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Shiga Toxin-Producing Escherichia coli Infection: Temporal and Quantitative Relationships among Colonization, Toxin Production, and Systemic Disease

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Abstract

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Comments

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Shiga Toxin–Producing *Escherichia coli* Infection: Temporal and Quantitative Relationships among Colonization, Toxin Production, and Systemic Disease

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Edema disease, a naturally occurring disease of swine caused by Shiga toxin–producing *Escherichia coli* (STEC), was used as a model for the sequence of events that occur in the pathogenesis of STEC infection. The mean time from production of levels of Shiga toxin 2e (Stx2e) detectable in the feces (day 1) to the onset of clinical disease (neurologic disturbances or death) was 5 days (range, 3–9). Bacterial colonization and titers of Stx2e in the ileum peaked at 4 days after inoculation in pigs without signs of clinical disease and at 6 days after inoculation in clinically affected pigs. Animals with the greatest risk of progressing to clinical disease tended to have the highest fecal toxin titers ($\geq$1 : 4096). Stx2e was detected in the red cell fraction from blood of some pigs showing clinical signs of edema disease but was not detected in the serum or cerebrospinal fluid.

Shiga toxin–producing *Escherichia coli* (STEC) cause diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) in humans. These strains of *E. coli* are often referred to as enterohemorrhagic *E. coli* (EHEC) because of their propensity to cause hemorrhage in the human intestinal tract. Infection is usually transmitted by the consumption of contaminated food or water or, secondarily, by person-to-person contact [1]. STEC produce several distinct Shiga toxins: Stx1, which is nearly identical to the toxin produced by *Shigella dysenteriae,* and Stx2 and its variants, Stx2c, Stx2d, and Stx2e. EHEC strains produce some combination of Stx1, Stx2, and/or Stx2c or Stx2d [2]. Strains of *E. coli* that cause edema disease of swine produce Stx2e [3, 4]. Shiga toxins are composed of an enzymatically active A subunit surrounded by a pentamer of B subunits that recognize specific glycolipid receptors [5, 6]. The A subunit enters the host cell and mediates cell death by the inhibition of protein synthesis at the ribosomal level [7]. Shiga toxins induce vascular damage [8–10], which can lead to the systemic complications of STEC infection: acute renal failure, hemolytic anemia, and thrombocytopenia in HUS and edema and vascular necrosis in edema disease of swine.

Different aspects of EHEC disease can be reproduced experimentally in rabbits, mice, neonatal calves, and gnotobiotic pigs [11–15]. STEC also cause naturally occurring dysentery in calves [16–19]. However, there is no evidence that the disease in calves involves toxemia or systemic vascular damage [15]. Idiopathic cutaneous and renal glomerular vasculopathy of greyhound dogs is thought to be caused by STEC infection [20, 21].

Edema disease is a naturally occurring disease of young pigs that involves systemic vascular damage as a result of intestinal infection with STEC that are host adapted to swine [22]. The hallmark of clinical edema disease is neurologic impairment and death. Gross lesions include edema of the eyelids, mesentery, and gastrointestinal tract [23, 24]. Necrosis of the arterioles in the intestinal tract and brain is a characteristic microscopic lesion [25, 26]. Like HUS, edema disease often has a prodromal phase of diarrhea. However, in edema disease, the diarrhea is due to heat-stable enterotoxin (STa and/or STb) and is only rarely associated with intestinal hemorrhage [24, 27]. Stx2e is not enterotoxigenic in pigs [28]. The *E. coli* strains that cause edema disease colonize the small intestines of pigs by fimbrial adhesion [29–32]. In contrast, EHEC strains are thought to colonize the colons of humans [33]. Furthermore, colonization by some EHEC is thought to be mediated, at least in part, by an attaching and effacing mechanism similar to that used by enteropathogenic *E. coli* [34–36]. However, EHEC strains that lack the attaching and effacing gene, *eae,* also cause hemorrhagic colitis and HUS in humans and presumably colonize by another mechanism(s) [37–39].

Although much is known about the pathogenesis of STEC

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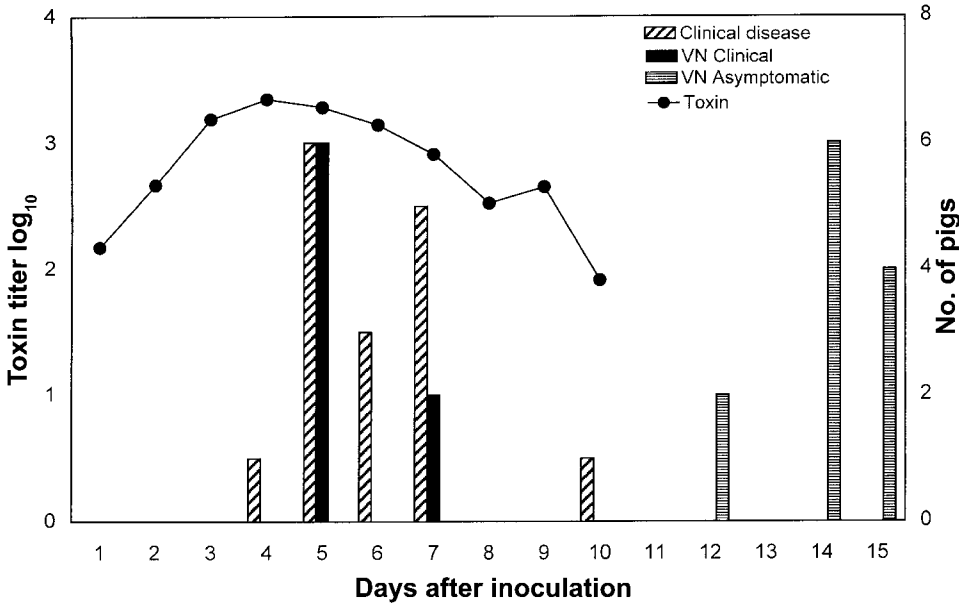

Figure 1. Clinical disease, vascular necrosis (VN), and geometric mean fecal Shiga toxin 2e titer (log_{10}) of pigs after inoculation with Shiga toxin–producing *Escherichia coli* strain S1191. Clinical, pigs ($n = 16$) that developed clinical edema disease; asymptomatic, pigs ($n = 34$) that did not develop clinical edema disease.

infections, the temporal and quantitative relationships between intestinal colonization, toxin production, toxemia, and the clinical manifestations of toxemia due to Shiga toxin have not been adequately defined. In this study, we used edema disease to define stages in the pathogenesis of STEC disease. We determined the times from inoculation to the peak of intestinal colonization with STEC and to the peak of Stx2e titers in the intestinal content and feces. We also sought to determine whether Stx2e titers in the feces and blood were predictive of systemic disease and the temporal and quantitative relationships between toxin production and systemic disease.

Materials and Methods

Bacterial strains. *E. coli* S1191 (serogroup O139) was first isolated from a clinical case of edema disease [3]. This strain is hemolytic on sheep blood agar (SBA) and produces Stx2e, heat-stable enterotoxin STb, and F18 fimbriae, which mediate attachment to the porcine small intestine [29–32]. S1191 is resistant to chloramphenicol. *E. coli* 123 (serogroup O43) was isolated from a healthy pig and is not pathogenic. Inocula were prepared as described elsewhere [40].

Experimental design. Three-week-old pigs (weaned at 14 days of age, all from the same commercial herd) were randomly assigned to groups of controls or principals. Pigs were acclimated to the diet of 21% protein for 4 days before challenge [41]. Rooms were kept at 21°C, and heat lamps were available. Pigs were weighed at −1, 7, and 14 days after inoculation. Rectal swabs were collected before inoculation and at days 2 and 4 after inoculation. The principals (a total of 50 from 3 replicates of the experiment) were orally inoculated with 10^{10} cfu of strain S1191 in a gelatin capsule, and controls ($n = 34$) were inoculated with 10^{10} cfu of strain 123.

Individual fecal samples were collected daily from all principals and 3 controls on days 1–10 after inoculation and assayed for free fecal Stx2e. Pigs were observed at least twice daily for signs of clinical edema disease and were killed and necropsied when advanced neurologic signs were detected. Randomly selected principal pigs without signs of clinical edema disease were killed and necropsied on days 2, 4, 6, 8, 10, 12, and 14 after inoculation (3/day on days 2–10 and 2/day on days 12–14). Randomly selected control pigs were killed and necropsied on days 1, 5, and 13 after inoculation (3/day on days 1 and 5 and 8/day on day 13). Blood samples (serum or whole blood with EDTA) were collected from some pigs before death. At necropsy, 5-cm sections of ileum and colon were collected for bacteriologic counts. Contents from ileum, colon, and rectum (feces) were collected for toxin assay. Tissue samples from ileum, brain stem, colon, and kidney were collected for histologic examination. Attempts to collect cerebrospinal fluid from clinically affected principals and from controls at necropsy yielded acceptable (blood-free) samples for toxin assay from 5 principals and 2 controls.

Pigs that died were also necropsied, and (except for 1 pig) tissue samples were collected for histologic examination. However, because of the potential for postmortem bacterial proliferation and toxin production, samples for bacteriology and toxin assay were not collected from pigs found dead. At the end of the experiment, 14–15 days after inoculation, the remaining principals were killed and necropsied, and their tissues were examined histologically.

Bacteriology. Intestinal samples were immediately placed on ice and processed within 2 h of collection. Samples for quantitative bacteriology were diluted in 20 mL of PBS and processed in a

Table 1. Titers of Shiga toxin 2e in feces of selected pigs after inoculation with Shiga toxin–producing *Escherichia coli* S1191.

Pig	Status	Vascular necrosis	Titer[a] at day after inoculation									
			1	2	3	4	5	6	7	8	9	10
727	Asymptomatic	+	NT	128	131,072	8192	65,536	131,072	131,072	32,768	8192	128
635	Asymptomatic	−	128	64	128	512	256	128	NT	512	64	64
730	Asymptomatic	+	128	512	4096	2048	16,384	NT	1024	1024	128	128
631	Clinical	−	128	2048	4096	4096[b]						
621	Clinical	−	256	8192	16,384	1024	4096[b]					
616	Clinical	+	64	512	2048	512	256	131,072	131,072[b]			

NOTE. Aymptomatic, pigs that did not develop clinical edema disease; clinical, pigs that developed clinical edema disease; NT, not tested.

[a] Reciprocal of toxin titer.

[b] Died or killed after developing signs of edema disease.

Stomacher laboratory blender (Seward Medical, London, UK) for 1 min. Samples were serially diluted 10-fold in PBS, inoculated onto SBA containing chloramphenicol (30 μg/mL), and incubated at 37°C overnight. Pigs were tested for preexisting infection with Stx2e$^+$ *E. coli* as follows. Rectal swabs from pigs at day −1 after inoculation were inoculated onto SBA without antibiotics. Hemolytic *E. coli* recovered were tested for the presence of genes encoding Stx2, by use of a multiplex polymerase chain reaction assay [42]. Rectal swabs from principals after challenge were inoculated onto SBA with chloramphenicol, to confirm that pigs were colonized by S1191.

Assay for Stx2e. Assays for Stx2e were done on monolayers of Vero cells as previously described [43, 44]. All samples were stored at 4°C until assayed. Pilot studies indicated that storage at 4°C or at freezing did not affect Stx2e titers of fecal or intestinal samples (unpublished data). Fecal and intestinal samples were processed by a modification of the method of Karmali et al. [45]. Samples were centrifuged at 7600 *g* for 10 min, and the supernatant was removed and centrifuged at 16,000 *g* for 10 min in a microfuge and then sterilized through a 0.22-μm filter. PBS (1 vol/1 vol feces) was added after the first centrifugation step if the sample did not contain 2 mL of supernatant. The sample was mixed well and recentrifuged twice. Intestinal and fecal samples were initially diluted 1 : 32, because preliminary studies indicated that there was nonspecific toxicity to Vero cells at lower dilutions in normal pig feces. Blood samples were centrifuged at 1500 *g* for 10 min. The plasma and buffy coat were removed, and the whole red blood cells were used in the assay. Preliminary in vitro studies, in which purified Stx2e was added to whole blood, indicated that the majority of the Stx2e was detected in the red blood cell fraction (unpublished data). Twofold dilutions of red blood cells were made in Hanks' balanced salt solution without Ca or Mg rather than in tissue culture medium. Blood samples were considered positive if the titer was >1 : 8, because there was nonspecific Vero cell toxicity at lower dilutions in the blood of a few normal pigs. Serum and cerebrospinal fluid were also assayed for Stx2e. All positive blood samples and at least 1 positive intestinal sample from each pig were neutralized with bovine polyclonal antibody that neutralizes Stx2 and Stx2e [46]. The intestinal samples were diluted to titers of ~1 : 100 before neutralization. Fetal calf serum was used as an antibody-negative neutralization control. Samples were considered neutralized and their Vero toxic activity to be due to Stx2e if the polyclonal antibody reduced the toxin titer by ⩾4-fold [47].

Histopathology. Samples were fixed in 10% formalin, processed by standard procedures, and stained with hematoxylin-eosin [25]. The slides were numerically coded and examined with a light microscope by 2 investigators who were not aware of the challenge strain or toxin levels for the pigs under examination.

Statistics. The weight gain of principal and control pigs was compared by use of a one-way analysis of variance. Pigs that died or were killed before day 7 were not included in the weight analysis. Average toxin titers were calculated as the geometric means of the reciprocal value. Differences in intestinal toxin titers among different tissue sites were compared by one-way analysis of variance (mixed model). Fecal toxin titers were analyzed with repeated-measures analysis of variance for days 1–5 after inoculation. Statistical analysis was not done on fecal toxin titers from days 6–10 after inoculation because of the small number of clinical pigs still surviving. Average colony-forming units of bacteria were calculated as the geometric means.

Results

Clinical signs. Nine of the 50 principal pigs exhibited ataxia and circling or became moribund and were killed. An additional 7 principal pigs did not exhibit neurologic signs during observation but were found dead. Edema disease (characterized by the presence of gross edema, fibrinous exudates, or microscopic vascular lesions) was determined to be the cause of neurologic signs or death in all 16 of these animals. Clinical edema disease (signs or death) occurred 4–10 days (mean, 6) after inoculation (figure 1). None of the control pigs exhibited signs of edema disease or died. Mild diarrhea of unknown cause occurred in some principal and control pigs before inoculation and sporadically in a few controls after challenge. Diarrhea occurred consistently in the principal pigs on days 2–8 after inoculation. The mean weight (±SD) of principals with clinical disease that survived to 7 days after inoculation tended to be less (5.3 ± 0.3 kg) than that of asymptomatic principals (no neurologic signs or edema; 6.2 ± 1.1 kg) and controls (6.3 ± 1.0 kg). The mean weights of asymptomatic principals and controls were not significantly different at 7 or 14 days after inoculation.

Microscopic vascular lesions. Vascular lesions were detected

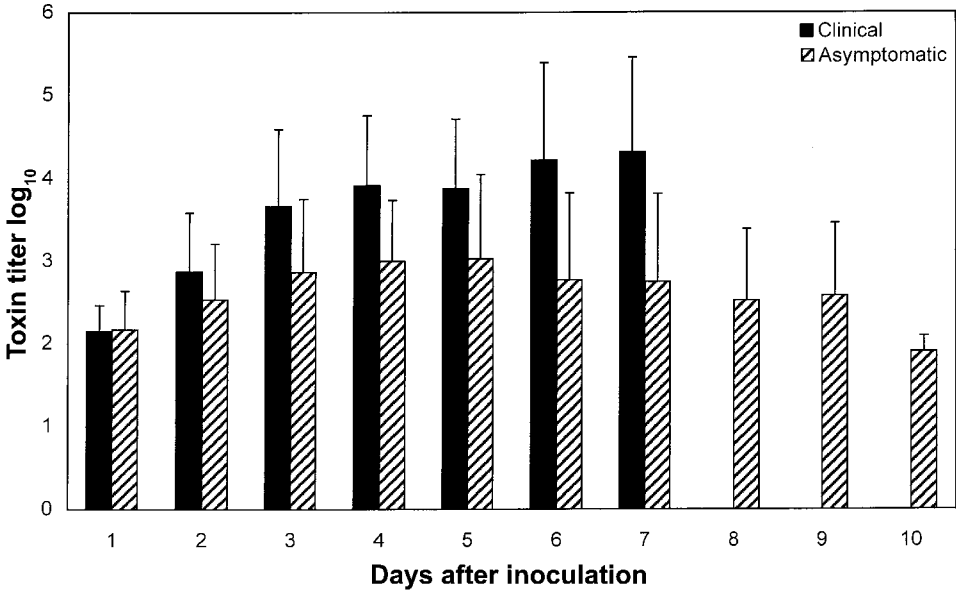

Figure 2. Geometric mean Stx2e titers (log_{10}) in feces of pigs inoculated with Shiga toxin–producing *Escherichia coli* strain S1191. Clinical, pigs ($n = 16$) that developed clinical edema disease; asymptomatic, pigs ($n = 34$) that did not develop clinical edema disease. Bars indicate SEs. Slope of mean fecal toxin titer for clinical pigs 1–5 days after inoculation was greater then that for asymptomatic pigs ($P < .05$). Data were analyzed by repeated-measures analysis of variance for days 1–5 after inoculation. Data from days 6–10 were not analyzed for statistical significance, because too few pigs remained in the clinical group.

in 8 of the 15 principals with clinical disease that were examined and in 12 of 34 asymptomatic principals. The lesions were detected in the brain, ileum, and colon but not in the kidney. The earliest detection of vascular necrosis was in clinical pigs at 5 days after inoculation and in randomly selected asymptomatic principals at 12 days after inoculation (figure 1). None of 14 control pigs necropsied had vascular lesions.

Fecal toxin titers. Stx2e was not detected in the feces of control pigs. Stx2e was detected in the feces of all principals on day 1 after inoculation. Thus, the mean time from production of Stx2e in intestine to the onset of clinical edema disease was 5 days (range, 3–9; figure 1). The range of fecal toxin titers varied widely both in pigs that developed and in those that did not develop clinical signs (table 1). For example, pig 727 attained fecal toxin titers of 1 : 131,072 for several days but did not exhibit any clinical signs of edema disease. In contrast, the peak fecal toxin titer of pig 631 was 1 : 4096 on day 3 after inoculation, and the pig was found dead from edema disease the following day (table 1). None of the 16 principals that attained maximal fecal toxin titers <1 : 4096 progressed to clinical disease. However, of 34 animals that attained fecal toxin titers ⩾1 : 4096, 16 (47%) progressed to clinical disease and 18 did not. The slope of the mean fecal toxin titer for principals with clinical disease was significantly greater than that for asymptomatic principals on days 1–5 after inoculation ($P <$ .05; figure 2). The mean fecal toxin titer of asymptomatic principal pigs that developed vascular necrosis was not significantly higher than that of the asymptomatic pigs without lesions.

Bacterial colonization and toxin titers in the intestine. $Stx2e^+$ *E. coli* were not detected in any of the pigs before inoculation. All the principals shed the inoculum strain in their feces 2–4 days after inoculation. Control pigs did not have detectable levels of $Stx2e^+$ *E. coli* in their intestinal tracts after inoculation. In randomly selected asymptomatic principals, the peak of both bacterial colonization and titer of Stx2e in the ileum occurred at 4 days after inoculation and fell sharply thereafter (figure 3). On this day, the geometric mean bacterial count of strain S1191 in the ileum was 1.6×10^8 cfu/cm, and the geometric mean toxin titer was 1 : 16,384. In the 9 pigs that were killed with clinical signs of edema disease, the greatest intensity of bacterial colonization and highest titer of Stx2e occurred at 6 days after inoculation. The geometric mean density of bacteria in these 3 pigs was 2.4×10^8 cfu/cm, and the geometric mean titer of Stx2e was 1 : 32,768. The toxin titers of the colon and rectal content from randomly selected asymptomatic pigs followed a course similar to that seen in the ileum (figure 4). During the early stage of the disease (days 2–4), the titer of Stx2e in the ileal content was significantly higher than that in the colon or rectal content ($P < .01$).

Titer of Stx2e in relation to the density of colonization by strain S1191. Although the titer of Stx2e in the ileum tended to increase with the number of STEC, there was not an absolute correlation between toxin titer and bacterial density (figure 5). The majority of animals (14/15) that had Stx2e titers ⩾1 : 4096 in their intestinal contents also had densities $>10^6$ cfu/cm of ileum. However, the individual animals that had the highest

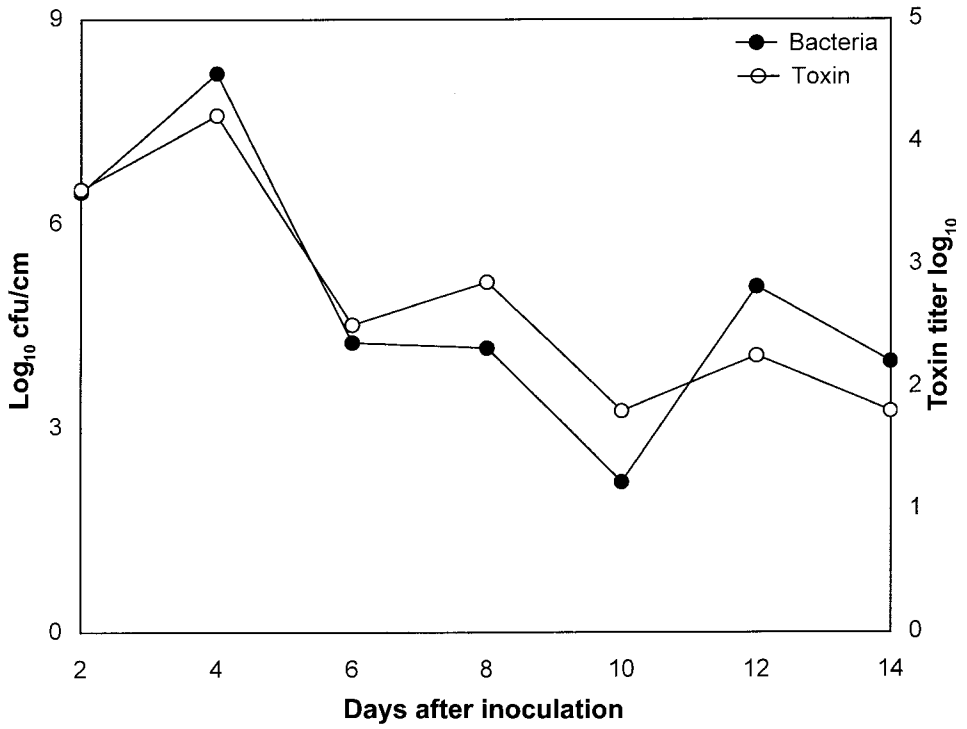

Figure 3. Geometric mean colony-forming units (log_{10}) of Shiga toxin–producing *Escherichia coli* strain S1191 and geometric mean Shiga toxin 2e titers (log_{10}) in ilea of randomly selected asymptomatic pigs (3 pigs/day on days 2–10 and 2 pigs/day on days 12–14).

titers of Stx2e did not have the greatest density of bacterial colonization. A high ileal toxin titer (≥1 : 8192) and a high density of bacterial colonization (≥10^6 cfu/cm) of the ileum occurred in most (8/9) of the clinically affected animals examined. The only clinical pig that had a relatively low ileal toxin titer and bacterial density at the time of necropsy (figure 5) attained a peak fecal toxin titer of 1 : 8192 at 4 days before necropsy.

Bacterial layers, characterized by aggregates of basophilic rod-shaped bacteria in immediate apposition to the epithelial brush border, were detected in the ileal sections of 8 of 34 asymptomatic principals and 6 of 15 clinical pigs. The mean bacterial count of strain S1191 in the ileum of these pigs with bacterial layers was 2.3×10^6 cfu/cm (range, <500 to 1×10^{10}). The bacterial layers were detected as early as 2 days and as late as 12 days (mean, 6) after inoculation in asymptomatic principals and 5–7 days (mean, 6) after inoculation in clinical pigs. Six of the 8 asymptomatic principals with adherent bacterial layers attained peak fecal toxin titers ≥1 : 8192. One of those 6 attained fecal toxin titers 1 : 131,072 on day 3 and remained clinically normal (pig 727; table 1). The mean bacterial count of strain S1191 in the ilea of pigs without adherent layers was 2.9×10^5 cfu/cm (range, <50 to 7.7×10^8). Bacterial layers were not detected in sections of colon. None of the 14 control pigs necropsied had detectable adherent bacteria in their intestinal sections.

Stx2e in blood and cerebrospinal fluid. Blood samples were obtained from 25 principal pigs, 7 of which were exhibiting clinical signs of edema disease at the time the blood was collected (before death). Stx2e was detected in the red cell fraction of blood from 5 of 7 animals with clinical edema disease (table 2). The mean titer of Stx2e in the blood of these 5 animals was 1 : 64 (range, 1 : 16–1 : 512). The ileal and peak fecal toxin titers of these 5 animals ranged from 1 : 8192 to 1 : 65,536 and from 1 : 16,384 to 1 : 131,072, respectively. The ileal and peak fecal toxin titers of the 2 clinical pigs that did not have detectable Stx2e in blood ranged from 1 : 64 to 1 : 32,768 and from 1 : 8192 to 1 : 16,384, respectively. The blood from the 18 asymptomatic pigs and 8 controls examined did not have detectable toxin. The cerebrospinal fluid samples from the 5 clinical and 2 control pigs examined were also negative. All the serum samples from the 7 clinical, 18 asymptomatic principal, and 8 control pigs examined were negative for Stx2e. Serum from 1 control animal was toxic to Vero cells, but that activity was not neutralized by bovine antibody to Stx2.

Serum samples collected at the end of the experiment were also assayed for Stx2e-neutralizing antibodies [44]. The geometric mean neutralizing titers for control pigs and asymptomatic principal pigs were 1 : 4 and 1 : 5, respectively.

In an experiment similar to that reported here but conducted as part of a different study, we detected Stx2e in the red cell

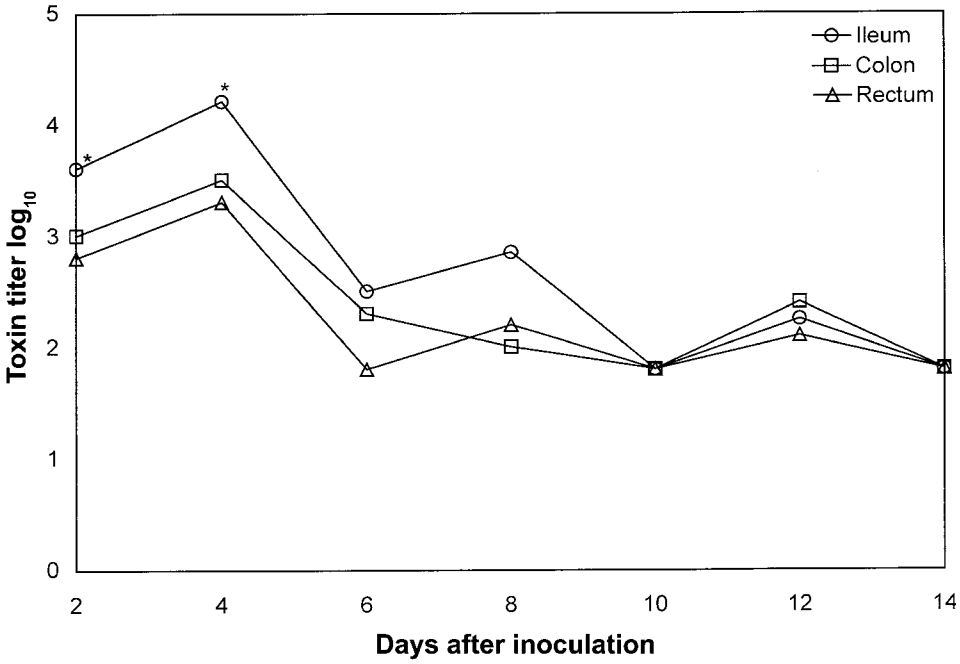

Figure 4. Geometric mean titers of Shiga toxin 2e (log_{10}) from ileal, colonic, and rectal contents of randomly selected asymptomatic pigs inoculated with Shiga toxin–producing *Escherichia coli* strain S1191 (3 pigs/day on days 2–10 and 2 pigs/day on days 12–14). *Titers were significantly greater in ileum than in colon or rectum on days 2 and 4 after inoculation ($P < .01$); data were analyzed by mixed-model analysis of variance.

fraction from the blood of 5 of 8 clinical and 2 of 12 asymptomatic pigs infected with strain S1191 (unpublished data).

Discussion

The reason(s) for the variable latent period of several days between the production of Stx2e in the intestine and the onset of systemic disease manifested as clinical signs or vascular necrosis (figure 1) is not known. Because the delay between intravenous administration of purified Stx2e and the onset of systemic disease in pigs is only 7–28 h [22], it seems likely that much of the latent period reflects the time required to attain critical levels of toxin in blood and/or vascular endothelium. Presumably, the time required to attain critical systemic levels of toxin is a function of the amount produced and the rate of transport across intestinal epithelium into blood [48, 49]. The onset of vascular necrosis in asymptomatic principals at 12 days after inoculation, well after the peak of toxin production (figures 1 and 3), leads us to speculate that Stx2e may act accumulatively during STEC infections. Whatever the mechanism(s) involved in the delay between the onset of toxin production and clinical disease, the occurrence of the delay suggests use of the edema disease model to determine if systemic disease can be prevented by intervention with antibody [50] or other anti–Shiga toxin therapy during the latent period.

The data indicate that fecal toxin titers were reflective of intestinal toxin titers (figure 4). Fecal toxin titers of clinical pigs as a group were significantly greater than those of asymptomatic principals (figure 2). Furthermore, none of the pigs that attained fecal toxin titers <1 : 4096 progressed to clinical disease, but nearly 50% of those that did attain titers ⩾1 : 4096 developed clinical disease. However, the range of toxin titers

Table 2. Titers of Shiga toxin 2e at time of death in ilea and blood of pigs inoculated with Shiga toxin–producing *Escherichia coli* S1191.

	Titer[a] in ileum			Titer[a] in blood		
Status	Range	Mean	No. positive/no. tested	Range	Mean	No. positive/no. tested
Clinical	64–65,536	16,384	7/7	16–512	64	5/7
Asymptomatic	64–16,384	709	17/17		ND	0/18
Control		ND	0/8		ND	0/8

NOTE. Clinical, pigs that developed clinical edema disease; asymptomatic, pigs that did not develop clinical edema disease; ND, none detected.

[a] Reciprocal of toxin titer.

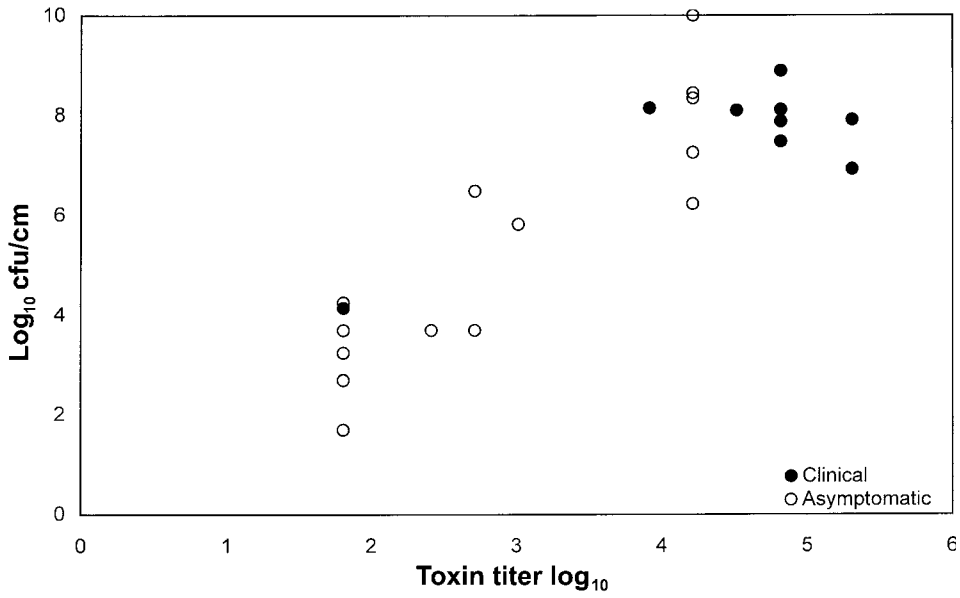

Figure 5. Titer of Shiga toxin 2e (log_{10}) relative to density of bacterial colonization (log_{10}) in ilea and occurrence of clinical edema disease in pigs inoculated with Shiga toxin–producing *Escherichia coli* strain S1191.

in individual pigs varied widely both in clinical and in asymptomatic principals (figure 2, table 1). Thus, fecal toxin titers tended to correlate with the relative risk of clinical disease for a group of pigs but (because of variable susceptibility of individual pigs to intestinal toxin) were not predictive for individual pigs. These data are consistent with the notion that the risk of clinical disease during human STEC infections increases with the level of intestinal toxin [51] but that intestinal toxin levels are not predictive of clinical outcome for individual patients [45, 52].

In general, the density of STEC colonization in the intestine was reflected by the titer of Stx2e in the gut contents. However, the individual animals with the highest titers of Stx2e did not have the greatest density of STEC colonization. One possible explanation for these results is that toxin production by strain S1191 is inducible in vivo and is stimulated by attachment to the intestinal wall or by interaction with metabolites in the intestinal lumens of some animals. We do not have any additional evidence for this hypothesis. In vitro–grown cultures of S1191 routinely produce an Stx2e titer of 1 : 1000 in culture supernatant, but sonic lysates of these same cultures have titers that are 100-fold higher [3]. Some of the pigs in this study had intestinal Stx2e titers similar to the titers of sonic lysates of S1191. Some EHEC strains produce a variant of Stx2 (Stx2d) that is activated by mouse or human intestinal mucus [53]. In the presence of this activator, toxin titers are increased 100-fold per cfu of bacteria. Stx2e produced by strain S1191 was not activated in vitro by mouse or human mucus, but porcine mucus was not tested [53]. However, the cytotoxicity of purified Stx2e [28] and the culture supernatant of strain S1191 (unpublished data) was not altered by incubation with intestinal fluid from pigs. The fact that Stx2e titers were higher in ileum than in colon or rectum suggests that small intestine may be the major site of Shiga toxin production in this model of STEC infection. The small intestine is the site of adhesion to intestinal epithelium (mediated by F18 fimbriae) during colonization by edema disease–causing strains of STEC [23, 27, 29–32]. We speculate further that STEC adherent to intestinal epithelium may be in a niche that enhances production, release, or activation of Shiga toxin. In contrast to the fimbria-mediated ileal colonization by STEC in edema disease, many (but not all) EHEC apparently colonize human colon, at least in part, via the attaching/effacing mechanism. Such differences in site and attachment mechanism among bacterial species may cause species differences in production, release, activation, or absorption of Shiga toxin. Therefore, results from edema disease may not be directly applicable to hemorrhagic colitis and HUS.

Early fecal samples greatly increase the recovery of *E. coli* O157:H7, a serotype of STEC frequently associated with human EHEC infections [54–57]. The data from the study reported here also emphasize the importance of fecal samples collected early in the disease. By 6 days after inoculation, the density of STEC in the ilea of randomly selected asymptomatic pigs had dropped to 10^4 cfu/cm, and toxin titers in the intestinal content were <1 : 500 (figure 3). Even in pigs with clinical signs, the mean bacterial count in the ileum dropped by 2 orders of magnitude, and the mean toxin titer dropped from 1 : 32,768 to 1 : 9506 between days 6 and 7. The delay in obtaining fecal samples may be one explanation for the comparatively low fecal

toxin titers reported from patients with EHEC infections [45, 52].

Some asymptomatic pigs had fecal toxin titers >10-fold higher (1 : 131,072) than the apparent minimum threshold level (1 : 4096) required for clinical disease (table 1). It may be that not all pigs transport an equal percentage of free toxin from the intestine into the blood. Previous studies indicate that purified Stx2e given intraintestinally does not cause clinical edema disease. However, when sodium deoxycholate is given to increase intestinal permeability, intraintestinal Stx2e causes clinical edema disease [49]. Alternatively, differences in the availability of receptors on erythrocytes and/or blood vessels may explain the variability among pigs in response to intestinal toxin loads [5]. We cannot exclude the possibility that variation in preexisting Stx2e antibody levels contributed to the variable susceptibility of pigs to clinical disease. However, we think that this is unlikely, because, in our experience [25, 41, 44], weanling pigs do not have detectable levels of serum Stx2e antitoxin before vaccination or challenge with STEC. Furthermore, assays of serum from the pigs used in the work reported here indicate little or no preexisting Stx2e antitoxin. Adherent layers of bacteria were demonstrated in the ilea of some but not all principals. It seems unlikely that this contributed to the variable susceptibility of pigs to toxin, for several reasons: first, adherent bacteria probably occurred segmentally and at varying times during the disease [23]. Adherent layers of STEC probably occurred at some site and time in most principals; otherwise, the STEC could not have colonized intensively enough to attain high toxin titers. Next, 6 of the 8 asymptomatic principals in which adherent layers of bacteria were found attained fecal toxin titers $\geq$1 : 8192, and 1 of those 6 attained a fecal toxin titer of 1 : 131,072 on 3 separate days. Finally, in a pilot experiment, we found that all 4 asymptomatic principals tested 17 days after inoculation with strain S1191 had ileal epithelial cell brush borders receptive to adhesion by $F18^+$ *E. coli* in vitro (unpublished data).

To our knowledge, this is the first report of Stx2e being detected in the red cell fraction of blood from individual STEC-infected animals. This provides further evidence that Stx2e contributes to the systemic damage seen in clinical edema disease [22]. It also supports the concept that Stx2e is transported from the gut, by the blood, to the site of tissue damage [5, 22, 49]. Previous work has demonstrated that Stx2e binds to pig erythrocytes and that the tissue distribution of intravenously administered toxin is dependent on the blood flow to particular sites [5, 58]. The fact that we detected Stx2e in the red blood cell fraction but not in the serum is consistent with the evidence that Stx2e binds to erythrocytes. Presumably, the Stx2e detected was a portion of that in equilibrium with the receptors on erythrocytes. In contrast to our results with edema disease, cytotoxic activity (presumed to be Shiga toxin) was detected in the serum of gnotobiotic pigs infected with EHEC strains [59, 60]. Concentrated serum samples from mice infected with EHEC also contained detectable Shiga toxin [61]. Our inability to detect Stx2e in the blood of most asymptomatic infected pigs that had high titers of Stx2e in their intestines may indicate that the toxin had not yet entered into the blood of these animals. Alternatively, erythrocyte receptors in most asymptomatic infected pigs may not have been sufficiently saturated to release detectable levels of toxin. In vitro studies with fluorescent-labeled Shiga toxin demonstrate that human erythrocytes also bind Shiga toxin, although the degree of binding varies considerably among donors of different blood group antigen types [62]. This suggests that variations in the toxin binding capacity of erythrocytes may cause variations in clinical outcome in edema disease, as has been suggested by some but not all studies of human STEC infections [62–66].

In conclusion, this study demonstrated a delay of several days from the production of Stx2e to the onset of clinical disease. It also demonstrated that fecal toxin titers could be used to identify a population of pigs that were at greatest risk for developing clinical disease after STEC infection. However, such titers were not predictive for individual animals. Stx2e was detected in the peripheral blood of pigs with clinical disease. This provides further evidence that Stx2e is transported, by blood, from the gut to sites of systemic tissue damage.

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References

1. Griffin PM, Tauxe RV. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli,* and the associated hemolytic uremic syndrome. Epidemiol Rev **1991**;13:60–98.
2. Melton-Celsa AR, O'Brien AD. Structure, biology, and relative toxicity of Shiga toxin family members for cells and animals. In: Kaper JB, O'Brien AD, eds. *Escherichia coli* O157:H7 and other Shiga toxin–producing *E. coli* strains. Washington DC: American Society for Microbiology, **1998**: 121–8.
3. Marques LRM, Peiris JSM, Cryz SJ, O'Brien AD. *Escherichia coli* strains isolated from pigs with edema disease produce a variant of Shiga-like toxin II. FEMS Microbiol Lett **1987**;44:33–8.
4. Weinstein DL, Jackson MP, Samuel JE, Holmes RK, O'Brien AD. Cloning and sequencing of a Shiga-like toxin type II variant from an *Escherichia coli* strain responsible for edema disease of swine. J Bacteriol **1988**;170: 4223–30.
5. Boyd B, Tyrrell G, Maloney M, Gyles C, Brunton J, Lingwood C. Alteration of the glycolipid binding specificity of the pig edema toxin from globotetraosyl to globotriaosyl ceramide alters in vivo tissue targeting and results in a verotoxin I–like disease in pigs. J Exp Med **1993**;177:1745–53.
6. Samuel JE, Perera LP, Ward S, O'Brien AD, Ginsburg V, Krivan HC. Comparison of the glycolipid receptor specificities of Shiga-like toxin type II and Shiga-like toxin type II variants. Infect Immun **1990**;58:611–8.
7. O'Brien AD, Holmes RK. Shiga and Shiga-like toxins. Microbiol Rev **1987**;51:206–20.
8. Obrig TG, Louise CB, Lingwood CA, et al. Shiga toxin–endothelial cell interactions. In: Karmali MA, Goglio AG, eds. Recent advances in vero-

cytotoxin-producing *Escherichia coli* infections. Amsterdam: Elsevier, **1994**:317–24.
9. Kaplan BS. Clinical and pathophysiological aspects of the hemolytic uremic syndrome. In: Karmali M, Goglio A, eds. Recent advances in verocytotoxin-producing *Escherichia coli* infections. Amsterdam: Elsevier, **1994**: 301–4.
10. Tesh VL, Samuel JE, Perera LP, Sharefkin JB, O'Brien AD. Evaluation of the role of Shiga and Shiga-like toxin in mediating direct damage to human vascular endothelial cells. J Infect Dis **1991**; 164:344–52.
11. Dykstra SA, Moxley RA, Janke BH, Nelson EA, Francis DH. Clinical signs and lesions in gnotobiotic pigs inoculated with Shiga-like toxin I from *Escherichia coli*. Vet Pathol **1993**; 30:410–7.
12. Francis DH, Moxley RA, Andraos CY. Edema disease–like brain lesions in gnotobiotic piglets infected with *Escherichia coli* serotype O157:H7. Infect Immun **1989**; 57:1339–42.
13. Gyles CL. VT toxemia in animal models. In: Karmali MA, Goglio AG, eds. Recent advances in verocytotoxin-producing *Escherichia coli* infections. Amsterdam: Elsevier, **1994**:233–40.
14. Wadolkowski EA, Burris JA, O'Brien AD. Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7. Infect Immun **1990**; 58:2438–45.
15. Dean-Nystrom EA, Bosworth BT, Cray WCJ, Moon HW. Pathogenicity of *Escherichia coli* O157:H7 in the intestines of neonatal calves. Infect Immun **1997**; 65:1842–8.
16. Hall GA, Reynolds DJ, Chanter N, et al. Dysentery caused by *Escherichia coli* (S102-9) in calves: natural and experimental disease. Vet Pathol **1985**; 22:256–63.
17. Moxley RA, Francis DH. Natural and experimental infection with an attaching and effacing strain of *Escherichia coli* in calves. Infect Immun **1986**; 53:339–46.
18. Schoonderwoerd M, Clarke RC, van Dreumel AA, Rawluk SA. Colitis in calves: natural and experimental infection with a verotoxin-producing strain of *Escherichia coli* O111:NM. Can J Vet Res **1988**; 52:484–7.
19. Wray C, McLaren I, Pearson GR. Occurrence of attaching and effacing lesions in the small intestine of calves experimentally infected with bovine isolates of verocytotoxic *E. coli.* Vet Rec **1989**; 125:365–8.
20. Hertzke DM, Cowan LA, Schoning P, Fenwick BW. Glomerular ultrastructural lesions of idiopathic cutaneous and renal glomerular vasculopathy of greyhounds. Vet Pathol **1995**; 32:451–9.
21. Fenwick B, Cowan L. Canine model of hemolytic uremic syndrome. In: Kaper J, O'Brien AD, eds. *E. coli* O157:H7 and other Shiga toxin–producing *E. coli* strains. Washington, DC: American Society for Microbiology, **1998**: 268–77.
22. MacLeod DL, Gyles CL, Wilcock BP. Reproduction of edema disease of swine with purified Shiga-like toxin II variant. Vet Pathol **1991**; 28:66–73.
23. Bertschinger HU, Pohlenz J. Bacterial colonization and morphology of the intestine in porcine *Escherichia coli* enterotoxemia (edema disease). Vet Pathol **1983**; 20:99–110.
24. Smith HW, Halls S. The production of oedema disease and diarrhoea in weaned pigs by the oral administration of *Escherichia coli:* factors that influence the course of the experimental disease. J Med Microbiol **1968**; 1:45–59.
25. Kausche FM, Dean EA, Arp LH, Samuel JE, Moon HW. An experimental model for subclinical edema disease (*Escherichia coli* enterotoxemia) manifest as vascular necrosis in pigs. Am J Vet Res **1992**; 53:281–7.
26. Kurtz HJ, Bergeland ME, Barnes DM. Pathologic changes in edema disease of swine. Am J Vet Res **1969**; 30:791–806.
27. Bertschinger HU, Gyles CL. Oedema disease of pigs. In: Gyles C, ed. *Escherichia coli* in domestic animals and humans. Wallingford, UK: CAB International, **1994**:193–219.
28. MacLeod DL, Gyles CL, Valdivieso-Garcia A, Clarke RC. Physicochemical and biological properties of purified *Escherichia coli* Shiga-like toxin II variant. Infect Immun **1991**; 59:1300–6.
29. Bertschinger HU, Bachmann M, Mettler C, et al. Adhesive fimbriae produced in vivo by *Escherichia coli* O139:K12(B):H1 associated with enterotoxaemia in pigs. Vet Microbiol **1990**; 25:267–81.
30. Imberechts H, Wild P, Charlier G, De Greve H, Lintermans P, Pohl P. Characterization of F18 fimbrial genes *fedE* and *fedF* involved in adhesion and length of enterotoxemic *Escherichia coli* strain 107/86. Microb Pathog **1996**; 21:183–92.
31. Vogel P, Bertschinger HU, Stamm M, et al. Genes specifying receptors for F18 fimbriated *Escherichia coli,* causing edema disease and postweaning diarrhoea in pigs, map to chromosome 6. Anim Genet **1996**; 27:321–8.
32. Zuniga A, Yokoyama H, Albicker-Ripppinger P, Eggenberger E, Bertschinger HU. Reduced intestinal colonization with F18-positive enterotoxigenic *Escherichia coli* in weaned pigs fed chicken egg antibody against the fimbriae. FEMS Immunol Med Microbiol **1997**; 18:153–61.
33. Griffin PM, Olmstead LC, Petras RE. *Escherichia coli* O157:H7–associated colitis: a clinical and histological study of 11 cases. Gastroenterology **1990**; 99:142–9.
34. Donnenberg MS, Tzipori S, McKee ML, O'Brien AD, Alroy J, Kaper JB. The role of the *eae* gene of enterohemorrhagic *Escherichia coli* in intimate attachment in vitro and in a porcine model. J Clin Invest **1993**; 92:1418–24.
35. Francis DH, Collins JE, Duimstra JR. Infection of gnotobiotic pigs with an *Escherichia coli* O157:H7 strain associated with an outbreak of hemorrhagic colitis. Infect Immun **1986**; 51:953–6.
36. Tzipori S, Gunzer F, Donnenberg MS, De Montigny L, Kaper JB, Donohue-Rolfe A. The role of the *eaeA* gene in diarrhea and neurological complications in a gnotobiotic piglet model of enterohemorrhagic *Escherichia coli* infection. Infect Immun **1995**; 63:3621–7.
37. Willshaw GA, Scotland SM, Smith HR, Rowe B. Properties of verocytotoxin-producing *Escherichia coli* of human origin of O serogroups other than O157. J Infect Dis **1992**; 166:797–802.
38. Dytoc MT, Ismaili A, Philpott DJ, Soni R, Brunton JL, Sherman PM. Distinct binding properties of *eaeA*-negative verocytotoxin-producing *Escherichia coli* of serotype O113:H21. Infect Immun **1994**; 62:3494–505.
39. de Azavedo J, McWhirter E, Louie M, Brunton J. EAE-negative verotoxin-producing *Escherichia coli* associated with hemolytic uremic syndrome and hemorrhagic colitis. In: Karmali M, Goglio A, eds. Recent advances in verocytotoxin-producing *Escherichia coli* infections. Amsterdam: Elsevier, **1994**:265–8.
40. Sarmiento JI, Casey TA, Moon HW. Postweaning diarrhea in swine: experimental model of enterotoxigenic *Escherichia coli* infection. Am J Vet Res **1988**; 49:1154–9.
41. Bosworth BT, Samuel JE, Moon HW, O'Brien AD, Gordon VM, Whipp SC. Vaccination with genetically modified Shiga-like toxin IIe prevents edema disease in swine. Infect Immun **1996**; 64:55–60.
42. Bosworth BT, Casey TA. Identification of toxin and pilus genes in porcine *Escherichia coli* using polymerase chain reaction (PCR) with multiple primer pairs [abstract B-509]. In: Proceedings of the 97th annual meeting of the American Society for Microbiology (Miami). Washington, DC: ASM, **1997**.
43. Gentry MK, Dalrymple JM. Quantitative microtiter cytotoxicity assay for *Shigella* toxin. J Clin Microbiol **1980**; 12:361–6.
44. Gordon VM, Whipp SC, Moon HW, O'Brien AD, Samuel JE. An enzymatic mutant of Shiga-like toxin II variant is a vaccine candidate for edema disease of swine. Infect Immun **1992**; 60:485–90.
45. Karmali M, Petric M, Lim C, Fleming P, Arbus G, Lior H. The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. J Infect Dis **1985**; 151:775–82.
46. Tesh V, Burris J, Owens J, et al. Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. Infect Immun **1993**; 61: 3392–402.
47. Karch H, Meyer T, Russmann H, Heesemann J. Frequent loss of Shiga-like toxin genes in clinical isolates of *Escherichia coli* upon subcultivation. Infect Immun **1992**; 60:3464–7.

48. Acheson DWK, Moore R, De Breucker S, et al. Translocation of Shiga toxin across polarized intestinal cells in tissue culture. Infect Immun **1996**; 64: 3294–300.
49. Waddell TE, Gyles CL. Sodium deoxycholate facilitates systemic absorption of verotoxin 2e from pig intestine. Infect Immun **1995**; 63:4953–6.
50. MacLeod DL, Gyles CL. Immunization of pigs with a purified Shiga-like toxin II variant toxoid. Vet Microbiol **1991**; 29:309–18.
51. Tarr PI. *Escherichia coli* O157:H7: clinical, diagnostic, and epidemiological aspects of human infection. Clin Infect Dis **1995**; 20:1–10.
52. Ramotar K, Henderson E, Szumski R, Louie TJ. Impact of free verotoxin testing on epidemiology of diarrhea caused by verotoxin-producing *Escherichia coli*. J Clin Microbiol **1995**; 33:1114–20.
53. Melton-Celsa AR, Darnell SC, O'Brien AD. Activation of Shiga-like toxins by mouse and human intestinal mucus correlates with virulence of enterohemorrhagic *Escherichia coli* O91:H21 isolates in orally infected, streptomycin-treated mice. Infect Immun **1996**; 64:1569–76.
54. Griffin PM, Ostroff SM, Tauxe RV, et al. Illnesses associated with *Escherichia coli* O157:H7 infections. Ann Intern Med **1988**; 109:705–12.
55. Tarr PI, Neill MA, Clausen CR, Watkins SL, Christie DL, Hickman RO. *Escherichia coli* O157:H7 and the hemolytic uremic syndrome: importance of early cultures in establishing the etiology. J Infect Dis **1990**; 162:553–6.
56. Wells JG, Davis BR, Wachsmuth IK, et al. Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Escherichia coli* serotype. J Clin Microbiol **1983**; 18:512–20.
57. Spika JS, Parsons JE, Nordenberg D, Wells JG, Gunn RA, Blake PA. Hemolytic uremic syndrome and diarrhea associated with *Escherichia coli* O157:H7 in a day care center. J Pediatr **1986**; 109:287–91.
58. Waddell TE, Coomber BL, Gyles CL. Localization of potential binding sites for the edema disease verotoxin (VT2e) in pigs. Can J Vet Res **1998**; 62: 81–6.
59. Tzipori S, Karch H, Wachsmuth KI, et al. Role of a 60-megadalton plasmid and Shiga-like toxins in the pathogenesis of infection caused by enterohemorrhagic *Escherichia coli* O157:H7 in gnotobiotic piglets. Infect Immun **1987**; 55:3117–25.
60. Tzipori S, Wachsmuth KI, Smithers J, Jackson C. Studies in gnotobiotic piglets on non-O157:H7 *Escherichia coli* serotypes isolated from patients with hemorrhagic colitis. Gastroenterology **1988**; 94:590–7.
61. Kurioka T, Yunou Y, Kita E. Enhancement of susceptibility to Shiga toxin–producing *Escherichia coli* O157:H7 by protein calorie malnutrition in mice. Infect Immun **1998**; 66:1726–34.
62. Bitzan M, Richardson S, Huang C, Boyd B, Petric M, Karmali MA. Evidence that verotoxins (Shiga-like toxins) from *Escherichia coli* bind to P blood group antigens of human erythrocytes in vitro. Infect Immun **1994**; 62: 3337–47.
63. Akashi Y, Yoshizawa N, Oshima S, et al. Hemolytic uremic syndrome without hemolytic anemia: a case report. Clin Nephrol **1994**; 42:90–4.
64. Orr PH, Dong V, Schroeder ML, Ogborn MR. P1 blood group antigen expression and epidemic hemolytic uremic syndrome. Pediatr Nephrol **1995**; 9:612–3.
65. Robson WL, Leung AK, Bowen T, Brant R, Ching E. The P1 blood group and the severity of diarrhea-associated hemolytic uremic syndrome. Clin Nephrol **1994**; 42:288–90.
66. Taylor CM, Milford DV, Rose PE, Roy TCF, Rowe B. The expression of blood group P1 in post-enteropathic haemolytic uraemic syndrome. Pediatr Nephrol **1990**; 4:59–61.

Note added in proof. Subsequent review of the histologic slides revealed vascular lesions in an additional asymptomatic principal at 8 days postinoculation.