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## Differences in Virulence among *Escherichia coli* O157:H7 Strains Isolated from Humans during Disease Outbreaks and from Healthy Cattle<sup>∇</sup>

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***Escherichia coli* O157:H7 causes life-threatening outbreaks of diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome in humans and significant economic loss in agriculture and could be a potential agent of bioterrorism. Although the prevalence of *E. coli* O157:H7 in cattle and other species with which humans have frequent contact is high, human infections are relatively uncommon, despite a low infectious dose. A plausible explanation for the low disease incidence is the possibility that not all strains are virulent in humans. If there are substantial differences in virulence among strains in nature, then human disease may select for high virulence. We used a gnotobiotic piglet model to investigate the virulence of isolates from healthy cattle and from humans in disease outbreaks and to determine the correlation between production of Shiga toxin 1 (Stx1) and Stx2 and virulence. Overall, *E. coli* O157:H7 strains isolated from healthy cattle were less virulent in gnotobiotic piglets than strains isolated from humans during disease outbreaks. The amount of Stx2 produced by *E. coli* O157:H7 strains correlated with strain virulence as measured by a reduction in piglet survival and signs of central nervous system disease due to brain infarction. The amount of Stx1 produced in culture was not correlated with the length of time of piglet survival or with signs of central nervous system disease. We suggest that disease outbreaks select for producers of high levels of Stx2 among *E. coli* O157:H7 strains shed by animals and further suggest that Stx1 expression is unlikely to be significant in human outbreaks.**

*Escherichia coli* O157:H7 causes both outbreaks and sporadic cases of diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS). *E. coli* O157:H7 isolates produce several factors that are believed to contribute to virulence, including Shiga toxin 1 (Stx1), Stx2, and several proteins encoded in the locus of enterocyte effacement pathogenicity island (18, 31, 44). Stxs are A-B-type toxins that inhibit protein synthesis in affected cells, particularly endothelial cells in small blood vessels (8, 9, 37, 38). Following bacterial colonization of the intestine, the toxins are thought to enter the systemic circulation by crossing the intestinal mucosa and to be carried systemically on the surfaces of polymorphonuclear leukocytes (1, 2, 61). Stxs are then transferred to endothelial cells and cause damage with fibrin deposition in and thrombosis of arterioles and other small- to medium-sized blood vessels in certain target organs, including the kidney, colon, and brain, which result in infarcts in these organs (14, 43, 44, 51, 60, 66).

Based on studies in various animal models, Stxs also appear to be capable of causing direct damage to tissues other than the endothelium, including the villous epithelium in the small intestine (26), the renal tubular epithelium (42, 67), neurons (19), and myelinated nerves in the central nervous system

(CNS) (21). Most patients fully recover from bloody diarrhea, but some develop life-threatening diseases of the kidneys and CNS; approximately 15% of the cases in children result in HUS (60). With early implementation of aggressive supportive therapy, including dialysis, the risk of death due to renal failure has been reduced substantially. However, about one-third of HUS cases involve significant encephalopathy, and brain injury is the leading cause of death associated with acute HUS (47, 54).

*E. coli* O157:H7 infections are typically contracted through consumption of contaminated food or contact with contaminated water, animal feces, or infected animals. Several disease outbreaks have been associated with contaminated beef, suggesting that cattle are an important reservoir for human infection (5, 15). However, despite the high prevalence of *E. coli* O157:H7 in cattle herds (16, 29, 55) and the low infectious dose in humans (57, 63, 64), the incidence in humans is quite low.

The discrepancy between the present and historic incidence in humans and the prevalence of *E. coli* O157:H7 in cattle today and in beef products in the past suggest that there is significant variability among the typical strains harbored by animals and the strains isolated from severe human infections. Kim et al. reported that *E. coli* O157:H7 strains isolated from diseased humans were members of a different lineage than strains typically isolated from healthy cattle (27). Gally et al. compared 30 *E. coli* serotype O157 strains from human disease cases in Scotland to a similar number of strains isolated from

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asymptomatic Scottish cattle for production of hemolysin, EspP, Tir, and EspD (32, 33, 49, 50). While few genotypic differences were observed among the strains from the two sources, strains isolated from infected humans produced significantly more EspD and Tir than strains isolated from cattle. Ritchie et al. compared *E. coli* O157:H7 strains isolated from sporadic cases of HUS with isolates obtained from cattle pastures and found that both basal and mitomycin C-induced Stx2 production by HUS-associated *E. coli* O157:H7 was significantly greater than production by bovine isolates (46).

We have reported that oral challenge of 1-day-old gnotobiotic pigs with *E. coli* O157:H7 strains expressing both Stx1 and Stx2 or only Stx2 and not Stx1 results in signs of CNS disease, vascular lesions, hemorrhage, and infarcts in the brain (18). Other workers reported similar observations (13, 65). Administration of anti-Stx2 serum protects gnotobiotic piglets inoculated with Stx2<sup>+</sup> *E. coli* O157:H7 against the development of brain vascular lesions, associated brain infarcts, and CNS clinical signs, providing evidence that the lesions are directly or at least indirectly caused by Stx2 (10, 12).

More recent studies have further shown that myelin degeneration occurs in the brain and that glomerular, arteriolar, and tubular lesions occur in the kidneys of gnotobiotic piglets inoculated with Stx2<sup>+</sup> *E. coli* O157:H7 (21, 42). In the kidneys, thrombotic microangiopathy (TMA), which is the morphological hallmark of HUS in humans, was consistently seen, and small preglomerular arterioles were demonstrated to express Gb3/CD77 (21, 42). Larger arteries and glomerular capillary endothelial cells were not found to express Gb3/CD77 (21). In these two studies, despite developing renal lesions, the piglets did not develop clinical signs of HUS (21, 42). In the one study in which they were investigated, clinical pathology laboratory parameters of HUS (uremia, hemolytic anemia, and thrombocytopenia) also were not detected (21); however, all of the piglets developed signs of CNS dysfunction, and five of six animals developed cerebellar microhemorrhages, lesions which were attributable to Stx-induced damage (21). In addition, the arachnoidea and endothelial cells of small arteries in the CNS expressed Gb3/CD77 (21). In the study of Pohlenz et al. (42), a retrospective histological examination of formalin-fixed kidneys from gnotobiotic piglets inoculated with different serotypes of Stx-producing *E. coli* was conducted. In addition to finding TMA and other vascular system-based lesions, as previously reported by Gunzer et al. (21), Pohlenz et al. (42) found that piglets frequently developed renal tubular cell apoptosis and necrosis. The sites of the renal tubular lesions matched the sites where Stx2 binding and Gb3/CD77 expression were identified immunohistochemically (21). Gb3/CD77 and Stx2 binding were also demonstrated in cells of the glomeruli and arteriolar endothelium (21). Piglets inoculated with other serotypes of *E. coli* that expressed variants of Stx2 (Stx2c, Stx2d, Stx2d1, and Stx2d2) had renal lesions similar to those caused by Stx2<sup>+</sup> *E. coli* O157:H7 (42).

In a previous study, we found that parenteral injection of Stx1 into gnotobiotic piglets results in the same vascular and ischemic lesions that are caused by Stx2<sup>+</sup> *E. coli* O157:H7. Thus, the differences between the two toxins in infectious disease appear to be due to relative toxicity (14). The vascular lesions in gnotobiotic pigs are essentially identical to those reported in human cases (65), suggesting that this model is

relevant to the study of human *E. coli* O157:H7 disease (17). Furthermore, in humans, as in the gnotobiotic pig model, Stx2 is more frequently associated with extraintestinal disease, including HUS and CNS disease, than Stx1 is (40, 68). Purified Stx2 is 1,000 times more toxic than Stx1 to human renal endothelial cells (30) and causes severe renal damage in mice at a lower dose than Stx1 (62). Administration of Stx2 to baboons also causes HUS, while comparable concentrations of Stx1 do not (53). Thus, substantial evidence has been presented that Stx2 is a major contributor to sequelae associated with *E. coli* O157:H7 infection.

Based on findings from previous studies, we hypothesized that *E. coli* O157:H7 isolates from cattle feces are, on average, less virulent than isolates from human patients with HUS. The main objective of the present study was to test this hypothesis using gnotobiotic piglets. A second objective was to determine the extent to which expression levels of Stx1 and/or Stx2 correlate with differences in virulence. A third objective was to test the hypothesis that CNS lesions but not renal lesions are correlated with the clinical demise of piglets.

#### MATERIALS AND METHODS

**Bacterial strains.** Ten *E. coli* O157:H7 strains originally isolated from human disease outbreaks were obtained from Timothy J. Barrett of the Centers for Diseases Control and Prevention in Atlanta, GA. Ten other *E. coli* O157:H7 strains originally isolated from healthy dairy cattle in the National Dairy Heifer Survey conducted by the National Animal Health Monitoring System of the U.S. Department of Agriculture were obtained from William Cray, Jr., of the National Animal Disease Center in Ames, IA (Table 1) (4). Strain EDL933, which is highly virulent in 1-day-old gnotobiotic piglets, was used as a positive control (18). All strains were shown by PCR to possess the *stx*<sub>1</sub>, *stx*<sub>2</sub>, and *eae* genes. Strains were stored in liquid nitrogen and cultured on blood agar plates (5% sheep blood in heart infusion agar [Difco Laboratories, Detroit, MI]). For piglet challenge, colony sweeps of bacteria were inoculated into 3 ml tryptic soy broth (Difco) and incubated for 18 h at 37°C to obtain a concentration of approximately  $1 \times 10^9$  CFU/ml. To assess the relative amount of Stx produced by each strain in vitro, the bacterial concentration of each strain was normalized to an optical density at 280 nm of 1.00.

**Gnotobiotic challenge studies.** Gnotobiotic piglets were derived by closed hysterotomy and reared in sterile isolators using standard procedures (34). One pig from each litter used in the study was inoculated with positive control strain EDL933 to limit strain-by-litter bias and to ensure the susceptibility of pigs in the litter to *E. coli* O157:H7. Litters containing control pigs that did not succumb to EDL933 within 8 days of challenge were removed from the study. Each of the 20 *E. coli* O157:H7 strains tested for virulence was inoculated into five gnotobiotic piglets. Litters of piglets were arbitrarily assigned to specific *E. coli* strains, and typically the piglets were divided for testing with three or four strains. To further reduce strain-by-litter bias, piglets from at least two different litters were used to evaluate each strain, and piglets within litters were randomly assigned to individual strains. All piglets were challenged per os with  $\sim 3 \times 10^9$  CFU at 24 to 30 h after birth, when it was demonstrated that the piglets were independently feeding. Following challenge, piglets were observed for clinical signs of illness, including diarrhea and CNS disease, at least three times daily for 8 days or until they exhibited signs of CNS disease or were unable to eat. Signs ascribed to CNS disease included head tilt, circling, lethargy, an inability to stand, lateral recumbency, and/or paddling (tonic-clonic convulsions). At 8 days postinoculation, or when signs of CNS disease were exhibited, piglets were euthanized and subjected to necropsy. At necropsy, pigs were examined for gross lesions, including intestinal hemorrhage and mesocolonic edema. Specimens of the jejunum, ileum, cecum, spiral colon, rectum, liver, lungs, heart, stomach, urinary bladder, kidneys, spleen, mesenteric lymph nodes, thymus, and spinal cord and the entire brain were collected for histological analysis. Brains were sectioned at the level of the medulla oblongata (olivary nucleus), cerebellum with medulla oblongata (cerebellar peduncles), midbrain (corpora quadrigemina), and two levels of cerebrum (interthalamic adhesion and genu of corpus callosum [18]). A section of the colon was also collected for aerobic and anaerobic bacterial culture to confirm the presence of the inoculated strain and to assess contamination.

TABLE 1. Relationship between Stx2 toxin titers for *E. coli* O157:H7 strains (in vitro) and the symptoms and survival of piglets infected with *E. coli*

Group	Strain	Reference or source	Stx2 titer <sup>a</sup>	Piglets			n
				Mean survival time (days) <sup>b</sup>	% with CNS disease	% with brain infarctions	
Control	EDL933	45	161	4.3	100	90	10
Human	3234-86	39	32	6.4	80	100	5
	B8763	41	25	5.2	100	80	5
	A7785	45	20	8.0	20	20	5
	C9490	3	32	5.2	60	20	5
	B1189	51	102	3.8	100	80	5
	C509	48	102	3.4	100	100	5
	C6183	T. J. Barrett	25	5.2	80	60	5
	C8779	3	32	4.0	100	80	5
	C7927	7	20	6.0	80	60	5
C4193	58	10	7.2	40	40	5	
Bovine	2890	4	64	5.2	80	80	5
	2893	4	<2	8.0	0	20	5
	2903	4	25	8.0	0	0	5
	2909	4	16	6.6	40	20	5
	3032	4	6	8.0	0	20	5
	2922	4	<2	8.0	0	0	5
	2918	4	<2	8.0	0	0	5
	2939	4	40	5.2	100	100	5
	2891	4	10	6.4	40	20	5
	2977	4	51	5.4	80	80	5

<sup>a</sup> Relative concentration of Stx2 (in arbitrary units) as measured with a verotoxigenic *E. coli* reverse passive latex agglutination assay.

<sup>b</sup> Up to 8 days postinoculation.

**Histological examinations.** Specimens collected for histologic analysis were fixed in 10% neutral buffered formalin and processed for histopathology by standard procedures, and tissues were stained with hematoxylin and eosin. Tissue sections were subjected to routine microscopic examination for lesions; however, in kidney tissue sections, the numbers of glomeruli and arterioles with lesions compatible with Stx-induced damage (16, 37) were counted, and the resulting data were included in the statistical analyses as separate counts. The pathologist conducting microscopic examinations of the tissues (R.A.M.) was blinded to the treatment groups of the piglets during the study.

**Vero cell cytotoxicity assay.** Bacterial strains were tested for Stx expression using a Vero cell cytotoxicity assay (20). Bacteria were inoculated into 3 ml of tryptic soy broth and incubated for 4 h at 37°C. Secreted toxins were extracted with polymyxin B (25). Vero cells (CCL-91; American Type Culture Collection) were plated with Dulbecco's minimum Eagle's medium (HyClone) containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) in 96-well U-bottom microtiter plates to obtain a concentration of  $2 \times 10^4$  cells/well and incubated until the cells were confluent (18 h at 37°C in 5% CO<sub>2</sub>). The concentration of toxin from each strain was determined in triplicate in each of five separate trials. Extracts containing toxin from each *E. coli* O157:H7 strain were added to Dulbecco's minimum Eagle's medium in the 96-well plates at an initial dilution of 1:6 and diluted further in twofold increments. The Vero cells in test wells were incubated with the diluted toxin extracts for 2 days. After incubation, detached cells, medium, and toxin were removed by vigorous shaking. The remaining cells were fixed with 2% formalin in 67 mM phosphate-buffered saline (pH 7.4) for 1 min, after which the fixative was removed. Attached cells were stained with 0.13% crystal violet in 5% ethanol-2% formalin-phosphate-buffered saline for 20 min. Excess stain was removed by rinsing the wells gently with distilled water. The plates were air dried, and the optical density at 620 nm of the attached cells was determined spectrophotometrically. The toxin titer (50% cytolethal dose) was calculated by determining the reciprocal of the dilution required to kill 50% of the Vero cells in a well of a 96-well U-bottom microtiter plate.

**Stx1 and Stx2 quantification.** Relative concentrations of Stx1 or Stx2 produced by *E. coli* O157:H7 strains during culture in tryptic soy broth at 37°C for 4 h were determined after polymyxin B extraction by measurement with a verotoxigenic *E. coli* reverse passive latex agglutination assay (Denka Seiken Co. Ltd., Tokyo, Japan) (24).

**Immunohistochemistry.** Immunohistochemical detection of *E. coli* O157 antigen in sections of urinary bladders from infected gnotobiotic piglets was conducted by a modification of methods described previously (6). Rabbit anti-O157 antiserum (1:1,000 dilution; *E. coli* Reference Laboratory, Pennsylvania State University) was used as the primary antiserum. Commercial goat anti-rabbit immunoglobulin G and a streptavidin-alkaline phosphatase kit (DakoCytomation LSAB2 System-AP) were used for subsequent antigen detection steps.

**Statistical analyses.** The statistical significance of data ( $P < 0.05$ ) was analyzed with Student's *t* test, by analysis of variance (ANOVA) for mean days for the "human" and "bovine" sources, and by analysis of covariance (ANCOVA) for mean days for the "human" and "bovine" sources, as well as the Stx1/Stx2 ratio. Logistic regression was used for data for "CNS," "diarrhea," "edema," "attaching and effacing (A/E) lesions," and "infarcts" since these data were binomially distributed. For both ANCOVA and logistic regression, the interaction between the source ("human" and "bovine") and the Stx1/Stx2 ratio was not statistically significant.

## RESULTS

***E. coli* EDL933 causes A/E lesions and CNS disease in gnotobiotic piglets.** Eleven litters of piglets were utilized in this study. A control piglet in one litter did not become clinically ill. This litter was removed from the study to limit the effect of piglet disease resistance on the assessment of strain virulence. For the remaining 10 litters, the positive controls inoculated with strain EDL933 exhibited signs of CNS disease or died 2 to 5 days postinoculation (mean  $\pm$  standard deviation,  $3.7 \pm 0.9$  days). Nine control piglets were euthanized, and one died spontaneously. Eight of 10 control piglets exhibited diarrhea. One of the two controls that did not develop diarrhea died 3 days postinoculation. The other control exhibited signs of CNS disease on day 3 and was euthanized. The early demise of these two control pigs may have precluded the development of di-



TABLE 2. Disease indicators for piglets infected with bovine or human strains of *E. coli* O157:H7<sup>a</sup>

Symptom	No. of piglets with symptom	
	Bovine strains (n = 50)	Human strains (n = 50)
Death or CNS disease	17 <sup>b</sup>	38
Brain infarcts	17	32
Diarrhea	45	49
A/E lesions	34	38
Mesocolonic edema	41	47

<sup>a</sup> Five piglets were infected with 10 strains of bovine origin and 10 strains of human origin. The potential postchallenge survival time, 8.0 days (termination of the study), was reduced to 6.9 and 5.4 days by bovine and human isolates, respectively ( $P < 0.03$ , ANOVA).

<sup>b</sup> Significantly different ( $P < 0.05$ , ANOVA).

arrhea in them. Nine of the 10 controls had microscopic evidence of brain infarction. Histological sections from the intestines of various control pigs exhibited attached bacteria with lesions consistent with microvillus effacement. Mesocolonic edema was observed at necropsy in euthanized control pigs. The results for the 10 litters support the proposition that the study measured differences in *E. coli* O157:H7 strain virulence rather than variation in piglet susceptibility among litters. Nine of 10 litters contained both piglets that developed CNS disease and piglets that failed to develop CNS disease (as a consequence of challenge with different *E. coli* strains). For only 1 of 20 strains (C9490) were the challenge outcomes different for pigs from different litters.

**On average, *E. coli* O157:H7 strains isolated from human outbreaks are more virulent in gnotobiotic piglets than strains isolated from healthy cattle.** Piglets inoculated with *E. coli* O157:H7 strains isolated from cattle and from patients involved in disease outbreaks varied in their manifestations of clinical signs and in the presence and extent of lesions (Table 1). Several strains were as virulent as or more virulent than EDL933, while other strains were relatively attenuated. Based on the development of CNS disease or death of inoculated piglets, all of the human isolates were virulent, while only one-half of the bovine strains were virulent. Despite great variability within the groups, the human isolates were significantly more likely to cause CNS disease and/or death (Table 2) (76% versus 34%;  $P = 0.023$ , logistic regression). In addition, the numbers of days that piglets survived after challenge were significantly less for pigs that received outbreak strains than for pigs that received bovine isolates (5.4 days versus 6.9 days;  $P = 0.032$ , ANOVA). Pigs inoculated with human disease strains showed a greater, but not a statistically significant greater, incidence of brain infarcts (64% versus 34%;  $P = 0.07$ , logistic regression). There was no significant difference between treatment groups in terms of mesocolonic edema, diarrhea, or A/E lesions.

**CNS disease symptoms are correlated with postmortem observation of brain necrosis (infarcts).** We previously observed that piglets typically die within several hours of manifestation of clinical signs consistent with disease of the CNS (18). To minimize piglet suffering and to preclude postmortem autolysis of tissues, piglets were euthanized immediately after CNS disease symptoms were observed. Appropriate recognition of signs of CNS disease was established by the high correlation

TABLE 3. Correlation between outcome variables and Stx1 and Stx2 production<sup>a</sup>

Stx	Outcome variable	P value
Stx1	Mean no. of days of survival	0.093
	CNS disease	0.623
	Diarrhea	0.832
	Mesocolonic edema	0.061
	A/E lesions	0.963
	Brain infarcts	0.955
Stx2	Mean no. of days of survival	<0.001
	CNS disease	0.003
	Diarrhea	0.078
	Mesocolonic edema	0.099
	A/E lesions	0.155
	Brain infarcts	0.003

<sup>a</sup> ANCOVA (mean number of days of survival) and logistic regression (all other variables) were used for assessment.

between signs of CNS disease and the postmortem observation of infarcts in sections of brain tissue ( $R^2 = 88.4\%$  and  $P < 0.001$ , ANOVA). Brain necrosis was not correlated with the presence of mesocolonic edema, diarrhea, or A/E lesions, nor was diarrhea correlated with the presence of mesocolonic edema or A/E lesions.

**Stx2 production is correlated with the virulence of *E. coli* O157:H7 strains.** The amount of Stx2 produced by *E. coli* O157:H7 strains was correlated with a reduction in survival to the end of the study ( $P < 0.001$ , ANCOVA) (Table 3 and Fig. 1). Stx2 production was also correlated with manifestation of signs of CNS disease ( $P = 0.003$ ) (Table 3) and brain necrosis (infarcts) ( $P = 0.003$ ) (Table 3), but not with mesocolonic edema, diarrhea, or A/E lesions. The correlation coefficients and significance were similar whether Stx2 data obtained from Vero cell cytotoxicity assays or enzyme-linked immunosorbent assays were used. The amount of Stx1 produced in culture was not correlated with the length of time that a piglet survived, with manifestation of CNS disease or brain infarcts, or with the

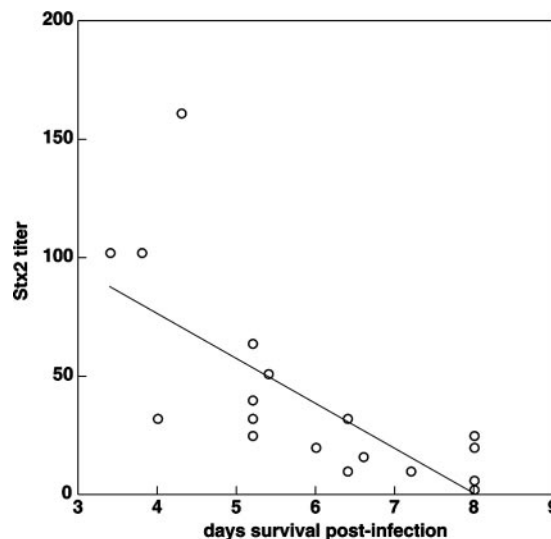


FIG. 1. Relationship between Stx2 titer and number of days of survival postinfection.

presence of mesocolonic edema, diarrhea, or A/E lesions (Table 3).

**Kidney lesions.** Lesions consistent with the lesions previously described (21, 42) were observed in the kidneys of most pigs in the study, including those challenged with positive control strain EDL933. Of 109 piglets whose renal tissue was examined histologically, 85% had lesions in the glomeruli, 59% had lesions involving arterioles, 52% had lesions in both glomeruli and arterioles, 61% had lesions involving tubules, 91% had lesions in either or both glomeruli and arterioles, and 95% had lesions involving one or more of the three areas. The lesions in the glomeruli included capillary endothelial swelling, capillary congestion, fibrin-platelet thrombi, thickening and fibroplasia of Bowman's capsule, and proliferation of mesangial cells. Each individual glomerulus that was counted as positive exhibited two or more of these lesions. Arterioles with lesions exhibited TMA with endothelial swelling and proliferation, concentric intimal thickening, fibrinoid change of the tunica intima and media, and fibrin thrombi. The tubular lesions included necrosis and/or apoptosis, dilatation, and luminal accumulation of sloughed epithelial cells or neutrophils. The number of tubular lesions per tissue section was not quantified since some of the changes (e.g., dilatation) were too numerous to count and not necessarily specific for toxin-induced damage. Statistical analysis did not establish a significant correlation between kidney glomerular or arteriolar lesions and CNS disease ( $P = 0.57$  and  $P = 0.098$ , respectively, Student's  $t$  test). Nor was a significant correlation observed between kidney lesions and Stx2 production by challenge strains (for Stx2 versus glomerular lesions,  $R^2 = 0.2\%$  and  $P = 0.28$ , as determined by regression analysis; for Stx2 versus arteriolar lesions,  $R^2 = 0.2\%$  and  $P = 0.28$ , as determined by regression analysis).

**Atypical lesions.** Lesions that were either uncommon or not previously described in this model were observed in some animals, but they did not appear to be due to a unique virulence attribute of any particular strain. Purulent cystitis that was mild to moderate and subacute to chronic was seen in 14 of 105 (13.3%) piglets whose urinary bladder was microscopically examined. Lesions in the urinary bladder were characterized by infiltration of the mucosal epithelium with neutrophils, by infiltration of the propria submucosa with lymphocytes, by smaller numbers of macrophages, and by granulation tissue formation. In 8 of 14 piglets with cystitis (57.1%), inflammatory lesions were associated with coccobacillary bacterial attachment to the surfaces of mucosal transitional epithelial cells. Immunohistochemical analysis to detect the O157 antigen (6) was conducted using urinary bladder sections from all piglets in which bacterial colonization was observed. Adherent bacteria, when present, stained positive for the O157 antigen in all cases, confirming that *E. coli* O157:H7 had infected the urinary bladder. In addition to the aforementioned renal lesions, two piglets had mild interstitial nephritis and two piglets had mild pyelitis (inflammation of the pelvic epithelium of the kidney); both of the latter piglets also had cystitis. Eight of 126 piglets (6.3%) developed bronchopneumonia, and there were significant lesions in two animals. Lung lesions appeared to have been caused by aspiration of small amounts of milk containing bacterial inoculum. Five piglets had mild erosive gastritis.

In addition to A/E lesions, other clinically significant lesions

were seen in the lower small and large intestines of some piglets. Necrosuppurative inflammation of the ileum, cecum, and colon was seen in five piglets (4.0%), and lesions were ulcerative in two of these animals. Three piglets (2.4%) had lesions suggestive of ischemic bowel necrosis. In one of these animals, lesions were associated with widespread thrombosis of small blood vessels in multiple organs, including the ileum, cecum, and colon. In the second piglet, coagulative necrosis of villi in the ileum was seen without widespread thrombosis in other organs. In the third piglet, lesions were extensive, with full-thickness necrosis of the ileal mucosa (Fig. 2) and necrohemorrhagic colitis (Fig. 3). Mucosal necrosis in the colon spared the deep portions of the crypts and was characterized by loss of the superficial portions and replacement by suffusive hemorrhage that filled the lamina propria.

## DISCUSSION

We showed that *E. coli* O157:H7 strains isolated from healthy cattle are, on average, less virulent in gnotobiotic piglets than *E. coli* O157:H7 strains isolated from human disease outbreaks. Virulence was highly correlated with in vitro production of Stx2 but not Stx1. We suggest that disease outbreaks may select for producers of high levels of Stx2 among *E. coli* O157:H7 strains shed by animals. This hypothesis is consistent with the findings of Ritchie et al., who reported that both the basal and mitomycin-enhanced Stx2 production by isolates from patients with HUS was greater than that by isolates from cattle (46). Gally et al. showed that strains isolated from sporadic or outbreak cases of disease had enhanced expression of EspD and Tir compared to bovine isolates, despite essentially identical strain genotypes (Stx production was not tested) (32, 33, 49, 50). Together, these observations suggest that there may be a general regulatory difference in virulence genes between strains with high virulence and strains with low virulence and that there is selection for greater virulence factor expression in more virulent strains.

A potential mechanism governing Stx2 expression differences is suggested by the observations of Muniesa et al. regarding Stx2 variability among outbreak strains (36). Isolated strains were grouped based on the amount of phage released after mitomycin C induction. Strains that released fewer phage were found to harbor two phage types,  $\phi$ LC159 and  $\phi$ SC370, although only  $\phi$ SC370 was detected in supernatants of induced cultures. When  $\phi$ SC370 was absent, large amounts of  $\phi$ LC159 were released, and the higher level of phage production was accompanied by an increased amount of Stx2 in the cultures. Some relationship was detected between phage production and the severity of the disease observed in patients from which the *E. coli* O157:H7 strains were isolated (36). These observations suggest that interaction between different phages may affect toxin regulation. Kim et al. revealed the existence of two distinctly different lineages of *E. coli* O157:H7 through octamer-based genome scanning (27). Human and bovine isolates were not randomly distributed between lineages. Restriction fragment length polymorphism analysis with lambdaoid phage genomes indicated that phage-associated polymorphisms segregate precisely along lineages predicted by octamer-based genome scanning. Thus, highly virulent strains (which also

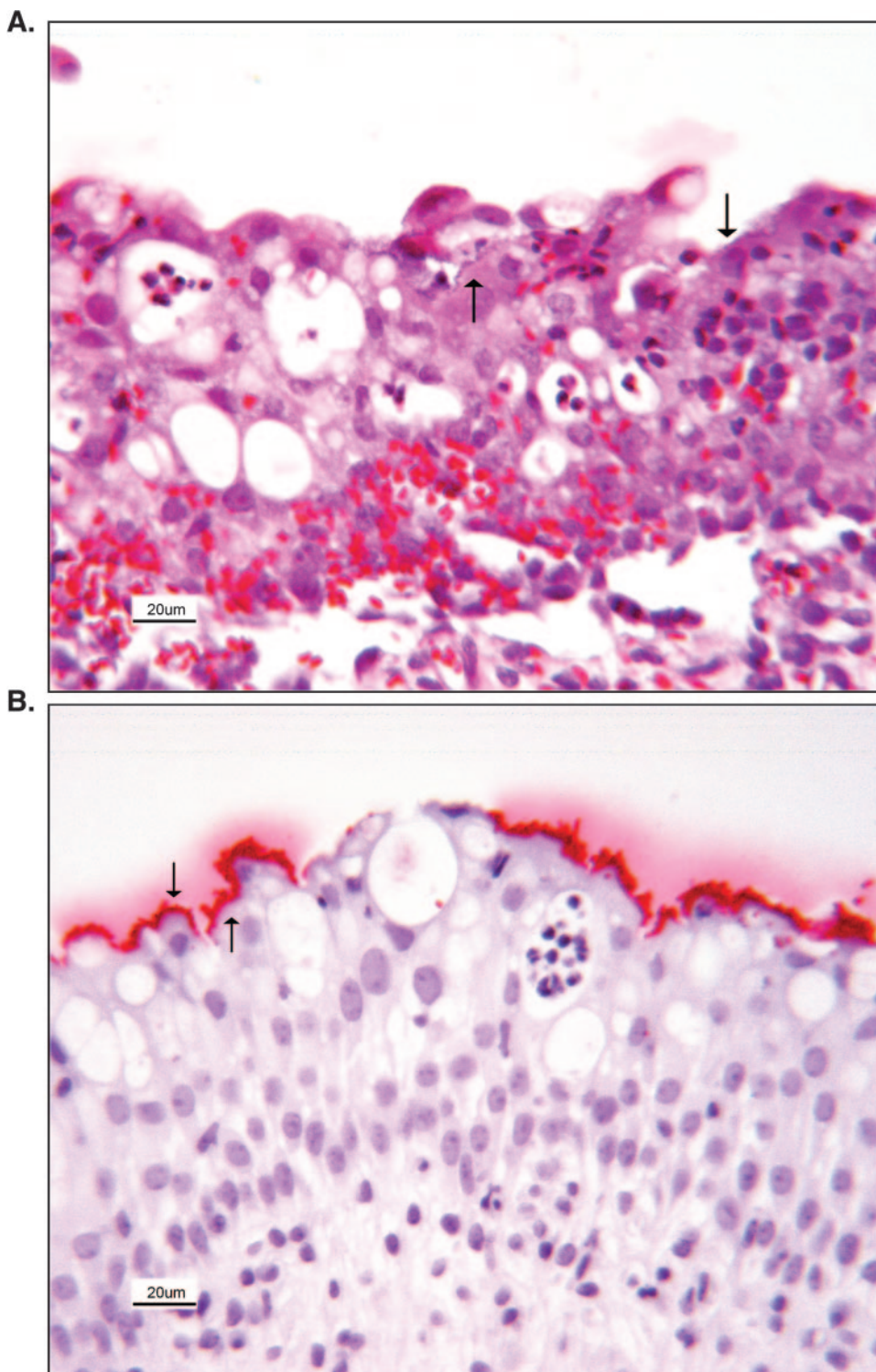


FIG. 2. Urinary bladder of a gnotobiotic piglet (animal 15627) infected with *E. coli* O157:H7. (A) Neutrophils have multifocally infiltrated the mucosa. The mucosa and propria submucosa are hemorrhagic. Foci of bacterial colonization (arrows) are present on the mucosal surface, with bacteria attached to transitional epithelial cells. (B) Additional sections were cut from the same block of paraffin-embedded tissue and stained immunohistochemically for *E. coli* O157 antigen. The bacteria attached to transitional epithelial cells and colonizing the mucosa were identified as *E. coli* O157; the red reaction product indicated a positive result (arrows). Tissue sections were counterstained with hematoxylin.



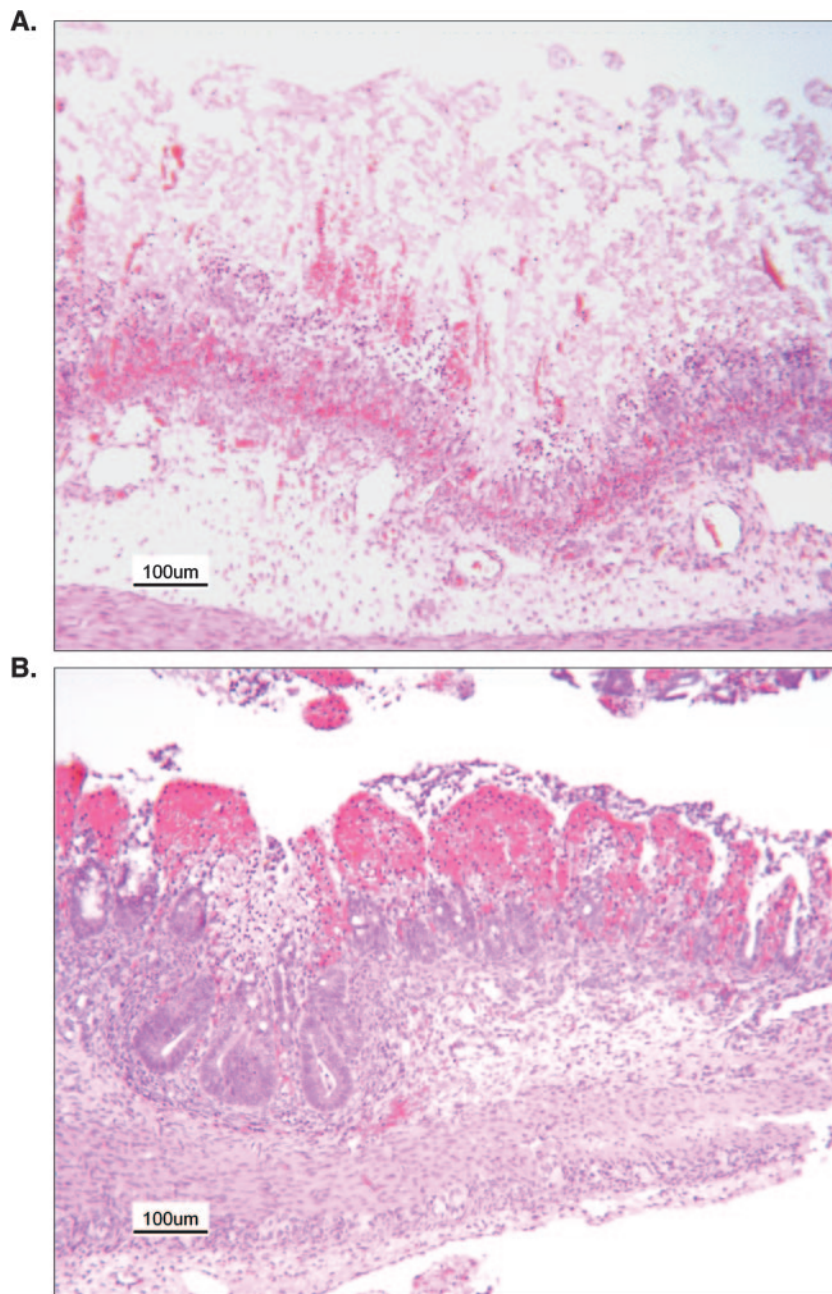


FIG. 3. Intestines of a gnotobiotic piglet (animal 12573) infected with *E. coli* O157:H7. (A) Ileum. There is coagulative necrosis involving the full thickness of the mucosa and remnants of surviving epithelium at the bases of some crypts in the process of regeneration. Hyperemia and hemorrhage are evident at the junction of the mucosa with the submucosa, but hemolysis obscures evidence of hemorrhage superficial to this region. Blood vessels in the mucosa are hyperemic, and some of them (not seen at this magnification) are necrotic and thrombosed. There is edema of the submucosa with associated lymphatic blood vessel dilatation. (B) The superficial mucosa has undergone necrosis and replacement with suffusive hemorrhage that fills the lamina propria. Necrosis has spared the deeper portions of the crypts in the mucosa. Blood vessels in the mucosa are hyperemic, and some of them (not seen at this magnification) are necrotic and thrombosed. The submucosa is edematous. Tissue sections were stained with hematoxylin and eosin.

produce high levels of Stx2) may represent a particular lineage of *E. coli* O157:H7 strains.

A few pigs developed lesions not previously described for gnotobiotic piglets infected with *E. coli* O157:H7. Most notable of these were cystitis and hemorrhagic colitis lesions. Infection by *E. coli* O157:H7 or other Stx-producing *E. coli* strains of the

urinary tract has not been reported in animal models but can be a prodrome to HUS in humans (11, 23, 52, 56, 59). Hemorrhagic colitis is a common feature in clinically recognized human infections, but this condition has not been previously reported to be an outcome of experimental infectious challenge in nonprimate animal models. The several instances of

necrotic and/or hemorrhagic lesions in the intestines of pigs in this study indicate that these animals are subject to these lesions, although they may be uncommon. Necrohemorrhagic intestinal lesions in the gnotobiotic piglet model were presumably secondary to ischemic necrosis and were essentially identical to the lesions which we saw previously in gnotobiotic piglets inoculated with Stx1 (14). They also closely resembled lesions seen in human patients with *E. coli* O157:H7 infections (22, 28, 35).

Recently, previously unrecognized lesions in the kidneys of gnotobiotic piglets infected with *E. coli* O157:H7 were reported (21, 42). Collectively in the two studies, renal lesions involved changes in the glomeruli, the afferent arterioles and small arteries, and the tubules. Glomeruli were affected with endothelial swelling, narrowing of the capillary lumina, congestion, and thrombosis. TMA preferentially affected afferent arterioles and, to a lesser extent, small arteries, whereas larger arteries were unaffected. Tubular epithelial apoptosis, degeneration, and necrosis were also seen (42). The locations of the vascular and tubular lesions matched the locations where Stx2 binding and the Stx2 receptor (Gb3) were identified immunohistochemically (42). In the present study, based on histological examination, 95% of the piglets had lesions consistent with those previously described which involved one or more of the three target sites (i.e., arterioles, glomeruli, and tubules). However, the presence of the lesions correlated with neither parameters of life-threatening clinical illness nor the relative amount of Stx2 produced by the challenge strains. Since previous studies have provided strong evidence that renal lesions in piglets are due to Stx2 and that these lesions resemble lesions seen in human patients with HUS, has been proposed that the gnotobiotic piglet is a relevant model for HUS (21, 42). However, a consistent finding in different studies, including the present study, is that the piglets die from brain damage and not from the clinical features of HUS (i.e., hemolytic anemia, thrombocytopenia, and renal failure). In the present study the hypothesis was that CNS lesions and not renal lesions are correlated with the clinical demise of the piglets; this was found to be the case. It may be that a piglet has greater susceptibility to Stx2-mediated damage in the brain and that the life-threatening nature of the lesions that result cause death before renal lesions have a chance to progress to clinical HUS. Alternatively, another possible explanation is that other putative factors necessary for the development of clinical HUS (60) are not present in the neonatal gnotobiotic piglet model.

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