CONCISE COMMUNICATION

Escherichia coli 0157:H7 Strains That Express Shiga Toxin (Stx) 2 Alone Are More Neurotropic for Gnotobiotic Piglets Than Are Isotypes Producing Only Stx1 or Both Stx1 and Stx2

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Infection with *Escherichia coli* O157:H7 can lead to hemolytic uremic syndrome (HUS) in some children. Epidemiologic data suggest that Shiga toxin (Stx) 2–producing strains are more frequently associated with HUS than are Stx1-producing strains. Less clear is whether strains that express Stx2 alone are more frequently associated with HUS than strains that express Stx1 and Stx2. Isogenic mutants 933*stx1*$^-$ and 933*stx2*$^-$ were produced from strain 933 (Stx1 and Stx2 producer), and 86-24*stx2*$^-$ was produced from strain 86-24 (Stx2 producer). Neurologic lesions or symptoms developed in 18 (90%) of 20 gnotobiotic piglets orally infected with strain 86-24, in 15 (85%) of 18 infected with mutant 933*stx1*$^-$, in 9 (31%) of 29 infected with strain 933, in 0 of 5 infected with mutant 86-24*stx2*$^-$, and in 0 of 6 infected with mutant 933*stx2*$^-$. It was concluded that strains expressing Stx2 alone are more neurotropic for piglets when fed orally than are those strains expressing Stx1 and 2, whereas Stx1-producing strains induce only diarrhea. It is also conceivable that strains that produce Stx2 may constitute a significant predictive risk factor for HUS in humans.

Enterohemorrhagic *Escherichia coli* (EHEC), the cause of hemorrhagic colitis and hemolytic uremic syndrome (HUS), are considered emerging pathogens in developed countries [1, 2]. HUS, the leading cause of acute renal failure in young children [1–3], is marked by microangiopathic hemolytic anemia, thrombocytopenia, and renal dysfunction. On rare occasions, neurologic complications may also occur [4, 5]. In contrast, hemorrhagic colitis occurs in people of all ages and is characterized by acute self-limiting illness of lower abdominal pain and watery diarrhea, which later may become bloody. Although *E. coli* O157:H7 is most frequently isolated [1–3], there are numerous other serotypes that are now classified as EHEC [1–3].

Most EHEC strains cause mucosal damage and diarrhea by intimate bacterial attachment and destruction of enterocytes and colonocytes. These lesions are readily demonstrated in newborn piglets [6, 7] and also presumably occur in humans. The gene locus that is involved in the formation of this bacterial attaching-effacing lesion is known as the locus of enterocyte effacement (LEE) [8]. EHEC strains, by definition, however, produce 1 or 2 Shiga toxins (Stx), which are closely associated with the development of HUS and possibly hemorrhagic colitis.

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In addition to humans, the pig is the only other known species that is naturally susceptible to the systemic effect of Stx produced by *E. coli* that proliferate in the gut. The syndrome, known as edema disease, is caused by Stx2e, a variant of Stx2 [9], and can be reproduced experimentally by parenteral inoculation of newborn piglets. In piglets (contrary to the case in children), the predominant symptoms of edema disease include edema of the eyelids, altered behavior, ataxia and staggering, tremor, paddling of limbs, convulsions, coma, and death. Microscopic lesions are associated with vascular injury and include vessel necrosis, perivascular edema and hemorrhage, and superficial colonic and cecal erosions. Vascular lesions are observed in the cerebellum, mucosa and submucosa of the stomach, cecum, and colon. These signs are quite distinct from endotoxemia [10]. Almost identical symptoms can also be induced by parenteral inoculation of piglets with Stx1 and Stx2 purified from EHEC strains. When gnotobiotic piglets are inoculated orally with O157:H7 strains, they develop diarrhea (largely due to the bacterial damage to colonocytes), and a proportion of them develop neurologic symptoms identical to those described above for edema disease, which are attributed to Stx [6, 11].

In humans, epidemiologic data suggest that *E. coli* O157:H7 strains that express Stx2 are more important than Stx1 in the development of HUS [5], and strains that express Stx2 alone are more likely to be associated with progression to HUS than are strains that produce both Stx1 and Stx2 [2]. However, the association of Stx2-only strains with progression to HUS has not been substantiated [13]. To determine the relative ability of

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Stx1, Stx2, or both toxins to induce systemic complications, we constructed a panel of isogenic strains that express either 1 or both toxins and then tested them in the gnotobiotic piglet model.

Materials and Methods

Bacterial strains and media. *E. coli* 86-24, an EHEC strain that produces Stx2, was isolated from an outbreak in Walla Walla, Washington [14]. TUV86-2 is a *stx2* deletion mutant of strain 86-24 [11]. *E. coli* strain 933 produces both Stx1 and Stx2 [3, 6]. *E. coli* SM10 λ*pir* is a lysogen of *E. coli* SM10 *(thi*-1 *thr*-1 *leu*B6 *sup*E44 *ton*A21 *lac*Y1 *rec*A$^-$::RP4-2-*Tc*::*Mu Km*^{r}*)* and contains the *trans*-acting factors needed to mobilize the plasmid pGP704 [15] and its derivatives. Bacteria were grown in Luria-Bertani (LB) broth and on MacConkey (Difco, Detroit), LB, or blood agar plates (Becton Dickinson Microbiology Systems, Cockeysville, MD) at 37°C or on LB/sucrose plates (10% sucrose, no NaCl) at 30°C. Ampicillin was added at 200 μg/mL, where needed. *E. coli* DH5α (F$^-$ϕ80d*lac*ZΔM15 Δ[*lac*ZYA-*arg*F]U169 *deo*R *rec*A1 *end*A1 *pho*A *hsd*R17(r_k^-, m_k^+) *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1; Life Technologies, Gaithersburg, MD) was used as the host strain for all other plasmids.

Plasmids and DNA primers. Derivatives of the suicide vector pGP704 [15] were used for the allelic exchange experiments. All other cloning was done in pUC18, using DH5α as the host strain. The sequences of the oligonucleotide primers used in this study are shown in table 1.

Construction of pTUV9. We used a 4-primer technique polymerase chain reaction (PCR) to construct an out-of-frame 500-bp deletion of *stx1* [16]. The primers used were UB-4, SI-3, SI-4, and FG-8. The deletion was constructed so that it removed active site residues of the toxin A subunit and extended into the B subunit. The deletion should create a protein that consists of the first 176 amino acids of the A subunit and an additional 10 amino acids before a stop codon is reached. The 1433-bp product was treated with restriction enzymes *Sac*I and *Xba*I and cloned into the *Sac*I and *Xba*I site of suicide vector pTUV5, a derivative of pGP704 containing the *sacB* gene cloned into the *Bgl*II site of pGP704. The recombinant plasmid, named pTUV9, was electroporated into *E. coli* SM10 λ*pir*.

Construction of isogenic toxin mutants of strain 933. The plasmid pTUV9 was introduced into EHEC strain 933 by plate mating, using strain SM10λpir (pTUV9) as the donor strain. The bacterial lawn was collected and spread on MacConkey agar–ampicillin plates. Lactose-positive, ampicillin-resistant colonies, representing potential *E. coli* strain 933 exconjugants, were picked and tested in a PCR using the primers SI-1 and SLT-I-1. Isolates that showed both the wild-type (wt) operon product (1210 bp) and the deleted operon product (710 bp) were selected for further study. Mutants that had lost the suicide vector either by phage curing or allelic exchange between the wt toxin alleles and the toxin deletion alleles were selected on LB plates containing 10% sucrose. These potential mutants were first tested by ELISA for toxin production. Culture supernatants were tested in triplicate in a toxin-capture ELISA, which was used for the detection of Stx1 monoclonal antibody 4D3 and Stx2 monoclonal 2B2, as described elsewhere [17].

Table 1. List of oligonucleotide primers used to study the ability of Shiga toxins 1 and 2 to induce systemic complications in piglets.

Primer	Sequence (5′→3′)[a]	Location [ref.], GenBank accession no.
UB-4	gctctagacgtaacagcattttgctctac, *Xba*I	49–69 [18], M16625
SI-3	ctgacgcagtctgtgggtaactggaaaggtgg	845–860 and 1361– 1376 [18], M16625
SI-4	ccacctttccagttacccacagactgcgtcag	Complementary to SI-3
FG-8	ccgagctcgcgcagttgcgcagccttatg, *Sac*I	1933–1953 [20], M23980
SLT-I-1	cagttaatgtggtggcgaag	381–400 [18], M16625
SI-1	cgcctgctattttcactgagc	1568–1588 [18], M16625
SLT-II-1	cttcggtatcctattcccgg	288–307 [19], XO7865
SLT-II-2	ggatgcatctctggtcattg	747–766 [19], XO7865

[a] Recognition sites for restriction endonucleases are underlined, and restriction endonucleases are indicated.

Southern blot analysis. C600(933W) and C600(933J) are 2 lysogens that harbor the Stx2- and Stx1-converting phages, respectively. Phage DNA was obtained from the 2 lysogens using the Qiagen Lambda kit (Qiagen, Santa Clarita, CA) and digested with the enzyme *Eco*RI. After digestion, the phage DNA was randomly prime-labeled using γ-^{35}S ATP and used as DNA probes for the Southern hybridizations. Bacterial genomic DNA from the wt parent and from representative isolates from the toxin-negative mutants of strain 933 were prepared using the QIAamp tissue kit (Qiagen). All genomic DNA was digested overnight with *Eco*RI, followed by separation on a 0.8% TAE agarose gel and subsequent transfer onto Zeta-Probe GT nylon membrane (Bio-Rad Laboratories, Hercules, CA). Hybridization was done under stringent conditions.

Animals and experimental procedures. Eighty-three gnotobiotic piglets were used in the experiments. The animals were derived by cesarean section from 10 litters and were maintained in microbiologic isolation throughout the experiment [6]. Piglets were fed 250 mL of milk replacer (Similac, Abbott Laboratories, Columbus, OH), which was given twice daily. The animals were grouped into 7 unevenly numbered experimental groups matched within each litter to minimize between-litter variation. Twenty-four hours after cesarean delivery, the 7 groups of animals were each given an oral challenge dose of 5×10^9 of 1 of 7 *E. coli* strains. Group 1 (20 piglets) received strain 86-24, and group 2 (29 piglets) received strain 933. Group 3 received strain Stx1$^-$ 933 mutants (5 received TUV933-2, which contains a deletion within the *stx1* genes; 13 received TUV933-2c, which is cured of the Stx1-converting phage). Group 5 (6 piglets) received TUV933-1 cured of Stx2-converting phage, and group 6 (5 piglets) received TUV86-2, a mutant with a *stx2* deletion. Group 7 (5 piglets) received a nonpathogenic human *E. coli* strain, HS. Animals were monitored several times daily for clinical signs of illness. They were killed when neurologic symptoms or wasting, as a consequence of severe diarrhea, were apparent, or 8 days after challenge. Piglets were killed, and formalin-fixed sections from the gut, viscera, and brain were prepared for histology. Gut contents and blood were cultured for bacterial growth at necropsy to determine the number and identity of bacteria.

Results

Construction of mutants. The initial objective was to create an isogenic *stx1* deletion of strain 933. To do this, we con-

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structed a suicide vector (pTUV9) that contained DNA from the upstream and downstream regions of the *stx1* genes and a 500-bp deletion of the *stx1* genes. From 3 independently isolated exconjugants, we isolated 3 mutants of strain 933 (TUV933-1, TUV933-2, and TUV933-2c), which were used in piglet experiments. Strain TUV933-1 has been phage cured of the converting phage that carries the *stx2* genes and hence was negative when analyzed by Southern blot using a DNA probe from the 933J phage. Strain TUV933-2 is an isogenic *stx1* deletion derivative of strain 933, and strain TUV933-2c is a derivative cured of the Stx1-converting phage. The phenotype and genotype characterizations of all the EHEC strains used in the piglet experiments are shown in table 2.

Clinical response. Table 3 summarizes the clinical and pathologic outcome. Two to 3 days after challenge, piglets infected with O157:H7 strain developed diarrhea, which varied considerably in severity, as described elsewhere [6, 7]; there was no correlation between the development of diarrhea and the specific O157:H7 strains with which the piglets were infected. Animals that developed severe diarrhea and wasting in <48 h were killed and were excluded from further analysis. Neurologic symptoms in affected animals appeared 2–7 days after challenge, and the time of appearance of these symptoms was related to the litter rather than to the type of bacterial strain given.

Table 3 shows that of the 38 animals challenged with strains that produced Stx2 only (strain 86-24, TUV933-2 and TUV933-2c), 33 (87%) developed neurologic symptoms. In contrast, only 9 (31%) of 29 piglets challenged with wt strain 933, which produces both toxins, developed neurologic symptoms. No such symptoms were observed in 6 piglets challenged with the Stx1-producing mutant TUV933-1, in 5 piglets challenged with mutant TUV86-2, or in 5 animals given the nonpathogenic *E. coli* (table 3). This indicated that strains producing Stx2 only were more likely to cause systemic complications, manifested by neurologic symptoms, than those infected with strains that produce both toxins. The failure of the Stx1-producing mutant TUV933-1 to induce neurologic symptoms was consistent with earlier observations made in this and other laboratories [7]. At necropsy, cerebellar and mesocolonic edema were the most prominent finding in these animals.

Microscopic lesions. Microscopic lesions caused by bacterial attachment-effacement were, with 1 exception, qualitatively similar in piglets infected with *E. coli* O157:H7, regardless of the strains or the mutants, and were similar to those previously reported in this model [6, 7]. Microscopic brain lesions were apparent in 31 (81.5%) of the 38 piglets challenged with the 3 Stx2-producing strains and in 11 (37%) of 29 challenged with strain 933, and were very mildly apparent in 1 of 6 piglets challenged with TUV933-1 ($Stx1^+$; table 3). Microscopic changes in the brain were most prominent in the cerebellar gray matter but were also occasionally apparent in the cerebellar white matter and the cerebral hemispheres. Although there was a strong correlation between animals with neurologic symptoms and lesions, there were 3 animals with neurologic lesions but no symptoms and 2 with symptoms but no apparent brain lesions.

Table 2. Phenotype and genotype characterization of *Escherichia coli* O157:H7 Shiga toxin (Stx) strains used to study the ability of Stx1 and Stx2 to induce systemic complications in piglets.

	Stx1			Stx2		
Strain	ELISA	PCR	Southern blot	ELISA	PCR	Southern blot
933	+	+	+	+	+	+
TUV933-1	+	+	+	−	−	−
TUV933-2	−	Δ+	+	+	+	+
TUV933-2c	−	−	−	+	Δ+	+
86-24	−	−	−	+	+	+
TUV86-2	−	−	−	−	+	+

NOTE. PCR, polymerase chain reaction; +, an ELISA reading above background, a PCR product, or a band by Southern blot analysis; −, a background ELISA reading, an absence of a PCR product, or the absence of a band by Southern blot analysis.

Bacterial culture. Bacterial counts in the small intestine ranged between 10^6 and 10^9, and between 10^9 and 10^{10} in the large intestine. This indicated that the *E. coli,* regardless of the strain, became well established in the gastrointestinal tract of infected piglets. The extent of bacterial colonization in the gut was not analyzed statistically, because animals were killed over 3–7 days. *E. coli* was cultured from the blood stream of 1 terminally ill piglet infected with 86-24. Organisms recovered from the gut were identified as those with which piglets were challenged.

Discussion

Although Stx2 does not cause HUS in piglets, the neurologic injury in piglets and HUS in children are consequences of systemic uptake of toxin from the gut. Therefore, it is more than likely that the early sequence of events of toxin translocation across the gut mucosa and the transport in the bloodstream are common pathogenic pathways shared by both species. Although the target organs are different neurologic involvement can occur in severely affected children [1, 4]. We therefore believe that investigations in the pig model can help elucidate the early sequence of events leading to HUS in children.

Studies on the pathogenesis of EHEC infections in piglets have revealed that of the 2 toxins, only Stx2 consistently induces neurologic complications [6, 7]. However, we have observed that a considerably higher proportion of piglets infected with Stx2-producing strains develop neurologic symptoms, compared with animals challenged with strains that produce both toxins. These observations differ from results of an earlier study that used nonisogenic strains tested in a smaller number of animals [7]. Epidemiologic observations in humans also suggest a similar trend with regard to HUS in children [12]. Whether a more severe form of HUS, with sequelae leading to neurologic complications and death, is also more frequently associated with

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Table 3. Summary of the clinical and histologic outcomes of 7 groups of gnotobiotic piglets challenged with 1 of 7 *Escherichia coli* strains.

Bacterial strains (toxin)	No. of animals per group	No. of animals with CNS involvement			No. of animals with gut lesions[a]
		Symptoms[b]	Lesions[c]	% Affected[d]	
86-24 (Stx2)	20	17	18	90	20
933 (Stx1 and Stx2)	29	9	11	37	28
Tuv933-2c (Stx2)[e]	13	11	9	84	12
Tuv933-2 (Stx2)[f]	5	5	4	100	5
Tuv933-1 (Stx1)	6	0	1[g]	16	6
TUV86-2 (Stx^-)	5	0	0	0	5
HS (control)	5	0	0	0	0

NOTE. Bacterial dose was 5×10^9 organisms/piglet. Animals were killed when neurologic symptoms developed or 8 days after challenge. CNS, central nervous system; Stx, Shiga toxin.

[a] Lesions were caused by bacterial attachment-effacement to enterocytes and colonocytes.

[b] Neurologic symptoms included ataxia, pressing head against objects, and recumbency.

[c] Neurologic lesions included petechial cerebellar hemorrhages.

[d] Fisher's exact test between-group comparisons: 1–2 ($P = .002$); 1–3 (not significant); and 2–3 ($P = .001$). Groups 1–3 differ from groups 4–6 ($P = .0001$).

[e] Piglets challenged with phage-cured mutant.

[f] Piglets challenged with Stx1-deleted mutants.

[g] Two discreet focal neurologic lesions were observed in 1 animal.

Stx2 remains to be substantiated. Although some studies have not found a statistically significant association between Stx2-only strains and progression to HUS [13], the similarity between the epidemiologic trend in humans and our observations in the piglet model prompted us to undertake these studies.

Isolated isogenic mutants of strain 933 were either cured of the converting phages or contained the phages but had a *stx1* gene deletion. Somewhat unexpected was the isolation of mutants that were cured of the Stx2-converting phage. There is ~50% DNA homology between the *stx1* sequences cloned into the suicide vector and *stx2* region sequences. This degree of homology may have allowed the suicide vector to insert itself into the *stx2* toxin gene. Because of the relatively low DNA homology between the DNA on the suicide vector and the DNA in the genome, phage curing was probably the predominant mechanism by which the suicide vector was lost from the exconjugant.

Using wt 0157:H7 strains and our laboratory-derived isogenic mutants, which are either cured of the toxin-converting phages or contain a toxin deletion and encode either Stx1 or Stx2, we demonstrated that Stx2-producing strains induced more systemic complications in piglets than did strains that produced both toxins. In contrast, Stx1 alone induced no apparent neurologic symptoms, as previously reported in piglets [7] and mice [21]. The Stx2-producing strains (86-24, TUV933-2, and TUV993-2c) induced distinct neurologic symptoms or lesions in 35 (~92%) of 38 challenged animals versus 11 (~38%) of 29 animals challenged with wt strain 933.

Although the calculated LD_{50}/kg body weight required to produce these symptoms in pigs is marginally less for Stx2 (1.5 ng/kg body weight) than for Stx1 [10], this, in our view, does not explain the contrast seen in the clinical outcome in piglets and possibly in humans. There are several possible explanations as to why strains that produce Stx2 were more neurotropic than those that produce both toxins. One explanation is that strains that express 2 toxins produce less Stx2 than do strains that express only 1 toxin. In vitro toxin production experiments however did not support this hypothesis since there was no difference in the amount of Stx2 produced by strain 86-24, TUV933-2c, and wt strain 933 (data not shown). Studies of toxin production within the highly complex conditions of the intestinal milieu, which may vary considerably among different isolates, were precluded because of the existence of a large number of uncontrolled variables. Another explanation is that since Stx1 has a higher Gb3 cell–receptor affinity than Stx2 [22], it is tempting to postulate that Stx1 molecules are retained largely by the gut mucosa. Immunohistochemistry studies of gut loops injected with either toxin showed that more Stx1 than Stx2 remained associated with the gut mucosa (unpublished data). Binding to receptors on the epithelial cells of the intestine may be a prerequisite for systemic entry of toxin. Occupation of intestinal receptors by Stx1 would therefore block Stx2 uptake.

Complementation of the toxin genes that uses plasmids was not done, since spontaneous phage induction contributes significantly to total Stx2 production [23] and since there are phage factors that mediate toxin expression. Thus, complementation that use plasmids would create strains that produce atypical amounts of toxin and are regulated, in terms of toxin production, very differently from the wt strains.

The production of either 1 or both toxins had no apparent effect on the occurrence or the intensity of mucosal lesions or diarrhea. As shown in table 3, there was a good correlation between the neurologic symptoms and the presence of cerebellar lesions. Identical brain lesions were observed in a child who died of HUS and neurologic complications and from whom Stx2-producing *E. coli* O157:H7 was recovered [4]. The detec-

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tion of mild lesions in a piglet challenged with TUV933-1 indicated that Stx1 is absorbed and can reach the brain in a few individuals but perhaps in insufficient amounts to induce serious lesions or symptoms.

In conclusion, we demonstrated that infection with *E. coli* strains that produce Stx2 leads to systemic complications in a greater proportion of piglets than infection with strains that produce both toxins Stx2 and Stx1. The study confirms that Stx1 was very mildly neurotropic for piglets and that production of Stx1 appeared to somehow reduce the degree of neurotropism of strain 933, which produces no less Stx2 than 86-24 or TUV933-2c. Since epidemiologic data recognize a similar trend in humans, this study strongly implicates Stx2-producing strains as potentially being more virulent for both species than strains that produce Stx1 or both Stx1 and Stx2. At this stage, we can only speculate that since both toxins compete for the same Gb3 cell receptor and since Stx1 has greater receptor affinity than Stx2, it is likely that the absence of Stx1 facilitates Stx2 systemic uptake from the gut.

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