

# Shiga Toxin-Producing *Escherichia coli* (STEC) in Fresh Produce—A Food Safety Dilemma

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**ABSTRACT** Produce contains high levels of mixed microflora, including coliforms and *Escherichia coli*, but occasionally pathogens may also be present. Enterotoxigenic *E. coli* and Shiga toxin-producing *E. coli* (STEC) have been isolated from various produce types, especially spinach. The presence of STEC in produce is easily detected by PCR for the Shiga toxin (Stx) gene, *stx*, but this is insufficient for risk analysis. STEC comprises hundreds of serotypes that include known pathogenic serotypes and strains that do not appear to cause severe illness. Moreover, Stx without a binding factor like intimin (encoded by *eae*) is deemed to be insufficient to cause severe disease. Hence, risk analyses require testing for other virulence or serotype-specific genes. Multiplex PCR enables simultaneous testing of many targets, but, in a mixed flora sample, not all targets detected may be coming from the same cell. The need to isolate and confirm STEC in produce is critical, but it is time- and labor-intensive due to the complexity of the group. Studies showed that only a handful of STEC strains in produce have *eae*, and most belonged to recognized pathogenic serotypes so are of definite health risks. Several *eae*-negative strains belonged to serotypes O113:H21 and O91:H21 that historically have caused severe illness and may also be of concern. Most of the other STEC strains in produce, however, are only partially serotyped or are unremarkable serotypes carrying putative virulence factors, whose role in pathogenesis is uncertain, thus making it difficult to assess the health risks of these STEC strains.

## INTRODUCTION

The worldwide trends for healthier lifestyles to reduce obesity and other complications arising from unhealthy diets have greatly increased the consumption of fresh fruits and vegetables. This increased demand, coupled with ever busier consumer lifestyles, also stimulated the growth of a “convenience” food industry and popularized

the concept of bagged salad vegetables and fruits. It has been estimated that several millions of bags of fresh produce are sold daily in the United States. Bagged produce, also referred to as “fresh cut” or “precut,” is often regarded as ready-to-eat (RTE) and consumed without further intervention steps. However, because produce is predominantly cultivated in soil in open fields, it is susceptible to contamination and can contain high levels of complex microbial populations, occasionally including bacterial pathogens. As a result, increases in fresh produce demand and consumption coupled with changes in production practices have also contributed to increases in incidents of food-borne illness. In the United States, about 0.7% of the infections in the 1970s were attributed to fresh produce, but this increased to 6% in the 1990s (1). Since “fresh cut” products are often mass produced, broadly distributed, and marketed worldwide, a single pathogen contamination event can have broadly impacting consequences, and several large, produce-related outbreaks have occurred in many countries (2, 3). In 2006, a large multistate outbreak in the United States

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that infected more than 200 persons was traced to bagged spinach contaminated with *Escherichia coli* O157:H7 (4). Several months later, another O157:H7 outbreak in a fast-food restaurant chain had initially implicated green onions but appeared to have been due to bagged lettuce. At about the same time, bagged lettuce was implicated in another O157:H7 outbreak that affected three states (5). Likewise, increased consumption of sprouts caused several outbreaks of *Salmonella* sp., *E. coli* O157:H7, and other Shigatoxin-producing *E. coli* (STEC) strains. STEC serotype O26:H11 strains caused an outbreak with alfalfa sprouts and, more recently, with clover sprouts, and the large outbreak of O104:H4 in 2011 in the European Union also implicated the consumption of sprouts (6). These large produce-related outbreak incidents worldwide have greatly raised concerns about the safety of fresh produce and about the microbiological and sanitary quality of fresh produce.

## MICROBIOLOGICAL QUALITY OF FRESH PRODUCE

### Total Microbial Populations

The basic fresh produce production practices have remained essentially unchanged as most produce is still cultivated in soil in a field and irrigated with available sources of water. This constant exposure of produce plants to the environment makes them susceptible to contamination, which can come from many sources, including soil, water, compost, animal wastes, and other environmental sources (<http://www.fda.gov/Food/RecallsOutbreaksEmergencies/Outbreaks/ucm235477.htm>).

As a result, it is not unusual to find high microbial counts on fresh produce in the field. Several studies examined the microbial content of preharvest produce plants in the field and observed that total bacterial counts ranged from  $10^4$  to  $10^7$  CFU/g (7–9). Bagged, “fresh cut” produce undergoes processing and is washed multiple times before and after shredding and before bagging and, therefore, would be expected to have lower microbial contents. However, it was surprising that these finished products can also contain high levels of bacteria. Microbiological surveys from several countries showed that total bacterial counts of bagged produce were similar to levels observed in plants in the field and ranged widely from  $10^3$  to  $10^7$  CFU/g (10). Furthermore, data from various countries showed that it was not unusual to have mean total microbial counts of  $10^7$  CFU/g or higher in both bagged salads and sprouts (11–14). Recent FDA survey studies of bagged produce in the

United States showed similar results and wide ranges, and in some cases, total counts as high as  $10^8$  CFU/g were observed in some bags of spinach (15, 16). Analysis of these microflora populations showed that the most frequently identified bacteria were *Bacillus subtilis*, *Pseudomonas fluorescens*, *Pantoea agglomerans*, and *Sphingomonas paucimobilis* (16).

### Coliform and Generic *E. coli* Populations

Coliform and *E. coli* have been used as indirect indicators of fecal contamination for more than 100 years, but there seems to be little correlation between the presence or the levels of indicator bacteria in food with the presence of pathogens. Still, these indicators continue to be of use in monitoring general sanitary conditions. For instance, some produce industries test their products to establish a baseline indicator level. In subsequent testing, the detection of any large deviations from baseline may be indicative of abnormal lots or processing conditions that warrant investigation.

Coliform and *E. coli* are ubiquitous in the cultivation environments and, therefore, are often found on produce plants. Surveys of produce in the field showed that coliform and generic *E. coli* levels often ranged from 10 to  $10^4$  CFU/g (7–9), and analogous to the findings with total bacterial counts, high levels of these indicator bacteria can persist in bagged finished products as well.

There are no microbial limits for coliforms and *E. coli* for bagged produce in the United States, but guidelines and limits exist in other countries. The Brazilian standard for minimally processed RTE vegetables has a fecal coliform limit of 100 CFU/g (13). The Public Health Laboratory Service of the United Kingdom has established microbiological quality guidelines for RTE foods, which include bagged produce, and has set an *E. coli* limit of <20/g as satisfactory, 20 to <100/g as acceptable, and  $\geq 100$ /g as unsatisfactory (17). Indicator bacteria are often enumerated by using the most probable number (MPN) method, and surveys from various countries showed that, like total counts, coliform levels in bagged salad varied greatly, ranging from undetected (<3.0 MPN/g) to  $10^3$  or  $10^4$  MPN/g (10, 15, 16). Similarly, generic *E. coli* may or may not be present, but, if found, the prevalence rate and the levels also varied greatly (10, 12, 15, 16). For example, surveys of lettuce samples at restaurants and university cafeterias in Spain showed that the prevalence rate of *E. coli* varied from 6.6% to ~26% (10, 18). A more startling finding is the study from Brazil that showed that 73% of the minimally processed vegetable salads examined had exceeded the fecal coliform limit of 100 CFU/g (13).

Likewise, *E. coli* can be prevalent in sprouts and at high levels, as a study from Spain showed that 40% of the sprout samples tested positive and, in several, the counts were  $>10^3$  MPN/g (11). Two large surveys in the United Kingdom tested approximately 3,000 samples of organic and conventional salad vegetables and showed that only 0.5% of the samples exceeded the *E. coli* limit of  $>100$  CFU/g and, therefore, were unsatisfactory (19, 20). Two surveys of bagged lettuce and spinach in the United States showed that *E. coli* was not detected in one study (16), and in the other, 16% of the samples had *E. coli*, but all were at levels of  $<10$  MPN/g (15). So, with a few exceptions where high levels were reported, *E. coli* levels in most bagged produce samples seem to be low and within acceptable limits (17). These findings show that coliforms are too prevalent in produce and their levels are too variable to be able to generate a useful baseline. However, *E. coli* does not appear to be part of normal flora in fresh produce, and because its presence and levels are usually low, it may be feasible to establish an *E. coli* baseline, where spikes in *E. coli* levels detected may be indicative of unsanitary or abnormal processing conditions.

These surveys showed that total bacteria and coliforms are prevalent in produce, and interestingly, some studies showed little differences in counts between organic versus conventional produce (9) or between imported and domestic produce (8). But one observation that is consistent is that the levels of total and coliform counts in produce vary greatly and can be unpredictable. One study showed that even seemingly identical products, of the same brand and “use by” dates and tested on the same day, can have as much as 2 to 3 log differences in counts (15). Such discrepancies have also been noted in RTE produce in other countries (10, 11, 13). Whether these large variations are due to the produce types, the complexity of produce microflora, seasonal or regional variations, or perhaps to inconsistencies or variations in processing parameters remains uncertain.

### PRESENCE OF PATHOGENIC *E. coli* IN FRESH PRODUCE

Considering the huge quantities of fresh produce that are harvested and consumed daily, the frequency of pathogen contamination in produce is very low. In response to increasing concerns about food-borne outbreaks associated with the consumption of fresh produce, the Agricultural Marketing Service of the U.S. Department of Agriculture initiated the Microbiological Data

Program (MDP) in 2001 to conduct surveillance of fresh produce samples collected from wholesale distribution centers across the country. On the average, about 10,000 to 15,000 produce samples of many varieties were tested yearly for the presence of *Salmonella* sp., enterotoxigenic *E. coli* (ETEC), *E. coli* serotype O157:H7, and other STEC strains. MDP analyses showed that many of these pathogens are found in various types of fresh produce, and the program provided one of the largest, publicly available databases on the presence of pathogens in fresh produce. Unfortunately, the program was affected by budget cuts and terminated in 2012. The yearly MDP reports are available at <http://www.ams.usda.gov/AMSv1.0/mdp>.

### Enterotoxigenic *E. coli*

ETEC is commonly known as the causative agent of traveler’s diarrhea; however, it is also an important diarrheal pathogen in infants. The two trait virulence factors of ETEC are the plasmid-encoded, heat-labile (LT) and heat-stable (ST) enterotoxins. The two serologically distinct LT types are designated LT-I and LT-II, but the latter is produced mostly by animal isolates and has not been associated with illness. There are also two distinct ST types; STa is produced by both human and porcine ETEC strains, but STb production is limited mainly to porcine strains (21).

ETEC infections are most often caused by the consumption of contaminated food and water, and so ETEC strains can be found in various foods, including produce and produce used in street-vendor food in Mexico (22). MDP analyses of produce showed that ETEC was isolated almost every year from samples including lettuce, parsley, cilantro, alfalfa sprouts, and spinach. Typically, only a handful of strains are isolated a year, but in 2009, 11 ETEC isolates were found and 4 came from cilantro and spinach samples, respectively. Considering that about 2,200 samples of each were tested yearly, the estimated ETEC prevalence rate in these products is  $\sim 0.18\%$  (23). Most of these ETEC isolates had STb, which is associated with porcine strains, but a few also had STa or LT. Although not surprising to find ETEC in fresh produce, ETEC has a fairly high infectious dose, which, based on volunteer feeding studies, has been estimated to be  $10^8$  to  $10^{10}$  cells. As a result, quantifying the levels of ETEC present in produce would be more useful than the presence or absence data in assessing the health risks of ETEC in fresh produce.

In the MDP produce testing scheme, a multiplex PCR assay was used to simultaneously screen produce samples for the presence of ETEC and STEC (24).

Interestingly, this assay detected some *E. coli* strains that carried both ETEC and STEC virulence factors. Although many of these strains were only partially identified and characterized, two strains from the 2004 MDP analysis were studied in detail. These two *E. coli* strains, found to carry both Stx1 and ST toxin genes, had an untypeable O antigen and H52 flagellar antigen and were isolated from fresh cilantro and cantaloupe, respectively. Analysis by pulsed-field gel electrophoresis showed that the XbaI profiles of both strains were similar and did not resemble those of selected Stx1-producing STEC or ST-producing ETEC strains examined, but they shared >90% XbaI profile similarity to two clinical Ont:H52 strains that had identical phenotypes and traits (24). Furthermore, multilocus sequence typing of all four strains showed that they had sequence type (ST) 274, which did not fall into any of the defined STEC clonal groups and, therefore, appeared to be a unique clone. The Stx1 and ST genes reside on phage and plasmid, respectively, and can be transferred, so it is uncertain if these Ont:H52 strains in produce were ETEC that was infected by the Stx<sub>1</sub> phage or, conversely, a STEC that acquired the ST-encoding plasmid.

### Shiga Toxin-Producing *E. coli*

STEC is characterized by the production of Stx, and there are two main types, designated Stx1 and Stx2. Within each are many subtypes, and currently, there are three known Stx1 (Stx1a, Stx1c, and Stx1d) and seven known Stx2 (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g) subtypes (25). There are estimated to be 300 to 400 known STEC serotypes that can produce any of the Stx or combination of Stx subtypes, but some subtypes seem to be found mostly in environmental or animal strains and have not affected humans (26); thus, not all STEC strains appear to cause illness. STEC has been found in various forms of wildlife (27) and in environmental sources like water and soil and is also common in farm or agricultural areas (<http://www.fda.gov/downloads/Food/FoodSafety/FoodborneIllness/UCM235923.pdf>).

STEC strains can also be found in meats and other foods (27–29), and fresh produce is no exception. During 2004 to 2009, European Union member states conducted a microbiological survey of produce and found that of the ~6,000 samples tested, 11 (0.18%) had STEC (<http://www.efsa.europa.eu/en/supporting/doc/166e.pdf>). Similarly, 10 years of MDP analyses showed that STEC strains were isolated from various types of produce in the United States, especially, spinach, lettuce, and cilantro (<http://www.ams.usda.gov/AMSV1.0/mdp>).

From MDP statistics on the number of STEC isolations per year in relation to the ~2,200 samples of each product tested yearly, it is estimated that STEC was present in 0.5 to 0.6% of the spinach, 0.3 to 0.5% of the cilantro, and 0.04 to 0.18% of the lettuce samples. These estimates, however, may not reflect the overall trend. For instance, the prevalence rate obtained for STEC in lettuce was based on MDP data from the past few years, which had low isolation rates, but there had been higher numbers of STEC isolations from lettuce in other years. These observations suggest that STEC prevalence may vary from year to year and depends on many factors, including, perhaps, regional and seasonal variations.

Spinach was included in the MDP testing scheme in 2008 in response to the 2006 spinach outbreak with *E. coli* O157:H7. Since then, this product has accounted for most of the STEC isolations, and the prevalence rate has remained fairly steady. For example, from 2009 to 2011, 11 to 14 STEC strains were isolated from spinach yearly, giving a prevalence rate of ~0.5 to 0.6%. But in 2012, there were 32 STEC isolations, 21 of which came from spinach (0.95%). This fairly consistent prevalence of STEC strains in spinach suggests that, perhaps, there may be some correlation between STEC and spinach plants or with spinach cultivation practices.

All STEC strains isolated by MDP were serotyped by the *E. coli* Reference Laboratory at Penn State University. However, more than 50% of the isolates could only be partially serotyped, when either the O or the H antigens or both could not be identified. Among those that were serotyped, there were strains from diverse serogroups, most of which were unremarkable, with no history of having caused human illness (Table 1). But there were strains from recognized pathogenic serotypes, including O157:H7, O26:H11, O121:H19, and O113:H21, as well as other serotypes like O165:H25 and O91:H21 (Table 2) (30) that, historically, have been implicated with severe human illness (31, 32).

### STEC—CHALLENGES IN PRODUCE TESTING AND IN RISK ANALYSIS

Testing for the presence of any pathogen in fresh produce is challenging. The heterogeneous distribution of pathogen contamination and the many types and varieties of fresh produce present problems to sampling and sample processing procedures. Furthermore, fresh produce can contain very high levels of microflora, which can mask or overwhelm the sporadic presence of often very low levels of pathogens. To complicate matters further, produce can contain inhibitors that interfere

**TABLE 1** Selected STEC strains and serotypes isolated from various produce commodities<sup>a</sup>

Product	Number	Serotypes <sup>b</sup>
Cantaloupe	3	Ont <sup>c</sup> :H11; Ont:H52; O88:H38
Cilantro	18	Ont:H16, H31, H49; O1:H+, O8:H16, H28; O113:H5; O139:H1; O153:H21; O168:H8; others
Coriander	3	Ont:H7; O2:H25; O119:H4
Hot peppers	3	O8:H9; O24:H11; O180:H14
Lettuce	28	Ont:Hnt, O6:H49; O8:H28; Ont:H2, H8; O136:H16; O143:H34; O163:H19; O168:H-, H8; O181:H49; others
Parsley	1	Ont:H38
Spinach	70	O8:H-, H28; O11:H15; O21:Hnt; O76:H+, O88:Hnt; O98:H36; O107:Hnt; O113:H36; O130:H11; O159:H19; O181:H49; many Ont with various H types
Sprouts (alfalfa)	3	Ont:H28, Hnt; O36:H14
Tomatoes	3	Ont:Hnt
Total	132	

<sup>a</sup>Table modified from reference 30.

<sup>b</sup>Excludes pathogenic serotypes listed in Table 2.

<sup>c</sup>nt, not typeable.

with assays; hence, fresh produce samples often have to be incubated in broth media containing antibiotics or other inhibitory substances to “enrich” for the target bacteria and to suppress the growth of background flora. Typical enrichment steps take 1 to 2 days, so these prerequisite produce sample preparation steps before testing are media-, time-, and labor-intensive.

Once the produce samples are media-enriched, they are often screened by antibody-based or PCR or real-time PCR (qPCR) assays that are specific for particular targets. Since STEC strains are characterized solely by the production of Stx, anti-Stx assays or the use of *stx*-specific PCR or qPCR assays is effective in screening produce enrichments. Although these assays are adequate to determine the presence of STEC in produce, such data alone are inadequate for making risk-assessment decisions.

To determine whether a bacterium poses health risk is difficult as it depends on many variables such as virulence factors, infectious dosage, the pathogenicity of the organism, etc., but it also depends on the health conditions and the susceptibilities of the human host. For instance, even generic *E. coli* strains are deemed as opportunistic pathogens as they can cause infections in immunocompromised hosts and, therefore, cannot be regarded as being “risk free.” Risk assessment for STEC is much more difficult due to the complexity of STEC pathogenesis, plus the group comprises a large diversity

of strains, from several hundred serotypes, that can produce any of the many Stx subtypes or combinations of subtypes. Though some of these subtypes such as Stx1e do not seem to affect humans, and infections by STEC strains with Stx1c tend to range from asymptomatic to mild diarrhea (33), nevertheless, they may only be regarded as low or minimal risk, but not without risk. On the other hand, infections by other STEC strains can cause severe diseases, such as hemorrhagic colitis (HC) that can lead to life-threatening complications like hemolytic-uremic syndrome (HUS). In these instances, the production of Stx alone is deemed to be insufficient, and an adherence factor that enables the pathogen to attach to epithelial cells is also needed for severe disease

**TABLE 2** Serotype and pathotype of selected produce STEC strains<sup>a</sup>

Commodity	Serotype <sup>b</sup>	Pathotypes <sup>c</sup>
Cherry tomato	O8:H19 <sup>d</sup>	<i>stx1, stx2, ehxA, subA/B</i>
Cilantro	O20:H19 <sup>d</sup>	<i>stx1, stx2, saa, ehxA</i>
	<b>O26:H11</b>	<i>stx1, eae, ehxA</i>
	<b>O165:H25</b>	<i>stx1, stx2, ehxA, eae</i>
	Untyped	<i>stx1, eae, ehxA</i>
Lettuce	O2:H27 <sup>e</sup>	<i>stx2, ehxA</i>
	<b>O121:H19</b>	<i>stx2, ehxA, eae</i>
	<b>O157:H7</b>	<i>stx2, eae</i>
	<b>O157:H7</b>	<i>stx2, eae, ehxA</i>
	O163:H19 <sup>d</sup>	<i>stx2, ehxA, subA/B</i>
	<b>O165:H25</b>	<i>stx1, stx2, ehxA, eae</i>
Spinach	O174:H21 <sup>d</sup>	<i>stx2</i>
	<b>O157:H7<sup>f</sup></b>	<i>stx2, eae, ehxA</i>
	O8:H19 <sup>d</sup>	<i>stx2, ehxA</i>
	O8:H19 <sup>d</sup>	<i>stx2, ehxA</i>
	<b>O26:H11</b>	<i>stx1, eae, ehxA</i>
	O82:H8 <sup>e</sup>	<i>stx2, ehxA, saa</i>
	O82:H8 <sup>e</sup>	<i>stx2, ehxA, saa</i>
	<b>O91:H21</b>	<i>stx2, ehxA, saa</i>
	O98:H36 <sup>g</sup>	<i>stx1, eae, ehxA</i>
	<b>O113:H21</b>	<i>stx2, ehxA, saa, subAB</i>
	<b>O113:H21</b>	<i>stx2, ehxA, saa, subAB</i>
	<b>O113:H21</b>	<i>stx2, ehxA, saa, subAB</i>
	O116:H21 <sup>h</sup>	<i>stx2, ehxA, saa, subAB</i>
<b>O157:H7</b>	<i>stx2, eae, ehxA</i>	
<b>O157:H7</b>	<i>stx2, eae, ehxA</i>	
O174:H2 <sup>d</sup>	<i>stx2, ehxA, saa</i>	
O174:H21 <sup>d</sup>	<i>stx1, stx2, ehxA, saa, subAB</i>	

<sup>a</sup>Table modified from reference 30.

<sup>b</sup>Known pathogenic serotypes are shown in bold type.

<sup>c</sup>*stx1*, Shiga toxin 1; *stx2*, Shiga toxin 2; *eae*, intimin; *ehxA*, enterohemolysin; *saa*, STEC agglutinating adhesin; *subAB*, subtilase cytotoxin.

<sup>d</sup>Serotype reported to have history of causing HUS (31).

<sup>e</sup>Serotype reported to have history of causing other illnesses (31).

<sup>f</sup>From the spinach outbreak of 2006. Included as reference.

<sup>g</sup>Serotype has no history of causing illness (31), but has *eae*.

<sup>h</sup>Serotype reported to have history of causing HUS (32).

to occur. Other general information on pathogenic *E. coli* can be found in a consumer-oriented report titled “*E. coli*: Good, Bad & Deadly” published by the American Academy of Microbiology and is available at <http://academy.asm.org/images/stories/documents/EColi.pdf>. The most notable adherence factor among pathogenic STEC is the intimin protein, encoded by the *eae* gene that resides on the locus for enterocyte effacement pathogenicity islands. The presence of *eae* and *stx2* has been found to be a good predictor that the STEC strain may cause HC or HUS (34). Hence, the term enterohemorrhagic *E. coli* has been used by some to designate a subset of STEC that comprises pathogenic strains, and most of these have *eae*. Among EHEC strains, serotype O157:H7 is the prototypic strain, but others in the serogroup O26, O111, O103, O145, to name a few, have also caused severe human illnesses. Also, other EHEC strains, such as strains in the serotype O113:H21, O91:H21, O104:H4, etc., do not have *eae* but have caused HUS (35, 36), so these pathogens appear to have other means of attachment. For example, the O104:H4 strain that caused the large HUS outbreak in the European Union in 2011 did not have *eae*, but it was an enteroaggregative *E. coli* strain, and its ability to attach and aggregate on epithelial cells, coupled with Stx2 production, is postulated to have caused the severity of infections.

The mechanisms of attachment used by other *eae*-negative EHEC strains are less certain. Analysis of the *eae*-negative O113:H21 strain that caused an outbreak of HUS in Australia identified the plasmid-borne *saa* gene that encodes for STEC agglutinating adhesin (Saa) (36). Saa was determined to be an adherence protein as a plasmid-cured, *saa*-negative O113:H21 mutant showed reduced adherence compared to wild type, and purified Saa protein enhanced adherence to HEp-2 cells (37). However, studies that examined the distribution of the *saa* genes found that over half of the STEC strains isolated from healthy cattle were positive for *saa* (38), and although it was also found in some clinical STEC strains, there was no significant correlation between the presence of *saa* and HUS (39).

Among the other factors that are often associated with pathogenic STEC strains are the plasmid-encoded enterohemolysin and subtilase cytotoxin. The *ehxA* gene that encodes for enterohemolysin has been found to be very common among STEC strains isolated from all sources, including clinical, environmental, and foods. The prevalence rate can range from 30% in STEC isolated from wildlife to as many as 70% of the STEC strains isolated from ground meats (40). However, the

prevalence of *ehxA* is not limited to STEC, as analysis of ~300 environmental isolates of generic *E. coli* from surface waters showed that almost all carried and expressed *ehxA* and none were STEC (41). Moreover, the role of enterohemolysin in STEC pathogenesis is uncertain. A study of ~300 clinical STEC isolates showed that 77% had the *ehxA* gene, but its presence could not be correlated with the occurrence of HUS or bloody diarrhea (34). In addition, the sorbitol-fermenting variants of O157:H7 (SFO157) do not express *ehxA* but are highly pathogenic and have caused many outbreaks of HUS in Europe (42). Despite the uncertainty of its role in pathogenesis, the expression of enterohemolysin has become a useful marker in STEC isolation procedures (see below).

The subtilase cytotoxin encoded by the *subAB* gene and produced predominantly by *eae*-negative STEC strains has been determined to be a potent toxin that is even more cytotoxic to Vero cells than Stx (43). The *subAB* gene has been found in ~50% of the STEC strains isolated from ground meats (40). Another study showed that 72% and 86% of the *eae*-negative STEC strains from patients with diarrhea and healthy sheep, respectively, had *subAB* (44), so it is very prevalent among STEC strains. However, other *eae*-negative STEC strains, like O91:H21 and O22:H8, have been implicated in severe illnesses, but ground beef isolates of these serotypes did not have *subAB* (40). So the role of this toxin in the pathogenesis of *eae*-negative STEC strains also remains elusive.

A recent study characterized ~130 STEC strains isolated from a variety of fresh produce samples over a period of about 10 years. The isolates were tested by PCR for the presence of *eae*, *ehxA*, *saa*, and *subAB* genes (30). The study showed that *eae* was present in about 8% of the isolates, and most of these were recognized pathogenic serotypes (Table 2) (30). The other genes were even more prevalent among STEC strains in produce, as ~60% of the STEC strains had *ehxA*, and 35% and 32% of the strains had the *saa* and *subAB* genes, respectively (30). Thus, considering the complexities of STEC pathogenesis, the variety of STEC serotypes that exist, and the wide distribution of various virulence and putative virulence genes among STEC strains in produce, it is clear that much more information beyond the production of Stx or the presence of *stx* is needed for STEC risk assessment.

Fresh produce has a short shelf life, so it is crucial to obtain relevant risk analysis data as quickly as possible. One such testing strategy is to use multiplex PCR assays to simultaneously screen for a combination of virulence

and putative virulence factor genes plus other relevant markers directly in produce enrichments. Others use sequential strategies, where samples positive for virulence factor genes are followed up by other trait-specific assays. The key STEC virulence genes tested by almost everyone worldwide are *stx* and *eae*, and if the samples are positive, they are often tested with assays specific for certain serotypes. While these strategies may seem straightforward, the selection of target serotypes is not. Studies showed that there are regional variations in the STEC serotypes that cause infections (45); hence, the follow-up serotype-specific assays may vary geographically. For example, serotype O157:H7 continues to be regarded as the most important EHEC pathogen, and so it is almost always included in all screening strategies. But other non-O157 STEC strains are increasingly being implicated in food-borne infections worldwide. In the United States, strains from serotypes O26, O111, O121, O103, O145, and O45 have been isolated most frequently from clinical infections, and so these six, commonly referred to as the “big 6,” have emerged as being important and, recently, declared as adulterants in meats. These strains are equally important in fresh produce as strains of O111, O121, and O145 have caused outbreaks in lettuce and O26 strains in alfalfa and clover sprouts. However, focusing on the “big 6” may be insufficient in produce testing, as other known pathogenic STEC serotypes have been isolated from fresh produce (30). Hence, for risk assessment of STEC in produce, it is essential that samples found to be positive for key virulence factors by initial screening be further tested to obtain additional relevant data to determine health risk.

While multiplex assays can generate many data points in one assay, there are drawbacks to using this approach to screen complex, mixed bacterial population samples, as all the gene targets detected may not be within the same bacteria. For example, a survey for *stx*, *eae*, and the “big 6” serotype-specific genes in another flora-complex food like ground meats showed that it was not uncommon to find bacterial strains that carried each of these gene targets independently (40). But when they were all present in the same sample, it gave positive multiplex results for all three targets and, thereby, the misleading impression that a pathogen with those attributes was present in the sample (40). Produce can contain equally complex, mixed microbial populations; hence, it is imperative that samples initially screened and found to be positive are plated onto selective or differential media to isolate the STEC strain and to verify that all the relevant genes targeted are within the same organism.

Without a doubt, isolation and confirmation of STEC strains from produce enrichments are time- and labor-intensive. Fortunately, these steps are actually easier and straightforward for O157:H7 strains due to the unique phenotypes expressed by this pathogen. Unlike typical *E. coli*, O157:H7 strains do not ferment sorbitol or express  $\beta$ -glucuronidase activity, so both traits are used extensively in plating agar media to differentiate O157:H7 colonies from others. Presence of the O157 and the H7 antigens has also eased identification, as simple latex agglutination tests can be used to identify O157:H7 strains quickly (21). An example of a method that uses these traits to identify O157:H7 is outlined in the FDA Bacteriological Analytical Manual (<http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm>).

In contrast, isolation and confirmation of the non-O157 STEC strains are much more complex, laborious, and time-consuming and, undoubtedly, the most problematic step in STEC testing. As mentioned, there are ~300 STEC serotypes that comprise diverse strains and serotypes, and not all appear to affect humans. Those strains that are known pathogens may share common virulence traits but do not uniformly exhibit unique phenotypes that can be used in differentiation and isolation. As a result, there are no methods that can select and isolate all the diverse strains existing within the pathogenic STEC group. Many have used various chromogenic substrates and combinations of phenotypes to develop plating media for differentiation of STEC strains. However, these media can be costly, are not very inclusive, and are useful only for selected serotypes and strains. Also, the high levels and the complex microflora present in produce samples can often mask or mimic pathogenic colonies on these media, thereby interfering with differentiation of STEC colonies. Similarly, to facilitate the isolation of selected serotypes from enrichment broths, specific antibodies have been used in immunomagnetic separation (IMS) methods to capture specific organisms. But the capture efficiency of IMS can vary greatly, depending on the antibodies used and on the type of produce samples being tested. In addition, the antibodies used in IMS often target serogroups rather than serotypes. For example, an anti-O157 IMS method is not specific for O157:H7, as it will capture O157 strains regardless of H type. There are many O157 strains that have H antigens other than H7, and they are not STEC nor are they pathogenic, but can be found in foods. As a result of these limitations and logistical problems, isolating a STEC strain from produce enrichments often entails the

laborious process of picking and pooling numbers of colonies from the plate, screening it for Stx or *stx* by antibody or PCR assays, and repeating the process until a pure STEC strain is isolated for serotyping and virulence testing. This process is not only labor-intensive and costly, it is time-consuming and does not provide timely data for making risk-assessment decisions.

The availability of good differential plating media would greatly simplify the process of STEC isolation from produce enrichments. As mentioned, even though its role in pathogenesis is uncertain, the majority of STEC strains produce enterohemolysin. Strains of O157:H7 almost always carry *ehxA* and express enterohemolysin, as do many of the other pathogenic STEC strains. Enterohemolysin activity can be visualized as a faint zone of hemolysis around the colonies on washed blood agar (WBA) medium, which is made up of tryptose blood agar base supplemented with 10 mM of CaCl<sub>2</sub> and 5% of defibrinated sheep blood (46). The WBA medium was later modified to become the WBMA medium by the addition of 0.5 µg/ml of mitomycin C, which has been shown to induce and enhance the visualization of enterohemolytic activity and also increased the detection rate of STEC strains (47). A similar medium called SHIBAM, which uses heart infusion agar base, is described in the FDA Bacteriological Analytical Manual. Although not all STEC strains express *ehxA*, the use of WBMA or SHIBAM has eased the recognition of STEC strains by the zones of hemolysis around the colonies and, thereby, facilitated the selection and isolation of STEC strains from complex, mixed-flora food enrichments like produce samples.

Once a STEC strain in produce has been isolated, characterized, and serotyped, subjectivity remains in making risk-assessment decisions. To illustrate these uncertainties, one study characterized ~130 STEC strains isolated from produce samples over a 10-year period (30). In some cases, risk-assessment decisions were straightforward. For example, some produce samples had O157:H7 strains, and these, without a doubt, are of serious safety concern. A few atypical O157:H7 strains were also isolated from produce, including strains that did not produce enterohemolysin or Stx. Both factors are encoded by mobile genetic elements that can be lost or transferred. The role of enterohemolysin in pathogenesis is uncertain and the SFO157 strains do not express enterohemolysin but are highly virulent, so an O157:H7 strain that is enterohemolysin negative should not be regarded lightly. The Stx encoding phage can be lost during culture and isolation, so the possibility that a toxigenic strain exists

in the product cannot be ruled out. Hence, in both instances, the presence of these atypical O157:H7 strains should also be regarded as being a health risk. Similarly, if a STEC strain is found to have *eae* and of a known pathogenic serotype, it is also a serious health risk; a handful of STEC strains in produce fit these criteria (Table 2). However, even if the serotype is unknown, a STEC strain that had *eae* should be regarded as a public health concern. Of the ~130 strains in produce tested in that study, 11 strains (~8%) had *eae*, and except for a strain of O98:H36 serotype and another that was untyped, all the other *eae*-bearing strains belonged to well-recognized pathogenic serotypes and, therefore, were of safety concern (Table 2) (30).

There are EHEC strains that do not have *eae* but have caused severe illness, and some of these, like strains of O113:H21 and O91:H21 serotype, have been found by MDP in fresh spinach samples. Characterization and comparison of the O113:H21 strains from spinach to strains that caused infections in Australia showed that although the produce strains had a different sequence type (ST223), they had identical traits and phenotypes as the pathogenic strains (48). A more detailed study used a microarray to analyze 41 pathogenic *E. coli* markers in 65 O113:H21 strains isolated from food, spinach and environmental sources worldwide and, showed that they could not be distinguished from the O113:H21 strains that caused HUS and therefore, are most likely pathogenic as well (49). Produce samples also contained several strains that belonged to serotypes like O2:H27, O82:H8, O116:H21, O174:H21, etc., that have been reported to have been isolated from human infections (31, 32). None of these strains carried *eae*, but many had *ehxA*, *saa*, and *subAB* (Table 2). In spite of the uncertain role of these genes in STEC pathogenesis, the historical association of these serotypes with illness should be considered when making risk-assessment decisions. Excluding the ~30 STEC strains in produce that fit the criteria mentioned and discussed above, risk assessment on the remaining ~100 STEC isolates from produce is more complex and difficult. These strains did not have *eae*, but ~60% had *ehxA*, 35% had *saa*, and 32% had *subAB*. Moreover, almost half of these only had partial O and H type data or could not be serologically typed. Knowing the serotype of the STEC isolates is important, but it is difficult to determine due to the large numbers and the complex combinations of O and H antigen types that can exist among *E. coli* strains. The limitations of using antibodies for serotyping, however, may be remedied in the future with genotypic assays, as a microarray was able to identify the serotype of many

untypeable STEC strains isolated from fresh produce (50). In the meantime, considering the complexities and uncertainties of these putative virulence factors in pathogenesis and the incomplete or lack of serotype identity, it is almost impossible to determine if these STEC strains in produce are of safety concern.

Thus, risk assessment on STEC isolates from produce can be made systematically and with some rationality in some cases, but most assessments tend to be inconclusive due to partial identity or the uncertain role of putative virulence factors detected. Even more critical is the inability to get timely data to make risk-assessment decisions on such short-shelf-life products as fresh produce. Occasionally, the ease of isolation and identification of O157:H7 makes it feasible to obtain data in time to decide to withdraw the product from the market. For most of the other STEC strains, by the time the strain is isolated, characterized, and serotyped, the product has either been consumed or passed its expiration date and has been taken off the market and discarded.

## CONCLUSION

Fresh produce is constantly exposed to the environment and, therefore, can contain complex microflora populations at levels that sometimes exceed  $10^8$  CFU/g. It is also common for produce to contain indicator bacteria, and the levels of coliforms can vary from undetected to as high as  $10^4$  CFU/g. The levels of generic *E. coli* in produce are much lower and, if *E. coli* is present, the level is usually  $<10$  CFU/g, but in occasional samples, it may exceed 100 CFU/g. Pathogenic *E. coli* can be found in produce samples but seems to be more prevalent in spinach where prevalence rates of  $\sim 0.2\%$  and  $0.5\%$  for ETEC and STEC, respectively, have been observed in some years. Screening for STEC in produce is straightforward, but there are no effective isolation methods; hence the confirmation process to isolate, characterize, and serotype the strains is time- and labor-intensive. Produce contains many different serotypes of STEC and many strains that could only be partially serotyped or are untyped. Only a handful of the STEC strains isolated from fresh produce carried the *eae* gene that encodes for the adherence protein, intimin, and can be regarded as a health risk. They included O157:H7 strains, a few “big 6” serotype strains like O121:H19 and O26:H11, but also included other pathogenic serotypes such as O165:H25. The majority of the STEC isolates from produce did not have *eae*, but some of them were of serotype O113:H21 and O91:H21 strains, which historically have been implicated with severe illness and,

therefore, may also be a safety concern. Risk assessment for the other STEC strains in produce is more difficult as many of these strains carried putative virulence factor genes that had uncertain roles in pathogenesis, only had partial serological identity, or belonged to serotypes that had no history of causing human illness. Thus, the logistical problems associated with testing for a complex group of bacteria like STEC in microflora-complex, short-shelf-life products like fresh produce have greatly limited the ability to obtain timely and relevant data for making risk-assessment decisions.

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