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Virulence Potential of Activatable Shiga Toxin 2d–Producing *Escherichia coli* Isolates from Fresh Produce

Angela R. Melton-Celsa^{1,*}, Alison D. O'Brien¹, and Peter C. H. Feng²

¹Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

²Division of Microbiology, U.S. Food and Drug Administration, College Park, Maryland 20740, USA

Abstract

Shiga toxin (Stx)–producing *Escherichia coli* (STEC) strains are food- and waterborne pathogens that are often transmitted via beef products or fresh produce. STEC strains cause both sporadic infections and outbreaks, which may result in hemorrhagic colitis and hemolytic uremic syndrome. STEC strains may elaborate Stx1, Stx2, and/or subtypes of those toxins. Epidemiological evidence indicates that STEC that produce subtypes Stx2a, Stx2c, and/or Stx2d are more often associated with serious illness. The Stx2d subtype becomes more toxic to Vero cells after incubation with intestinal mucus or elastase, a process named “activation.” Stx2d is not generally found in the *E. coli* serotypes most commonly connected to STEC outbreaks. However, STEC strains that are *stx*_{2d} positive can be isolated from foods, an occurrence that gives rise to the question of whether those food isolates are potential human pathogens. In this study, we examined 14 STEC strains from fresh produce that were *stx*_{2d} positive and found that they all produced the mucus-activatable Stx2d and that a subset of the strains tested were virulent in streptomycin-treated mice.

Shiga toxin (Stx)–producing *Escherichia coli* (STEC) infection may lead to watery, bloody diarrhea or hemorrhagic colitis and, in some patients, result in hemolytic uremic syndrome (HUS). STEC strains can produce two main Stx types, designated Stx1 and Stx2, which share the same mode of action and epithelial cellular receptor, but antiserum to one toxin does not cross-neutralize the other toxin type. There are three established Stx1 subtypes, Stx1a (the prototype), Stx1c, and Stx1d (28). A newly described subtype, Stx1e, was recently found in an *Enterobacter cloacae* strain that caused watery diarrhea (26). There are seven Stx2 subtypes, Stx2a (the prototype), Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g (28). STEC strains can produce any Stx type, subtype, or combination of subtypes, but some subtypes are produced mostly by animal and environmental STEC strains and have seldom caused human illness (13, 22). The Stx2 subtypes most often implicated in severe human illness are Stx2a, Stx2c, and Stx2d (10). The Stx2d subtype was first found in 1990 in strain B2F1, a STEC isolate of serotype O91:H21 (15). Stx2d was originally thought to be a variant of Stx2a, as it had lower Vero cell toxicity than Stx2a but had equivalent lethality to

* Author for correspondence. Tel: 301-295-9612; Fax: 301-295-3773; angela.melton-celsa.ctr@usuhs.edu.

mice (19). It was later found that the Stx2d made by strain B2F1 became 10- to 1,000-fold more toxic for Vero cells after incubation with intestinal mucus (23). This mucusenanced toxicity was termed “activation,” and Stx2d proteins shown to be activatable are designated Stx2dact. The factor in mucus that increased the toxicity of Stx2d was later found to be elastase (16). STEC strains that produce Stx2dact have been implicated in outbreaks of bloody diarrhea and HUS in various countries (2, 5).

STEC that produce Stx alone, without an adherence factor, are unlikely to cause severe diseases like hemorrhagic colitis or HUS. Intimin, an outer membrane protein that is encoded by *eae* (31), is the most common adherence factor found in STEC strains that have caused outbreaks. The presence of both *eae* and *stx₂* in a strain is associated with the capacity of that organism to cause HUS (3, 8). STEC strains that encode *stx_{2d}* usually do not have *eae* but can cause HUS; therefore, they have a different adhesin and, possibly, additional virulence factors besides the toxin. One possible alternate adhesin to intimin is Saa, the STEC agglutinating adhesin (32). Another toxin found in some STEC strains is the subtilase cytotoxin encoded by *subAB*, though the possible contribution of subtilase to STEC pathogenesis is not clear (25). In addition, many STEC strains produce enterohemolysin, encoded by *ehxA* (1), but the role of this toxin in pathogenesis is also unclear. To date, *stx_{2d}* has not been found in O157:H7 strains or, until recently, in any of the “big 6” non-O157 STEC serotypes that cause most of the infections in the United States (27). A recent publication reported that some O26:H11 strains that had *stx_{2d}* and *eae* were isolated from several HUS cases in France (7). Similarly, an O80 strain that carried *stx_{2c}*, *stx_{2d}*, and *eae* was reported to have caused HUS and bacteremia (21). These examples show the potential for acquisition and transfer of important STEC virulence factors, which can give rise to unusual strains that can express a variety of virulence genes.

STEC strains that produce Stx2dact have been isolated from healthy cattle and sheep at slaughter (30). A recent study examined the Stx subtypes of 132 STEC strains isolated from fresh produce in the United States and found 14 strains that carried only *stx_{2d}* (9). To determine whether the toxins produced by these environmental strains were Stx2dact, we examined these strains for mucus-activated cytotoxicity in assays on Vero cells. We also tested selected strains for lethality in streptomycin-treated BALB/c mice.

Materials and Methods

Strains and characterization

The 14 STEC strains were isolated from fresh produce by the U.S. Food and Drug Administration or by the Microbiological Data Program of the U.S. Department of Agriculture, which surveyed fresh produce for various microbial pathogens. All 14 strains carried only *stx₂* and were determined to be the *stx_{2d}* subtype by the PCR typing method of Scheutz et al. (28). The strains were also tested by various PCR assays for the presence of *eae* and other putative virulence factor genes, including *saa*, *subAB* (encoding subtilase cytotoxin), and *ehxA* (encoding enterohemolysin) (9). The strains were serotyped by the *E. coli* Reference Center at Pennsylvania State University; however, 10 of the 14 strains were untypeable or only had partial serotype data. Hence, these strains were genetically serotyped with the U.S. Food and Drug Administration *E. coli* Identification (ECID) microarray (17).

Cytotoxicity and activation assays

We tested the supernatants from overnight cultures of each strain for the presence of Stx2d by using Vero cells as described previously (11, 29). Briefly, serial dilutions of the supernatants were overlaid onto Vero cells seeded 24 h previously into 96-well plates. The plates were incubated for an additional 48 h at 37°C in an atmosphere of 5% CO₂. The cells were then fixed in formalin and stained with crystal violet, and the A_{630} measured. The 50% cytotoxicity dose (CD₅₀) was calculated as the reciprocal dilution of the supernatant that killed half of the Vero cells. The capacity of Stx2d in the culture supernatants to be activated by mouse intestinal mucus was determined as described previously (23). Briefly, bacterial culture supernatants were incubated with either HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid] buffer or mucus for 2 h at 37°C, and then those samples were overlaid onto Vero cells.

Mouse studies

All animal studies were approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences and were conducted in strict accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals* (6). The streptomycin-treated mouse model (24) was used to determine the virulence of a subset of the strains, except that male BALB/c mice were used rather than CD-1 mice. Briefly, the mice were fed water with streptomycin (5 g/liter) ad libitum and fasted. The next day, the mice were fed the strain of interest in a total of 50 µl (given as 25 µl twice) by pipette. The mice were observed at least twice daily for 16 days for morbidity or mortality.

Results and Discussion

We analyzed 14 isolates from fresh produce that were *stx*_{2d} positive (*stx*_{2d}⁺) by PCR, and all 14 produced Stx, as the supernatants from overnight cultures were toxic to Vero cells. The toxin levels produced by each strain ranged from 10² to 3 × 10³ CD₅₀s per ml of culture supernatant. These toxicity levels were consistent with that of Stx2d, because the cytotoxicity of Stx2d is lower than that of Stx2a on Vero cells due to two amino acid differences in the B subunit (19). All of the strains were further found to produce Stx2dact, as the CD₅₀s measured in the supernatants increased 6- to 21-fold after incubation with mouse intestinal mucus (Table 1). None of these strains had *eae*, and fewer than half contained *saa*, *subAB*, or *ehxA* (Table 1).

Four of the 14 strains examined in this study were fully serotyped by serology, and most of the other 10 strains had known H types but no O type or had OX types, which are used to designate strains unclassified by standard O antisera (12). We serotyped these strains with the ECID microarray (17) and verified the H types of all strains except that of strain 11-27, which was serotyped as H16 but was determined to be H2 by the ECID array (Table 1). It is not uncommon to find environmental strains that have been mistyped by serology (17). We were unable to determine the O type for three isolates, but 6 of 10 strains tested had the antigen sequences of *Shigella boydii* type 9 (OB9). This result is not unusual, as *E. coli* and *Shigella* species are closely related genetically and share many O antigens (20). Also, strains

that have OB9 antigen seem to be fairly common, as they composed almost 10% of the STEC strains isolated from produce (17). The last isolate was typed as O174:H28, a serotype that has been associated with cattle (4, 14).

We tested nine of the isolates for virulence in the streptomycin-treated mouse model. We found that 6 of 9 strains were lethal to 20 to 60% of the mice (Fig. 1). Although these Stx2dact⁺ isolates were not as virulent to mice as the Stx2dact⁺ human isolate B2F1 (18), the virulence data supported the hypothesis that these Stx2dact⁺ STEC isolates from produce have pathogenic capability. The virulence capacity of the produce isolates correlated with that of Stx2dact and did not appear to depend on the presence of *saa*, *subAB*, or *ehxA*, as some strains that had all three genes were not lethal to all mice, and conversely, strains that had none of these genes were mouse lethal. There is also evidence from a study of human isolates that the presence of Stx2dact in a strain is cause for concern. A large German study examined 922 STEC strains isolated from clinical patients with various symptoms and identified 60 strains that produced Stx2dact (2). None of the 60 isolates had *eae*; Stx2dact was the sole toxin subtype produced by 31 of the 60 strains, and those strains accounted for 13 of 16 cases of hemorrhagic colitis and HUS. It should be cautioned, however, that the presence of Stx2dact alone in these produce isolates may not correlate directly with virulence in the streptomycin-treated mice, and it is likely that additional factors, such as the level of toxin production, the capacity to grow in intestinal mucus, and others, may also play a role in pathogenesis.

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Figure 1.

Percentages of streptomycin-treated mice surviving after oral infection with STEC produce isolates that make Stx2dact. The numbers of mice used per strain were 5 for strains 09-02, 695-5, and 695-41, 10 for strains B2F1, 10-48, 11-28, 11-37, 11-40, and 695-35, and 15 for strain 1E2.

Table 1
Sources, activation ratios, partial genotypes, and serotypes of strains^a

Strain	Source	Fold activation ^b	<i>saa</i>	<i>subAB</i>	<i>ehxA</i>	Serotype	ECID serotype
05-613	Lettuce	21.5	-	-	-	O174:H36	ND
09-02	Spinach	11.4	+	+	+	OX25	Ont:H11
10-48	Spinach	11.3	w+	-	+	Ont:H2	OB9:H2
11-28	Spinach	6.2	w+	+	+	Ont:H2	Ont:H2
11-37	Spinach	16.2	w+	w+	-	Ont:H16	OB9:H2
11-40	Spinach	15.4	-	w+	-	Ont:H2	OB9:H2
12-05	Cilantro	14.1	-	-	-	O168:H8	ND
12-49	Spinach	10.0	+	+	+	O181:H49	ND
12-57	Spinach	10.2	+	+	+	O181:H49	ND
695-5	Spinach	14.6	-	-	-	Ont:H2	OB9:H2
695-35	Spinach	17.8	-	-	-	OX23:H2	OB9:H2
695-41	Spinach	10.7	-	-	-	Ont:H2	OB9:H2
300879-14	Sprouts	14.4	-	-	-	ND	O174:H28
1E2	Cilantro	21.0	ND	-	-	ND	Ont:H2

^a-, negative; +, positive; w+, weakly positive; ND, not done.

^bFold activation is the ratio obtained by dividing the CD50s per milliliter after incubation of culture supernatant with mucus by the CD50s per milliliter after incubation of the supernatant with HEPES buffer alone.