Survival of O157:H7 and Non-O157 Serogroups of *Escherichia coli* in Bovine Rumen Fluid and Bile Salts

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Abstract

While Shiga toxin-producing *Escherichia coli* (STEC) reside asymptotically within ruminants, particularly cattle, these strains pose a serious health risk to humans. Research related to STEC has historically focused upon O157:H7. However, with an increase in foodborne outbreaks of non-O157 origin and recent changes in testing for non-O157 by the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS), there is now a critical need to understand the biological activity of non-O157 serogroups. The focus of this study was to determine whether variations exist in the ability of different serotypes of STEC to survive within bovine rumen fluid medium and bile salts. The results of this study demonstrated through viable plate count analysis that the five serotypes tested (O157:H7, O111:H8, O103:K:H8, O145:H28, and O26:H11) were capable of growing in rumen fluid medium. However, the concentrations of the serotypes O103:K:H8 and O26:H11 after 24 h were significantly less ($p < 0.05$) than that observed for the other serotypes tested. A significant decrease ($p = 0.03$) in the survival of O103:K:H8 in 50 mg/mL of bovine bile salts in comparison to the other STEC strains tested was also observed. Collectively, these data suggest that non-O157 serogroups of *E. coli* respond differently to the environment of the bovine gastrointestinal tract. Further research is needed to elucidate how these differential physiological variations correlate with alterations in colonization success within ruminants and how they may impact human illnesses.

Introduction

SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC) are able to cause hemorrhagic colitis when consumed by humans, which can lead to the potentially fatal hemolytic uremic syndrome (HUS) (Kaper *et al.*, 2004). Young children, older adults, and people who have a weakened immune system or gastrointestinal disorders are at the highest risk of becoming infected with *E. coli* (Boyce *et al.*, 1995; Nataro and Kaper, 1998). Recently, an increasing incidence of non-O157 STEC associated outbreaks has been observed. The Centers for Disease Control and Prevention (CDC) estimates that nearly 265,000 cases of STEC occur annually within the United States, with only about 33% of these being attributed to the O157:H7 serotype (CDC, 2011). Due to the increased prevalence of these non-O157 associated outbreaks, the USDA-FSIS has expanded testing of beef products to include the serogroups O26, O103, O111, O45, O121, and O145 in addition to O157.

It is well known that ruminants are asymptomatic carriers of STEC, with cattle being considered the primary reservoir (Ferens and Hovde, 2011; Wells *et al.*, 1991). Both *E. coli* O157:H7, as well as non-O157 serogroups, are commonly found in the gastrointestinal tract of cattle (Arthur *et al.*, 2002; Low *et al.*, 2005; Matthews *et al.*, 2006; Oporto *et al.*, 2008). Studies have implicated cattle in non-O157 STEC infections due to consumers either coming into direct contact with infected animals or through consumption of contaminated meat (Caprioli *et al.*, 1994; Ethelberg *et al.*, 2009). However, data implicating cattle to incidence of non-O157 STEC infections in humans is sparse and inconsistent. Therefore, it is critical to thoroughly examine the physiological differences between these serogroups that may affect their survival in the ruminant gastrointestinal tract. The goal of this study was to determine whether the O26:H11, O103:K:H8, O111:H8, and O145:H28 serotypes survive as well as O157:H7 within an *in vitro* ruminal fluid fermentation system and within various concentrations of bovine bile salts.

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Materials and Methods

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 1 and were purchased through the American Type Culture Collection (ATCC, Manassas, VA). Where genotypic information was incomplete from ATCC, the multiplex polymerase chain reaction (PCR) assay was performed to identify the presence of the *stx1*, *stx2*, *eaeA*, and *hlyA* genes, as previously described by Paton and Paton (1998) and shown in Table 1. All *E. coli* strains were routinely cultured in Luria-Bertani (LB) medium at 37°C. In order to select for these specific strains in a mixed ruminal microorganisms medium, each strain was transformed with the plasmid pXen-13. This plasmid confers ampicillin resistance to Gram-negative bacteria. For transformation with the plasmid pXen-13 (Caliper Life Sciences, Hopkinton, MA), all strains were made competent by washing mid-logarithmic phase cultures four times with ice cold 10% glycerol. Competent cells were then transformed with pXen-13 by electroporation and cultured in LB supplemented with 100 µg/mL of ampicillin (amp; filter sterilized) using standard techniques (Sambrook and Russell 2001). All strains were found to grow identically with and without pXen-13 in LB broth medium, indicating that the presence of the plasmid did not affect viability (data not shown).

Survival in bovine rumen fluid

Ruminal fluid was collected from the rumen ventral sac of three ruminally cannulated steers on a total mixed ration diet at the Henry Leveck Animal Research Center at Mississippi State University. The rumen fluid medium was prepared from each steer separately as previously described (Russell and Martin, 1984). Briefly, 1 L of medium was freshly prepared with the following components: 0.8 mM K₂HPO₄·3H₂O, 1 mM KH₂PO₄, 2.4 mM (NH₄)₂SO₄, 5.4 mM NaCl, 0.3 mM MgSO₄·7H₂O, 0.3 mM CaCl₂·2H₂O, 0.2 mM cysteine HCl, 24 mM Na₂CO₃, and 33.3% strained rumen fluid. The pH of the rumen fluid medium was adjusted to 6.5 with 1 N NaOH and incubated in a shaker incubator for 16 h prior to use. Rumen fluid medium was verified to be ampicillin sensitive by plating 0.1 mL of freshly prepared medium on LB supplemented with 100 µg/mL amp, followed by a 16-h incubation at 37°C. All strains of *E. coli* harboring pXen-13 were grown to an OD₆₀₀ of 0.3 in 2 mL of LB supplemented with

100 µg/mL amp. Cells were pelleted via centrifugation for 2 min at 10,000 × g, and then resuspended in an equal volume of rumen fluid medium and incubated at 37°C. At 0, 1, 2, 3, 4, 8, and 24 h post-inoculation into the three different rumen fluid media, aliquots were removed and diluted in a 10-fold serial dilution in 1 × PBS, and dilutions were plated onto LB agar with 100 µg/mL amp. Plates were incubated overnight at 37°C prior to performing colony counts. Data from three independent replicates from each of the three individual rumen fluid media prepared from three steers were analyzed and averaged prior to statistical analysis.

Survival in bovine bile salts

Cells were grown to an OD₆₀₀ of 0.3 in 2 mL of LB broth, at which time cultures were supplemented with 0, 50, or 100 mg/mL bovine bile salts (B8331; Sigma Aldrich, St. Louis, MO) and incubated at 37°C. At 0, 3, and 6 h post-bile salt treatment, aliquots of cells were serially diluted in 1 × PBS, dilutions were plated onto LB agar, and plates were incubated overnight at 37°C prior to performing colony counts. Data from three (*n* = 3) independent replicates were analyzed for each treatment.

Statistical analysis

Summary statistics were calculated for log₁₀ CFU/mL and averaged across each treatment. Data were analyzed by analysis of variance (ANOVA) for repeated measures with the PROC MIXED procedures of SAS (version 9.2; SAS Institute Inc., Cary, NC). The initial model for rumen fluid medium included animal, strain, and time and animal × strain × time; results indicated there was no difference due to animal (*p* = 0.74). Animal was removed from the model and the data was pooled and only strain, time and strain × time were included in the final model. For bovine bile salts the model included strain, time, and strain × time. For both rumen fluid medium and bile salts, least significant means were calculated, and when *F*-tests were significant (*p* ≤ 0.05), treatment means were separated using the method of least significant difference.

Results and Discussion

Survival of STEC in bovine rumen fluid

In spite of the presence of volatile fatty acids in the rumen (Wolin, 1969), *E. coli* O157:H7 has been frequently isolated

TABLE 1. BACTERIAL STRAINS USED IN STUDY

ATCC designation (other designation)	Serotype	stx1	stx2	eaeA	hlyA	Isolation source (location)	References
43895 (CDC EDL 933)	O157:H7	+	+	+	+	Raw hamburger meat (United States)	Wells <i>et al.</i> , 1983
23982 (NCDC H515b)	O103:K:H8	–	+	+	+	Human feces (Denmark)	ATCC, this study
BAA-2129 (TWO7865)	O145:H28	–	+	+	+	Feces from calf with diarrhea (Germany)	ATCC, this study
BAA-1653 (EH1534)	O26:H11	+	–	+	–	Feces from outbreak associated with contaminated ice cream (Belgium)	De Schrijver <i>et al.</i> , 2008
BAA-179 (CDC 1997–3215)	O111:H8	+	+	+	+	Feces from patient with HUS (United States)	ATCC, this study
43888 (CDC B6914-MS1)	O157:H7	–	–	+	–	Human feces (unknown)	ATCC, this study

ATCC, American Type Culture Collection; HUS, hemolytic uremic syndrome.

from ruminal fluid (Rasmussen *et al.*, 1999). Previous studies have proposed that non-O157 *E. coli* strains behave similarly to O157:H7 in the ruminant gastrointestinal tract (Jacobsen *et al.*, 2009). To investigate this possibility further, the strains O111:H8, O26:H11, O145:H28, O103:K:H8, O157:H7 Δ stx1stx2, and O157:H7 were analyzed for their ability to survive within a ruminal fluid *in vitro* system. Rumen fluid medium was prepared separately from three steers, and the microbes collected were retained within the medium. These strains were chosen to represent five of the serogroups put forth by the USDA-FSIS new policy and also to determine whether variations in the stx capacity would influence survival in environments likely to be encountered in the rumen gastrointestinal tract.

To ensure that all cells were at similar physiological states, cells were grown to mid logarithmic phase prior to growth in the rumen fluid medium. Table 2 presents the bacterial concentrations (in log₁₀ CFU/mL) for each strain analyzed in rumen fluid media. All strains were able to replicate in the bovine rumen fluid media, with no significant differences evident by 24 h of growth in comparison to O157:H7. Differences were evident between the O26:H11 and O103:K:H8 strains in comparison to the O111:H8, O145:H28, and the O157:H7 Δ stx1 stx2 (Table 2).

Variations were evident in the initial adaptation to this environment. The O111:H8 serotype had a significant increase ($p=0.03$) in populations in comparison to O157:H7 at both 1 and 2 h in bovine rumen fluid media, suggesting this serotype might be able to adapt to this environment more efficiently than O157:H7 (Table 2). A comparison of the least squares (LS) means for percent change in relation to time 0 h also revealed differences with the serotypes O145:H28 and O103:K:H8 in relation to O157:H7 (data not tabulated). The calculation of LS means for percent change indicated that O145:H28 serotype had a lower percent change in comparison to O157:H7 at both 8 h (32.8% compared to 65.6%, respectively, $p=0.03$) and 24 h (84.3% compared to 114.3%, respectively, $p=0.05$). Additionally, the percent change of the O103:K:H8 strain was lower than O157:H7 at 8 h (33.2% compared to 65.6%, respectively, $p=0.03$) and 24 h (76.7% compared to 112.6%, respectively, $p=0.02$) post-inoculation into rumen fluid media.

Together these data suggest that, although these serotypes of *E. coli* can grow in rumen fluid, significant variation is exhibited in their capability to survive in this environment. The

survival rates of O26:H11 and O111:H8 serotypes in ruminal fluid were comparable to O157:H7. However, the O103:K:H8 and O145:H28 serotypes had reduced survival in comparison to the other strains examined. It is critical to determine whether these serotypes exhibit similar survival capabilities in an *in vivo* environment.

Survival of STEC in bovine bile salts

Bile salts are the antibacterial component of bile (Hofmann and Small, 1967), and resistance to bile salts has been suggested to be key to the ability of enteric bacteria to survive within the gastrointestinal tract (Allerberger *et al.*, 1989; Begley *et al.*, 2005; Gahan and Hill, 2005; Jacobsen *et al.*, 2009; Merritt and Donaldson, 2009). An abundance of evidence exists that indicates that *E. coli* is able to resist the bactericidal properties of bile (Ma *et al.*, 1995; Nikaido and Vaara, 1985; Thanassi *et al.*, 1997). *E. coli* O157:H7 have been found to be able to colonize within the gallbladder, which contains the greatest concentration of bile salts (Jeong *et al.*, 2007). However, the prevalence of *E. coli* O157:H7 in gallbladders was found to be low (0.56%) in a study that analyzed 933 cattle at slaughter (Reinstein *et al.*, 2007). Nevertheless, this pathogen has the capability to survive high concentrations of bile salts, but it is not known how non-O157 serogroups survive in these environments.

As performed in the rumen fluid assay above, the *E. coli* serotypes O111:H8, O26:H11, O145:H28, O103:K:H8, O157:H7 Δ stx1 stx2, and O157:H7 were initially grown to mid-logarithmic phase to ensure that all cells were in the same physiological state. Cells were then transferred to media supplemented with a mixture of conjugated and non-conjugated bovine bile salts at concentrations of 0 (non-treated), 50, or 100 mg/mL bile salts. These concentrations were chosen as they reflect the range of bile salts found throughout the intestinal tract for most animals, including cattle (Coleman *et al.*, 1979).

Variations were observed with regard to the ability of each *E. coli* serotype to survive in bile salts. When treated with 0 mg/mL bile salts (non-treated), all strains had either no significant change or an increase in survival rate by 6 h (Table 3). When treated with 50 mg/mL bile salts, both strains of the O157:H7 serotype tested had an increase in growth from 3 to 6 h ($p=0.04$ for O157:H7 and $p=0.007$ for O157:H7 Δ stx1 stx2). The O103:K:H8 serotype had a decrease in

TABLE 2. LEAST SIGNIFICANT MEANS OF *ESCHERICHIA COLI* O157:H7 AND NON-O157 SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC) POPULATIONS (LOG₁₀ CFU/mL) IN BOVINE RUMEN FLUID MEDIA^a

Time (h)	Log ₁₀ (CFU/mL)						p-value
	O157:H7 Δ stx1 stx2	O26:H11	O103:H8	O111:H8	O145:H28	O157:H7	
0	6.0	5.2	5.4	5.2	5.1	5.0	0.10
1	6.8 ^c	5.9 ^{b-d}	5.5 ^{b,d}	6.6 ^{b,c}	6.0 ^{b-d}	5.4 ^d	0.03
2	6.5 ^b	5.6 ^b	5.6 ^b	7.8 ^c	5.6 ^b	5.6 ^b	0.03
3	6.9 ^b	6.5 ^{b,c}	6.0 ^{b,c}	5.7 ^c	6.5 ^{b,c}	5.8 ^{b,c}	0.05
4	7.7 ^c	6.3 ^b	6.2 ^b	6.9 ^{b,c}	7.3 ^{b,c}	6.3 ^b	0.03
8	8.4 ^{b,c}	8.1 ^{b,c}	7.3 ^b	8.6 ^c	8.9 ^c	8.2 ^{b,c}	0.02
24	11.0 ^c	9.6 ^b	9.3 ^b	10.8 ^c	10.9 ^c	10.3 ^{b,c}	0.02

^aData reported in this table are pooled data from rumen fluid medium ($n=3$) made from three individual steers. Animal, as a main effect, was not significant ($p=0.74$).

^{b,c,d}Values without a common superscript letter within a row differ ($p\leq 0.05$).

TABLE 3. LEAST SIGNIFICANT MEANS OF *ESCHERICHIA COLI* O157:H7 AND NON-O157 SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC) POPULATIONS (LOG₁₀ CFU/mL) IN BOVINE BILE SALTS

Time (h)	Bile salt (mg/mL)	Log ₁₀ (CFU/mL)						p-value
		O157:H7 Δ stx1 stx2	O26:H11	O103:H8	O111:H8	O145:H28	O157:H7	
0	—	7.6	8.2	8.0	8.3	7.8	8.5	0.18
3	0	7.9	8.7	9.1	8.9	8.6	8.6	0.08
6	0	9.4	9.2	9.7	9.1	9.6	9.2	0.37
3	50	7.6	8.1	7.2	8.3	8.1	8.0	0.08
6	50	9.0 ^a	8.4 ^a	7.0 ^b	8.7 ^a	9.0 ^a	9.1 ^a	0.03
3	100	7.5	7.8	6.8	7.5	7.3	7.4	0.11
6	100	8.1 ^a	8.0 ^a	6.5 ^b	8.0 ^a	7.3 ^{a,b}	7.0 ^{a,b}	0.03

^{a,b}Values without a common superscript letter within a row differ ($p \leq 0.05$).

viability at 6 h post-bile salt treatment in comparison to populations collected at time 0 h ($p=0.04$, Table 3). Interestingly, O103:K:H8 was also the only serotype tested that had a decrease in viability in comparison to O157:H7 after 6 h of 50 mg/mL bile salts ($p=0.003$). The population of the O103:K:H8 serotype was also less than that of the O157:H7 Δstx1 stx2 ($p=0.0034$), O26:H11 ($p=0.03$), O111:H8 ($p=0.01$), and O145:H28 ($p=0.003$) after 6 h of growth in 50 mg/mL bile salts (Table 3). These data indicate that O103:K:H8 was not able to survive at 50 mg/mL bile salts as efficiently as the other serotypes tested.

When treated with 100 mg/mL bile salts, O157:H7 had a decrease in survival in relation to cells not treated with bile salts (0 mg/mL bile salts) at both 3 h ($p=0.004$) and 6 h ($p=0.006$) post-exposure to bile salts (Table 3). Serotype O103:K:H8 also had a decrease in survival in comparison to the non-treated cells at both 3 h ($p=0.01$) and 6 h ($p=0.004$) post-exposure to bile salts. Populations of O103:K:H8 were less than those for O111:H8 ($p=0.03$), O26:H11 ($p=0.03$), and O157:H7 Δstx1 stx2 ($p=0.02$) after 6 h of exposure to 100 mg/mL bile salts; survival of O103:K:H8 was not significantly different in comparison to both O157:H7 and O145:H28 ($p>0.05$).

These data indicate that the O157:H7 Δstx1 stx2 had the most efficient means of surviving elevated concentrations of bile salts. The serotype O103:K:H8 had a significant decrease in survival within both 50 and 100 mg/mL bile salt treatments at 6 h in comparison to exposure to non-treated cells (0 mg/mL bile salts), thus indicating that it may take longer periods for resistance mechanisms in this serotype to counteract bile salt toxicity in these stressful environments in comparison to other STEC.

Conclusion

The results of this *in vitro* study suggest that variations do indeed exist in the ability of *E. coli* O157:H7 and non-O157 STEC to survive within the rumen and gastrointestinal tract of cattle. Furthermore, key differences may exist in the physiologic responses to environmental stressors that are correlated to the colonization of non-O157 STEC strains in the ruminant gastrointestinal tract. Most importantly, these data suggest that other STEC serotypes, most notably O103:K:H8, may not survive well within the ruminal gastrointestinal tract in comparison to the serotype O157:H7. Because *E. coli* O157:H7 has been isolated from the bovine gallbladder (Jeong *et al.*, 2007), it is critical to explore

whether these non-O157 STEC exhibit similar colonization capabilities *in vivo*.

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Disclosure Statement

No competing financial interests exist.

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