Differential virulence of clinical and bovine-biased enterohemorrhagtic *E. coli* O157:H7 genotypes in piglet and Dutch Belted rabbit models

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ABSTRACT:

Enterohemorrhagic *E. coli* O157:H7 (EHEC O157) is an important cause of food and waterborne illness in the developed countries. Cattle are a reservoir host of EHEC O157 and a major source of human exposure through contaminated meat products. Shiga toxins (Stx) are an important pathogenicity trait of EHEC O157. The insertion sites of the Stx-encoding bacteriophages differentiate EHEC O157 isolates into genogroups commonly isolated from cattle but rarely from sick humans (bovine-biased genotypes, BBG) and commonly isolated from both cattle and human patients (clinical genotypes, CG). Since BBG and CG share the cardinal virulence factors of EHEC O157 and are carried by cattle at similar prevalence, the infrequency of BBG among human disease isolates suggests that they may be less virulent than CG. We compared the virulence potential of human and bovine isolates of CG and BBG in newborn conventional pig and weaned Dutch Belted rabbit models. CG-challenged piglets experienced more severe disease accompanied by earlier and higher mortality than BBG-challenged piglets. Similarly, CG-challenged rabbits were more likely to develop lesions in kidney and intestine when compared with the BBG-challenged rabbits. The CG strains used in this study carried *stx*2 and produced significantly higher amount of Stx, whereas the BBG strains carried *stx*2c gene variant only. These results suggest that BBG are less virulent than CG and that this difference in virulence potential is associated with the Stx 2 sub-type(s) carried and/or the amount of Stx produced.
INTRODUCTION:

Enterohemorrhagic *E. coli* O157:H7 (EHEC O157) is a major cause of food- and water-borne illnesses characterized by bloody diarrhea, hemorrhagic colitis (HC) and life-threatening hemolytic uremic syndrome (HUS) in developed nations across the globe (27, 38, 43). The lack of specific treatment leads to 2 to 5% mortality among HUS patients. The annual incidence of EHEC O157 infection is estimated to be >70,000 cases in the US (19). The CDC surveillance data shows that the number of EHEC O157 outbreaks increased recently from 27 in 2006 to 39 in 2007 (8, 9). In 2008, the CDC reported 1.12 cases of foodborne EHEC O157 infections per 100,000 population in the USA (7). The infectious dose of EHEC O157 is estimated to be very low (10-100 bacteria) (54), and transmission occurs primarily via contaminated food, water, or direct contact with infected animals (18, 20, 25, 47). Cattle are considered a major reservoir of EHEC O157 due to the frequent associations of EHEC O157 outbreaks with the consumption of contaminated beef and dairy products, confirmed by molecular epidemiologic evidence of identical strain types (3, 5). Cattle colonized with EHEC O157 remain asymptomatic while shedding up to $10^5$ cfu / g of feces typically for periods of days to weeks (51). EHEC O157 is defined as an adulterant when identified in foods, and therefore, in addition to its public health significance, this organism is responsible for significant economic losses to the food industry due to mandatory recalls of contaminated meat and meat products.

Nearly all EHEC O157 isolates harbor two cardinal virulence factors: production of one or more antigenically distinct Shiga toxins (Stxs) and the presence of a
chromosomal pathogenicity island referred to as the Locus of Enterocyte Effacement (LEE) (34, 43). Stxs are encoded in late genes of one or more lambdoid bacteriophages that are integrated into the EHEC O157 bacterial chromosome at specific sites (11, 22, 31, 32, 41, 45, 48). Shiga toxins are important virulence factors associated with the systemic effects of EHEC infection including HC and HUS (28, 56). The LEE encodes a type III secretion system (T3SS), including an effector molecule Tir (translocated intimin receptor), and Intimin, an EHEC O157 surface expressed molecule that interacts with Tir and that is also a putative adhesion factor (44). The LEE is required for EHEC O157 colonization and formation of attaching and effacing (A/E) lesions on intestinal epithelium (15, 40).

Diverse genotyping systems for EHEC O157 strains (4, 29, 30, 36, 41, 52, 62, 63, 65) emphasize the role of bacteriophage, including Stx-encoding bacteriophages, in generating genetic diversity in EHEC O157. Several of these methods identified genotypes occurring at different frequencies in cattle and human host (4, 29, 36, 63). Recent studies comparing some of these genotyping methods suggest that they result in generally concordant classifications (33, 60). Using Stx-encoding bacteriophage insertion (SBI) sites as a marker for strain differentiation, EHEC O157 isolates can be classified into two genogroups: bovine-biased genotypes (BBG, predominantly SBI genotypes 5 and 6, found primarily in cattle and rarely isolated from sick humans) and clinical genotypes (CG, predominantly SBI genotype 1-3, found in both cattle and humans) (4, 52, 61). Both BBG and CG carry and express the cardinal virulence factors of EHEC O157 (4). Despite the similar prevalence of both genogroups in cattle, indicating approximately equal human exposure, the relatively rare occurrence of BBG as a cause of
human disease suggests that BBG may be less virulent than CG. Therefore, this study was designed to evaluate the virulence potential of human and bovine strains belonging to BBG and CG, using a newborn conventional pig (NCP) and Dutch Belted rabbit (DBR) models. We show that strains of EHEC O157 belonging to BBG are less virulent and cause less severe disease in both animal models. This difference in virulence potential is associated with the presence of different Stx 2 sub-type(s) and/or the quantity of Stx produced by BBG strains.

MATERIALS AND METHODS

Piglet husbandry and experimental challenges
All animal experiments were conducted according to the protocols approved by the WSU Institutional Animal Care and Use Committee (IACUC). Piglet challenges were conducted as described previously (14) with a few modifications as follows. Piglets were obtained from WSU swine center at ~4 h after birth, and confirmed EHEC O157-free by testing fecal swabs obtained prior to challenge by using immunomagnetic separation (Dynabeads anti-\textit{E. coli} O157, Invitrogen Dynal AS, Oslo, Norway). Piglets were orally treated with nalidixic acid (25 mg) followed by sodium bicarbonate (10 ml, 10% w/v) 1 h later. Immediately after sodium bicarbonate treatment, piglets were orally challenged with nalidixic acid resistant EHEC O157 (~10^{10} cfu, Nal^R). Piglets were fed Enfamil infant milk formula (Mead Johnson & company, IN USA) three times daily beginning at 210 ml/day and gradually increasing to 450ml/day on day 6 and 7 post infection (PI). Nalidixic acid (25 mg every 8 h) was orally administered to each piglet throughout the
experiment. To avoid confounding by potential genetic factors, each piglet cohort (4 – 6 piglets from single litter) was randomly allocated equally to CG and BBG strain challenges. Piglets were observed daily for EHEC O157-associated clinical signs (Table 1) for up to 7 days PI. Fecal samples from each piglet were collected for quantification of EHEC O157. Piglets were treated with oral fluids and electrolytes (Enterolyte H.E., Oral Powder, Pfizer) and flunixin meglumine (Banamine 1 mg/lb BW IM q24 h) when showing signs of diarrhea and moderate CNS disease (shivering, tremors or mild seizures). Piglets exhibiting severe clinical signs (severe seizures, lateral recumbency, or paresis) were humanely euthanized. At 7 days PI, all surviving piglets were humanely euthanized. Complete necropsies were performed on all piglets, and internal organs were collected and processed for bacteriological, histopathological and electron microscopic examination. All clinical and pathologic assessments were conducted by veterinarians blind to the challenge genotype.

Bacterial strains for NCP

Twenty EHEC O157 strains (Table 2) were randomly selected from the bank of SBI-genotypes of EHEC O157 at Washington State. EHEC O157 strain Sakai (CG-3, kindly provided by Dr. Thomas S. Whittam, NFSTC-MSU) isolated from an outbreak in Japan served as positive control. In order to generate spontaneous nalidixic acid resistant variants (Nal\(^R\)), each EHEC O157 strain was inoculated into brain heart infusion (BHI) broth, incubated at 37°C overnight (~16 h), and 1 ml was plated on Sorbitol MacConkey agar plates (Hardy Diagnostics, Santa Maria, CA, USA) supplemented with nalidixic acid (30 µg/ml) (SMAC-N). For animal challenge studies, Nal\(^R\) strains were inoculated in 200
ml of trypticase soy broth (TSB) and incubated at 37°C overnight with shaking at 200 rpm. Aliquots of these cultures were then stored in glycerol (10% v/v) stocks at -80°C until use.

**Bacteriological culture for piglet challenge**

EHEC O157 were detected and enumerated in fecal samples and intestinal tissues of each piglet by decimal dilution plating on SMAC-N agar plates using a spiral plater (Whitley Automated Spiral Platter, Don Whitley Scientific Ltd, UK). Ten sorbitol non-fermenting colonies from each specimen were tested for lactose fermentation (MacConkey’s agar, Becton, Dickinson and Company, Sparks, MD, USA) and for beta-glucuronidase activity (EC-MUG agar, Hardy Diagnostics, Santa Maria, CA, USA).

Presumptive EHEC O157 colonies (sorbitol negative, lactose positive and beta-glucuronidase negative) were confirmed by O157 latex agglutination test (*E. coli* PRO O157, Hardy Diagnostics, USA). One confirmed EHEC O157 colony recovered from each sample was stored (18% buffered glycerol in BHI, -80°C) for subsequent comparisons with their respective challenge strains by SBI-genotyping (61) and by pulsed field gel electrophoresis (PFGE) following XbaI restriction digestion (6, 12).

**Histopathological examination of piglet tissues**

Duodenum, jejunum, ileum, cecum, spiral colon, distal colon, recto-anal junction (RAJ), kidney and brain collected at necropsy were immediately fixed in 10% neutral buffered formalin, and subsequently paraffin-embedded, sectioned, and stained with hematoxylin and eosin for histopathologic examination. Brain tissue sections were also stained with Luxol-fast blue stain (59). Immunohistochemistry was performed on selected tissues
using a mouse IgG1 anti-O157 monoclonal antibody and the AEC detection kit (SIGNET, Covance, CA). For EM, tissues were fixed in McDowell’s and Trump’s 4F:1G (37). Histopathologic scoring was based on observed bacterial attachment, necrosis, inflammation, vasculitis, and presence or absence of fibrin. Scoring was performed independently by two ACVP board-certified pathologists (KP and KL) and by SS blinded to the identity of the challenge strain. The histopathological lesions in brain and kidney were scored by two observers (KP and KL), and in intestine by three observers (KP, KL, and SS).

**Rabbit experiments**

All rabbit experiments were approved by the IACUC at Massachusetts Institute of Technology. Three challenge experiments were performed using weaned 7 to 8-week-old Dutch Belted rabbits (DBR). Each challenge experiment included five rabbits each for positive controls inoculated with EHEC O157 strain EDL933 (CG-3) and four to five rabbits each for negative controls inoculated with PBS, along with six rabbits each inoculated with the CG or BBG challenge strain of EHEC O157. In each experiment, rabbits were fasted overnight and sedated prior to blood collection and orogastric intubation. Experimental and control rabbits were inoculated with 10 ml of 10% sterile sodium bicarbonate to neutralize gastric acidity. Experimental rabbits received $1-2 \times 10^9$ colony-forming-units of EHEC O157 re-suspended in sterile PBS and control rabbits received sterile PBS (vehicle only). Food and water were provided *ad libitum* following inoculation. Rabbits were monitored closely for clinical signs and body weight measurements were recorded. Fecal cultures were performed on days 1 and 3 PI. Rabbits
were euthanized on day 6 or 7 PI; however, rabbits exhibiting severe clinical signs were euthanized before the end of the study (day 4 PI). Prior to euthanasia, blood samples were collected for clinical pathological evaluation. Complete necropsies were performed on all the rabbits and tissue samples from various organs including cecum and kidneys were collected for histopathological evaluation.

**Bacterial strains for DBR**

Five challenge strains were selected for the rabbit infection studies (Table 2). Stock inocula containing ~ $10^{10}$ cfu was prepared and stored in 1 ml aliquots at -80°C until use.

**Bacteriological culture for DBR**

Challenge strains were detected…

**Histopathological examination of rabbit tissues**

Tissues were fixed in formalin, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. Kidney sections were also stained with Carstairs’ to detect fibrin deposition. Kidney and intestinal (cecal) sections were evaluated by a board-certified veterinary pathologist (SM) blinded to the identity of the challenge strain.

Scoring criteria for histopathological evaluation included renal glomerular (capillary thickening, luminal constriction, fibrin thrombi, white blood cell infiltration, mesangial deposits, changes in bowman’s capsule and space) and vascular (intimal swelling, mural degeneration, perivascular edema, and red blood cell fragmentation) criteria as well as assessment of intestinal vasculopathy (17).
C-reactive protein analysis

The concentration of C-reactive protein (CRP) was measured in serum samples from rabbits in experiment 3 using a commercially available rabbit C-reactive protein ELISA (Immunology Consultants Laboratory, Inc., Newberg, OR) following the manufacture’s directions. Rabbit serum samples were diluted 1:400 in assay diluent buffer for testing. Samples were adjusted to a 1:10,000 dilution and retested if they did not fall within the working range of the assay’s standard curve. The normal range of rabbit CRP is 0-31 µg/ml (Gentry PA, 1999).

stx2 gene typing

The stx2 gene variants present in the challenge strains were determined using PCR-RFLP (13). In this method, restriction patterns generated by HaeIII differentiate stx2 from stx2c while restriction pattern generated by PvuII differentiate stx2 and stx2c from other stx2 variant types.

Stx Quantitation

Stx production in Nal\(^\text{R}\) challenge strains and their nalidixic acid susceptible wild-type parents (WT-Nal\(^\text{S}\)) were assessed using ELISA (Premier EHEC test kit, Meridian Bioscience, Cincinnati, OH). Briefly, for each strain, single colonies from blood agar plates were inoculated into 10 ml of TSB, incubated at 37\(^\circ\)C overnight and used to prepare fresh TSB subcultures by inoculating 500 µl of overnight culture into 10 ml of fresh TSB. This subculture was processed for bacterial counts and three aliquots (1 ml each) were transferred into three wells of 96-well culture plates followed by addition of
1) carbadox (0.5 µg/ml, Sigma-Aldrich, St. Louis, MO), 2) nalidixic acid (30 µg/ml) or 3) no inducing agent. The plates were incubated overnight at 37°C with shaking at 250 rpm.

Overnight cultures were diluted 1:100 in sterile TSB immediately followed by 1:2 dilution in sample diluent provided in the ELISA kit and 100 µl aliquots of diluted samples were tested by ELISA according to the manufacturer’s protocol.

Statistical analysis

Statistical analyses were performed using NCSS 2007 version 07.1.19 (23). For NCP challenge study, the genogroups were compared using following statistical tests. The clinical scores were analyzed using one-way ANOVA. The five clinical scoring categories (diarrhea, CNS disease, appetite, vomiting and abnormal vocalization) were analyzed using two-way generalized linear method (glm). Survival data was compared using Kaplan-Meier survival analysis and the Log-Rank test. Bacterial load recovered from five segments of intestine (ileum, cecum, spiral colon, distal colon and RAJ) of piglets was compared using two-way glm. Scores for histopathological lesions in intestinal tract were analyzed using two-way (genogroup and pathologist) glm.

Association of development of brain lesions in the piglets with the development of CNS disease or with the genogroup of the EHEC O157 strain used to challenge the piglets was assessed using Fisher’s exact test. In the DBR model, statistical analyses were performed using GraphPad Prism. The Mann-Whitney two-tailed test was used to compare the lesion scores of experimental versus control rabbits. All rabbits inoculated with EDL933 (positive controls) and those inoculated with PBS (negative controls) in the three challenge experiments were grouped for the statistical analyses. Association between stx
gene typing and genogroup of the challenge strain was assessed using chi-square test. Stx
ELISA OD\textsubscript{450} scores were analyzed using three-way (growth condition, nalidixic acid
susceptibility, and genogroup) glm. Multiple comparisons were only done when the
overall statistical model was significant using Tukey-Kramer test. Each statistical test
was conducted at the significance level of $P \leq 0.05$.

**RESULTS**

*NCP Model*

**Clinical Signs**

Piglets were scored for disease using a standard scoring system (Table 1). The piglets
cohorts did not differ significantly ($P=0.138$). Piglets challenged with the clinical
genotype strains (CG-1 and CG-3) exhibit higher clinical scores when compared with the
piglets challenged with bovine-biased genotype strains (BBG-5 and BBG-6) ($P=0.005$,
Table 3). Piglets challenged with CG strains and BBG strains had clinical scores that did
not differ significantly from the positive control Sakai strain and sham inoculated piglets,
respectively ($P>0.439$). In individual genotype comparisons, CG-3 challenged animals
had significantly higher clinical scores than BBG-6 inoculated piglets ($P=0.023$, Fig 1),
while CG-1 and BBG-5 challenged piglets showed intermediate clinical scores. Diarrhea,
CNS symptoms, appetite, vomiting, and abnormal vocalizations were scored separately.
Analyses of these categories suggested that CNS disease scores were significantly higher
for piglets challenged with CG strains when compared with the piglets challenged with BBG strains ($P=0.004$, Table 3). No other scoring category differed significantly between CG and BBG challenged piglets.

**Mortality**

Challenge with CG strains resulted in earlier and higher mortality than challenge with BBG strains ($P=0.002$, Fig 2 and Table 3). Sham-inoculated piglets survived until the end of experiment. CG-challenged piglets showed mortality similar to the piglets challenged with positive control strain Sakai ($P=0.852$). Out of 10 piglets challenged with CG strains only one piglet (challenged with strain E12064) survived until the end of the experiment. On the contrary, out of 10 piglets challenged with BBG strains only two piglets (challenged with strain E12053 and E12156) died on days 2 and 4 PI.

**Magnitude of bacterial load**

The mean±SEM $\log_{10}$ cfu/cm² of EHEC O157 recovered from intestine (ileum, cecum, spiral colon, distal colon and RAJ) were significantly higher for piglets challenged with CG strains, when compared with the piglets challenged with BBG strains ($P=0.003$, Table 3). The mean±SEM $\log_{10}$ cfu/cm² of EHEC O157 recovered from ileum, cecum, spiral colon, distal colon and RAJ were 3.86±0.40, 4.92±0.32, 4.56±0.22, 5.00±0.26 and 5.14±0.27, respectively. Although the number of bacteria recovered from large intestine was higher when compared with small intestine, statistically significant differences were only observed between RAJ and ileum.

Negative control piglets were housed in separate cages within the same room and at the same time as EHEC challenged piglets. EHEC O157 was recovered from some negative control piglets, albeit in small numbers, indicating the likely cross-transmission
of EHEC O157 between challenged and sham-inoculated animals. Therefore, SBI genotypes and PFGE profiles of challenge strains were compared with the strains recovered from each piglet to determine the level of cross-transmission, if any. In all but two cases, only the challenged strain was recovered from each experimental piglet, indicating that cross-transmission was uncommon. Both of the cross contaminated piglets were challenged with BBG-6 strain. In one of these BBG-6 challenged piglets, one out of 6 recovered isolates belonged to a genotype (CG-3) that was different from the genotype of the challenge strain. In other BBG-6 challenged piglet, all seven isolates recovered belonged to non-BBG-6 genotypes (BBG-17, one isolate; BBG-5, one isolate; CG-1, five isolates). However, the clinical scores of these two piglets were lower than the piglets that were challenged with the cross-contaminating CG strains in the same cohort, suggesting that this transmission had minimal effect on the clinical outcomes. Isolates recovered from challenged animals occasionally differed in PFGE profiles by one or two bands from the challenge or contaminating strain. Two genotypes that were not included in the study (genotype 9, negative for all the six PCR fragments in SBI genotyping, and genotype 17, positive for only yehV-left junction PCR fragment in SBI genotyping) were recovered from two piglets. These piglets had been either challenged or contaminated by BBG-5 strain (positive for stx2 gene and yehV-left junction PCR fragments in SBI genotyping) suggesting loss of stx2 encoding bacteriophage or loss of stx2 gene either in-\textit{vivo} or during the isolation process. However, the PFGE profiles of these recovered isolates were closely related to the challenge or the contaminating BBG-5 strain, confirming their origin.

\textbf{Histopathology}
The histopathological scores for intestinal tract in CG and Sakai challenged piglets were higher than BBG and sham inoculated piglets but did not reach statistical significance ($P=0.071$, Table 3). However when the two genogroups (CG and BBG) were compared the histopathological scores of intestinal tract for CG-challenged piglets were significantly higher than BBG-challenged piglets ($P=0.02$). Brain lesions were scored as present or absent (Fig 3). The number of piglets in which brain lesions were observed by at least one observer were 5/10, 2/10, 2/4, and 0/4 in CG, BBG, positive and negative control groups, respectively. The number piglets showing brain lesions did not differ significantly between CG and BBG challenged piglets ($P=0.159$). However, 7 out of 11 piglets that died or were euthanized following severe CNS signs did exhibit brain lesions while none of 9 piglets that did not exhibit severe CNS signs or mortality developed brain lesions ($P<0.003$). No kidney lesions were observed in any of the piglets.

**DBR Model**

Three challenge experiments were performed to compare the virulence potential of CG and BBG strains in the DBR model. The most severe clinical signs were observed in rabbits from experiment three. Rabbits in this experiment were challenged with EDL933, E3046 and E5252. Three rabbits (one in each infection group) was euthanized early (at 4 days PI) due to the severity of diarrhea. The % change in body weight of these three rabbits infected with EDL933, E3046, and E5252 at day 4 PI was -1.8%, -4.6%, and 11.5%, respectively. Pre- and post-inoculation clinical pathological parameters including hematocrit, white blood cell count, percent heterophils, platelet count, blood urea nitrogen, total protein,
and albumin were compared for rabbits in each group; however, analyses did not reveal consistent changes associated with disease. In experiment three, increased serum concentration of CRP was detected in rabbits that were euthanized on day 4 PI due to severe disease: 451.4 µg/ml (EDL933), 162.1 µg/ml (E3046), and 399.5 µg/ml (E5252). These values were approximately 4-fold higher than the group mean and were consistent with published rabbit values (65-350 µg/ml) indicative of an acute phase response (Gentry PA, 1999).

On gross necropsy, infected rabbits exhibited serosal hemorrhages near the cecocolic junction (Fig. 4). Specifically, these hemorrhages were observed in approximately 33% (5/15), 17% (1/6), and 83% (5/6) of rabbits infected with EDL933, E3046, and E5252, respectively. Serosal hemorrhages were not observed in other infected groups or uninfected controls.

Significant lesions in the cecum and kidneys of rabbits are summarized in Table 4. Relative to sham-inoculated control rabbits, significant intestinal vasculopathy (Fig.5 B and C) was observed in most O157:H7 infected rabbits except in those infected with strain E5880. Rabbits infected with E5252 developed the most renal lesions that were significant and included both glomerular and vascular lesions such as glomerular capillary thickening (Fig.5E), luminal constriction (Fig.5F), erythrocyte fragmentation (Fig.5F), intimal swelling and perivascular edema. Rabbits infected with E3046 developed significant renal vascular lesions that included intimal swelling and perivascular edema.

stx2 gene typing
All the CG strains tested in this study carried \( stx2 \), and two of the five CG-1 strains also carried \( stx2c \). In contrast, all BBG strains carried \( stx2c \) alone \( (P<0.001) \).

**Stx Quantitation**

The number of bacteria in the initial subculture used for induction by nalidixic acid or carbadox or left un-induced was \(~10^7\) cfu/ml (range, \( 1.6\times10^7 \) cfu/ml to \( 7.8\times10^7 \) cfu/ml). There was no significant difference in the bacterial counts (mean±SEM \( \log_{10} \) cfu/ml) of BBG \( (7.54±0.06) \) when compared with CG \( (7.65±0.03) \) at the time of induction \( (P=0.289) \). Induction with carbadox resulted in a significant increase in the Stx ELISA OD\(_{450} \) scores irrespective of the genogroup \( (P=0.05) \), while induction with nalidixic acid did not result in a significant increase in Stx ELISA OD\(_{450} \) scores by any group, probably due to the Nal\(^R \) trait of all challenge strains. CG strains produced significantly higher Stx ELISA OD\(_{450} \) scores than BBG strains under non-inducing and carbadox inducing conditions \( (P=0.05) \). In individual genotype comparisons, challenge strains belonging to CG-1 and CG-3 produced significantly higher Stx ELISA OD\(_{450} \) scores than BBG-5 and BBG-6 under carbadox inducing conditions \( (P=0.05, \text{Fig. 6}) \). However, under non-inducing conditions, challenge strains belonging to CG-1 produced significantly higher Stx ELISA OD\(_{450} \) scores than BBG-5 and BBG-6. Nalidixic acid induction resulted in no significant difference between genotypes. There was no difference in production of Stx between of Nal\(^S \) and Nal\(^R \) strains within the genotypes under any of the tested growth conditions, suggesting that the use of nalidixic acid to suppress the normal flora of the experimental piglets did not affect \textit{in vivo} Stx production during these challenge experiments.
DISCUSSION

The relatively decreased virulence of BBG strains support the hypothesis that not all EHEC O157 SBI genotypes are equally pathogenic. This is evident based on the higher clinical disease severity, earlier and higher mortality, and more severe histopathological lesions observed for piglets challenged with CG strains. Similar results were obtained in rabbits where animals challenged with CG strains exhibited significantly higher histopathological scores. In contrast, the BBG strains generally showed little virulence in either animal model. Our observations are consistent with the previously published study where EHEC strains from healthy cattle were, on average, less virulent in gnotobiotic piglets than EHEC isolated from human disease outbreaks (2). Here we can refine these observations to show that virulence in animal models is associated with specific EHEC O157 genotypes, and that these differences in virulence correlate with the frequency of occurrence of these genotypes among clinical isolates rather than in the animal reservoir.

Further, we show that the CG and BBG genotypes tested in these animal models are characterized by consistent differences in their Stx2 variant gene content, with \( stx2 \) consistently present in the CG strains (with or without \( stx2c \)) while \( stx2c \) is consistently present and \( stx2 \) is consistently absent in the BBG strains.

Our piglet model differed in several ways from that of previously published reports (2). In contrast to the gnotobiotic piglet model, in which ~24 h old piglets were inoculated with ~10^9 cfu of EHEC O157, we used ~4 h old nalidixic acid treated conventional piglets that were pretreated with 10% sodium bicarbonate before challenge.
with $\sim 10^{10}$ cfu of EHEC O157. The piglet model closely reproduces some aspects of EHEC O157-induced systemic disease of human infection. However, one of the important clinical manifestations of EHEC O157 infection in piglets is CNS disease, which is often associated with brain lesions (2). In this case, the animal model demonstrates lesions in a tissue (brain) typically unaffected in infected humans; however, the brain lesions and CNS disease are arguably analogous to Stx-mediated renal disease in humans as they are the result of Stx being absorbed into circulation, damaging Stx-receptor-expressing endothelial cells resulting in microangiopathy and target tissue damage (14, 56). CG strains induced more severe CNS symptoms (seizures, convulsions, paresis, and lateral recumbency) as compared with BBG strains. In addition, there was a significant association between CNS disease and the presence of histopathological lesions in the brain.

Several recent studies have shown that EHEC O157 infected piglets also develop kidney lesions that appear to be associated with Stx production (2, 21, 46). However, kidney lesions were not observed in the piglets infected with either BBG or CG strains in this study. Perhaps due to the rapid onset of severe CNS disease in piglets, the renal disease is only minimally expressed before death (2). Genetic differences (the breed of pigs), the age at the time of infection and time of euthanasia after the appearance of first CNS signs may have contributed to the observed differences in the severity of brain lesions and lack of kidney lesions in our study compared with the previous studies using piglet model.

We present direct evidence of some cross-transmission in our piglet studies; however the direction and degree of cross-transmission were not sufficient to confound
the strong bacterial genotype effects on piglet virulence observed here. Cross-
transmission of EHEC O157 from infected to uninfected piglets has been described
earlier by Cornick and Vukhac (10), suggesting that EHEC O157 was readily transmitted
among swine via contaminated aerosols and suggesting that a very low dose is required
for piglet infection. Similarly, minor differences in bacteriophage content and PFGE
pattern between challenge strains and fecal isolates as reported here have been reported
previously, for example in experimental bovine infections (64). The infrequency of these
observations in our study demonstrates the overall stability of genotypes during piglet
infections.

The DBR model for EHEC O157 exhibits renal lesions that mimic HUS in
humans (17). Therefore, we used this model to evaluate the relative virulence of BBG
and CG strains and their capacity to induce intestinal and renal lesions. Rabbits
experimentally infected with different EHEC O157 genotypes developed a wide range of
intestinal and renal histopathological changes suggesting that the virulence of EHEC
O157 varies depending on the strain and/or genotype. More specifically, CG-3 strain
E5252 appeared to be the most pathogenic based on the extent and severity of the renal
and intestinal lesions. Furthermore, the increased serum concentration of CRP in the most
clinically affected rabbits suggested that this acute phase protein may represent a
potential biomarker of severe EHEC infection and disease. CRP has been considered a
predictive factor for hemolytic uremic syndrome development in humans with *E. coli*

Several studies have associated the amount of Stx produced and/or type of *stx*
gene present to the virulence of EHEC O157 strains (2, 16, 39, 42). Therefore, we
compared the \( stx2 \) gene subtype present and the quantity of Stx produced by the EHEC O157 strains used in this study. CG strains carry \( stx2 \) alone or in combination with \( stx1 \) or \( stx2c \) genes while BBG strains carry only \( stx2c \) gene. Moreover, under bacteriophage inducing conditions using carbadox, CG strains produced higher Stx ELISA OD\(_{450}\) scores when compared with the BBG strains. These results suggest that the presence of \( stx2 \) gene and/or production of higher amounts of Stx may be contributing to the increased virulence of CG strains. Although, similar relationship between amount of Stx produced and virulence has been reported earlier (2, 16, 39, 42), the quantification of Stx depends on the specificity of antibody used in the kit, which detects Stx1 or Stx2 and Stx2c with different sensitivity (26, 42). In our study, we observed higher Stx ELISA scores for the CG strains when compared with the BBG strains. However, it was not possible for us to attribute this difference solely to the amounts of toxin produced by these strains as they also differ in the type of \( stx2 \) gene content. Therefore, a careful interpretation is needed while using ELISA to compare the amount of Stx produced among EHEC O157 strains.

HUS/severe disease in humans have been linked to the strains that carry \( stx2, stx2c \) or in combination (1, 42). 9 out of 10 piglets challenged with strains carrying \( stx2 \), whether alone or in combination with \( stx1 \) or \( stx2c \), died. However, only 2 out of 10 piglets challenged with the strains carrying \( stx2c \) alone died. These results suggest that the EHEC O157 strains that carry \( stx2c \) alone are likely to be less pathogenic when compared with the strains that carry \( stx2 \) alone or in combination with \( stx2c \) or \( stx1 \). The higher amount of Stx produced suggested by higher Stx ELISA OD\(_{450}\) scores of CG strains carrying \( stx2 \) gene could explain the severe CNS signs that were observed in CG challenged piglets when compared with the BBG challenged piglets. Other researchers
have observed the histopathological lesions in the brain and kidney and have attributed these to the systemic effect of Stx (2, 21, 46). In addition to the amount of Stx produced, differences in \textit{stx2} gene content could result in differences in the affinity of the toxin to the receptors in the brain and eventual differences in their ability to cause disease or Stx-induced damage to the brain (42).

Besides inducing systemic disease, recent studies suggest that Stx2 production in the intestine can also promote colonization, by enhancing the expression of host cell intimin receptors, using \textit{in vitro} cell culture and mouse model (35, 50). In contrast to this, similar studies conducted in cattle and rabbit suggested no effect of Stx on colonization in intestine (49, 53). Thus the effect of \textit{stx2} gene and more specifically its subtype on colonization in intestine needs more investigation.

CG strains induced more severe intestinal histopathological lesions when compared with the BBG strains in the piglet and rabbit models, suggesting differences in their ability to attach to and efface the intestinal mucosa. Recently, whole genome expression microarray has been used to compare the differential gene expression between BBG and CG strains (58). The important virulence factors, including the genes encoded on chromosomal LEE, and several genes encoded on pO157 plasmid (the enterohemolysin \textit{ehxA}, \textit{toxB}, and \textit{etp} genes necessary for T2SS) showed increased expression in the CG when compared with BBG strains. In contrast, the genes essential for survival in the environment, such as acid resistance and stress response, were up regulated in the BBG when compared with the CG strains (24, 55, 57, 58). This study supports our observations suggesting that differential expression of LEE-encoded virulence genes \textit{in vivo} have led to the differences in the magnitude of intestinal bacterial load and histopathological
lesions in the intestines between the piglets and rabbits challenged with CG and BBG strains. This initial damage to the intestinal epithelium may facilitate the absorption of Stx into the bloodstream where it may cause more severe systemic damage.

In conclusion, we have demonstrated that BBG and CG EHEC O157 differ in virulence potential using two animal models, consistent with the hypothesis that not all EHEC O157 strains are equally pathogenic. The amount of Stx and/or type of stx2 gene present are correlated to the virulence differences of EHEC O157 genotypes and more studies are needed in order to clarify the role of these bacterial factors in pathogenesis and virulence of specific EHEC O157 genotypes.

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Table 1. Scoring system for clinical signs applied to neonatal piglets.

<table>
<thead>
<tr>
<th>Diarrhea (consistency, frequency, straining)</th>
<th>CNS signs</th>
<th>Vomiting</th>
<th>Loss of appetite</th>
<th>Abnormal Vocalization</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0-3)</td>
<td>(0-5)</td>
<td>(0-1)</td>
<td>(0-2)</td>
<td>(0-1)</td>
</tr>
<tr>
<td>None (formed feces) (0)</td>
<td>None (0)</td>
<td>None (0)</td>
<td>None (0)</td>
<td>None (0)</td>
</tr>
<tr>
<td>Loose (not formed), no straining or blood (1)</td>
<td>Lethargy (1)</td>
<td>Vomiting (1)</td>
<td>Slight (1)</td>
<td>Present (1)</td>
</tr>
<tr>
<td>Thick liquid, more than twice, mild straining and staining of hind limbs with feces (2)</td>
<td>Splayleg, hind limb weakness, thinner appearance (2)</td>
<td>Complete (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Runny watery- with/without blood, severe straining and staining of hind limbs with feces (3)</td>
<td>Shivering, tremors (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seizures, convulsions (4)</td>
<td>Grand mal seizures or lateral recumbency or paralysis of legs (5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Table 2.** EHEC O157 strains used in this study and their origin.

<table>
<thead>
<tr>
<th>EHEC Bank #</th>
<th>SBI Genotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Year&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Source and Location of collection&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Cohort&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDC EDL 933</td>
<td>CG-3</td>
<td></td>
<td>Raw hamburger meat implicated in hemorrhagic colitis outbreak</td>
<td>NA</td>
</tr>
<tr>
<td>E2325</td>
<td>CG-1</td>
<td>1995</td>
<td>Bovine fecal, WA USA</td>
<td>R1</td>
</tr>
<tr>
<td>E3046</td>
<td>CG-3</td>
<td>1995</td>
<td>Bovine fecal, WA USA</td>
<td>R3</td>
</tr>
<tr>
<td>E5252</td>
<td>CG-3</td>
<td>1998</td>
<td>Bovine fecal, OR, USA</td>
<td>R3</td>
</tr>
<tr>
<td>E5880</td>
<td>BBG-5</td>
<td>1999</td>
<td>Water, USA</td>
<td>R2</td>
</tr>
<tr>
<td>E6996</td>
<td>BBG-6</td>
<td>2000</td>
<td>Bovine fecal, WA, USA</td>
<td>R2</td>
</tr>
<tr>
<td>E12000 (Sakai)</td>
<td>CG-3</td>
<td>1996</td>
<td>Sakai City, Osaka Pref. Japan</td>
<td>NA</td>
</tr>
<tr>
<td>E12053</td>
<td>BBG-5</td>
<td>1993</td>
<td>Bovine fecal, USA</td>
<td>P3</td>
</tr>
<tr>
<td>E12056</td>
<td>BBG-5</td>
<td>1999</td>
<td>Water, USA</td>
<td>P1</td>
</tr>
<tr>
<td>E12057</td>
<td>BBG-6</td>
<td>1999</td>
<td>Bovine fecal USA</td>
<td>P2</td>
</tr>
<tr>
<td>E12058</td>
<td>BBG-6</td>
<td>2000</td>
<td>Bovine fecal, WA, USA</td>
<td>P1</td>
</tr>
<tr>
<td>E12059</td>
<td>BBG-5</td>
<td>2001</td>
<td>Bovine fecal, USA</td>
<td>P2</td>
</tr>
<tr>
<td>Code</td>
<td>Strain</td>
<td>Year</td>
<td>Location</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>--------</td>
<td>------</td>
<td>---------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>E12061</td>
<td>CG-1</td>
<td>2004</td>
<td>Human Clinical WADOH, WA USA</td>
<td>P2</td>
</tr>
<tr>
<td>E12062</td>
<td>CG-1</td>
<td>2004</td>
<td>Human Clinical WADOH, WA USA</td>
<td>P5</td>
</tr>
<tr>
<td>E12063</td>
<td>CG-3</td>
<td>2004</td>
<td>Human Clinical WADOH, WA USA</td>
<td>P2</td>
</tr>
<tr>
<td>E12064</td>
<td>CG-1</td>
<td>2005</td>
<td>Human Clinical WADOH, WA USA</td>
<td>P1</td>
</tr>
<tr>
<td>E12065</td>
<td>CG-3</td>
<td>2005</td>
<td>Human Clinical WADOH, WA USA</td>
<td>P3</td>
</tr>
<tr>
<td>E12066</td>
<td>CG-1</td>
<td>2006</td>
<td>Human Clinical WADOH, WA USA</td>
<td>P3</td>
</tr>
<tr>
<td>E12067</td>
<td>CG-3</td>
<td>2006</td>
<td>Human Clinical WADOH, WA USA</td>
<td>P1</td>
</tr>
<tr>
<td>E12068</td>
<td>BBG-5</td>
<td>1996</td>
<td>Bovine fecal, TX, USA</td>
<td>P5</td>
</tr>
<tr>
<td>E12149</td>
<td>BBG-6</td>
<td>1995</td>
<td>Bovine fecal, WA USA</td>
<td>P4</td>
</tr>
<tr>
<td>E12153</td>
<td>BBG-6</td>
<td>2001</td>
<td>Bovine fecal, WA, USA</td>
<td>P4</td>
</tr>
<tr>
<td>E12154</td>
<td>CG-1</td>
<td>2004</td>
<td>Human Clinical WADOH, WA USA</td>
<td>P5</td>
</tr>
<tr>
<td>E12155</td>
<td>CG-3</td>
<td>2006</td>
<td>Human Clinical WADOH, WA USA</td>
<td>P5</td>
</tr>
<tr>
<td>E12156</td>
<td>BBG-5</td>
<td>1994</td>
<td>Bovine fecal, WA, USA</td>
<td>P5</td>
</tr>
<tr>
<td>E12157</td>
<td>BBG-6</td>
<td>1995</td>
<td>Bovine fecal, OR, USA</td>
<td>P3</td>
</tr>
<tr>
<td>E12377</td>
<td>CG-3</td>
<td>2004</td>
<td>Human Clinical WADOH, WA USA</td>
<td>P5</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td>--------</td>
<td>-------------------------------</td>
<td>----</td>
</tr>
</tbody>
</table>

- **a** CG=Clinical genotypes, BBG=Bovine-biased genotypes
- **b** WADOH=Washington State Department Of Health
- **c** Year of the strain isolation or time deposited in WSU EHEC bank.
- **d** P=Litter cohorts of piglets, R= Experimental cohorts of Rabbits, NA= Not Assigned
Table 3. Clinical scores, clinical scores for CNS disease, survival data, intestinal bacterial load and histopathological scores for intestine in the piglets challenged with BBG strains, CG strains, positive (Sakai) and negative (sham-inoculated) control piglets.

<table>
<thead>
<tr>
<th>Disease Outcome</th>
<th>Groups of piglets (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BBG</td>
</tr>
<tr>
<td>Clinical scores</td>
<td>1.5 ± 0.45  a</td>
</tr>
<tr>
<td>CNS disease Scores</td>
<td>0.69 ± 0.25  a</td>
</tr>
<tr>
<td>Survival time (days)</td>
<td>6.20 ± 0.55  a</td>
</tr>
<tr>
<td>Intestinal bacterial load (cfu)</td>
<td>4.30 ± 0.18  a</td>
</tr>
<tr>
<td>Histopathological scores of intestine</td>
<td>1.08 ± 0.23</td>
</tr>
</tbody>
</table>

Groups not sharing superscript letters differed significantly ($P$≤0.05).
**Table 4.** Intestinal and renal lesions in rabbits infected with various *Escherichia coli* O157:H7 strains*

<table>
<thead>
<tr>
<th>O157:H7 strain</th>
<th>Intestinal vasculopathy</th>
<th>Capillary thickening</th>
<th>Luminal constriction</th>
<th>Erythrocyte fragmentation</th>
<th>Intimal swelling</th>
<th>Perivascular edema</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDL933</td>
<td>P&lt;0.002</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>E5252</td>
<td>P&lt;0.003</td>
<td>P&lt;0.03</td>
<td>P&lt;0.008</td>
<td>P&lt;0.008</td>
<td>P&lt;0.003</td>
<td>P&lt;0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(P&lt;0.006)</td>
<td></td>
<td>(P&lt;0.0003)</td>
<td></td>
</tr>
<tr>
<td>E3046</td>
<td>P&lt;0.01</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>P&lt;0.03</td>
<td>P&lt;0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(P&lt;0.004)</td>
<td>(P&lt;0.006)</td>
</tr>
<tr>
<td>E2325</td>
<td>P&lt;0.007</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>E6996</td>
<td>P&lt;0.04</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>E5880</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>P&lt;0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* P values are relative to sham-inoculated controls; NS, not significant; P values in parenthesis are relative to EDL933-infected rabbits.

**Figure 1.** Clinical scores of piglets challenged with EHEC strains belonging to different genotypes. Groups not sharing specific letters differed significantly ($P=0.023$).
Figure 2. Kaplan-Meier survival curves for piglet groups challenged with different EHEC O157 genogroups.
Figure 3. Piglet challenged with positive control Sakai strain. (A) Section of the brain stained with LFB stain showing affected blood vessel (arrows, magnified in fig. 3B and 3C) with vacuolation of adjacent tissues (arrow head) caused by focal myelin degeneration. (B) Affected blood vessel in brain showing microhemorrhage (long arrow), pyknosis (short arrow) and hyperplasia (arrow head). (C) Affected blood vessel in brain showing perivascular edema. Bar=100µm.
Figure 4. Serosal hemorrhage in the area of the distal cecum adjacent to the junction with the proximal colon in a Dutch Belted rabbit infected with *Escherichia coli* O157:H7 strain EDL933.
Figure 5. A, Sham-inoculated normal rabbit cecum. B, O157:H7 strain E5252-infected rabbit cecum with submucosal edema and necrotizing vasculitis (arrow). C, Higher magnification of submucosal vascular lesion in “B” showing heterophilic vasculitis and perivasculitis with fibrinoid vascular degeneration/necrosis and intimal proliferation. D, normal rabbit glomerulus. E, Glomerulus of a rabbit infected with O157:H7 strain E5252 demonstrating mild capillary thickening (arrows). F, Glomerulus of a rabbit infected with O157:H7 strain E5252 showing global edematous swelling, luminal constriction, decreased numbers of erythrocytes (“bloodless glomerulus”), and fragmentation of erythrocytes (arrow).
Figure 6. Stx ELISA OD$_{450}$ scores by *E coli* O157:H7 genotypes following uninduced, nalidixic acid induced, or carbadox induced growth in broth enrichment culture. All strains are nalidixic acid resistant.