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General Interest

Hazard Identification and Characterization: Criteria for Categorizing Shiga Toxin–Producing *Escherichia coli* on a Risk Basis[†]

FAO/WHO STEC EXPERT GROUP

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ABSTRACT

Shiga toxin–producing *Escherichia coli* (STEC) comprise a large, highly diverse group of strains. Since the emergence of STEC serotype O157:H7 as an important foodborne pathogen, serotype data have been used for identifying STEC strains, and this use continued as other serotypes were implicated in human infections. An estimated 470 STEC serotypes have been identified, which can produce one or more of the 12 known Shiga toxin (Stx) subtypes. The number of STEC serotypes that cause human illness varies but is probably higher than 100. However, many STEC virulence genes are mobile and can be lost or transferred to other bacteria; therefore, STEC strains that have the same serotype may not carry the same virulence genes or pose the same risk. Although serotype information is useful in outbreak investigations and surveillance studies, it is not a reliable means of assessing the human health risk posed by a particular STEC serotype. To contribute to the development of a set of criteria that would more reliably support hazard identification, this review considered each of the factors contributing to a negative human health outcome: mild diarrhea, bloody diarrhea, and hemolytic uremic syndrome (HUS). STEC pathogenesis involves entry into the human gut (often via ingestion), attachment to the intestinal epithelial cells, and elaboration of Stx. Production of Stx, which disrupts normal cellular functions and causes cell damage, alone without adherence of bacterial cells to gut epithelial cells is insufficient to cause severe illness. The principal adherence factor in STEC is the intimin protein coded by the *eae* gene. The aggregative adherence fimbriae adhesins regulated by the *aggR* gene of enteroaggregative *E. coli* strains are also effective adherence factors. The *stx_{2a}* gene is most often present in locus of enterocyte effacement (*eae*)–positive STEC strains and has consistently been associated with HUS. The *stx_{2a}* gene has also been found in *eae*-negative, *aggR*-positive STEC that have caused HUS. HUS cases where other *stx* gene subtypes were identified indicate that other factors such as host susceptibility and the genetic cocktail of virulence genes in individual isolates may affect their association with severe diseases.

Key words: Characterization; Risk criteria; Shiga toxin–producing *Escherichia coli*

Shiga toxin–producing *Escherichia coli* (STEC) is a large, complex group of *E. coli* strains that vary greatly in phenotypic, serologic, and genotypic characteristics. STEC pathogenesis is highly complex, requiring multiple virulence factors to cause severe diseases. Some of these virulence factors have many subtypes or alleles, not all of which seem to affect humans. Many of these STEC proven and putative virulence factors reside on mobile genetic elements and can be lost or transferred. As a result, strains of the same serotype may have different virulence genes and pose different health risks. The Food and Agriculture Organization of the United Nations (FAO) and the World Health

Organization (WHO) were asked to provide advice to the Codex Committee on Food Hygiene on a number of aspects related management of STEC in the food chain, one of which was the identification of the hazard. A Joint FAO/WHO Expert Meeting on Microbiological Risk Assessment (JEMRA) Group was convened to address this issue. The JEMRA Group decided that a set of criteria and/or a decision tree (<http://www.fao.org/3/ca0032en/CA0032EN.pdf>) based on current knowledge of factors known to be required in STEC pathogenesis and of phenotypes historically linked with disease should be developed to provide a harmonized risk-based approach for characterization of STEC isolated from a food or along the food chain. A database of strains and serotypes could be developed to facilitate application of the decision tree. For example, the database could include information on strains that have certain patterns when assessed against the criteria used in the decision tree and historically linked in different regions with various levels of health risk from severe to minimal or when no known risk has been reported. This characterization,

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together with other factors such as knowledge of the intrinsic nature of the food, further handling that may affect survival, food preparation practices before consumption, and whether the food is to be provided to known high-risk consumer groups, could be used for determining the potential human health risk posed by an STEC strain found in the food chain.

Pathogenicity of STEC is complex, but in general infection entails three features: ingestion of a contaminated food or other vehicle, colonization of intestinal epithelial cells by STEC, and production of Shiga toxins (Stx), which disrupt normal cellular functions and cause cell damage. The evidence suggests that production of Stx alone without adherence of bacterial cells to gut epithelial cells is insufficient to cause severe illness. STEC infection can be asymptomatic. Most people who come to medical attention have diarrhea, which is often bloody and can be hemorrhagic (hence the term enterohemorrhagic *E. coli*). Hemolytic uremic syndrome (HUS) is the most important complication (58), and some patients with HUS develop chronic renal failure. People with STEC infection with or without HUS can die. This risk-based discussion focuses on mild diarrhea, bloody diarrhea (BD), and HUS.

ADHERENCE FACTORS

The majority of STEC known to cause BD or HUS have virulence factors that enable attachment to intestinal epithelial cells, so adherence factors are generally considered essential for severe illness and perhaps even for non-BD. The main adherence factor in STEC is the intimin protein coded by the *eae* gene that resides on the locus of enterocyte effacement (LEE) pathogenicity island. Intimin is also a virulence factor of enteropathogenic *E. coli* (EPEC) and is crucial in the attaching-effacing lesion that has been associated with EPEC and LEE-positive STEC strains (78). The *eae* gene is highly polymorphic, with over 34 genetic variants (alleles) (72, 89), which are designated by Greek letters. For example, *E. coli* O157:H7 carries γ (gamma) *eae*, serotype O26:H11 often has β (beta) *eae*, and serotype O121:H19 has ϵ (epsilon) *eae*. The presence of both *eae* and the Stx gene *stx₂* is a reliable predictor that the STEC strain may cause BD or HUS (40).

LEE-negative (i.e., *eae*-negative) STEC has been implicated as a cause of severe disease (112). For example, an STEC O113:H21 strain was first isolated from a child with HUS in 1983 (83), and this serotype later caused a cluster of HUS cases in Australia (122). STEC O91:H21 strains that are also LEE negative have been implicated in HUS cases in Germany (99, 100). LEE-negative STEC strains probably have other means or mechanisms for adherence (36). The O113:H21 strains have the STEC agglutinating adhesin (121). The *sab* gene that codes for an outer membrane autotransporter protein that enhances biofilm formation (70) is also thought to be an adherence factor. Evaluations of other STEC strains have revealed the presence of *paa*, *efal*, *ompA*, *lpfA*, and other genes that code for adhesins (78). The plasmid-borne *toxB* gene also codes for an adhesin and is found in O157:H7 and many LEE-positive STEC strains, including strains of the O26, O121, and O145 serogroups, and in EPEC strains (154). The *toxB*-encoded adhesin is thought to contribute to the adherence

properties of the O157:H7 serotype. However, like other adhesins, the role of the *toxB* adhesin in STEC virulence has not been fully determined, so this adhesin is often regarded as a putative virulence factor, and its prevalence differs among STEC strains (42). In a recent study, an 86-kb mosaic pathogenicity island (PAI) named the locus of adhesion and autoaggregation (LAA) was reported, which is composed of four modules of 80 genes, including those that code for novel and known virulence factors associated with adherence and autoaggregation (104). Phylogenomic analysis revealed that LAA appears to be exclusively present in a subset of emerging LEE-negative STEC strains, including strains from hemorrhagic colitis and HUS cases. LAA acquisition may be a recent evolutionary event, which may have contributed to the emergence of these STEC strains (104).

By far the most compelling evidence that adherence is critical to severe disease outcome is the enteroaggregative *E. coli* (EAEC) O104:H4 strain, which caused the large HUS outbreak in Germany in 2011 (48). EAEC strains do not have *eae* but have the aggregative adherence fimbriae (AAF) adhesins regulated by the *aggR* gene. The ability of O104:H4 strains to aggregate on epithelial cells coupled with Stx₂ production resulted in a remarkably high HUS rate of 22% (20). This outbreak indicated that an adherence factor other than *eae*, in combination with *stx_{2a}*, can produce severe disease (8). Some public health agencies are now testing STEC for both *eae* and *aggR* to detect EAEC strains that have acquired the ability to produce Stx. Because the *aggR* genes reside on plasmids that can be lost after disease is produced, chromosomal markers such as the *aaiC* gene have also been used to identify EAEC strains (38).

Key Points on Adherence Factors

1. Adherence factors are critical factors for STEC pathogenicity.
2. The principal adherence factor in STEC is the intimin protein coded by the *eae* gene.
3. The AAF adhesins regulated by the *aggR* gene of EAEC are also effective adherence factors.
4. Other putative STEC adherence factors include those coded by genes *saa*, *sab*, *paa*, *efal*, *ompA*, *lpfA*, *toxB*, and the LAA PAI.

STX TYPES AND SUBTYPES

STEC are characterized by the production of Stx, of which two main types have been described, Stx1 and Stx2, with three Stx1 (Stx1a, Stx1c, and Stx1d) and seven Stx2 (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g) subtypes (137). A novel subtype of Stx1, Stx1e (GenBank accession no. KF926684), which has limited reactivity with anti-Stx1 antibodies, has been found in *Enterobacter cloacae* (128). Provisional designations also have been proposed (88) for two new Stx2 subtypes, encoded by genes *stx_{2h}* (GenBank AM904726) and *stx_{2i}* (GenBank FN252457), but the proposed sequence of *stx_{2h}* was found to be identical to the already published variant *stx_{2e}*-O8-FHI-1106-1092 (137). STEC strains can produce any of the Stx or a combination of Stx subtypes, but not all subtypes have

been implicated in severe illness (71, 96). For example, among the Stx1 group, little is known about the clinical significance of the Stx1d subtype. Stx1c is the most common subtype in strains isolated from sheep, wild deer, and wildlife meats (24, 71, 106); these strains often do not produce intimin and tend to cause asymptomatic infection or mild diarrhea (51). The Stx1a subtype is often produced by LEE-positive STEC strains that have caused severe infections, including serotypes O157:H7, O26:H11, and O111:H8. Brooks et al. (25) found that 83% of O26, 50% of O111, and 100% of O103 strains that caused BD in the United States carried *stx*₁ and *eae*, but of these, only one O111 strain was implicated in HUS. Consistent with those observations, O103:H2 is the second most common STEC causing infection in Norway but is not associated with HUS (110). These three O groups have been declared as adulterants in raw nonintact beef and intact beef products intended for nonintact use in the United States (156). Some STEC serotypes with *stx*_{1a} and *eae* are found in foods (45) but have not been implicated in human infections, suggesting that not all STEC that produce Stx1a and have *eae* pose the same health risk. STEC with *stx*_{1c}, either alone or together with *stx*_{2b}, is often isolated from wild ruminants. Most of these strains are *eae* negative (71), so their presence in humans has not received much attention. However, in some studies 10 to 15% of clinical samples from patients with diarrheal illnesses have been positive for *stx*_{1c} and/or *stx*_{2b} (22, 26, 32, 47).

Stx2 is more important than Stx1 in the development of HUS (35). Among the Stx2 group, the subtypes most often associated with severe disease are Stx2a, Stx2c, and Stx2d (50, 124). Some researchers have suggested that other subtypes may also cause severe infections. Some Stx2 subtypes share high gene sequence similarities and have probably been misidentified in some reports. The nomenclature for Stx subtypes is continually being refined. Increased use of whole genome sequencing should help to clarify the associations of Stx subtypes with severe diseases. Whole genome sequencing results have also indicated that different *stx* subtypes are associated with different virulence profiles. In a study from The Netherlands, the genes *ehxA* and *ureC* were significantly associated with HUS-associated STEC strains but not with the presence of *eae* (49), suggesting that these genes could be important pathogenicity markers next to *eae* and *stx*_{2a}.

The Stx2b subtype was proposed to designate a strain subtype with a variant of the *stx*_{2c} gene but that did not cause HUS (124). Analysis of STEC in Europe revealed that *stx*_{2b} alone or together with *stx*_{1c} is common in STEC from deer droppings and wildlife populations (71, 106) but does not appear to cause severe human illness (21, 27, 32, 47). The Stx2e subtype is mostly found in isolates from pigs and pork meat (9) and is commonly associated with pig edema disease (7). STEC with *stx*_{2e} have been isolated from fresh produce (45) and rarely from humans; in one study the frequency of isolation of STEC with *stx*_{2e} was similar among people with and without diarrhea (50). In another study, isolation of Stx2e-producing STEC was not correlated with diarrheal illness (7), suggesting that Stx2e-producing strains are generally not pathogenic for humans. However, Fasel et

al. (41) reported the isolation of an STEC O51:H49 strain with *stx*_{2e} and *eae* from a 65-year-old patient with HUS in Switzerland, and in another study, *stx*_{2e} was found in STEC O9abH⁻ and O101:H⁻ strains isolated from an HUS patient (151).

The Stx2f subtype has a genetic sequence that is very distinct from that of the other Stx2 subtypes, and the designation Stx2f was first applied to STEC strains isolated from pigeons (139), although this subtype was first reported as Shiga-like toxin IIva from an STEC strain isolated from an infant with diarrhea (56). Analyses of STEC isolates from the wild, from bovine farm environments, and from humans have seldom found Stx2f (50, 71, 103). Some studies suggested that an STEC strain that produce Stx2f can cause mild diarrhea or can be asymptomatic (54, 126), but it appears to be rare (71, 124). However, in a recent study an STEC O8:H19 strain that carried both *stx*_{2f} and *eae* was isolated from an HUS patient in The Netherlands (53), and others have also reported isolating Stx2f-producing STEC strains from HUS patients (63). Additional information is needed to understand the association between Stx2f and severe illness.

STEC with the Stx2g subtype was first isolated from bacteriophages in water with fecal contamination (57). The Stx2g subtype was also found in 8.4% of the STEC strains isolated from farm environments (103) and in some STEC strains isolated from foods (9). STEC strains with *stx*_{2g} have rarely been isolated from human samples (9). Such a strain was isolated from German patients with diarrhea, fever, and abdominal pain but has not been implicated in severe diseases (125).

Several researchers have indicated that subtypes Stx2a or Stx2d are significantly associated with the risk of BD, HUS, or both (22, 26, 40, 94, 99, 124). These subtypes were at least 25 times more potent than Stx2b and Stx2c in cytotoxicity assays using primary human renal proximal tubule epithelial cells and Vero cells (55). In mice, the potencies of Stx2b and Stx2c are similar to those of Stx1, whereas Stx2a and Stx2d were 40 to 400 times more potent than Stx1 (55).

In STEC O157:H7, four major and two minor subtypes of *stx*₂-encoding bacteriophages have been studied to determine the production of Stx2a (115). One of the two bacteriophage subclades in clade 8, a hypervirulent lineage of serotype O157:H7, confers the highest Stx2a production in the host strain (115). Striking phage-related variability in toxin production has been observed in clinical isolates of O157 as well as in other O groups (O83, O111, and O145). The genotype of the bacteriophage and host strain factors are relevant to STEC pathogenesis (158) as was recently demonstrated in a whole genome sequencing comparison of Stx2f-producing STEC strains, some of which were isolated from patients with HUS (63). In that study, only the three strains isolated from HUS patients had the EPEC-associated *efal* gene, which resides on the PAI OI122, the STEC plasmid genes *ehxA*, *espP*, and *katP*, and intimin type ξ (ξ) or β . The *stx*_{2f} STEC strains isolated from patients with diarrhea but without HUS and the strains isolated from pigeons lacked these genes (63). Although some of these genes, such as *ehxA* that codes for enterohemolysin, are

prevalent in STEC strains that have caused severe infections, their role in STEC pathogenesis remains undetermined. Nevertheless, this example suggests that the genotype of the host strain can have an effect on disease outcomes.

The Stx2d subtype has been suggested as an indicator for severe clinical outcomes such as BD or HUS (12). This subtype used to be known as *stx*_{2d} activatable because it was activated by elastase in mucus to become 10- to 1,000-fold more cytotoxic (102). In a French outbreak caused by a hybrid STEC–extraintestinal pathogenic *E. coli* (ExPEC) strain of serotype O80:H2, *stx*_{2d} in combination with other *stx* subtypes was found in 69% of the 52 strains isolated from HUS patients. Among the isolates, 62% had *stx*_{2c/2d}, 7% had *stx*_{2a/2d}, and 31% had unique variants of *stx*_{2a} (22%) or *stx*_{2d} (9%). All 52 strains had the intimin variant *eae*-ξ, and 87% carried the *ehxA* gene (95). All 52 O80:H2 strains examined also shared the four genes (*sitA*, *cia*, *hlyF*, and *ompT*) that are characteristic of the ExPEC pS88 plasmid and other ExPEC traits, 98% carried the *iss* and *iroN* genes, 96% had the *cvaA* gene, and 61% had the *iucC* and *etsC* genes (142). In a study in Spain, 236 STEC strains isolated from patients with HUS, diarrhea, or both were evaluated. Of these, 193 were *eae* positive, 43 were *eae* negative, and 7 (3%) carried *stx*_{2d} (135). Further analysis revealed that six of the *stx*_{2d}-bearing strains were *eae*-negative STEC of serotypes O73:H18, O91:H21, O148:H8, O181:H49, and ONT:H21 and one was an O157:H7 strain that was also positive for *stx*_{2c} and *eae*. In a study of 32 isolates of O26:H11 sequence type 29 recovered from patients with HUS between 2010 and 2013 in France, 7 isolates were positive for *stx*_{2d}, *eae*-β, and SP_26_E (using a CRISPR-based assay) but lacked any of the usual plasmid genes associated with O26 strains (33). Although these findings indicate that Stx2d causes severe infections, not all STEC strains with *stx*_{2d} may cause severe disease. For example, nine patients in Norway infected with an *stx*_{2d}-positive STEC strain did not develop HUS (22). In an outbreak of gastroenteritis in Japan, both *Escherichia albertii* and STEC O183:H18 that were *stx*_{2d} positive were isolated, but none of the 44 patients examined developed BD or HUS (21, 118). In a large study of 626 STEC infections in Germany, none of the 268 HUS patients were infected with an STEC strain that was positive for *stx*_{2d} (50). At least 18 genetic variants of the *stx*_{2d} subtype have been identified, and eight of the strains tested were variable in being activatable by elastase (137), which may account for the variability in clinical outcomes associated with Stx2d strains.

Because of sequence similarities, genes *stx*_{2a}, *stx*_{2c}, and *stx*_{2d} can be quite difficult to discern and identify (137). The *stx*_{2c}-positive strains had been thought to cause severe disease and HUS (37, 50, 124), but recent information has doubts about this assumption. For example, the European Food Safety Authority (EFSA) Panel on Biological Hazards (37) stated that O111 strains isolated from HUS patients (162) were *stx*_{2c} positive. However, the alignment of the two sequenced strains revealed 100% homology with the *stx*₂ sequences found in O157:H7 strain EDL933, which is known to have *stx*_{2a} but not *stx*_{2c} (137). Persson et al. (124) examined 20 STEC strains isolated from HUS patients and found only 1 strain that had *stx*_{2c} alone. That strain has since

been sequenced (unpublished data) and identified as belonging to clade 8 of O157:H7, which is known to have *stx*_{2a} but not *stx*_{2c} (115). Friedrich et al. (50) did not find a significant difference in the prevalence of *stx*_{2c} between STEC isolates from patients with HUS versus those from patients with diarrhea ($P = 0.49$) nor between isolates from patients with HUS versus those from asymptomatic patients ($P = 0.74$). Additional data obtained with discriminating molecular subtyping methods may clarify whether *stx*_{2c} is strongly associated with severe disease.

Large genotypic differences in *stx* phage have also been observed in LEE-negative STEC strains (146). The virulence potential of STEC is quite likely determined by a combination of factors, including bacteriophage clade, *stx* subtype, and genotype of the bacterial host. Consistent with those assumptions, identification of the specific *stx* subtype, the bacteriophage mechanisms that control Stx production, and selected virulence genes carried by a particular STEC strain would be useful for assessing health risks, especially considering that not all Stx subtypes appear to affect humans and that some subtypes are more often associated with severe illnesses than are others.

Key Points on Stx

1. Twelve subtypes of Stx have thus far been identified: Stx1a, Stx1c, Stx1d, Stx1e, and Stx2 subtypes Stx2a to Stx2g, and Stx2i, encoded by genes *stx*_{1a}, *stx*_{1c}, *stx*_{1d}, *stx*_{1e}, *stx*_{2a} to *stx*_{2g}, and *stx*_{2i}, respectively.
2. The gene *stx*_{2a} is often found in LEE (*eae*)-positive STEC and has consistently been associated with HUS.
3. The gene *stx*_{2a} has also been found in *eae*-negative, *aggR*-positive STEC that have caused HUS.
4. The gene *stx*_{2d} in LEE-negative strains has to a lesser degree been reported from cases of HUS, but not all STEC strains with *stx*_{2d} may cause severe disease.
5. HUS cases associated with other Stx subtypes have been identified, indicating that other factors such as host susceptibility, the genetic cocktail of virulence genes in individual isolates, and other (bacteriophage related) factors may affect the association of other Stx subtypes with severe disease.

SEROTYPES AND REGIONAL DIVERSITY

E. coli strains are typically identified serologically by two surface antigens; the somatic (O) and the flagellar (H), of which there are ~186 and 53 types, respectively. The serotype identity of STEC strains has been used widely to identify STEC strains with the potential to cause severe diseases, but serotype is not a virulence factor and *E. coli* strains can carry any combination of O and H antigens; thus, the number of *E. coli* serotypes is very large. An estimated 470 STEC serotypes have been reported (105) that can produce any one of the 12 Stx1 and Stx2 subtypes or combinations of these subtypes. However, not all Stx subtypes appear to cause human illness, possibly because these strains lack known adherence factors associated with human illness. The estimated number of STEC serotypes that causes human illness ranges from >60 (4) to >100 (77).

Incidences of STEC strains causing foodborne infections have been reported from numerous countries worldwide (77). Serotype O157:H7 is the prototypic STEC that has caused infections worldwide. Serotype O26:H11 also seems prevalent and has caused infections in many countries, but other serotypes have caused infections only in a particular country or region (5, 98, 120), suggesting that there may be regional variations in STEC serotypes of importance. For example, in 2009, the EFSA (37) identified STEC with *stx* and *eae* from five O groups (O157, O26, O103, O111, and O145), also known as the “big 5,” as being of health concern in the European Union (EU). Similarly, in the United States, six O types (O26, O45, O103, O111, O121, and O145), or the “big 6,” have been found to account for >75% of clinical non-O157 STEC infections (25, 68). As a result, in 2011, the U.S. Department of Agriculture Food Safety Inspection Service (156) declared the “big 6” STEC O types that carry *stx* and *eae* and the STEC O157 strains as adulterants in raw nonintact beef and intact beef products intended for nonintact use. Although many of these O types of importance identified by various public health agencies are the same, types O121 and O45, which are on the U.S. priority list, are not listed as types of concern in other countries (77).

The evidence for geographic clustering and divergence seems to apply to both different STEC serotypes and strains within serotypes. Mellor et al. (101) used multilocus genotyping to examine O157:H7 strains isolated in the United States versus Australia and found that the strains differed in both genotype and genetic markers and virulence genes. Feng et al. (43) used multilocus sequence typing to characterize O113:H21 strains that have caused HUS in Australia versus environmental and clinical strains isolated elsewhere in the world and found that even though all the strains were within the same STEC clonal group, the Australian O113:H21 strains were of sequence type 820, which was not observed in the other strains.

STEC serotypes are evolving and moving among countries, partly because of the ease of worldwide travel, the vast international commerce of foods, and migration of wildlife (106). For example, an atypical O157:H7 variant that ferments sorbitol (SFO157) was first identified in Bavaria, Germany, in 1988 (80) and has now been found in other EU countries, including Finland (39), Austria, Czech Republic (17), and Scotland (2). SFO157 strains seem to be better able to adhere than do other O157 STEC (52, 132). Perhaps related to this difference are reports that a higher percentage of individuals with SFO157 infections develop HUS than do individuals with other O157 STEC infections (2, 132). Analysis of SFO157 strains isolated from various EU countries revealed identical or near identical pulsed-field gel electrophoresis (PFGE) profiles, suggesting that the same strains may have spread between the countries (44). SFO157 strains have thus far not been isolated in the United States but have been found in Australia (6), Egypt (134), and Korea (90); however, some of the strains described in those reports had genetic traits different from those of the German SFO157 strain, including the presence of *stx*₁. Most STEC O26:H11 strains were initially found to produce only Stx1, but isolates obtained later produced both Stx1 and Stx2.

Since the mid-1990s, a new clone of O26:H11 that produces only Stx2 has emerged in Europe and has caused several outbreaks of severe disease (1, 18, 31, 79, 92, 119, 141, 157, 163, 164). STEC O26 strains have also been isolated from cases of HUS in Argentina (131), and O26 was the most common non-O157 STEC serotype isolated from 1983 to 2002 (25) and 2000 to 2010 (62) in the United States. Among the O26 human isolates in the United States from 1983 to 2002, 13% had *stx*₂, of which only 2% had *stx*₂ alone and the other 11% also had *stx*₁ (25). Another example of changing regional clustering is STEC O121, which was not listed as being of concern in many countries (77), but together with O26, O103, O111, O117, and O145, O121 was listed as the third most common among the top six non-O157 STEC serogroups associated with serious illness in Canada (29). A strain of serotype O121:H19 (*stx*₂ positive) was implicated in a 2017 Canadian outbreak suspected to have been associated with contaminated flour (108). STEC O104 became a concern in the United States (77) because of an outbreak of BD in 1994 associated with milk contaminated with a strain of O104:H21 (30). However, the large outbreak of O104:H4 infection in Germany and France in 2011 (48) quickly raised our awareness of the health risks of this serotype and sent a cautionary message concerning the difficulties of anticipating STEC serotypes that may emerge to cause severe illness. The O104:H4 outbreak strain has not been found in the United States except for one strain isolated from a patient who had travelled to Germany during the outbreak period, thus highlighting the risk of pathogen spread via travel.

Identification of the serotype of STEC causing a particular group of infections is important in epidemiology for detection, investigating outbreak incidences, and tracking global emergence. However, *E. coli* serotyping is complex because of the large number of O and H antigens that exist, and not all *E. coli* isolates can be serotyped. Studies of STEC and enterotoxigenic *E. coli* (ETEC) strains isolated from fresh produce revealed that >50% of the isolates could not be typed or yielded only partial serotypes (45, 46). Because many STEC virulence genes are on mobile genetic elements that can be lost or transferred, it is not unusual to find STEC strains of the same serotype that carry different virulence genes and pose different health risks. As a result, although serotype data can be useful for identifying STEC strains, these data should not be assessed independently but rather evaluated along with the other attributes when determining health risks.

Key Points on Serotypes and Diversity

1. At least 470 STEC serotypes can produce any one or more of the 12 known Stx subtypes.
2. The number of STEC serotypes that cause human illness differs depending on reports but is probably >100.
3. Serotype is not a virulence factor and does not (necessarily) predict the virulence profile and health risks associated with a particular STEC but is useful in outbreak investigations and for prevalence surveillance.

OTHER FACTORS THAT AFFECT VIRULENCE CHARACTERIZATION

Horizontal gene transfer. Mobile genetic elements such as plasmids, bacteriophages, transposons, PAIs, and insertion sequence elements play a major role in the evolution of *E. coli* (161). Plasmids are highly diverse and may possess genes for antibiotic resistance, virulence, regulation, and adhesins. Through the process of conjugation, plasmids can transfer small or large fragments of DNA between bacteria and convey various traits to the recipient. Some bacteriophages have the capacity to mobilize genes, as indicated by the enormous proportion of fecal phage particles that contain bacterial DNA. Through lysogenic conversion of resident intestinal bacteria, phage may introduce new phenotypic traits, such as antibiotic resistance and the ability to produce exotoxins (23). Stx-converting bacteriophage (Stx phage) carry the *stx* gene and can lysogenize nonpathogenic bacterial strains and convert them into STEC (69). Stx phage, therefore, represent highly mobile genetic elements that play an important role in Stx expression, in horizontal gene transfer, and in STEC genome diversification. One example is the Stx-producing EAEC O104:H4 strain, which caused a large outbreak in Germany in 2011 (48). Some researchers have hypothesized that this strain originated from a genetically primitive lineage of *E. coli* in a confined geographical area but evolved via several independent streams of horizontal gene exchange (11, 14, 130).

Evidence from Central Europe and Italy indicates that O26:H11 strains have been shifting from carrying *stx*₁ only to carrying *stx*₁ and *stx*₂ and now to carrying *stx*₂ only and that those strains with only *stx*₂ are more virulent than the other O26 strains (1, 13, 18). As a further complication, loss and gain of Stx-encoding phage has been observed in O26:H11 strains (15). In the United States, mostly *stx*₁-bearing O26 strains have been found in foods, and isolation of strains with *stx*₂ alone has thus far not been common.

Frequent loss of *stx* genes in clinical STEC isolates has been observed upon subcultivation (81), and *stx*-negative *E. coli* O157:H7/H⁻ variants may occur at a low frequency in patients with diarrhea or HUS (34, 138). The loss and gain of Stx-encoding phage from *E. coli* in the human intestine or during cultivation can result in strains with different pathotypes. Such strains can present challenges for DNA fingerprinting techniques (such as PFGE), resulting in variable diagnostic results, and can have clinical, epidemiological, and evolutionary implications.

Free and infectious *stx* phage can be found in high densities in fecal samples from healthy humans, in environments polluted with human and animal feces, and in foods (73, 97, 109). As a result, molecular detection of *stx* genes in a sample merely reflects the presence of *stx* genes (phage), and results will have to be confirmed through isolation and characterization of the STEC strain. Other enterobacterial species also known to acquire *stx* phage are *Shigella dysenteriae* type 1, *Shigella flexneri*, *Shigella sonnei*, *Citrobacter freundii*, *E. albertii*, *Acinetobacter haemolyticus*, *Aeromonas caviae*, and *E. cloacae* (3, 10,

21, 28, 64, 69, 84, 117), and these genes may also be detected by *stx*-specific assays. Usually, detection of one or more *stx* genes in foods associated with an outbreak coupled with supporting epidemiological data provide sufficient information to link the food to human illness. However, free *stx* phage found in foods may result in false-positive findings. Alternative methods that can eliminate or significantly reduce the detection of free *stx* phage may allow more specific detection of STEC in foods (129).

More than 170 PAIs carrying important virulence properties have been annotated as genomic islands in the sequences of the STEC O157:H7 strains EDL933 and Sakai (67, 123). One of these PAIs carries the LEE, which has the *eae* gene necessary for the attaching and effacing lesion. Another PAI, designated O island 122 (OI-122), carries the large virulence gene cluster *efal-lifA* (85, 113, 145) and has frequently been found in STEC strains associated with severe human disease (82, 87, 107). OI-122 has multiple other functions and appears to be involved in cell adhesion, immunosuppression, disruption of epithelial barrier function, and intestinal colonization (86).

Another important PAI is OI-57, which harbors *adfo*, a putative virulence gene for adhesion, and *ckf*, which encodes a putative killing factor for bacterial cells. OI-57 is present in the majority of the STEC genomes and in a proportion of human EPEC, suggesting that this PAI could be involved in the attaching and effacing colonization of the intestinal mucosa (74).

A more complete description of many of the additional mobile genetic elements is beyond the scope of this assessment, but a few examples of recombinant strains derived from these elements, also referred to as hybrid strains, deserve mention here.

EAEC-STEC. *E. coli* O104:H4 from the German outbreak in 2011 has *stx*_{2a} and EAEC pAA (the virulence plasmid with genes coding for AAF/I, AggR, and SepA), extended-spectrum β -lactamase antibiotic resistance plasmid, and chromosomal genes for Aat (dispersin translocator), SigA (IgA protease-like homolog), and Pic (serine protease precursor) (19, 20).

EPEC-STEC. *E. coli* serotypes O26:H11, O55:H9, and O80:H2 from HUS patients in Austria and Italy have *stx*_{2f}, the EPEC-associated *efal* gene that resides on PAI OI-122, the STEC plasmid genes *ehxA*, *espP*, and *katP*, and intimin types ξ or β (63).

ExPEC-STEC. *E. coli* O80:H2 strains reported from France and Spain have *stx*_{2a}, *stx*_{2c}, or *stx*_{2d}, intimin gene *eae*- ξ , and at least four genes characteristic of pS88 (*sitA*, *cia*, *hlyF*, and *ompT*) and other genes associated with ExPEC virulence (*iss*, *iroN*, and *cvaA*) (142). Thirteen O2:H6 strains with sequence type 141 had *stx*_{2b}, *saa*, and ExPEC-associated genes *vat*, *clb* island, *cdiAB*⁻, and *ybt* clusters; 12 also had *iro*, 10 had α -*hly*, *cnfI*, the *pap* cluster, and *hek*, and 9 also had the *sfaII* cluster (16).

ETEC-STEC. An *E. coli* O2:H27 strain with *stx*_{2a}, *ehxA*, and *estla* (gene for ETEC heat-stable toxin) was isolated from two people (one had diarrhea and one was asymptomatic), and an O101:NM strain with *stx*_{2a}, *ehxA*, *estla*, and *eae* was isolated from a patient with HUS in Finland (114). *E. coli* O159:HUT sequence type 171, with *stx*_{2a}, *elt* (gene for ETEC heat-labile toxin), and ETEC colonization factor *CS12*, was isolated from a patient with diarrhea in Korea (116). Four O15:H16, five O175:H28, two O136:HNM, and one ONT:H16 human clinical isolate from Germany were positive for *stx*_{2g} and *estla* (the O15:H16 strains were also positive for the plasmid-encoded *astA* and *espP*) (125).

A less well-characterized *stx*_{2f}-positive O8:H19 isolate from a patient with HUS in The Netherlands was also positive for the *eae* gene but negative for *ehxA* (54). STEC O8:H19 strains do not usually have *stx*_{2f}.

In summary, mobile DNA and horizontal gene transfer in *E. coli* can transfer virulence genes to other bacteria and pose an ongoing challenge in diagnostic procedures and detection methodology and in making health risk determinations for STEC found in foods.

Key Points on Horizontal Gene Transfer

1. Independent streams of horizontal gene exchange play a major role in STEC diversity.
2. Mobile DNA and horizontal gene transfer in *E. coli* poses an ongoing challenge for diagnostic and detection methods and for the risk assessment of STEC found in foods.
3. Other diarrheagenic *E. coli* pathotypes are also known to acquire *stx* phage.
4. Other species of *Enterobacteriaceae* are also known to acquire *stx* phage.

Dose-response assessment for STEC virulence types. *Stx* is the main STEC virulence factor, but it is seldom produced in foods unless the food has undergone severe time and temperature abuse. Significant production of *Stx1* can occur in milk and ground beef when these have been subjected to vigorous aeration at 37°C for 48 h (159). However, these conditions are seldom encountered in normal food production processes because they result in spoilage, which will render the food unfit for consumption. Foodborne STEC infections typically occur from ingesting STEC-contaminated food and other vehicles. The STEC cells then bind to intestinal epithelial cells and express *Stx*. The severity of disease outcomes in STEC infections may also depend on the number of STEC cells ingested. The infectious doses of STEC are suspected to be low but can differ depending on serotype and strain. Disease outcomes also can differ depending on the individual's susceptibility.

Limited information is available on the dose-response of STEC. The risk of life threatening illness in humans and the absence of an animal model that replicates human pathology preclude experimental determination of the STEC dose-response. Estimates of dose-response have been made for STEC O157:H7 based levels of the pathogen in food and

consumption data from patients in outbreaks. Exposure to <100 cells of STEC O157:H7 may be sufficient to cause infection. Exposure estimates have been reported from three outbreaks in which the level of STEC O157:H7 in the food at consumption could be determined: 2 to 45 cells in salami (152), <700 cells in beef patties (155), and 31 to 35 cells in pumpkin salad with seafood sauce (150). These estimates are supported by reports of STEC O157:H7 levels (expressed either as CFU or most probable number [MPN]) in a variety of foods involved in outbreaks: raw milk cheeses, 5 to 10 CFU/g (147) and 0.0037 to 0.0095 MPN/g (60), and beef patties, 1.45 MPN/g (65) and 0.022 MPN/g (59). The probability of infection after exposure to a single viable STEC O157 cell is significant. In one foodborne outbreak, the median probability was estimated as 25% for children and 17% for adults (150). The frequency of transmission in childcare centers and among family members also suggests that the probability of infection per cell is significant.

It is not known whether the dose-response of STEC strains that use intimin for attachment differs among serogroups, although because of the known genetic and physiological variability of STEC the differences can be presumed to be significant. However, it is not currently possible to identify STEC strains that have a higher probability of causing infection than does STEC O157:H7. An investigation of an STEC infection outbreak involving serotypes O145:H28 and O26:H11 in ice cream revealed levels of 2.4 MPN/g for O145 and 0.03 MPN/g for O26 (27). In an outbreak of STEC O111:H⁻ infection associated with fermented sausage, the estimated exposure dose was 1 cell per 10 g (120). Thus, the probability of infection upon exposure to other STEC strains may approach that of O157:H7.

In addition to STEC strain factors, host factors very likely also affect dose-response relationships and disease outcomes. Individuals with weakened immune systems, such as frail or elderly persons, and individuals that lack acquired immunity, such as young children, have the highest rate of illness and HUS (66). In one study in Germany, examination of the relation between major STEC O groups and the affected individual's age and severity of illness revealed that age was a relevant factor in the severity of STEC illness (127). In another study in Germany, in children younger than 3 years of age, the relevant risk factors were contact with ruminants and consumption of raw milk; foods such as meats and sausages were not STEC risk factors until the children were at least 10 years old (160). These factors should be considered when extrapolating dose-response estimates to different demographic groups or epidemiological scenarios. Heterogeneity in exposure as influenced by infectivity, dose, attack rates, host susceptibility, food, etc. also needs to be taken into account when determining dose-responses in O157:H7 outbreaks (149).

Key Points on Dose-Response

1. The severity of disease outcome from infections may depend on the number of STEC cells ingested.

2. The infectious dose of STEC is suspected to be low but can differ among serotypes and among strains.
3. Disease outcome severity differs depending on an individual's susceptibility.

Human and other factors. Although selected STEC traits may be used to assess potential health risks, they provide no conclusive prediction on the outcome or the severity of disease. STEC pathogenesis is highly complex, and aside from STEC virulence traits other factors may also play a role in disease outcome. For example, coculturing O157:H7 strains with commensal *E. coli* can increase Stx2 production and the virulence of O157:H7 strains in mice, suggesting that there is a synergistic effect of STEC and the typical intestinal bacteria (61). Some clades of O157:H7 overexpress Stx2 and are more often associated with severe human infections (111). Severity of STEC infection outcomes can also be affected by synergistic effects with other organisms. In a 2001 to 2010 survey of 1,800 non-O157 STEC infections, 3.6% of the cases had multiple etiologies (93). In some cases, patients were coinfecting with a non-O157 STEC and O157:H7, *Cryptosporidium*, or *Campylobacter*. Coinfections with pathogenic *E. coli* and other pathogens have been characterized by severe diarrhea (153).

The occurrence and severity of STEC infections are also affected by human factors and genetics, which can affect STEC colonization and thus the severity of STEC infection outcomes (133). The impact of human individual susceptibility is also indicated by reports of asymptomatic STEC carriers (144). In a study of fecal samples from 5,590 asymptomatic workers from the Swiss meat processing industry, 3.5% of the samples were positive for *stx* genes, 47 STEC strains were isolated, and some strains also had the *eae* gene, including one isolate of the O157:H7 serotype (143). In a study in northern Italy of fecal samples from 350 asymptomatic farm workers from 276 dairy farms and 50 abattoir workers from seven facilities, 1.1% of the farm workers were infected with O157:H7 strains that had *eae* plus *stx*₁, *stx*₂, or both (140). All of these individuals were adults, and although they were asymptomatic, they could have posed health risks to younger individuals. An asymptomatic mother with an *eae*-negative O146:H28 strain with *stx*_{2b}, a Stx subtype usually associated with asymptomatic carriage (143), transmitted the strain to her child, resulting in a neonatal case of HUS (148).

Other evidence on the effects of human factors include a case in Finland, where an *eae*-negative, *stx*_{1c}-positive O78:H⁻ strain was isolated from the fecal samples of all five family members (91). The Stx1c subtype is most prevalent in STEC strains from sheep (24), and infections by Stx1c-producing strains tends to be mild or asymptomatic (51). Accordingly, the parents and the older siblings had no symptoms, but the 2-year-old child developed HUS. In another study, 3-year-old identical twins were infected with the same O157:H7 strain but had different outcomes; one twin developed HUS but the other did not (75). The authors speculated that differences in inoculum size may have impacted the disease outcomes. These examples suggest that

human genetics and individual susceptibility can greatly contribute to disease outcomes. Hence, no STEC strain may be “without risk”; all STEC strains probably pose some health risk to some individuals but maybe not to everyone. Thus, instead of the commonly used terms such as pathogenic and nonpathogenic, perhaps STEC strains should more appropriately be designated as having a low or high health risk. Such terminologies have been proposed and advocated by others for distinguishing the health risk associated with STEC strains (88, 136).

Medical histories can reveal that a particular STEC serotype has caused severe infections and outbreaks; therefore, serotype information may be useful when characterizing the STEC health risks, but such data need to be interpreted with caution. STEC strains of serotype O8:H19 have been found in flour in the United States and are common in cattle (76), and an O8:H19 strain also was reported to have caused HUS in a boy in The Netherlands (54). Most O8:H19 strains do not have *eae* and can have *stx*_{1a}, *stx*_{2a}, or both genes, but the HUS-causing strain from The Netherlands was unusual in that it had *eae* and *stx*_{2f}. Most of the STEC virulence genes reside on mobile genetic elements that can be transferred between strains and, as evident from various studies, strains with the same serotype can have different virulence genes and therefore can differ in their potential to cause severe illnesses. The case in The Netherlands also shows that under certain circumstances *stx*_{2f} can cause severe disease, supporting the conclusion that all STEC strains can pose health risks to certain individuals. The fact that strains of the same serotype can differ in pathotype greatly complicates health risk decision making and indicates the difficulty in establishing uniform criteria that can be used to determine whether an STEC has the potential to cause severe disease. Future research may identify better traits that can be used in STEC health risk characterization, in which case the critical health risk criteria currently used will need to be modified accordingly.

Key Points on Human and Other Factors

1. Human factors are thought to play a role in the outcome and severity of STEC diseases, but this role is undetermined.
2. All STEC strains have the potential to cause diarrhea and pose some health risks, but those that carry certain virulence traits are regarded as high risk and can cause HUS.

CONCLUSIONS

1. Adherence factors are critical for STEC pathogenicity.
2. The principal adherence factor in STEC is the intimin protein coded by the *eae* gene.
3. The AAF adhesins regulated by the *aggR* gene of EAEC are also an effective means for adherence.
4. Other putative adherence factor genes are *saa*, *sab*, *paa*, *efal*, *ompA*, *lpfA*, *toxB*, and the LAA PAI.
5. Twelve subtypes of Stx have been identified: Stx1a, Stx1c, Stx1d, Stx1e, Stx2a to Stx2g, and Stx2i, encoded

by genes *stx*_{1a}, *stx*_{1c}, *stx*_{1d}, *stx*_{1e}, *stx*_{2a} to *stx*_{2g}, and *stx*_{2i}, respectively.

6. The gene *stx*_{2a} is most often present in LEE (*eae*)-positive STEC and has consistently been associated with HUS.
7. The gene *stx*_{2a} has also been found in *eae*-negative, *aggR*-positive STEC that have caused HUS.
8. The gene *stx*_{2d} in LEE-negative STEC has, to a lesser degree, been reported from HUS cases, but not all STEC strains with *stx*_{2d} cause severe disease.
9. HUS cases in which other Stx subtypes were identified indicate that other factors such as host susceptibility and the genetic cocktail of virulence genes in an individual isolate may affect the association of these other Stx subtypes with severe diseases.
10. An estimated 470 STEC serotypes can produce any one or more of the 12 known Stx subtypes.
11. The number of STEC serotypes that cause human illness differs depending on the individual report and is probably >100.
12. Serotype is not a virulence factor and does not (necessarily) predict risk or virulence profile of an STEC strain but is useful in outbreak investigations and for prevalence and surveillance studies.
13. Independent horizontal gene exchanges play a major role in STEC pathogenicity and diversity.
14. Mobile DNA and horizontal gene transfer in *E. coli* transfers virulence genes to other bacteria and poses an ongoing challenge to diagnostic procedures and detection methodologies and to the risk assessment of STEC findings.
15. Other diarrheagenic *E. coli* pathotypes are known to acquire *stx* phage.
16. Other species of *Enterobacteriaceae* are also known to acquire *stx* phage.
17. The severity of disease outcome in infections may depend on the number of STEC cells ingested.
18. The infectious dose of STEC is suspected to be low but can differ between serotypes and strains.
19. Disease outcomes differ depending on individual susceptibility.
20. Human factors are thought to play a role in STEC diseases, but this role is undetermined.

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