine carcasses, other on-farm animals, insects, and abiotic surfaces on the farm and in the processing plant environment are not well understood. Nevertheless, how O157 adheres to or colonizes such surfaces may be critical to identifying ways to prevent food contamination. In fact, introduction of the pathogen into the food chain comes not only from colonized animals but also from hair coat and abiotic surface contamination. For example, when 20% of the cattle are colonized as determined by fecal pen–prevalence (the percentage of pen floor fecal samples that test positive for O157), as high as 80% of the cattle have contaminated hides *(335)*. Thus, low levels of fecal prevalence can cause contamination of hides of non-colonized animals. These combined sources of contamination lead to higher carcass contamination with O157 in the processing establishment *(71)*. The farm/feedlot environments impact cattle carriage of O157, but little is known about how the bacteria adhere to the various surfaces in these settings. However, it is likely that the A/E lesion and fimbrial proteins that direct intestinal attachment play a role. For example, flies that carry O157 have cellular injuries on their mouthparts similar to intestinal A/E lesions seen in animals *(147)*.

To summarize our current understanding, O157 intestinal colonization likely begins with connections between the bacterial surface and host extracellular membrane proteins such as laminin, collagen IV, and/or fibronectin. The bacteria then attach closely to host cells when

intimin interacts with Tir and host nucleolin. A/E lesions form when the translocated bacterial protein EspFu interacts with several host proteins to cause host-cell actin rearrangement and microvilli effacement. We predict that other eae-encoding organisms have similar mechanisms of host colonization. The mechanisms by which STEC without eae colonize remain to be elucidated. Furthermore, how STEC attach to produce, animal hair, or abiotic surfaces has not been defined.

C. Shiga toxin (Stx)

C.1. Background and characteristics. A link between Stx, STEC and HUS was initially made in 1983 by Karmali et al. *(143)* when that group found Vero toxin (also known as Stx)-producing *E. coli* in the stool of a patient who died from HUS. Soon after that discovery came the finding that the Vero toxin produced by similar diarrhea-inducing *E. coli* was the same as Stx made by *S. dysenteriae* type 1 *(223)*. Additional research by the latter group showed that *E. coli* can produce two antigenically distinct types of Stx *(298)*, Stx1 and Stx2. These prototype toxins are now called Stx1a or Stx2a, respectively.

The Stxs are AB_5 toxins in which the A subunit is an enzyme that depurinates the 28S rRNA and ultimately kills the target cell. The pentameric B moiety mediates the binding of the holotoxin to the receptor globotriaosylceramide or Gb3 [see review *(201)*]. A protease sensitive loop in the A subunit allows asymmetric cleavage of that molecule into an A_1 subunit that retains catalytic activity and an A_2 peptide that joins A_1 to the B pentamer. The A_1 and A_2 portions of the molecule remain linked by a disulfide bridge. The crystal structures of Stx from *S. dysenteriae* type 1 (same as, or with only a single amino acid difference from, Stx1a) and the *E. coli* Stx2a were solved *(88, 89)* and showed that the toxins are markedly similar, (Fig. 8). Among the differences between Stx and Stx2a noted by the latter researchers were that the Stx2a active

site is more accessible and that there is a difference in conformation of one of the receptor-binding sites (there are 3 Gb3 binding sites per B monomer) *(89)*. The variances in structure also appear to translate to differences in biological activity of the toxins. For example, Stx1a binds with greater affinity to Gb3 than does Stx2a and is more toxic than Stx2a to Vero (monkey kidney) cells [reviewed in *(201)*]. In contrast, Stx2a is more active against intestinal and renal endothelial cells [see review *(8)*]. In animals, the lethal dose 50% (LD_{50}) for Stx2a in mice is approximately 100-fold lower than for Stx1a, 1 ng as compared to 100-400 ng, respectively *(290, 303)*.

Both Stxs from *E. coli* have subtypes (a, c, d for Stx1 and a-g for Stx2), based mostly on biological differences (Table 3). As mentioned above, the prototype toxins are now known as Stx1a and Stx2a *(276)*. As assessed by in vitro and in vivo assays, the biological differences among the Stx2 subtypes have been studied more extensively than have the variations among the Stx1 subtypes. For example, Stx2c and Stx2d are antigenically distinct from and have lower specific activities for Vero cells than does Stx2a *(36, 169, 280)*. In addition, Stx2d becomes more toxic on Vero cells after incubation with intestinal mucus *(204)*, a phenotype called activation. Perhaps not surprisingly, given its association with HUS, Stx2d has a low LD_{50} (1.8 ng) in mice that is similar to that of Stx2a (1 ng), while the LD_{50} of Stx2c in mice is higher, about 14 ng *(36, 303)*. The higher LD_{50} of Stx2c compared to Stx2a or Stx2d is likely due to its reduced molecular stability *(36)*. Stx2e binds to globotetraosylceramide (Gb4), a different, but related, cellular receptor compared to the other Stxs and is found in STEC strains that cause edema disease of swine *(308)*. The genes for the toxin subtypes are differentiated based on a PCR typing scheme *(276)*.

Most importantly from the perspective of the response to the NACMCF charge, and based on epidemiological data, the toxin subtypes of an infecting STEC strain appear to impact disease severity. Specifically, STEC that make Stx1a, Stx2a, Stx2c, and Stx2d have all been linked to HUS [see review *(273)*]. However, *E. coli* that produce Stx2a are more commonly associated with HUS than isolates that make both Stx2a and Stx1a, while those that express Stx1a only occasionally cause severe disease. Stx2d is generally found only in non-O157 strains and was only recently identified in combination with *eae* in a group of strains associated with HUS *(64)*. A further point of note is that STEC linked to human disease produce higher levels of Stx (with or without induction with ciprofloxacin) as measured in vitro than isolates from food or animals *(220, 284, 341)*.

How the small differences in the crystal structures of Stx (same as Stx1a as mentioned earlier) and Stx2a translate into the reason that Stx2a is linked to more severe disease than is Stx1a remains a subject of some controversy. One possibility is that the catalytic activity of the A_1 subunit of Stx2a is higher than that of the A_1 subunit of Stx1a *(7)*. Alternatively, studies with chimeric toxins show that the B pentamer defines the overall toxicity pattern for the Stxs: a toxin with the Stx1a A subunit and the Stx2a B pentamer has the same specific activity as Stx2a, just as the reverse hybrid has the activity of Stx1a *(265)*. These comparison studies of toxin activity do not explain why strains that make both Stx1a and Stx2a are less likely to lead to HUS than isolates that make just Stx2a. However, the recent finding that oral administration of both Stx1a and Stx2a to mice causes less illness than when just Stx2a is given *(264)* suggests that Stx1a has the capacity to interfere with the toxicity of Stx2a.

Once the toxin reaches the circulation, it can bind to Gb3 present on tissues in the kidney and the central nervous system (CNS). The target cells in the kidney are tubular epithelial and

glomerular endothelial cells [see review *(8)*]. The Stx- mediated damage to the ribosome halts protein synthesis and the cell subsequently undergoes apoptosis [see review *(302)*].

C.2. Stxs are phage-encoded. The genes encoding Stxs are carried within the genomes of lysogenic bacteriophages (prophages). The Stx-encoding phages can exist either integrated into the bacterial chromosome (main lifestyle) or as free phage particles after activation of the lytic replication cycle. These lambdoid Stx-encoding phages are transmissible. The mobile nature of *stx*-converting phages means that *E. coli* or other phage-susceptible organisms can acquire or lose the capacity to produce Stx. The Stx-phage genomes are diverse and generally organized like lambda (λ) phage, with a single copy of the stx_1 or stx_2 operon (tandem A and B subunit genes) located in the late gene region of the phage genome. Expression of the Stx genes is primarily under the control of the late phage promoter p_R', and the encoded toxins are not produced and released in significant quantities until prophage induction, such as after exposure to ciprofloxacin, has resulted in toxin gene amplification and phage-mediated lysis of the host bacterium. This mechanism of toxin regulation highlights the direct role that Stx-phages themselves play in pathogenesis to regulate the amount of toxin produced and released during infection *(3, 174, 277, 321)*. Stx-phages may also influence the amount of toxin produced by transducing susceptible members of the commensal microbial population *(98)*.

Stx-phages have a broad host range as exemplified by the capacity of these toxin-converting phages to infect over 400 serotypes of *E. coli (273)* and *Shigella (13, 109, 111, 222)*, as well as other species in the *Enterobacteriaceae* [e.g., *E. albertii (28, 227), Citrobacter freundii (278) , Enterobacter cloacae (238, 252),* and *Acinetobacter heamolyticus (110)*]. The stability of these transductants can vary, with Stx-phages being readily lost or transiently infecting some hosts *(18, 110, 240, 278)*.

Within *E. coli*, the transduction of Stx-phages has driven the emergence of new hybrid pathotypes (groups of STEC that can cause disease), notably a hybrid pathotype between STEC and EAEC. Members of this hybrid pathotype produce Stx2a except where noted, and include *E. coli* O104:H4, which caused a large outbreak of bloody diarrhea and HUS in Europe in 2011 *(260, 274)*; *E. coli* O111:H2, which caused a small outbreak of HUS in France in 1992 *(211)*, and *E. coli* O111:H21 (Stx2c+), which caused a household outbreak in Northern Ireland involving three individuals with one requiring hospitalization *(58)*. Other STEC/EAEC hybrids include *E. coli* O59:NM, which was isolated from a case of bloody diarrhea *(251)* and *E. coli* O86:NM, which was isolated from two individuals in Japan (one with HUS and another with bloody diarrhea) *(130)*. Hybrid pathotypes have also evolved from Stx2-phages transducing *E. coli* serotypes O2:H6 and O80:H2 isolates that are phylogenetically associated with strains causing extra-intestinal infections. The clinical features of patients infected with these pathotypes (urinary tract infection and bloody diarrhea for serotype O2:H6 and bacteremia and HUS for serotype O80:H2) are consistent with the virulence traits of the merged pathotypes *(19, 184)*.

STEC strains may be lysogenized with either single or multiple closely-related functional Stx-phages, or with non-functional remnants of Stx-phages, as well as functional and non-functional phage (without *stx*) genomes from prior lysogenic infections. Following induction, Stx-phages can be transduced into susceptible bacteria in human feces *(98)*, in the gastrointestinal tract of various animals *(1, 53, 282)*, in food, in water *(126, 246)*, and in biofilms *(291)* to generate new STEC serotypes or Stx-producing bacteria *(187)*. The modular, repeated regions of homologous sequences provide sites for recombination to continually drive the diversification and genetic variability of Stx-phages for potential expansion of their host range.

In addition to phage acquisition, STEC can occasionally lose phage during infection, an event which complicates their identification as STEC *(16, 18)*.

In summary, elaboration of Stx is absolutely required for development of HC and HUS. Production by E. coli of certain Stxs are more likely to lead to HUS than others, i.e., Stx2a, Stx2d, and probably Stx2c. Stx1a is also associated with progression to HUS but much less frequently than the other Stxs, and may in fact, reduce the pathogenicity of STEC that express other Stxs. Since the Stxs are phage-encoded, these mobile elements can be transferred into other E. coli such as EAEC or into more typical STEC to cause a shift in the toxin profile of those strains.

D. Other toxins

D.1. Subtilase cytotoxin (SubAB). Subtilase (SubAB) is a potent AB_5 toxin in which the A subunit is a highly specific serine protease *(237)*. SubAB is toxic to Vero and primary human renal tubular epithelial cells, though Stx2a is more potent for both cell types *(185)*. Wang et al. *(324)* showed that intraperitoneal injection of SubAB into mice caused renal histopathology and altered blood parameters in mice that shared characteristics with human HUS. However, SubAB has yet to be associated with pathogenesis in an oral infection animal model. Furthermore, SubAB-coding genes are primarily detected in *eae*-negative (LEE-negative) STEC strains *(207)*. However, other *eae*-negative STEC strains that have caused serious human illness do not have *subAB*. Therefore, the role of this toxin in the pathogenesis of STEC strains is not clear *(78)*.

D.2. Enterohemolysin (EhxA). Many STEC strains have a large plasmid-encoded enterohemolysin, *ehxA* (also called *ehlyA*) that is related to α-hemolysin *(14, 173)*. Enterohemolysin causes small turbid zones of hemolysis around the bacterial colonies after 18 to

24 h incubation on blood agar containing washed erythrocytes. The gene for EhxA gene is frequently found in STEC associated with diarrheal disease and HUS, but is also found in environmental and food samples as well as *E. coli* that do not carry s*tx (78, 173).* For example, an analysis of 338 wastewater effluent samples for generic *E. coli* from dispersed regions of the U.S. showed that almost all carried *ehxA* and expressed EhxA. However, none of these *E. coli* isolates were STEC, although many did have *eae (23).* A role for enterohemolysin in virulence has not been demonstrated. Indeed, STEC cured of the large plasmid do not lose virulence in mice *(61, 202, 320).* Nevertheless, *ehxA* can serve as a marker for the large plasmid found in many EHEC.

In summary, toxins other than Stx that are expressed by O157 and other STEC are not proven virulence factors. Moreover, not all STEC encode these other toxins. However, it is possible that such toxins may contribute to the virulence of certain subsets of STEC (156). Additionally, some of these other toxin genes, such as ehxA, might serve as epidemiological markers for STEC.

E. Acid tolerance

Acid tolerance allows microorganisms to survive in acidic foods, animal feed, and food-processing treatments, and travel through the digestive tract *(167).* The low oral 50% infectious dose (ID_{50}) of O157 (estimated ID_{50} of <50 or <700 depending on food) supports the hypothesis that these organisms are acid-tolerant *(11, 79).* However, two studies showed that acid tolerance among STEC is highly variable *(11, 327).*

In summary, although there may be a role for acid tolerance in STEC survival in certain environments and during initial infection, the variable acid resistance among STEC strains makes it difficult to specifically correlate acid resistance with pathogenesis.

F. Approaches for predicting the capacity of STEC to cause severe illness

One of the early models for predicting risk of severe disease from STEC infections was developed by Karmali et al. in 2003 *(141)*. This approach empirically grouped STEC serotypes into five groups, designated seropathotypes (SPTs) A through E, according to their relative incidence, frequency of involvement in outbreaks, and association with severe disease (defined as HUS or HC). The authors also reported a significant association between the numbers of four virulence markers from pathogenicity O island 122 (OI-122) and the level of pathogenicity ascribed to the different SPTs. Subsequent studies looked for the presence in STEC strains of *stx*$_1$, *stx*$_2$, *eae*, several plasmid genes, plus additional virulence genes from OI-122 and other PAIs. They not only confirmed the earlier association of these markers with significant illness but also illustrated the additive effects of a variable repertoire of virulence genes on the capacity of isolates to cause severe disease *(52, 333)*.

A later phylogenetic approach that used a seven-gene multilocus sequence typing method to link the public health significance of STEC infections to the genotype of the isolates that cause those illnesses was also reported, and found to successfully separate strains capable of causing severe disease (SPTs A, B, and C) from those causing mild or no disease in humans (SPTs D and E) *(346)*. While the serotypes in SPTs A to C still represent the main serotypes of concern today, the seropathotype classification scheme has its limitations. The prevalence of rare serotypes may increase and new, hybrid strains have emerged to blur the boundaries between seropathotypes.

For example, the hybrid EAEC/STEC O104:H4, recently described extraintestinal STEC serotypes O80:H2, which caused HUS in an adult with associated bacteremia *(184)*, and serotype O2:H6, which triggered diarrhea and a urinary tract infection *(19)*. In response to the outbreak of EAEC/STEC O104:H4, the European Food Safety Authority (EFSA) recommended the adoption of a detection algorithm that screens isolates for the presence of selected serogroups (O157, O26, O103, O145, O111, and O104) in combination with *stx*, *eae*, a gene for a type 6 effector (*aaiC*), and *aggR (70)*.

Exploration of virulence differences between and within seropathotypes related to the presence or absence of specific virulence genes *(35)*, PAIs *(105)*, and lineage differences *(129, 155, 180, 197)* are areas of active research. While an exact genetic definition of a pathogenic STEC is likely to be elusive and to need continual refinement as horizontally transferred virulence determinants are recognized, epidemiologic studies show that stx_2 (including specific subtypes stx_{2a}, stx_{2c}, and stx_{2d}) and *eae* are clearly associated with severe disease *(15, 24, 31, 73, 114)*.

F.1. Models to predict virulence

F.1.1. Cell culture

F.1.1.1. Adherence. Both cell and tissue culture are used to model STEC adherence patterns in vitro. The terminal end of the ileum and the colon are the main regions for colonization of EHEC bacteria in humans *(48)*. And, indeed, human epithelial cell lines of intestinal origin have been used to demonstrate adherence capacity of STEC, Table 4 *(164, 198, 239, 250)*. Adherence of O157 and some other STEC strains is easily demonstrated in some of these model systems and for certain other types of human epithelial cells (e.g., HEp-2, laryngeal

969 in origin). A/E lesion formation that may appear as microcolonies of bacteria can also be seen
970 *(41, 192)*. However, levels of adherence vary by cell line and by STEC isolate *(156, 250)*. Both
971 polarized cells and in vitro organ culture may also be used to model STEC adherence *(25, 163)*.
972 **F.1.1.2. Intoxication.** Multiple epithelial cell lines are
973 sensitive to Stxs, e.g. Vero, HeLa, and primary human renal tubular cells.
974 Although epithelial cells such as renal tubular cells are likely damaged by Stx
975 during infection, damage to the microvascular endothelial cells, particularly
976 those in the glomeruli, are the cells that seem to contribute most to the HUS
977 triad. Human endothelial cells from the umbilical vein and of renal
978 microvascular origin have been used to show relative toxicity of Stxs *(305)*.
979 As a way to model Stx movement across the gut, various human polarized
980 intestinal cell lines have been used *(25, 123, 265, 306)*.
981 ***Cell culture systems allow for in-depth investigations of certain aspects of STEC***
982 ***virulence such as intimin-based adherence and Stx potency and trafficking. The conclusions***
983 ***from these in vitro studies can then be verified through the use of animal models and***
984 ***extrapolated as appropriate to humans. As cell culture systems become increasingly complex,***
985 ***these in vitro systems can more closely reflect actual animal and human conditions and thus***
986 ***allow researchers to analyze multiple virulence components/factors.***
987 **F.2.1. Animal models.** Several small animal models are used to assess relative
988 potency of the Stxs or the virulence of STEC [see review *(205)*]. Injection of the toxin into mice,
989 rabbits or baboons demonstrates that both Stx1a and Stx2a are exquisitely toxic in vivo.
990 Furthermore, in contrast to Vero cells, animals show increased sensitivity to Stx2a compared to
991 Stx1a, with LD_{50} values in mice, for example, of about 1 ng or 100 ng, respectively *(290, 303)*.

992 In certain models, injection or gavage of toxin may lead to a HUS-like syndrome, and/or
993 diarrhea, or neurological symptoms *(100, 209, 270, 287, 292, 295)*. In contrast to the other
994 models, the LD_{50} for Stx1a (20 ng/kg) was found to be lower than that of Stx2a (884 ng/kg) in
995 Japanese white rabbits *(95)*.

996 Mouse STEC infection models have provided a significant amount of information
997 pertinent to human diseases but do not reflect the full spectrum of illness in people. Murine
998 STEC infection models generally require either that the animals are treated with antibiotics
999 (streptomycin, ampicillin, or streptomycin and mitomycin C) or that the mice are germ-free to
1000 allow the infecting strain to colonize *(69, 96, 320, 341)*. In those infection models, Stx2a-, Stx2c-
1001 or Stx2d-mediated renal tubular damage and lethality can be demonstrated. Careful examination
1002 of brain sections by electron microscopy may show neuronal or CNS damage as well *(96)*.
1003 Furthermore, the in vivo role of Stx2a-phage induction in enhancing STEC pathogenesis was
1004 demonstrated in a germ-free mouse model *(310)* and in streptomycin-treated mice to which the
1005 phage-inducing antibiotic ciprofloxacin was administered after infection *(343)*. Alternative
1006 mouse models for STEC infection and disease include those in which the diet of the animals has
1007 been altered to either deplete protein calories *(152)* or increase the amount of butyrate in the gut
1008 *(347)*. The alteration of diet in both models enhances sensitivity to STEC infection. In infant or
1009 older Dutch belted or New Zealand White rabbits, experimental infection with STEC can lead to
1010 Stx2a-mediated diarrhea and kidney damage that appears to be strain-dependent *(99, 232, 234,*
1011 *256, 286)*. In Dutch belted rabbits that had a naturally acquired Stx1a+ STEC infection, severe
1012 kidney damage was observed *(101)*. Gnotobiotic or neonatal piglets, or antibiotic-treated pigs
1013 infected with STEC may exhibit intestinal lesions, diarrhea, CNS damage, brain lesions, and/or
1014 death *(61, 67, 286)*. In a primate model of oral infection with an Stx1a+ Stx2a+ O157 strain,

monkeys had brief, watery diarrhea. The bacteria colonized throughout the intestine and A/E lesions were detected. Kidneys from the infected primates showed moderate tubular but no glomerular damage *(138)*.

Taken together, animal intoxication models show that Stx2a and Stx2d are the most potent Stxs, that antibody to Stx2 is protective [not discussed above, see (168, 203, 265, 270)], and that the Stxs can directly cause diarrhea, kidney damage, CNS involvement, and death. Infection with STEC leads to similar outcomes, though diarrhea is more difficult to demonstrate. In summary for cell and animal models, both in vitro and in vivo models are used to assess function and virulence potential of factors that may contribute to the pathogenicity of STEC. None of the models replicate all aspects of STEC pathogenesis. The type of model used depends on the question that is being asked (see Table 4).

Overall chapter summary:

Predictions of the pathogenic potential of STEC can be made (Fig. 7): the most likely combination of virulence factors that could lead to severe disease is the presence of *stx*$_{2a}$ in an EAEC background, or with *eae* in the O157 serogroup, followed by that same combination (*stx*$_{2a}$ and *eae*) in O26, O45, O145, O103, O111, or O121. The combination of *stx*$_{1a}$ and *stx*$_{2a}$, or *stx*$_{2a}$ and *stx*$_{2c}$, or *stx*$_{2d}$ with *eae* is also of particular concern. The lack of *eae* suggests a reduced potential for human disease except when *aggR* or *stx*$_{2d}$ is present.

Chapter 3: Methods to detect and characterize STEC

A. Introduction

The purpose of this chapter is to: 1) summarize the test strategies applied by the USDA FSIS in non-intact raw meats, the FDA to detect high-risk STEC in fresh produce and other

foods, and by PHLs to detect STEC in clinical samples; 2) provide more details on the specific practices used to support those approaches; 3) outline the methods used by industry for the same purpose; 4) relate the basic tests employed by clinical and public health laboratories to detect STEC in patient samples; and, 5) discuss new and developing molecular techniques to rapidly identify high-risk STEC in food regulated by the FDA.

B. Overview of protocols currently used by USDA FSIS, FDA, or clinical and PHLs for the detection of STEC

Robust and validated testing methods are required by regulatory agencies for food surveillance, compliance, enforcement, and to support outbreak investigations. Currently, both the USDA FSIS and the FDA screen foods for the presence of STEC, using methods that are described in their respective manuals - USDA FSIS Microbiology Laboratory Guidebook (MLG) (http://www.fsis.usda.gov/wps/portal/fsis/topics/science/laboratories-and-procedures/guidebooks-and-methods/microbiology-laboratory-guidebook/microbiology-laboratory-guidebook) and the FDA Bacteriological Analytical Manual (BAM) (http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm). Both methodologies are similar in that culture enrichments of foods are screened by real-time PCR assays (RT-PCR) for specific STEC genes. However, the FDA regulates the safety of foods including fresh produce and cheeses, both of which have been found to contain diverse STEC serotypes *(81)*. Hence, that agency has taken an inclusive (both agencies consider *stx*+, *eae*+ STEC of the big 6 plus O157 as high risk) but broader approach than USDA FSIS, in part because many products like fresh produce and cheeses are often eaten uncooked. Thus, the presence of certain STEC isolates in FDA-regulated foods (e.g., *eae*+, stx_{2a}+) may be deemed a

safety concern even if the strain is not among the seven STEC serogroups considered adulterants by USDA FSIS.

B.1 USDA FSIS method (Fig. 9). An enrichment step is followed by RT-PCR to screen for O157:H7 or *eae* and *stx*. If the enrichment is positive for O157:H7, an attempt is made to isolate the organism and confirm the finding of O157:H7. If both *eae* and *stx* are found in the enrichment, RT-PCR is used to test for the big 6 non-O157 serogroups. Samples that are positive for any of the big 6 non-O157 O serogroups are subjected to immunomagnetic separation (IMS) for the particular O group (s) for culture confirmation.

B.2 FDA method (**Fig. 10).** An enrichment step is followed by multiplex RT-PCR to screen for stx_1, stx_2, and the O157-specific single nucleotide polymorphism (SNP) in the *uidA* gene or the O157 *wzy* gene. The differentiation of the two *stx* types is useful, as Stx2 is regarded as the more potent toxin and is most often implicated in severe diseases (see chapters **1** and **2** above). Enrichment samples that are positive for all of these targets are suspected to contain O157 and, therefore, are plated on sorbitol MacConkey or other chromogenic media. From these plates, presumptive O157 colonies are isolated, identified biochemically as *E. coli*, serotyped, and retested by PCR to confirm the presence of the specific genes. Samples positive for stx_1 and/or stx_2 but negative for *uidA* SNP or O157 *wzy* are suspected to contain non-O157 STEC and are plated on various selective and differential media for isolation and confirmation. Presumptive STEC colonies are tested with the *E. coli* identification (ECID) array (see section F.4) and subjected to WGS for genetic serotyping and characterization for health risk relevant attributes such as *eae* and *aggR* and *stx* subtypes.

B.3 Clinical laboratories/PHLs recommended methods (Fig. 11). Clinical stool specimens are tested simultaneously for O157 by culture on chromogenic agar and placed in an

enrichment broth for STEC detection. After enrichment, the broth is tested for the presence of Stxs or *stx*s. If O157 is isolated, the strain is sent to the PHL. If O157 is not found but Stx is detected in the broth, the broth is sent to the PHL. At the PHL, the toxin findings are verified and the broth is plated to isolate STEC. Suspect colonies are screened for Stx by immunoassay or *stx* by PCR. If a colony is positive for Stx or *stx*, the strain is biochemically identified as *E. coli*, serotyped, and tested for additional virulence genes. In addition, at the PHL all STEC are tested by PFGE and by 2018 will be sequenced; the data are then uploaded to NCBI and analyzed in PulseNet. Clinical laboratories are now rapidly adopting PCR diarrheal syndromic panels that can identify STEC proprietary gene targets but do not yield an isolate. To preserve access to isolates for public health purposes, specimens that are positive for STEC by these assays should be sent to the PHL for culture *(128).*

C. Advantages and disadvantages of methods currently used by FDA, USDA FSIS, or clinical and PHLs for the detection of STEC

C.1 Enrichment.

Due to generally low levels of STEC contamination of food and patient samples and the fact that the ID_{50} of O157 is estimated to be very low and may be similar or only slightly higher for other STEC serotypes *(84)*, almost all assays include cultural enrichment (requiring up to 24 hours) to increase bacterial numbers. Enrichment allows pathogens to replicate to detectable levels, resuscitates injured or stressed cells, differentiates viable from nonviable cells, and can also dilute the effects of background microbiota and assay-inhibitory components in food *(257).* Enrichment media often include antibiotics that select for the target pathogens and suppress other microorganisms. In addition to being time-consuming and labor-intensive, the effectiveness of

enrichment may vary depending on the strain, the food type, the media, the incubation temperature, and the types and concentrations of antibiotic(s) *(9, 32)*. As a result of these efficacy differences, various STEC enrichment media and conditions exist for different strains and for different foods *(139, 317)*. Therefore, a single medium to enrich for all STEC from all types of foods has not been described, although such a medium would be very useful.

C.2 Multiplex RT-PCR. The FSIS assay uses multiplex PCR to test for *stx*, *eae* and various O type genes. For the FDA assay, the initial targets for RT-PCR from the enrichment broth are stx_1, stx_2, and the O157 *uidA* SNP or O157 *wzy*. If the enrichment broth is positive for *stx* only, the agency proceeds to try to isolate STEC and further checks for serotype, *stx* subtypes, and other virulence genes (see below). Multiplex PCR assays done on enrichment can detect the target genes, but there is no assurance that the loci detected are from the same cell.

C.2.1 Limitations to PCR. PCR-based assays are sensitive and used extensively in STEC testing. However, non-viable bacteria in a sample can retain DNA and be amplified by PCR, which may result in false positives for living organisms. Adding an enrichment step to increase target cell count can provide some assurance that only viable cells are detected. Alternatively, reverse transcriptase-PCR using mRNA as the target for amplification may also be used to differentiate viable and non-viable cells. The predicted short half-life (seconds) of mRNA in a cell has prompted much attention on the use of mRNA as a marker of viability. Several STEC targets have been examined by reverse transcriptase-PCR, including *stx (191)*, the serogroup-specific *rfbE* gene for O157 or *wzx* for O26 *(307 2010)*, *eae*, *fliC* encoding the flagellar H7 antigen, and *ehxA (65)*. However, mRNA can persist in a detectable form for many hours after cell death *(20)*, and therefore, may not be well correlated with cell viability. The

instability of mRNA in samples and assay reproducibility are other problems encountered with the use of reverse transcriptase-PCR on food samples.

C.3 Culture. When the enrichment sample is positive for an O157 marker, the enrichment sample is plated on chromogenic media. Selective and differential culture media for non-O157 STEC may be the same as those for O157 or are variations of the media *(249, 315).* Other media useful for STEC isolation are those that detect enterohemolysin, such as washed sheep's blood agar with calcium chloride and mitomycin C *(166, 314).* Although the role of enterohemolysin in STEC pathogenicity remains uncertain, most STEC produce enterohemolysin. Thus STEC can be recognized by a faint turbid zone of hemolytic activity around the colonies on blood agar. Comparative studies showed that some agars may be suitable for STEC isolation; however, no single plating medium is effective for the isolation of all STEC strains *(119, 144, 312).*

C.4 Toxin immunoassays. Most clinical and PHLs use an immunoassay to screen stool broths for the presence of Stxs. These include EIA kits, which have detection limits of <100 pg/mL and allow for simple and quick, serotype-independent screening of Stxs in stools *(106, 323).* Others use Lateral Flow Immunoassays (LFIA) *(47)* that do not require washing, manipulations, or additional equipment and, as such, are simple to use and relatively inexpensive. LFIA results can be obtained minutes after cultural enrichment, and some kits can differentiate between Stx1 and Stx2 at the ng/mL levels.

C.4.1 Limitations to toxin immunoassays. Direct testing of feces for Stx is not recommended as levels of free toxin are often below the limit of detection for toxin immunoassays *(106).* An enrichment step is recommended and in addition most kit manufacturers suggest including polymyxin B or mitomycin C in the enrichment to induce *stx*

phage and increase Stx levels. False negative results can occur as *stx* phage can be lost, resulting in the loss of Stx production. In addition, false positive results were noted in two norovirus outbreaks, in the absence of Stx, suggesting that the antibody cross-reacted with noroviruses, or something else in the stool *(45, 46)*. Another limitation is that not all Stx subtypes (i.e., Stx2d and Stx2e) are detected by various kits *(334)*. Finally, toxin tests can be positive in the absence of STEC because bacteria such as *Acinetobacter heamolyticus*, *Citrobacter freundii*, *Enterobacter cloacae*, and *Shigella* can occasionally produce Stx *(109, 110, 241, 252)*.

C.5 IMS. This technique uses antibody-coated paramagnetic nanobeads that are added to food enrichment to selectively capture specific O serogroups *(323)*. A magnet physically separates the bead-antibody-antigen complex from non-target microbes and other potential inhibitors to obtain a fairly clean, though not a pure, culture of the target *(56)*. The IMS-treated sample can be plated on selective agars for isolation or testing by other methods. IMS has been used for STEC isolation in a variety of foods including fresh produce, meat, and dairy *(56, 68, 103, 226)*. IMS recovery efficiency can vary depending on the STEC strain, the level of O antigen expression, the affinity of the antibody used, and the food matrix. Presently, USDA FSIS uses antibodies to the big 6 STEC O types conjugated to beads for capture, followed by acid treatment and plating on modified Rainbow agar for isolation *(314)*. The lack of specific and high-affinity antibodies for the large number of other STEC O types has precluded wider application of IMS in STEC isolation *(68)*.

C.6 Biochemical traits. Bacterial isolates are often identified by biochemical traits in addition to serology (discussed below). Two useful biochemical traits of most O157 strains are the absence β-glucuronidase (GUD) activity and delayed sorbitol fermentation, so these attributes are often used for the isolation and presumptive identification of O157. There are

however, atypical O157 strains that express GUD and have caused infections *(212)*, as well as O157 strains that ferment sorbitol, express GUD, are non-motile and have caused HUS in various EU countries *(140)*. These phenotypic variants are not detected by media or tests used for O157. There are many manual or automated commercial assays that use biochemical and other phenotypic traits to identify isolates as *E. coli*, but to confirm STEC requires testing for Stx or *stx*. Non-O157 STEC, on the other hand, do not have universally distinguishing biochemical characteristics. Also, some STEC isolates, such as the O121:H19 strain that caused the outbreak associated with flour in 2016, are atypical in that they do not ferment lactose without induction; thus, these *E. coli* can be missed on differential media.

C.7 Serotyping. *E. coli* identification may entail serological typing of somatic (O) and flagellar (H) antigens for epidemiological purposes, because selected serotypes are more often implicated in STEC infections and outbreaks *(31)*. Serotyping *E. coli* is complex due to the existence of 187 O type and 53 H types, so complete serology is a cumbersome procedure that takes a few weeks to do. Moreover, only a few laboratories like the *E. coli* Reference Center at Penn State University and the CDC have the capacity to perform these serological typing assays.

Most STEC serological methods only test for specific O groups, although not all serotypes within a serogroup are STEC. For example, O157:H7 is an STEC of health significance, but O157 with other H types have not yet been found to have *stx (80)*. However, bacteria of these serotypes are found in foods and will react with anti-O157 sera. So, H typing can be useful for identification of STEC that may be pathogenic. Most clinical and public health laboratories are not required to test for H type, though some will test for a few well known H types if the assays are available. For the most part, reagents for many H types are not readily

available, and, furthermore, H typing can be confounded by non-motile (NM) or H-negative strains.

Many *E. coli* isolates from food and environmental sources cannot be serotyped or are mistyped by antisera *(78, 81, 153)*. Moreover, serotyping with antisera is time-consuming, especially when dealing with products like fresh produce that have a shelf life of only a few weeks. As a result, some laboratories are phasing out the use of antibodies for serotyping *E. coli* and replacing that method with DNA-based assays. Examples of genetic serotyping assays include the ECID which tests for 122 O types and all 53 H types *(72, 153, 236)* and a genoserotyping array which identifies 94 O types and 47 H types *(104)*. The serotype of STEC strains can also be identified by querying raw WGS data on the SerotypeFinder program of the Center for Genomic Epidemiology.

Serotype data are useful to assess whether such serotypes have been linked to serious human illness; however, serotype alone does not indicate risk without further information about whether the isolate carries *stx* and *eae* or *aggR*. Conversely, serotypes that have never been implicated in human disease may well carry *stx* and *eae* or *aggR* and may be of high risk. Thus, the combination of virulence genes, not the serotype, is predictive of risk.

D. Overview of the protocols currently used by the food industry for the detection of human disease-linked STEC

Sampling and testing are essential components of an effective and comprehensive food safety system. Although the objectives may be the same, the process and how the outcomes are handled may vary by industry. There is no standardized testing process. Thus, the various manufacturers may implement their own systems as suggested by consultants and testing firms or other industry best practices. All aspects of testing (lot size, sampling, sample size, detection methods, etc.) are

based on continually refined industry best practices. For example, the ground beef industry usually uses a "test and control" process, whereby the product is not delivered to market until a negative test result is obtained.

When testing, the three primary attributes considered by the industry are as follows: assay specificity and sensitivity; time-to-result, and cost. It is important that testing methods are validated for specificity and sensitivity for the target bacteria in the product matrix. Although testing methods and platforms vary in the food industry, there has been a shift from affinity-based antibody screening methods (i.e. lateral flow assays) to DNA-based assays like PCR, which have been formatted into fast, easy-to-use platforms. Although PCR assays are typically higher in cost, the expense is offset by the advantages of increased specificity and decreased time-to-results. Thus, to maintain production flow of fresh products, a company may make safety decisions (to divert or destroy) based on presumptive positive sample results without awaiting confirmation. The reason for making safety decisions based on a presumptive positive finding is that confirmation often takes 5-7 days, a timeframe that is inconsistent with shelf life of fresh products. In contrast, when testing processed products with a longer shelf life, presumptive test results can be taken to confirmation.

A positive finding of a pathogen in a sample incurs significant financial burden on the industry, but disposition of such a positive lot can vary within the industry. For example, when a lot of ground beef tests positives at a processing plant, the lot is diverted and can be sold to an establishment with approved HACCP and validated cooking procedures to treat the product. But, when a lot of fresh produce tests positive, there is no recourse, and the entire lot is removed from the food chain.

The industry has clear objectives for STEC testing, namely, lot acceptance/rejection, process validation and verification, or trend analysis. Many new advanced technologies such as WGS (see below) have become widely available to better characterize and trace the pathogens. For example, investigators have devised a rapid environmental STEC characterization assay for beef washes using single nucleotide polymorphism (SNP)-based subtyping *(221)*. However, portions of the industry remain hesitant and uncertain as to how to incorporate these molecular tools into their routine testing objectives or to justify the increased costs. The regulatory ramifications to the industry on generating these extensive pathogen data sets are also unknown.

D.1 General considerations that apply to the detection of STEC in foods. The effectiveness of methods to detect the presence of STEC in foods is affected by many variables including sample numbers and size, the screening methods, the sensitivity of these methods, and the anticipated distribution of the organism in the commodity. Also, most screening assays use multiplex PCR that detect several targets simultaneously. However, since most foods contain mixed microbiota, there is no assurance that all the targets detected originate from the same bacteria. As a result, it is essential to obtain an isolate to verify that all critical target genes are in the same strain.

Sensitivity and sampling are critical and mutually dependent factors in testing for pathogens in foods. The typical desired sensitivity for food testing is usually 1 CFU in 25 grams of food, but many assays do not achieve that sensitivity. For example, 10^2-10^4 CFU/ml are required for PCR detection *(118)* and lateral flow assays require >10^4-10^5 CFU/ml *(345)*. Furthermore, pathogens can be present at levels <1 CFU/25g food. The time for enrichment remains a significant bottleneck to rapid pathogen testing in foods.

Non-homogeneous distribution of bacteria and limitations of sampling present challenges to making health risk decisions. The International Commission on Microbiological Specifications for Foods (ICMSF) has published tables on the statistical confidence of accepting a contaminated lot based on the number of samples tested *(85)*. Intuitively, the lower the level of contaminant in a lot, the higher the number of samples per lot that need to be tested to have confidence in detection. Similarly, a larger sample size or an increased number of samples tested would also increase the odds of pathogen detection and the level of confidence in the results. However, sample sizes that are too large are difficult to handle logistically and the number of samples that can be reasonably collected and tested is limited. The only way to have 0% probability of accepting a positive lot with very low (>0 and <0.1%) pathogen prevalence is to test the entire lot, consequently there would be no food left for consumption.

E. Detection of virulence genes plus serogroup/serotype: two schemes with different approaches. Note: confirmation that virulence genes detected in enrichment broths all belong to the same organism is critical to both strategies outlined below.

Approach #1: Both the European Food Safety Authority (EFSA) and USDA use a strategy in which STEC strains with *stx* and *eae* genes and which belong to selected O types are deemed to be of public health concern. However, it is critical to confirm that all the genes are within the same cell. For example, Bosilevac and Koohmaraie tested 4,133 ground beef samples with an *stx* PCR and found 24.3% of the samples to be positive *(27)*. But, follow-up PCR of the 3338 *stx*+ isolates revealed that only six strains also had *eae*, and belonged to the big 6 non-O157 O types that are of USDA concern.

Approach #2: An example of an alternate method is the FDA BAM scheme which tests for O157-specific markers and also for *stx*$_1$ and *stx*$_2$ to detect all STEC. The STEC isolates are then characterized for the presence of *eae*, *aggR,* for specific *stx* subtypes, and serotype.

The Stx subtype genes most often associated with severe illnesses are *stx*$_{1a}$, *stx*$_{2a}$, *stx*$_{2c}$ and *stx*$_{2d,}$ Table XI *(90, 243)*, so determining the Stx subtype gene(s) produced by a strain provides useful health risk information. Feng et al. examined the specificity of various anti-Stx reagents and *stx* PCR primers and found great variations in specificity to different *stx* subtypes *(79)*. Subsequently, Scheutz et al. developed an *stx* subtyping PCR scheme that was tested in academic and PHLs using blinded samples of different stx subtypes *(276)*. With this scheme, even *stx*$_{2c}$ and *stx*$_{2d}$ can be distinguished if the correct annealing temperature is used. An array-based DNA subtyping assay from Alere Technology differentiates the Stx subtypes of 446 STEC strains isolated from various sources *(104)*. Additional subtypes such as *stx*$_{2h}$ and *stx*$_{2i}$ have been reported *(154)*.

E.1 Advantages and limitations in health risk predictions of the two approaches.

The advantage of the first strategy above is that the targets are well defined, the procedure straight forward, the objectives and the intended applications are clear, namely to ensure that STEC of selected O types with *stx* and *eae* genes are absent in foods and in the case of FSIS, in non-intact beef. Hence, the finding of a STEC strain that has *stx, eae* and belongs to selected O types will result in regulatory action, but the decisions may be less certain if all 3 targets were not detected. For example, finding a strain with *stx* and *eae* but that does not belong to the selected O types, is deemed a negative result. However, it is potentially dangerous to assume that the strain is not of public health concern. For example, an O80:H2 *eae stx*$_{2c}$ *stx*$_{2d}$ strain that caused HUS *(184)* might not trigger regulatory action since O80 is not one of the big 6

serogroups. Also, an *eae*-negative, *stx*$_{1c}$-positive O78:H- strain was isolated from the fecal samples of all five members of a family in Finland *(165)*. Infections by STEC strains with *stx*$_{1c}$ tend to be mild or asymptomatic *(91)*; accordingly, the parents and the older siblings had no symptoms, but the two-year-old child developed HUS. Similarly, an *eae*-negative O146:H28 strain with *stx*$_{2b}$, a subtype usually associated with asymptomatic carriage *(293)*, was transmitted from an asymptomatic mother to her child, resulting in neonatal HUS *(296)*. Furthermore, *eae*-negative STEC strains from serotypes like O113:H21 and O91:H2 that have caused HUS, as well as the O104:H4 strain that uses *aggR*-regulated adherence factors, would be ignored.

Other drawbacks are the lack of H typing and *stx* subtyping, both of which are useful for risk analysis. The strategy that begins with *eae* and *stx* detection will identify all strains with these genes regardless of serotype. Health risk decisions then become more complex and subjective as factors like the Stx subtype carried, the serotype or the H type of the strains, past history of having caused severe illness, etc. are taken into consideration. Also, to obtain this latter information requires additional testing and some, like serotyping, are not easy to perform, nor are they always productive. Hence, critical health risk data are often not available in a timely manner. Use of the ECID microarray and WGS can facilitate the process of getting these critical health risk data but, as our knowledge of STEC virulence mechanism expands, additional traits may need to be factored into the determination of potential for the isolate to cause human illness. Thus, the health risk criteria used in the strategy are fluid.

STEC pathogenesis is highly complex and aside from STEC virulence traits, other factors such as dose of STEC ingested may also play a role in disease outcome. Similarly, human factors and genetics may also affect colonization and the severity outcome of STEC infections *(137, 263)*. These examples suggest that human genetics and individual susceptibility can greatly

affect disease outcome. All STEC could have some risk to some individuals. Hence, terms such as "pathogenic" or "non-pathogenic" STEC may be misnomers and perhaps, should be replaced with "low" or "high" health risk STEC. Such a position and terminology would be consistent with those proposed by Scheutz for distinguishing the health risk of STEC strains *(273)*. Finally, past history that strains with the same serotype have caused severe infections and outbreaks may be useful to consider in risk prediction. However, serotype alone as such a predictor needs to be interpreted with caution because most STEC virulence genes reside on mobile genetic elements. Therefore, even STEC strains in the same serotype can have very different pathotypes that differ in their potential to cause severe illnesses. As a result, there are no uniform criteria that can be applied for determining the health risk of STEC that lack *stx*$_{2a}$ and *eae* or *aggR*, and so the process often results in a review on a case-by-case basis.

F. New and developing high throughput methods

F.1 WGS and epidemiology. PFGE had been the gold standard for linking patient isolates to each other and to isolates from contaminated food, but PFGE lacks the capacity to discriminate closely related strains any may also separate closely related strains. Currently, federal and state agencies are sequencing STEC isolates from outbreaks, inspections and surveillance samples. These sequenced genomes, which are publicly available, are uploaded in real-time and analysis results include a phylogenetic tree with epidemiologically relevant metadata providing the closest match to each genome at the single nucleotide level (see http://www.ncbi.nlm.nih.gov/pathogens/). To enhance strain resolution in food monitoring, the FDA created "GenomeTrakr", a WGS network comprised of state, federal, international, and industry food laboratory partners that submit DNA sequences of bacterial isolates from foods or food environments. The network is the first of its kind to use genomic data to characterize and

trace foodborne pathogens back to their source. Clinical isolates are now starting to be sequenced in public health laboratories of the parallel national network called PulseNet, which has used molecular subtyping since 1996 to identify clusters of infections with closely related strains and thus target investigation of possible outbreaks *(299).* All of the PulseNet sequences are added to the same National Center for Biotechnology Information (NCBI) database. This system together with USDA FSIS sequence data and GenomeTrakr enable rapid detection and accurate investigation of foodborne outbreaks, faster recall of contaminated foods, and more effective monitoring of preventive controls for food manufacturing environments *(2).* With international partners, the network has provided a rapid surveillance system to support effective public health responses to foodborne outbreaks worldwide.

To generate the datasets in the network, federal agencies have adopted the Illumina MiSeq platform to sequence of major foodborne pathogens from environmental, food, and clinical sources. The data are stored at the NCBI where sequences of environmental and food strains can be easily compared in GenomeTrakr to uncover new contamination events. In 2016, the CDC began expanding WGS technology to state PHLs and updated PulseNet to ultimately replace PFGE. To ease local data management and analysis and to generate a uniform schema for naming patterns, PulseNet relies on core genome multilocus sequence typing (cgMLST), as well as markers for serotype, antibiotic resistance, and virulence. The Bionumerics 7.6 software that supports PulseNet has also been updated to facilitate rapid upload of sequences to the NCBI database for analysis using cgMLST as well as SNP-based methods. As these genomic databases expand, the networks will continue to provide high-resolution detection of outbreaks, better source attribution, improved risk predictions, and monitoring of follow-up sampling after contamination events. The WGS landscape will need better representation of global

environmental and commensal strains to balance the clinical/disease bias in the existing available genomes.

A major advantage of WGS is that it is not limited by the need for *a priori* knowledge of existing or future molecular attributes and Stx subtypes that may emerge as risk-relevant markers. Thus, serotyping markers, *stx* subtypes, virulence, and adherence genes are inherently included in the sequence dataset generated for each isolate. Fast and relatively uncomplicated analysis of raw sequence datasets can also be achieved with a web-based portal at the Center for Genomic Epidemiology at the Technical University of Denmark (CGE; http://genomicepidemiology.org/). CGE offers tools like SerotypeFinder and VirulenceFinder for identification of many serotypes and known virulence attributes, as well as tools for pathogen identification, antibiotic resistance genes, etc., without the need for sequence manipulation or bioinformatic expertise. While useful, the identifications are limited by the scale and breadth of the query database contained at the portals. For example, serologically there are 187 O types, genetically there are many more O types, with more being identified. Databases will need continuous improvement to account for phenotypic predictions from genotypic data.

It is uncertain how rapidly or broadly WGS will be adopted by the food industry as a whole. Presently, WGS technology is still evolving and is not rapid or cost-effective enough for routine product testing, though it is used for source attribution and other investigations. Furthermore, how WGS would fit in with the "test and control" objective of the industry and the potential regulatory repercussions of an extensive genomic database on pathogens or potential pathogens in their products remains of industry concern.

F.1.1 Limitations to WGS as a rapid method. WGS requires that an isolate be obtained, and that process slows decisions making by regulatory agencies. The ability to perform

WGS directly on stool or food source or enrichment broth would obviate the initial requirement for an isolate; however, current technology does not allow us to be certain that the genes identified came from the same organism. A major drawback of screening mixed microbial samples using multiplex assays is that the different targets detected may be from different bacteria. In other words, a sample positive for *stx*, *eae* and a particular O type may give the impression that the sample contains that target pathogen, but the *stx* may be from a STEC or another enteric that carries *stx*, the *eae* may be from another STEC, an EPEC or atypical EPEC, and the O type may be from a generic *E. coli* strain. As a result, it is critical to isolate the organism to verify that all three targets detected are within the same cell. As mentioned previously, STEC isolation is extremely time- and labor-intensive, and often, the strain can't be isolated. Assays able to specify that *stx*, *eae* and a gene for the specific O type are from a single pathogen present in a mixed microbe sample would be very useful for the EFSA and FSIS screening strategies and ultimately for clinical diagnosis as well. It would also be beneficial and cost effective for the industry by reducing unnecessary product disposal or re-processing based solely on the presumptive finding of all three targets.

F.2 Digital PCR. This technology has the potential to discriminate target source without an isolate. Digital PCR systems can distribute a PCR reaction into ~20,000 tiny droplets, each of which holds only a single bacterial cell. By reading the labelled signals from each droplet, the assays detect the specific target amplified and can improve confidence that the signals are within the same genome. Using primers to *stx*, *eae* and the big 6 O serogroups, these assays detected the three targets in spiked cattle feces and showed that all the signals were within the same cell *(171)*. At present, these assays can only determine whether two to three genes are present in the same genome. Although this molecular technique would be useful as a screening

assay, STEC health risk predictor may require additional data such as H type, serotype, and the *stx* subtypes carried by the strain. Thus, isolates will still be needed for characterization. Similarly, isolates will still be required for epidemiological investigations, for PFGE typing, and for WGS for phylogenetic analysis.

F.3 Biosensor. NeoSeek (Neogen, Lansing, MI) is an example of a biosensor platform that combines DNA-based targeting with mass spectrometry to identify and differentiate STEC pathogens in 24-36 hours, starting from an enrichment broth. The initial PCR amplification generates amplicons (~100 bp) that are differentiated by mass spectrometry. Formatted into a 384 Sequenom MassArray chip, the assay detects O-types 26, 45, 103, 111, 121, 145, and 157, and H-types 2, 6, 7, 8, 9, 10, 11, 16, 19, 21, 25 and 28, as well as the presence/absence of a proprietary set of target SNPs to generate a molecular profile for identification. The O type targets currently detected are focused on FSIS priorities, but the technology has the capacity for expansion to include new targets and enables the user to custom build a 'molecular profile' for the bacteria of interest.

F.4 Microarray. The FDA ECID (Affymetrix GeneAtlas) incorporates genetic signatures from over 250 whole genome sequences, resulting in an assay that detects 41,932 *E. coli* gene targets and 9,984 single nucleotide polymorphisms (SNP) to provide a near true representation of the *E. coli* pangenome *(236)*. The array targets include virulence factors of various pathogenic *E. coli* groups, such as *eae* and *aggR*, the 10 subtypes of stx_1 and stx_2, many putative virulence genes, and a molecular serotyping component for the various O and H antigens *(153)*. The capacity of the ECID array can be expanded to include additional O antigen probes, but better means of distinguishing closely related antigen sequences would allow more precise identification of *E. coli* serotypes. The ECID microarray is currently undergoing a single

lab validation study using a panel of reference strains for serotyping and identifying the relevant genetic targets in STEC. The assay has a turn-around assay time of 24 – 48 hours with complete strain-to-data analysis. Lastly, it allows various entities such as industry and academia to enter the genomics arena without specialized bioinformatics or "big data" expenses for personnel and software typically associated with next-generation short-read sequencing technologies.

F.5 Limitations on establishment of rapid high-throughput methods. The molecular platforms mentioned above offer significant dynamic range in targets but can differ extensively in throughput. For example, gene target capacity can range from hundreds (NeoSeek) to thousands (microarray) with rapid assay time-to-result in 24 hours for either platform. However, throughput is constrained by the peg setup on a standard microarray and is limited to a handful of strains (i.e. four for the ECID), whereas multiplexing for 384 strains can be accomplished with the NeoSeek Sequenom MassArray chip. On the contrary and depending on cycling conditions and intended read lengths, WGS requires a minimum 24 hour run time in addition to significant hands-on time for DNA preparation and library construction. The methodology for either the NeoSeek or Affymetrix systems is less labor intensive by comparison. In addition, WGS requires data processing and bioinformatic analyses which can involve significant time and computational resources. Nevertheless, tens of strains can be sequenced on a standard Illumina MiSeq platform.

G. Genomic clusters or lineages that can be used to predict whether an STEC isolate is likely to cause serious humans disease

G.1 Genomics and risk prediction. The FDA has committed significant analytical resources to the genomic analyses of STEC to improve risk characterization. This is especially critical with unknown STEC strains isolated from foods, where regulatory actions may be

pending on the assessment of potential pathogenicity. Currently, one of the factors used by the FDA for risk determinations is the serotype of STEC to assess its past history of having caused human illness. Similar associations could be identified and further refined using genomic clusters or lineages (molecular identifiers).

As it is newly applied to WGS, analyses of genomic clusters or lineages are focused on understanding and relating core- and accessory-genomes of *E. coli* strains. Specifically, the core genome focuses on genes that are common in all *E. coli* which can be used to establish evolutionary relationships through phylogenetics. In contrast, the accessory-genome contains all the transitory genes, including horizontally acquired genes such as *stx* on a bacteriophage. The core genome phylogeny can be overlaid with elements of the accessory-genome tailored for a particular pathogenic group such as STEC and thus can be used to assess the potential for human health concern. These aggregate landscapes can reveal potential "hotspots" for toxin and adhesion factor genes that can contribute to the emergence of lesser-known serotypes or hybrid strains in disease, such as occurred in 2011 with the O104:H4 EAEC/STEC strain. It is noteworthy that next-generation genomic sequencing technologies along with rapid public access of data were pivotal in providing a genetic snapshot of this new pathogen in real-time *(33, 133, 196, 254, 260)*. These genomic landscapes also enable the identification of environmentally-derived cryptic lineages *(322)*, circulating virulence factors, and most recently, a novel plasmid found in hybrid STEC/enterotoxigenic *E. coli* strains *(160)*. With respect to STEC, the complexity of the group poses additional challenges to qualitatively mine and identify the virulence content that are potentially indicative for severe disease such as HUS.

G.2 CIDTs based on DNA sequencing. A potential vision for CIDTs in the regulatory setting may be the use of metagenomics, where DNA *in toto* within foods or clinical

samples are sequenced and analyzed for appropriate DNA signatures of a pathogen. Such metagenomic strategies are highly dependent on the existence of deeply populated phylogenies, such as GenomeTrakr as a subset of the NCBI pathogen detection website. These databases will be essential to finding unique genomic signatures of virulent lineages and their associated virulence genes. Furthermore, as discussed above, another paramount obstacle in metagenomics would be to determine whether the key virulence signatures are contained within a single viable organism. In other words, and in adherence to legal requirements, it will be essential to show that the genes for the relevant virulence traits are contained within one organism as opposed to originating from two or more separate organisms in the sample.

Another challenge to the metagenomic approaches to CIDTs will be to determine the sensitivity and specificity of next generation sequencing needed to capture complete representation of DNA in a sample. In other words, the sequencing depth or the numbers of reads generated per sample needed to avoid false negatives. A proof-of-concept study was recently done by the FDA and showed that 10 CFU of O157 spiked into 100g of spinach could not be detected directly from the food at a depth of 10,000,000 reads. However, adding an eight hour enrichment step enabled metagenomic detection of key virulence determinants with significant coverage of the Sakai genome *(159)*. This has been demonstrated in other foods as well *(230)*. Similar studies show promise in determining the core genome lineages of non-O157 STEC among the complex metagenomes that are found on spinach *(160)*. While there are many other potential uses for metagenomics, the major disadvantage remains the cost and the sensitivity of high-throughput sequencing, factors which are dependent on the sequencing depths needed. These limitations can be somewhat mitigated if combined with enrichment to decrease required depths and sample multiplexing per sequencing run, both of which will lower costs. Regardless,

sequencing-based CIDTs will require further research and development but hold significant promise from a variety of perspectives. Over the coming years, federal and state agencies will standardize and adopt standard operating procedures (SOPs) for metagenomic approaches.

H. Transcriptomics and proteomics

Failure of a clinical STEC isolate to produce Stx is rare, and, when it occurs, is generally due to the loss of the *stx*-converting phage. However, as mentioned above, STEC isolates from food may have reduced Stx expression. The simplest solution to determine if an isolate is producing Stx is to do an ELISA or LFIA for the toxin. However, if protein levels are too low, detection of the toxin transcript by quantitative reverse transcriptase-PCR might be helpful. Whether a complete transcriptome analysis of an STEC isolate can help provide signatures of virulence has not been determined. The analysis itself (RNAseq) can be labor intensive and require significant bioinformatics expertise. Moreover, the culture conditions for such analyses would need to be standardized because *E*. coli transcript profiles can vary depending on growth phase and media composition *(235).*

There have also been studies to try to identify STEC by proteomic assays. One group found that serogroups O157, O26 and O111 could be distinguished from other *E. coli* serogroups and that three biomarkers, ribosomal proteins S15 and L25 and the acid stress chaperone HdeB, were effective biomarkers for O157*(225).* Additionally, the DNA-binding protein H-NS allowed the differentiation of O26 and O111 from other O types. A semi-automated pattern-matching approach that used these biomarkers allowed discrimination of 57 O157, 20 O26 and six O111 strains with 100% reliability, regardless of the sample conditions. Similarly, Christner et al. used MALDI-TOF mass spectrometry to analyze 294 *E. coli* isolates from clinical samples collected during the 2011 EAEC/STEC O104:H4 outbreak in northern Germany and identified two

characteristic biomarkers that specifically identified all 104 O104:H4 isolates examined during the outbreak *(49)*.

Although proteomic assays seem to have potential in STEC identification, the majority of biomarkers found were ribosomal proteins that were difficult to distinguish from other closely related genera and species such as *Shigella* and other *E. coli (266).* Also, there are conflicting reports as to whether proteome profiles change under different growth conditions *(39, 316)*, and, if so, standardization of test conditions would be critical. Lastly, proteomic assays have not been used to examine STEC virulence proteins to determine their capacity to distinguish among Stx subtypes. The mobility of *stx* genes will also affect spectral profiles and pose challenges to using proteomic assays to type STEC. While knowing whether a STEC virulence gene is actually expressed would be desirable and have relevance in making health risk decisions, at present, proteomic assays are not readily available or cost-effective for practical use. Furthermore, the additional time required for proteomic analysis of strains would further extend test time and delay timely health risk decisions, especially for short shelf-life commodities like fresh produce.

Overall chapter summary:

USDA FSIS, FDA, PHLs and industry use similar methods to detect STEC. Because methods are not sensitive enough to detect low level contamination, an enrichment step precedes all testing methods and is a bottleneck to rapid testing. Traditional DNA-based PCR screening followed by culture, isolation, and biochemical and/or serological identification is being replaced by new and developing high-throughput CIDTs. For example, an advantage of WGS is the potential for fast and uncomplicated analysis of raw sequence datasets that are not limited by existing or future molecular attributes that may emerge as risk-relevant markers. However, limitations still exist that preclude utilization of

these rapid genomic techniques as standalone procedures. Among the challenges are the required enrichment culture, cost, time-to-result, and the need to assure that genomic targets are from the same and viable STEC cell. Techniques like droplet PCR combined with proteomics may ensure the identified genomic sequences belong to the same living cell and that the genes are expressed. As STEC virulence is better understood and genomic libraries of pathogenic and environmental STEC increase, these techniques will likely be adopted and advance the STEC risk determination process.

Chapter 4: Gaps and recommendations

- **How do we improve the usefulness of STEC isolate characterization data (clinical, food, and animal sources) and patient interview data (epidemiologic and clinical) to compare virulence profiles across years and countries?**
  - **Recommendations:**
    - **Standardize epidemiological and clinical information collected as part of routine public health surveillance of sporadic cases and reported outbreaks (including standard definition of severe disease).**
    - **Standardize descriptive information about source of food isolate and circumstances of collection of food samples.**
    - **Apply WGS to characterize all STEC isolates collected as part of routine public health surveillance and food monitoring.**
    - **Link epidemiological, clinical, source and WGS data from STEC isolates to monitor trends in recognized and emerging virulence attributes such as Stx type and phage profile.**
    - **Encourage the WHO and Food and Agriculture Organizations (FAO) expert committees to define ways to gather better data in developing countries.**
- **How can sample collections be expanded to include a variety of non-clinical sources that are not routinely monitored by public health agencies and to compare the data from those isolates to data from clinical strains?**
  - **Recommendations:**
    - **Expand systematic sampling of food, animals, food contact and non-food contact environmental surfaces, and water for STEC.**

- **Encourage state and local agencies that are doing routine sampling of foods for STEC to upload WGS results to NCBI.**
- **Explore ways for industry to share test data anonymously.**

- **How does public health surveillance adjust to changing diagnostic testing strategies such as the growing use of CIDTs without access to cultures?**
  - **Recommendations:**
    - **Report test methods used along with results as part of surveillance.**
    - **Encourage submission of isolates or clinical material to PHLs, when Stx is detected by a culture-independent assay.**
    - **Develop future CIDTs for doing serotyping, virulence gene determination, and high-resolution subtyping directly from clinical specimens.**

- **What human host factors influence the outcome of STEC infection?**
  - **Recommendation**
    - **Encourage funding for academic research on host factors that influence the outcome of STEC infection, including the composition of the gut microbiome.**

- **Is there a way to predict and measure toxin levels from assembled WGS data or by other methods?**
  - **Recommendations:**
    - **Investigate the regulation of toxin expression on a genome level.**
    - **Determine whether particular phage insertion sites or multiple phage are associated with higher toxin levels and whether the genomic location of one *stx*-phage affects the toxin level produced from another *stx*-phage.**

- **Develop a rapid, quantitative method to detect toxin levels from isolates grown *in vitro* and assess whether those levels are predictive of virulence.**

- **What gene or genes are required for *eae*-negative STEC strains to colonize humans?**
  - **Recommendations:**
    - **Develop a colonization model reflective of the human system (e.g., a human biochip system or perhaps in a humanized mouse model system).**
    - **Define the genes and gene products responsible for colonization in those models.**
    - **Conduct epidemiological analyses of *eae*-negative STEC strains associated with severe human illness to attempt to identify the colonization factors most commonly found in these isolates.**
- **What promotes *stx*-encoding phage mobility that leads to emergence of new Stx-producing bacteria?**
  - **Recommendations**
    - **Determine the transmissibility of *stx*-encoding phage within known pathogenic serogroups of *E. coli* and to other bacterial genera.**
    - **Address the reason that lysogeny with one *stx*-phage does not prevent lysogenization with another *stx*-phage.**
    - **Determine what promotes *stx*-phage loss which may result in subsequent negative tests for *stx*.**
- **Why is serotype O157:H7 more highly associated with outbreaks and apparently sporadic illness in the United States than other STEC serotypes?**
  - **Recommendations:**

- **Compare the ID_{50} and virulence of O157 to that of other STEC that have caused severe human disease, as assessed in appropriate animal models.**
- **Compare the adherence of O157 to that of other STEC that have caused severe human disease in standardized *in vitro* models.**
- **Determine whether O157 are more persistent in the natural (water, soil), farm or food production environment in the U.S. or on surfaces than other serogroups, and, if so, why.**
- **Assess whether O157 are more tolerant to agents or processes used to reduce contamination on produce, meat and other food commodities than other STEC serogroups; and, if so, why?**

- **Why are some Stx subtypes linked to more severe disease in humans?**
  - **Recommendations:**
    - **Develop an oral infection model that closely mimics human disease (diarrhea, bloody diarrhea, HUS) and addresses relative virulence of toxin subtypes in that model.**
    - **Assess the site of Stx subtype binding in that model and in a kidney model system.**

- **What are the mechanism(s) of adherence and persistence of STEC on fresh produce and abiotic food-contact surfaces?**
  - **Recommendations:**

- **Identify STEC and fresh produce characteristics that facilitate or inhibit binding and persistence of the bacteria and their internalization into intact produce.**
- **Identify characteristics of abiotic surfaces that facilitate or inhibit binding and persistence of STEC.**

- **Can enrichment and isolation protocols be developed that can be broadly used for all STEC and would be applicable to all foods?**
  - **Recommendations**
    - **Develop a new enrichment medium that can be broadly used for all STEC in any food.**
    - **Develop methods that will shorten, simplify, and improve the isolation of STEC from the enrichment.**
- **Are there better ways to group STEC by genomic methods?**
  - **Recommendations**
    - **Replace traditional serotyping with genomic technologies such as WGS to more effectively determine the serotype (O and H) and the Stx subtype of the STEC strain.**
    - **Evaluate a classification scheme for *E. coli* based on genomic clusters rather than DNA-based serotyping.**
- **Are there high-throughput assays that can be used to characterize large numbers of diverse STEC strains or to test directly from food or environmental samples?**
  - **Recommendations**

- **Explore high-throughput methods that can assess health risk directly from enrichment medium to eliminate the need to isolate the bacteria and also expedite decision making.**
- **Develop/improve methods that can ascertain that all critical markers detected are within the same cell, to eliminate the need to isolate the organism.**
- **Implement high-throughput methods that are flexible and can be modified as other markers emerge as critical risk criteria.**
- **Develop/improve high-throughput assays to make them economical and widely available.**

- **Can WGS data obtained from virulent lineage studies and identification via SNP typing or other molecular profiles be used to determine the virulence potential of STEC?**
  - **Recommendations**
    - **Improve/encourage standardization of sequencing platforms, protocols, and bioinformatics to enable comparisons of sequence data worldwide.**
    - **Increase usage/awareness of programs such as VirulenceFinder and SerotypeFinder (http://genomicepidemiology.org/) which can be queried with raw sequence data to derive information on presence of risk critical traits.**
    - **Identify additional genetic markers that are more inclusive of STEC that have the potential to cause severe illness.**
    - **Generate additional fully assembled and closed whole genome sequences of STEC for reference databases.**

- **Develop/improve and reduce cost of the equipment and methodology technology for long-read sequencing.**
- **Enhance and support a publicly available, curated, annotated, and searchable sequence database of STEC linked to disease.**

- **Would it be feasible to use proteomic assays that measure gene expression to provide a more precise assessment of health risk?**
  - **Recommendations**
    - **Develop proteomic assays that can detect different adherence proteins, different serotypes and to discriminate Stx subtypes.**
    - **Develop a database that can be used to evaluate the proteomic profile of STEC strains.**

Tables

Table 1. Most common serogroups of human STEC isolates, Foodborne Diseases Active Surveillance Network, United States, 2008–2014[a]

Serogroup	No. of isolates (%)
O157	3,420 (50.1)
O26	868 (12.7)
O103	696 (10.2)
O111	470 (6.9)
O121	177 (2.6)
O45	105 (1.5)
O145	102 (1.5)
O118	84 (1.2)
Other	451 (6.6)
Undetermined/unknown	451 (6.6)
Total	**6,824 (100.0)**

[a]CDC FoodNet Fast (https://wwwn.cdc.gov/foodnetfast)

Table 2. Demographic and clinical characterics of patients with STEC infection, by most common serogroup, Foodborne Diseases Active Surveillance Network, U.S., 2008–2014[a].

Characteristic	All STEC (N=6,755)	O157 (N=3,420)	Non-O157 (N=3,335)	Big Six					
				O26 (N=868)	O103 (N=696)	O111 (N=470)	O121 (N=177)	O45 (N=105)	O145 (N=102)
Patient demographics									
Median age, years	17	16	18	15	18	14	21	20	18
Female sex, %	55	54	56	55	58	58	50	53	56
Hispanic ethnicity, %	12	8	15[b]	17[b]	18[b]	18[b]	8	4	11
Exposures, %									
Outbreak-associated	12	20	4[b]	7[b]	2[b]	7[b]	7[b]	0[b]	5[b]
International travel	8	3	14[b]	9[b]	18[b]	18[b]	4	1	4
Clinical characteristics, %									
Bloody diarrhea	69	84	54[b]	60[b]	53[b]	51[b]	75[b]	76	54[b]
Hospitalization	27	40	15[b]	12[b]	14[b]	17[b]	23[b]	30[b]	26[b]
HUS	8	15	1.4[b]	0.5[b]	0.8[b]	1.6[b]	3.8[b]	1.6[b]	9.7

[a]*(128)*

[b]Indicates statistically significant (p-value<0.05) difference compared with O157 STEC based on Wilcoxon-Mann-Whitney test (age) or Fisher's exact test (all other variables).

Table 3. Characteristics of STEC toxin subtypes

Toxin group	Toxin subtype	Distinguishing feature(s) compared to prototype	Association with HUS	Reference(s)
Stx1	Stx1a[a]	Prototype toxin	Yes (much less common than Stx2a)	*(183, 273)*
	Stx1c	Less toxic than Stx1a; antigenically distinct	Rare (1 case in a bacteremic patient)	*(165, 342)*
	Stx1d	Antigenically distinct	No	*(37)*
Stx2	Stx2a	Prototype toxin	Yes (most common)	*(24, 90, 229, 273)*
	Stx2b	Identified by the failure to amplify the B subunit gene with traditional primers	Rare	*(247, 296)*
	Stx2c	Antigenically distinct; less toxic to Vero cells	Yes	*(29, 273, 280)*
	Stx2d	Antigenically distinct; less toxic to Vero cells ; activatable by intestinal mucus	Yes	*(15, 169, 204, 280)*

	Stx2e	Binds globotetraosylceramide (Gb4) preferentially; immunologically distinct	Rare (one case in a bacteremic patient)	*(63, 77, 269)*
	Stx2f	Antigenically distinct	Rare (only one published case)	*(92)*
	Stx2g	Lower capacity to inhibit translation	No	*(115, 279)*

[a]Same as Stx from *S. dysenteriae* type 1.

1728 **Table 4. Models used to assess function and/or pathogenic potential of STEC or toxins**

1729 **produced by STEC**

General model type	Questions that can be answered	Specific model
In vitro	Adherence capacity & localization; A/E lesions (not T84 cells)	HEp-2, T84, HCT8 in vitro organ culture (pediatric intestinal tissue, HEp-2 cells, T84 cells)
	Stx specific activity; relative toxicity of Stxs; toxicity of SubAB	Epithelial cells (Vero cells; HeLa cells[a]; HRTECs[b])
	Relative toxicity of Stxs	Vero cells, endothelial cells (HRMECs[c], HUVECs[d])
	Stx translocation	Transwells (T84, HCT-8, Caco-2)
In vivo	Relative Stx potency (injection); HUS-like model (Stx2a injection); colonization capacity in non-antibiotic treated mice (infection)	Mouse
	Relative pathogenicity of STEC strains after oral infection; relative Stx potency	Mouse (germ-free or antibiotic treated); neonatal pig

	as delivered from STEC	
	Enterotoxicity (ileal loops); diarrhea-inducing capacity)	Rabbit
	Colonization capacity, A/E lesion, relative pathogenicity of STEC strains or mutants	Pig, rabbit
	Bloody diarrhea, TTP	Greyhound
	Colonization capacity	Rabbit, cattle
	Relative Stx toxicity; HUS model	Baboon

[a]HeLa cells are less sensitive to Stxs, particularly Stx2e

[b]HRTEC: primary human renal tubular epithelial cells

[c]HRMECs: human renal microvascular endothelial cells

[d]HUVECs: human umbilical vein endothelial cells

Table 5: Virulence factors and markers of STEC from case studies in Appendix 1[a]

Case #	Year	Location	*E. coli* serotype or serogroup	Vehicle	Relevant genotypes
1	1982	Michigan and Oregon, U.S.	O157:H7	Ground beef from quick serve restaurants	*stx*$_{1a}$, *stx*$_{2a}$, *eae*, *ehxA*
2	1993	West coast states, U.S.	O157:H7	Ground beef from quick serve restaurants	*stx*$_{1a}$, *stx*$_{2a}$, *eae*, *ehxA*
3	1996	Sakai City, Japan	O157:H7	White radish sprouts	*stx*$_{1a}$, *stx*$_{2a}$, *eae*, *ehxA*
4	2006	Multistate, U.S.	O157:H7	Spinach	*stx*$_{2a}$, *stx*$_{2c}$, *eae*, *ehxA*
5	2009	Multistate, U.S.	O157:H7	Raw cookie dough	*stx*$_{2a}$, *eae*, *ehxA*
6	1999	Texas, U.S.	O111:H8	Mixed	*stx*$_{1a}$, *stx*$_{2a}$, *eae*, *ehxA*
7	2010	Multistate, U.S.	O145:NM	Romaine lettuce	*stx*$_{2a}$, *eae*, *ehxA*
8	2011	Germany	O104:H4	Sprouts	*stx*$_{2a}$, *aggR*, *eae* and *ehx* negative
9	2011	Japan	O111:H8 and O157:H7	Yukhoe (raw beef dish) in restaurants	O111: *stx*$_{2a}$ (+) and *stx*- O157: *stx*$_{1a}$, *stx*$_{2a}$, *stx*$_{1a}$ and *stx*$_{2a}$
10	2015	Multistate, U.S.	O26:H11	Mexican-style quick serve restaurant	*stx*$_{1a}$, *stx*$_{2a}$, *eae*
11	2016	Multistate, U.S.	O121:H19	Flour	*stx*$_{2a}$, *eae*

[a]See Appendix 1 for outbreak-specific references; all other microbiologic features from unpublished CDC data.

Figures

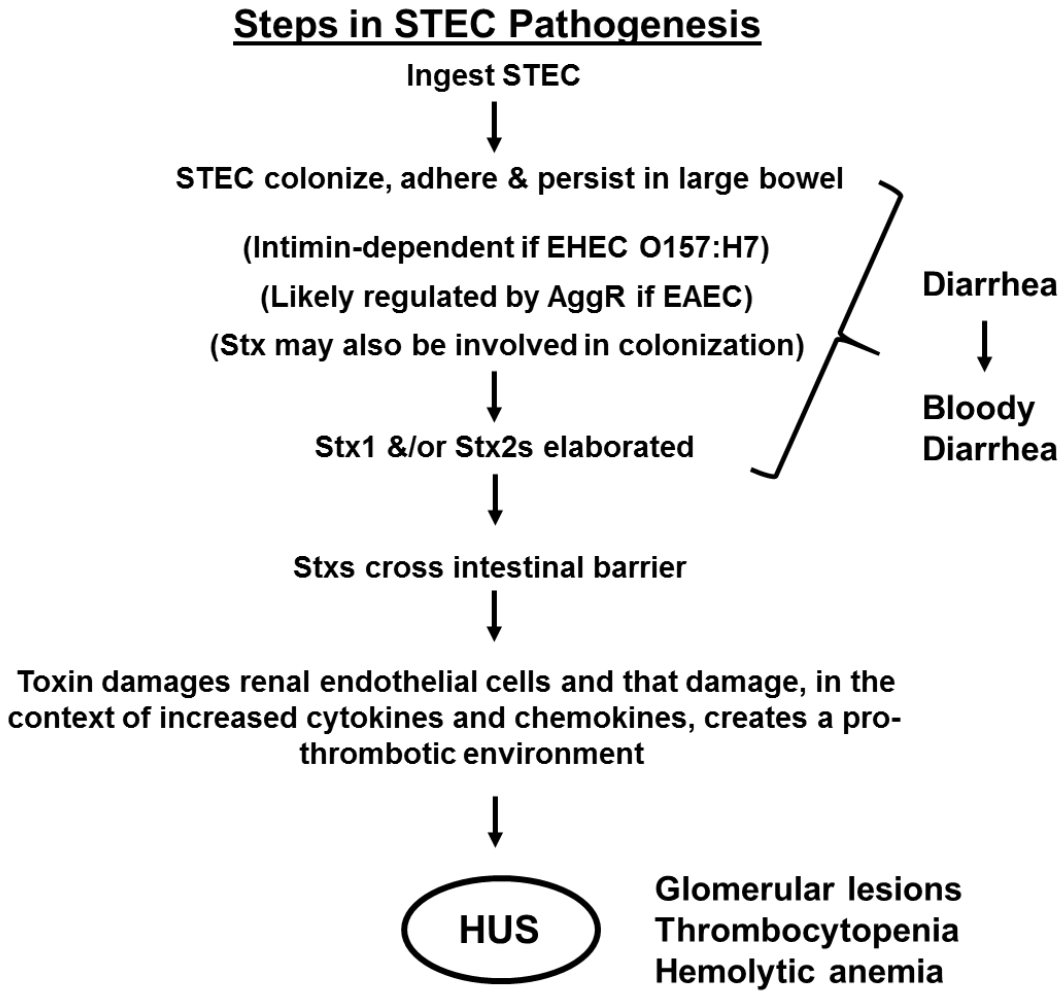

FIGURE 1. Steps in STEC pathogenesis. STEC are ingested in contaminated food or water and establish colonization (attach and persist long enough to cause disease) within the large intestine and elaborate Stx(s). The combination of adherence and toxin production lead to bloody diarrhea. Some of the Stx passes from the intestine to the bloodstream to sites where the toxin receptor may be found (kidney and sometimes, CNS). Adapted from Melton-Celsa et al. *(200).*

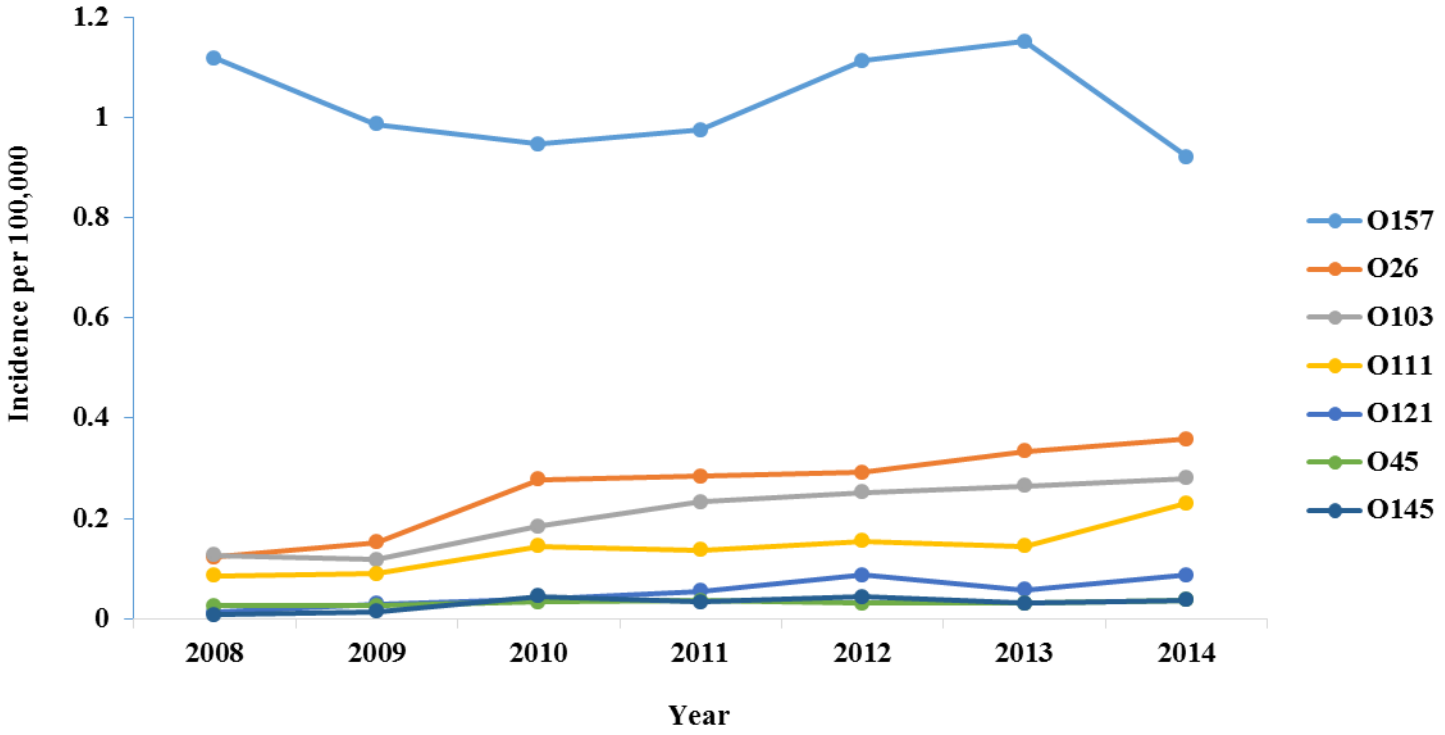

FIGURE 2. Incidence of STEC infections, by serogroup and year. Data from Foodborne Diseases Active Surveillance Network, U.S. 2008–2014 and CDC FoodNet Fast (https://wwwn.cdc.gov/foodnetfast).

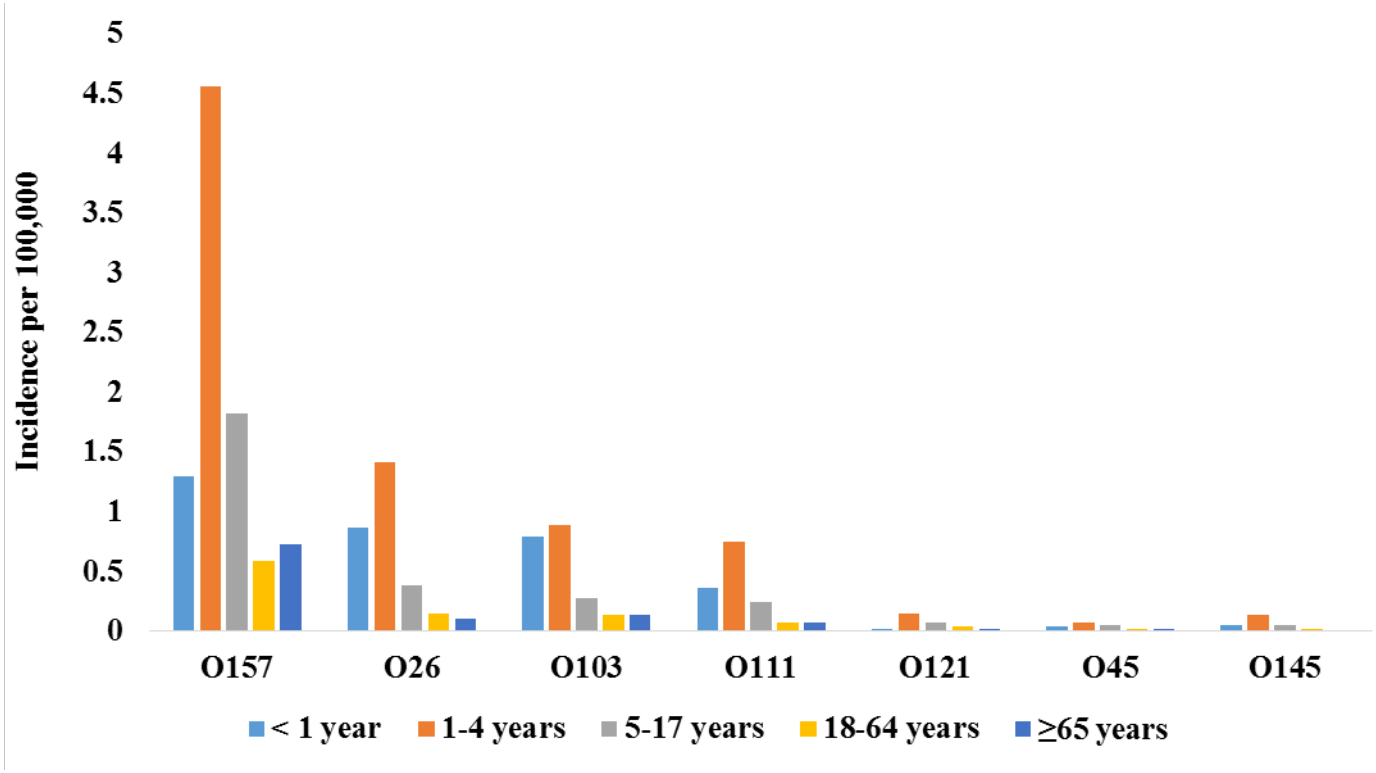

FIGURE 3. Average annual incidence of STEC infections, by serogroup and age group, Foodborne Diseases Active Surveillance Network, U.S. 2008–2014, *(128)*.

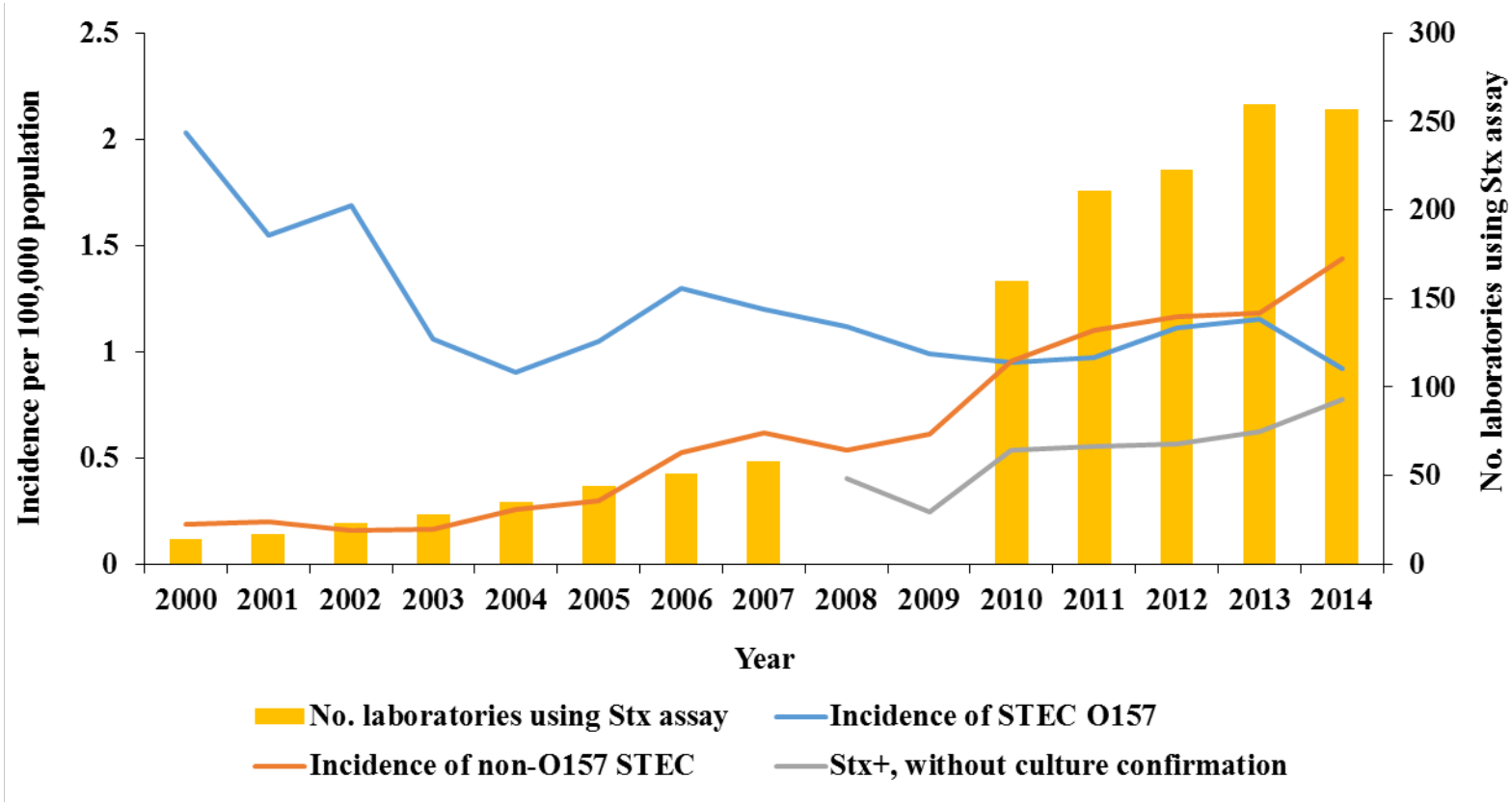

FIGURE 4. Incidence of STEC infections and number of laboratories using Stx assays, by year, Foodborne Diseases Active Surveillance Network, 2000-2014, *(120, 128)*.

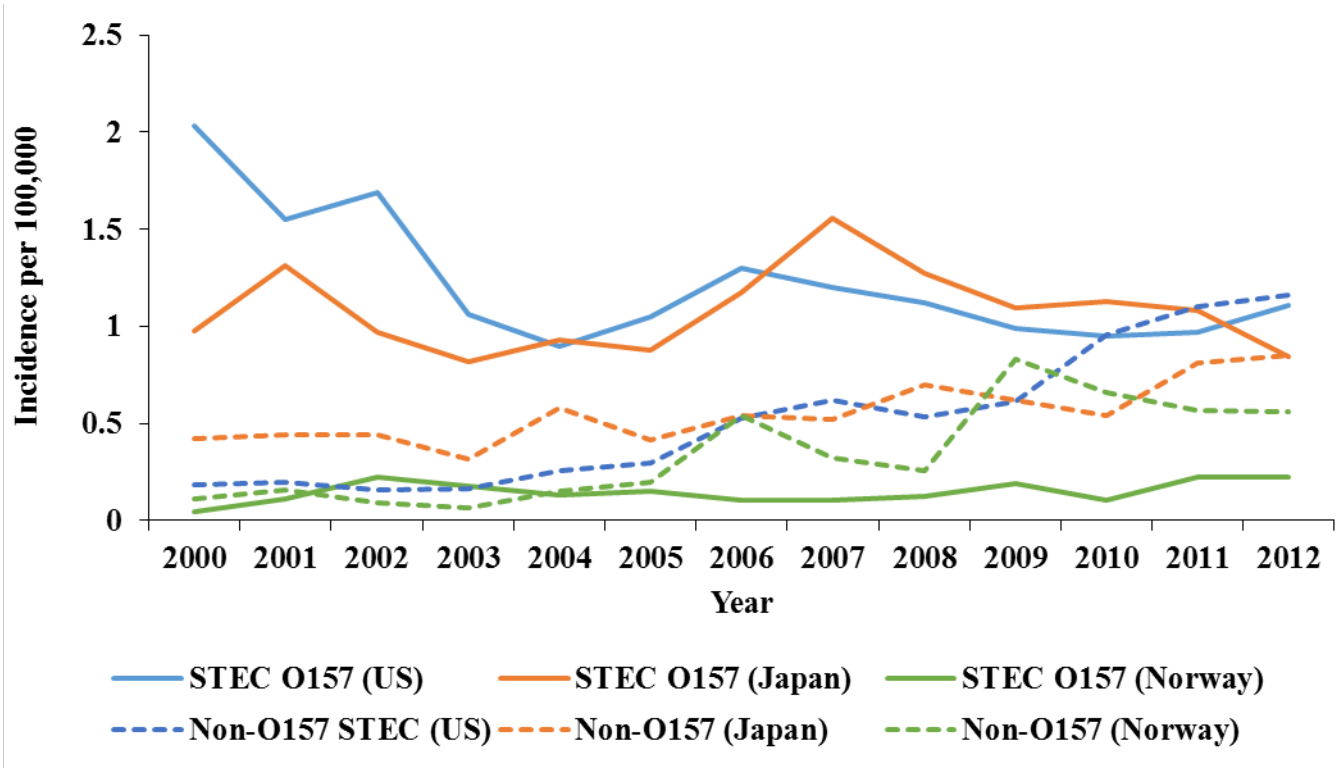

FIGURE 5. Multiyear trendlines of U.S., Norway, and Japan showing O157 and non-O157 STEC incidence, CDC FoodNet Fast (https://wwwn.cdc.gov/foodnetfast), *(29, 214)*.

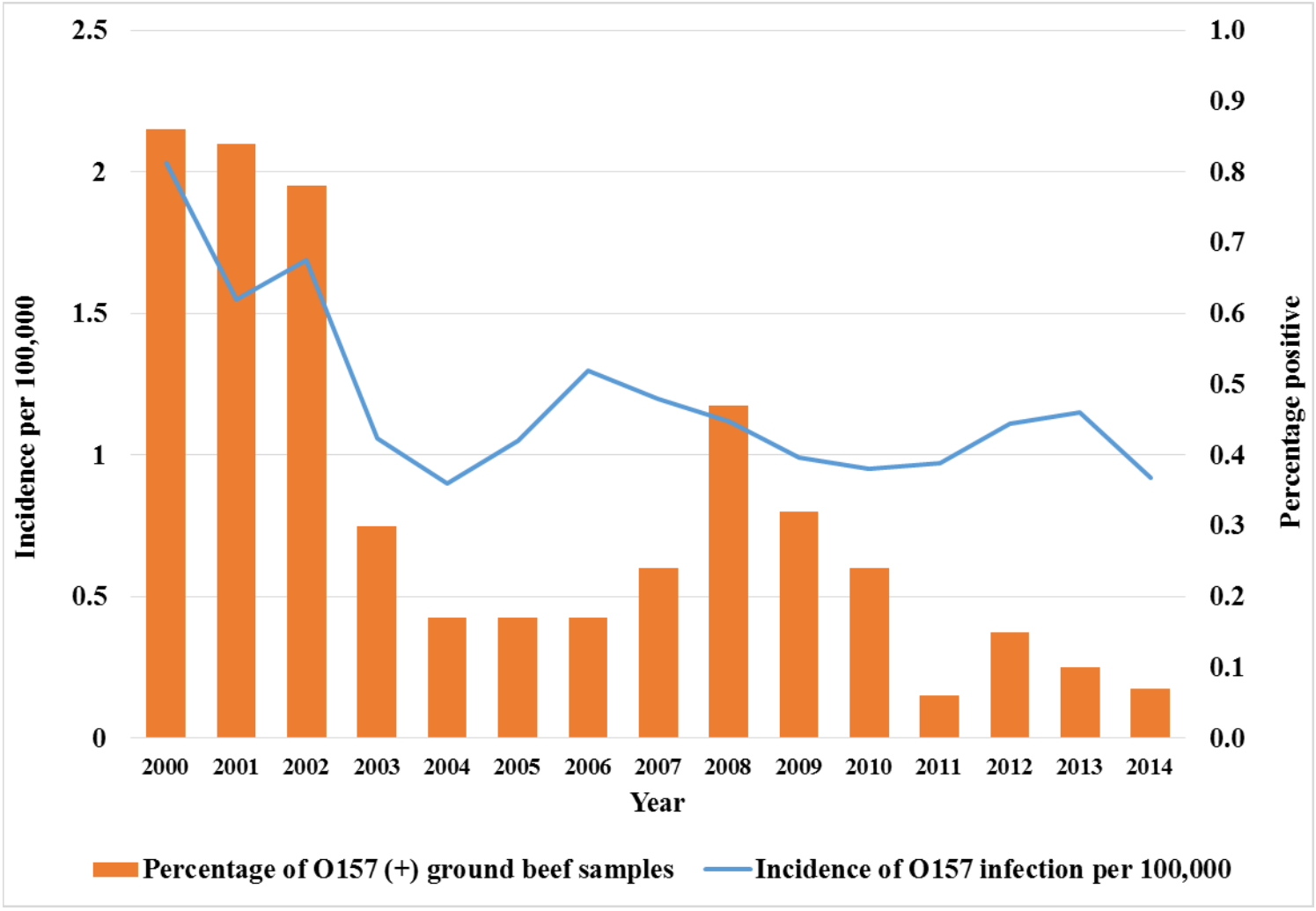
2.5
2
1.5
1
0.5
0
Incidence per 100,000
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Percentage positive
2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014
Year
Percentage of O157 (+) ground beef samples
Incidence of O157 infection per 100,000

FIGURE 6. Trends in overall O157 infection incidence and in contamination of ground beef with *E. coli* O157 in the U.S. Data from 2003-2010: https://www.fsis.usda.gov/wps/portal/fsis/topics/data-collection-and-reports/microbiology/ec/summary-data/ec-summary-data-1994-2010; from 2011-2013: https://www.fsis.usda.gov/wps/portal/fsis/topics/data-collection-and-reports/microbiology/ec/stec-annual-report; from 2014: USDA FSIS unpublished

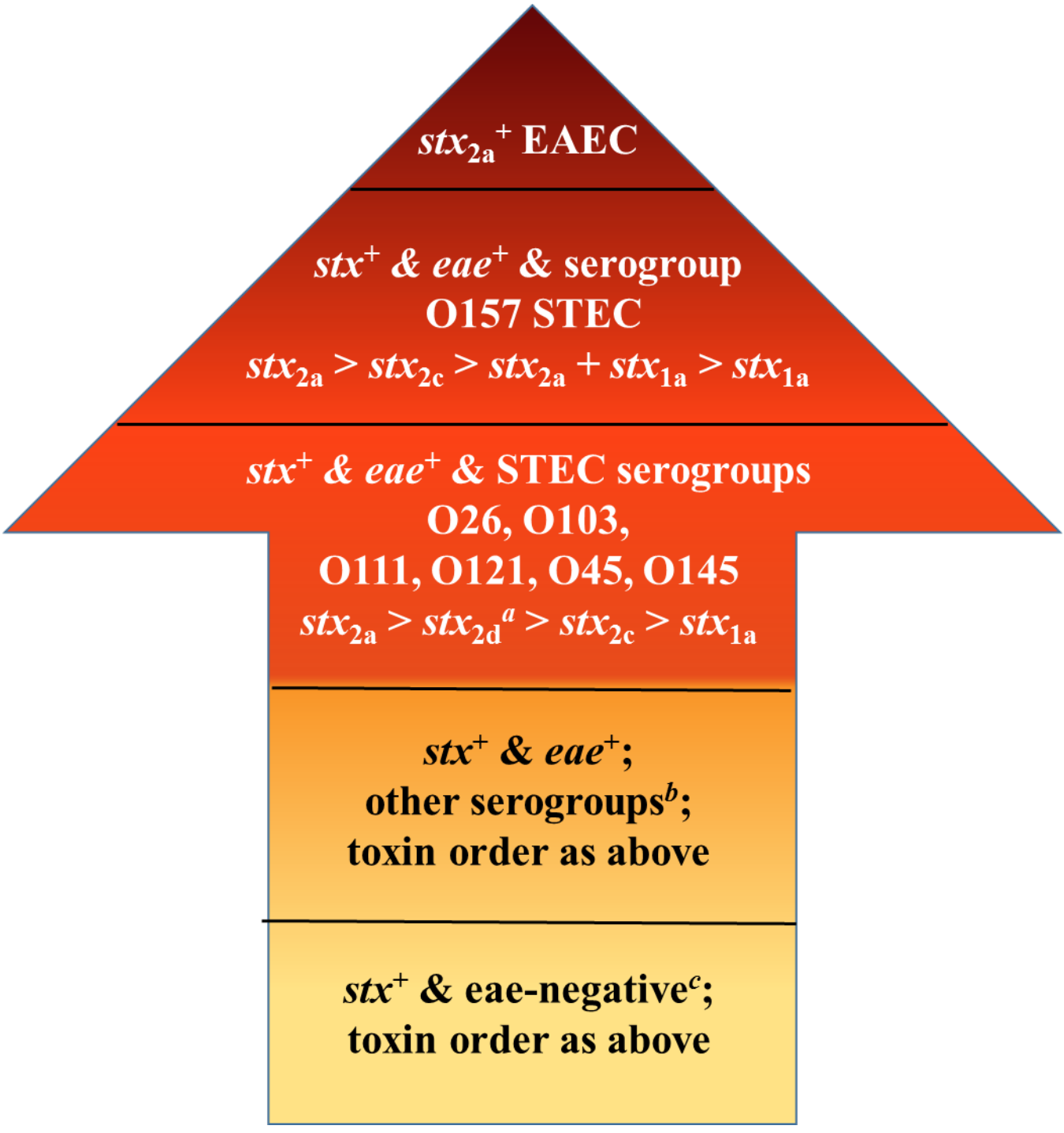
stx_{2a}^{+} EAEC
stx^{+} & eae^{+} & serogroup O157 STEC
$stx_{2a} > stx_{2c} > stx_{2a} + stx_{1a} > stx_{1a}$
stx^{+} & eae^{+} & STEC serogroups O26, O103, O111, O121, O45, O145
$stx_{2a} > stx_{2d}^{a} > stx_{2c} > stx_{1a}$
stx^{+} & eae^{+}; other serogroups[b]; toxin order as above
stx^{+} & eae-negative[c]; toxin order as above

FIGURE 7. Relative risk characteristics of STEC and Stx-producing EAEC. Increasing risk is indicated from bottom to top and from yellow to dark red. So far, only *stx*$_{2a}$ and *stx*$_{2c}$ *(58)* have been found in EAEC; if an EAEC acquired any subtype of *stx*, it should be considered a health risk. [a]Of the serogroups listed, *stx*$_{2d}$ has only been reported in O26. [b]STEC that are *eae*-positive in serogroups other than the big 6 may be as virulent as those in the big 6; however the incidence of these is lower, so relative pathogenicity compared to the big 6 is unknown. [c]other adhesins discussed in text (chapter **2**, **B.1.5**)

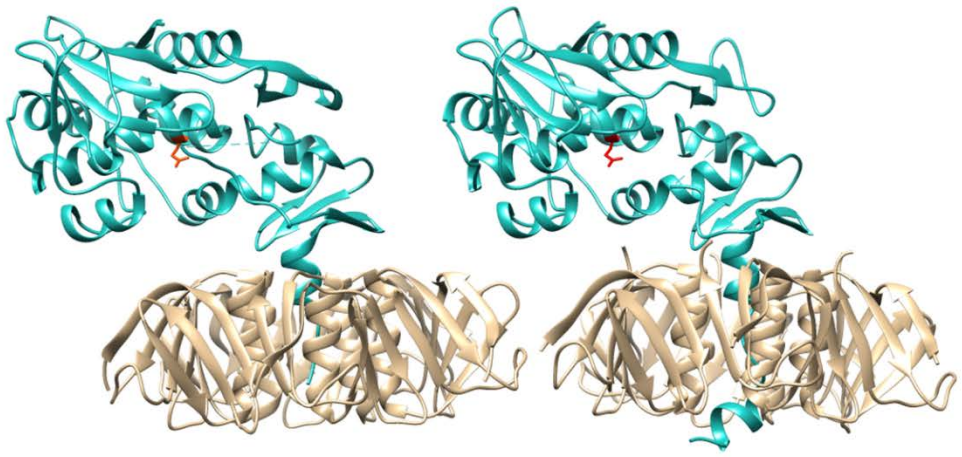

FIGURE 8. The crystal structures of *Shigella dysenteriae* type 1 Stx (left; same sequence as Stx1a) and Stx2a (right) are highly similar. The A subunit is colored blue with the active site glutamic acid colored red. The B pentamer is shaded tan. This figure was generated with the University of San Francisco Chimera package *(244)*.

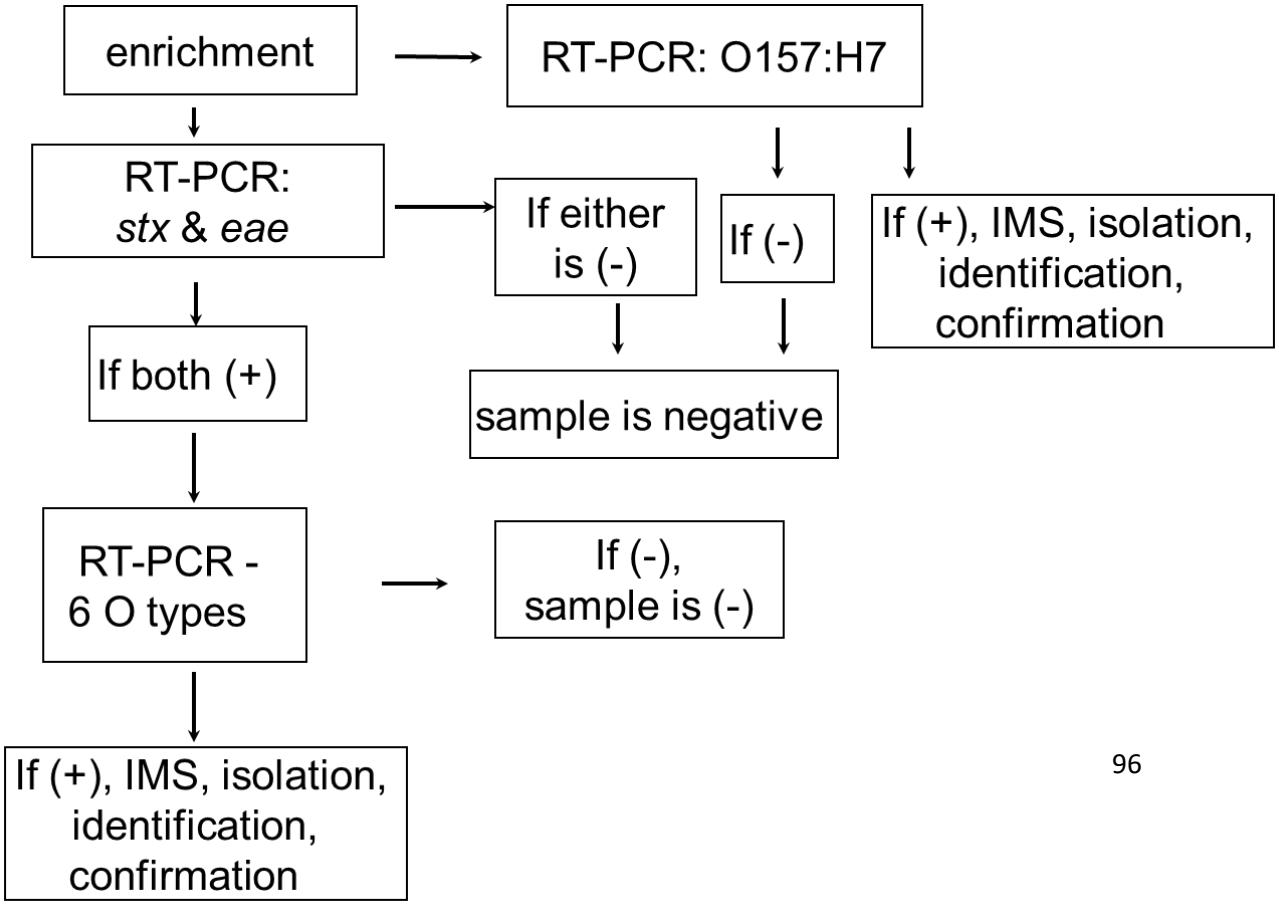

FIGURE 9. The USDA FSIS STEC testing strategy. The USDA FSIS tests enrichment broth by real-time (RT)-PCR for O157:H7 and uses a separate RT-PCR for *stx* and *eae*. If the assay is negative for O157:H7 and either *eae* or *stx* is absent, the samples are considered negative. Samples that are positive O157:H7 are subjected to IMS, isolation and confirmation. Samples positive for both *eae* and *stx* are tested by RT-PCR for the big 6 serogroups. If the sample is positive for one of the big 6 serogroups, it is subjected to IMS, isolation and confirmation.

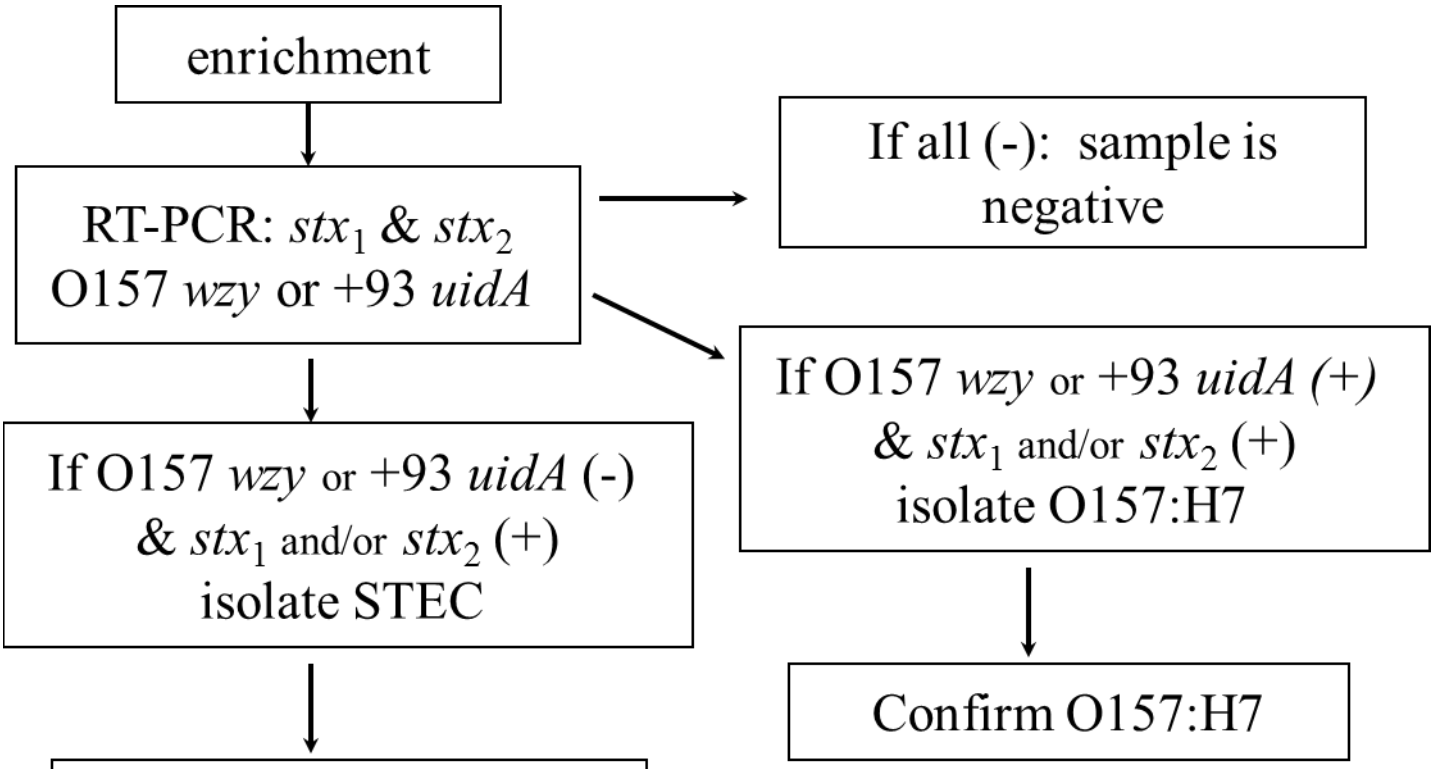

Figure 10. The FDA STEC testing strategy. The FDA tests enrichment cultures for STEC isolates by real time (RT)-PCR for stx_1, stx_2, the SNP in *uidA* specific for many O157 strains, or O157 *wzy*. If the sample is positive for any of those genes, the enrichment is cultured on selective media for isolation. Samples that are negative for O157 are further tested for *aggR*, *eae*, *stx* subtype, and serotype to assess health risk.

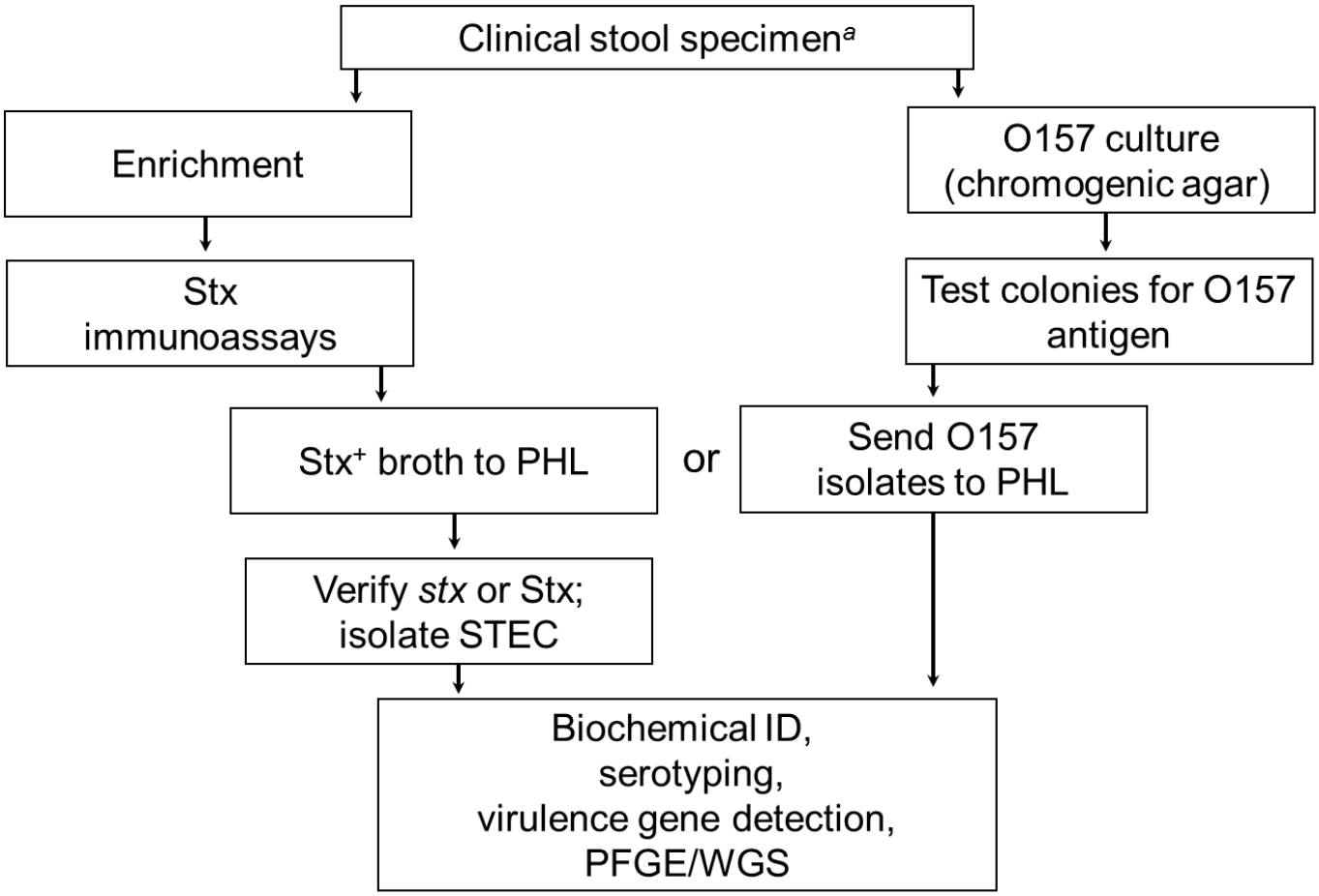

FIGURE 11. STEC testing strategies in the clinical and public health laboratory (PHL).

Clinical stool specimens are simultaneously cultured for O157 and placed in an enrichment broth to detect non-O157 STEC. If O157 is isolated it is sent to the PHL and the broth discarded. If O157 is not isolated and Stx is detected in the broth, the broth is sent to the PHL, where STEC is isolated and further characterized *(6)*. All STEC are currently tested by PFGE and by 2018 will be sequenced, uploaded to NCBI, and analyzed in PulseNet. *Clinical specimens tested with PCR syndromic panels that identify the presence of STEC should be retained and sent to the PHL for culture.

Appendix 1: Case studies of selected major STEC outbreaks (See Table 5 for Stx subtypes and virulence factors)

O157 STEC outbreaks

1. Oregon and Michigan ground beef outbreak O157 (1982)

In the summer of 1982, O157 caused illness in 47 patrons at two retail locations of the same quick-serve restaurant chain. The bacterial strain was first recognized as pathogenic in the course of the investigation. The *E. coli* O157 serogroup had only been isolated previously in the U.S. in one sporadic case of a "bloody diarrhea syndrome" in 1975, but in this outbreak it was isolated from 9 out of the 12 stools collected within four days from onset of illness *(255, 328)*. Grossly bloody diarrhea with little to no fever was highly correlated (Oregon [$p<0.005$] and Michigan [$p= 0.0005$]) with eating burgers at a quick-serve restaurant. No HUS was recognized.

2. West coast outbreak O157 (1993)

The highly publicized O157 outbreak from a national fast-food chain affected patrons in 4 Western States of the U.S. in the summer of 1993, particularly children. It was the largest O157 outbreak at the time with 732 cases, of whom 25% were hospitalized, 7.5% developed HUS, and 4 children died. Illness was associated with eating ground beef *(10, 253)*. The publicity surrounding the outbreak resulted in a large hamburger recall, prompted the decision to make O157 a nationally notifiable disease, and contributed to many subsequent changes in food safety *(54)*.

3. Sakai City, Osaka, Japan outbreak O157 (1996)

In Japan, an outbreak of *E. coli* O157:H7 infections affected school children in one town in 1996. The outbreak affected 47 schools and caused 9,451 illnesses and 12 deaths (the infections of 129 children were culture confirmed). Cases with the same PFGE patterns occurred in two outbreaks in the nearby community *(208, 326).* School and community outbreaks were linked to white radish sprouts from a single producer that were included in the school lunches. At that time, this was the largest STEC outbreak recorded *(97).*

4. Large multistate spinach outbreak O157 (2006)

In 2006, a 27-state outbreak in the U.S. emerged with 191 confirmed O157 cases *(283).* This outbreak offered complete clinical and exposure data, with stx_2 detected in stool samples from all patients, in addition to demographic, clinical, and food exposures. Consumption of a specific brand of bagged spinach that had been contaminated in the fields of Salinas Valley, California, was associated with O157 infection. A high percentage of cases in this outbreak were hospitalized (56%) and progressed to HUS (19%), and 5 died (2%) *(283)*. The risk of infection was not influenced by washing greens before consumption *(107).* Unlike the three preceding outbreaks, in which the O157 strains carried both stx_2 and stx_1 genes, this O157 only carried stx_2, without any detectable stx_1 genes. This toxin gene profile may have accounted for the higher-than-average incidence of HUS associated with this outbreak. This outbreak highlighted the link between STEC and fresh produce, and the importance of preventing pre-harvest produce contamination.

5. *Multistate O157 raw refrigerated, prepackaged cookie dough (2009)*

Ready-to-bake prepackaged cookie dough was the vehicle for a 2009 outbreak of O157 that caused at least 77 infections in 30 states *(219)*. Those with illness ranged in age from 2 to 65 years (median = 15 years of age). Females were more likely than males to become sick (71%), as were people under the age of 19 (66%). Thirty-five were hospitalized (55%) and 10 developed HUS (18%) *(219)*.

Extensive sampling of product, processing plants, and ingredients did not identify the outbreak strain. Based on the epidemiologic evidence, raw cookie dough was determined for the first time to be the vehicle for STEC transmission, raising the awareness of the processed food industry to this food safety hazard. The investigation led to a nationwide recall of 47 products (3.6 million packages in total) and their reformulation, making this brand of cookie dough not only ready-to-bake, but also ready-to-eat *(219)*. While raw cookie dough contains many individual ingredients, flour (a raw agricultural product) was indicated as the most likely contaminant due to it being the only component lacking a kill step, but the link between flour and illness was not definitively made *(219)*.

Non-O157 STEC outbreaks

6. Texas cheerleader camp O111:H8 (1999)

While O157 often receives much attention, outbreaks from other STEC highlight the need for continued surveillance and research on non-O157 STEC. An outbreak of STEC O111:H8 at a 1999 youth camp infected 11% of attendees, two of which developed HUS. Diarrheal illness caused by STEC O111:H8 occurred throughout the event, and was associated

with the salad served at the first meal and later with the ice provided in a large barrel on the last day *(30)*.

7. Multistate romaine lettuce O145:NM (nonmotile) (2010)

In 2010, 31 cases (26 confirmed and five probable) of STEC O145:NM infections were reported from five states, linked to shredded romaine lettuce. The case hospitalization rate was 35% and three developed HUS *(301)*.. This added further impetus to improve produce safety regulations, which became a reality the following year with the FDA Food Safety Modernization Act of 2011. (https://www.fda.gov/Food/GuidanceRegulation/FSMA/default.htm).

8. Sprout-associated outbreak in Germany and France O104:H4 (2011)

In 2011, an extremely serious outbreak linked to fenugreek sprouts erupted in northern Germany *(34)*. Among 3,816 cases reported in Germany, 845 patients (22%) developed HUS, and there were 54 deaths (case fatality rate = 1.4%). Most patients were adults (88%; median age, 42), and women (68%) *(86)*. Illness was linked to eating raw fenugreek sprouts made by a local sprouter using imported seeds. Cases also occurred in other countries among people that visited Germany, and in France, where the same seeds were sprouted and served. The outbreak strain carried stx_{2a} and though it was *eae*-negative, it had genetic features of EAEC which provided an alternate pathway for attachment.

In response to the outbreak, Germany increased the speed of case reporting for HUS and other high priority conditions. As a consequence of the outbreak, the European Commission introduced new requirements, including traceability and source certification for seeds intended for sprouting, approval of sprouting facilities, and microbial criteria for sprouts themselves (Regulations 208-211/2013 of 11 March 2013, published in Official Journal of the EU volume

56 (12 March 2013) at: http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=OJ:L:2013:068:TOC).

9. Japan raw beef O111:H8 and O157 (2011)

In 2011, a deadly Japanese outbreak involving two strains of STEC was reported. The source was a traditional beef dish (yukhoe) served raw at a popular barbecue restaurant chain *(337)*. Among 181 confirmed cases, 34 (19%) developed into HUS, 21 developed acute encephalopathy and five died. Most cases were due to O111:H8, and a small number were caused by O157:H7. Both *E. coli* O111 *stx*$_2$-positive and *stx*-negative strains were detected in the implicated meat. Although the O157 strains had three *stx* gene profiles (*stx*$_1$ and *stx*$_2$ alone and combined), based on PFGE data, researchers believe that these isolates originated from a single clone that lost an *stx*-converting phage during *in vitro* growth *(325)*. As a result of this outbreak, serving raw beef in restaurants has since been banned in Japan.

10. Multistate Mexican-style quick serve restaurant O26:H11 (2015)

At the end of 2015 and early 2016, STEC O26 infections occurred in people eating at many different outlets of a Mexican-style restaurant chain *(42)*. It was reported as two outbreaks caused by different strains of STEC O26, 55 cases from one strain and 5 from the other strain. Among the 60 confirmed cases, 22 (37%) were hospitalized, and none developed HUS or died. Patients ate a variety of foods at the restaurants, and the investigation did not determine the ultimate source of STEC O26. Cross-contamination of foods in these restaurant kitchens was suspected to have played a role *(42)*.

11. Multistate flour outbreak of O121:H19 infections (2016)

In 2016, flour from a manufacturer in Kansas City, Missouri, was the source of an outbreak of STEC O121:H19 infections that resulted in 38 cases and 10 hospitalizations *(43)*. The outbreak was detected by PulseNet using PFGE and trace-back helped to identify the source. A wide range of ages was affected (1-95 years; median = 18), and 78% of cases were females. There were no HUS cases. Illness was highly correlated with cooking with flour, playing with dough, or eating raw dough. Trace back of implicated flour led to one large flour mill, and the outbreak strain was found in the flour. The ultimate source of the contamination has not yet been determined. Together with the 2009 cookie dough outbreak in which flour was suspected as the source, this outbreak highlights the challenge of flour safety, as flour is made from a raw agricultural commodity (wheat) that is not ready-to-eat, and may be contaminated with pathogens. *(43)*.

Appendix 2: List of acronyms, terms, and definitions

Acronym	Term	Definition/explanation or association
A/E lesion	Attach and efface lesion	A lesion at the site of attachment of LEE-expressing *E. coli*, characterized by effacement of the villi.
aggR	Regulator of aggregative adherence in EAEC	A global regulator of virulence-associated traits in EAEC
BAM	Bacteriological Analytical Manual	FDA manual of approved microbiological methods
CDC	Centers for Disease Control and Prevention	The national public health agency of the U. S. with a mission to fight disease.
CGE	Center for Genomic Epidemiology	Technical University of Denmark
CIDTs	Culture independent diagnostic tests	Term for test(s) that do not require an isolate. Done with or without enrichment
CNS	Central nervous system	
CSTE	Council of State and Territorial Epidemiologists	An organization that works with CDC and epidemiologists at the state and local level to influence public health programs and policy.
	Curated genomic databases	WGS databases that link deposited genome sequences to annotations of encoded protein

		and genomic architectures. Additionally, various metadata are linked including epidemiologically and phenotypically significant attributes and publications.
eae	*E. coli* attaching and effacing gene.	Encodes the adhesin intimin found in EPEC and STEC with the LEE locus.
EAEC	Enteroaggregative *E. coli*	
ECID	*E. coli* identification array	A custom DNA microarray developed by the FDA for DNA-based serotyping, virulence profiling, and phylogenetics
EFSA	European Food Safety Authority	
EHEC	Enterohemorrhagic *E. coli*	A subset of STEC that can cause HC and HUS, have a large plasmid, and adhere closely to the mucosal surface of the bowel with subsequent effacement of the microvilli.
EhxA	Enterohemolysin	A hemolysin encoded on the large plasmid of many STEC.
ehxA	Gene encoding EhxA	*ehxA* can serve as a marker for the large plasmid found in many EHEC.
EIA	Enzyme immunoassay	Diagnostic test.

EPEC	Enteropathogenic *E. coli*	*E. coli* that adhere to the enterocytes of the small intestine and form A/E lesions.
ESRD	End-stage renal disease	
EU	European Union	
FAO	Food and Agriculture Organizations	An agency of the United Nations that leads international efforts to defeat hunger
FDA	Food and Drug Administration	Responsible for the safety of FDA-regulated human and animal food products.
FDOSS	Foodborne Disease Outbreak Surveillance System	A CDC surveillance system that provides information about the agents and foods that cause illness and the settings where contamination occurs.
Gb3	globotriaosylceramide	The receptor to which Stx binds.
	GenomeTrakr	A distributed network comprised of state, federal, international, and industry partners that submit whole genome bacterial sequences for foodborne pathogen tracking and analysis.
GUD	β-glucuronidase	
H antigen	Flagella associated antigenic proteins	A determinant of the *E. coli* serotype, H groups and types
HC	Hemorrhagic colitis	Frank blood evident in the diarrheal stools.

HUS	Hemolytic uremic syndrome	Sequela of some STEC infections. Consists of acute renal injury, thrombocytopenia, and hemolytic anemia.
ID_{50}	infectious dose 50%	The dose required to infect 50% of a population.
IFSAC	Interagency Food Safety Analytics Collaboration	A web resource for food safety data collection, analysis, and use with a focus on foodborne illness source attribution.
IMS	Immunomagnetic separation	
	Intimin	A 94- to 97-kDa outer membrane protein (adhesin) produced by all EHEC strains and encoded by the gene *eae.* Intimin is required, but not sufficient, to induce A/E lesions *in vitro* and *in vivo.*
LEE	locus of enterocyte effacement	A large pathogenicity island (section of chromosomal DNA) which carries the genes necessary for the formation of A/E lesions including the *eae* gene.
Lpf	Long polar fimbria (Lpf)-1	One type of fimbria.
	Metagenomic(s)	The study of DNA that is extracted directly from communities in environmental samples representing a complex mixture from

		multiple organisms.
MLG	Microbiology Laboratory Guidebook	USDA FSIS manual of approved methods
NACMCF	National Advisory Committee for the Microbiological Criteria for Foods	An ad hoc advisory committee to several federal government agencies.
NCBI	National Center for Biotechnology	Houses a repository for genomic, genetic and biomedical data; part of the U.S. National Library of Medicine at the National Institutes of Health (NIH)
NMFS	National Marine Fisheries Service	A US federal agency, responsible for the stewardship of national marine resources.
O antigen	Somatic (cell wall) associated antigenic proteins,	A determinant of the *E. coli* serotype, O groups and types
OI-122	Pathogenicity O island 122	Contains four STEC virulence markers
O157:H7/O157	STEC serotype/serogroup	The STEC serotype associated with the most outbreaks in the U.S.
Pathotype		A group of STEC that cause disease
PFGE	Pulsed Field Gel Electrophoresis	A laboratory technique used to genetically characterize bacterial isolates. PFGE patterns for a variety of isolates associated with foodborne disease are stored in the

		PulseNet database.
PHL(s)	Public Health Laboratory/Laboratories	
PR/HACCP	"Pathogen Reduction; Hazard Analysis and Critical Control Point System" – a rule published by the USDA FSIS	HACCP is a food safety management program. HACCP was adopted by the USDA FSIS for the reduction of *E. coli* O157 and STEC contamination in beef
RT-PCR	Real-time PCR	
saa	A gene encoding an adhesin protein	STEC autoagglutinating adhesin
SMAC agar	Sorbitol MacConkey agar	A primary isolation medium for the detection of *E. coli* O157.
SNP(s)	single nucleotide polymorphism(s)	A variation of a single base pair in a DNA sequence.
SOP	Standard Operating Procedure	
SPTs	Seropathotypes	STEC serotypes are grouped into five groups A through E based on predicted risk of severe disease from STEC infection.
STEC	Shiga toxin-producing *E. coli*	Any *E. coli* which has the genetic elements for the production of one or more Shiga toxins.
Stx	Shiga toxin	An AB_5 toxin that kills target cells by

		inhibition of protein synthesis. There are two primary variants of the toxins that are immunologically distinct, Stx1 and Stx2. Stx1a is the prototype toxin for Stx1. Stx2a is the prototype toxin for Stx2. Also known as Vero toxin.
stx	Shiga toxin operon	Encodes for Stx
stx-phage	A lysogenic bacteriophage that carries *stx*	The genes encoding Stxs are carried within the genomes of lysogenic bacteriophages
SubAB	Subtilase cytotoxin	A potent AB_5 toxin produced by some non-O157 STEC. The A subunit is a highly specific subtilase-like serine protease.
subAB	Gene encoding SubAB	
Tir	translocated intimin receptor	The receptor for intimin. Encoded in the LEE locus.
T3SS	Type III secretion system	A complex protein structure used by some pathogens to inject proteins into host cells.
uidA	Gene for β-D-glucuronidase	A SNP in *uidA* is specific for many O157 strains.
USDA FSIS	United States Department of Agriculture Food Safety Inspection Service	Responsible for the food safety of commodities regulated by the USDA including beef

cgMLST	Core genome multilocus sequence typing	A method to perform molecular typing of microorganisms using genome-wide typing of conserved genes in a given species.
WGS	Whole Genome Sequencing	A laboratory technique used to determine the complete DNA sequence of an organism's genome.
WHO	World Health Organization	An agency of the United Nations concerned with international public health.

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