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Abstract

The virulent types of *Escherichia coli* including enterohemorrhagic *E. coli* are life-threatening foodborne pathogens. Asymptomatic cattle can be a natural reservoir of these pathogens. Although various dietary and breeding regimens influence colonization of these *E. coli*, there is insufficient knowledge about the carriage of the pathogens. In this study, the effect of grazing on fecal shedding of *E. coli* and the virulence-associated with *E. coli* in cattle were investigated. The fecal shedding of *E. coli* and total coliforms enumerated on the Chromocult agar did not vary before and after grazing. The number of sorbitol-fermenting cells on sorbitol MacConkey agar containing cefixime and tellurite significantly decreased ($P < 0.05$) in cattle samples after grazing. Using PCR targeting the *eaeA*, *slt-I* or *slt-II* gene, four cattle tested positive for non-O157 enterohemorrhagic *E. coli* in pre-grazing fecal samples, three of which did not test positive after grazing. Our data from 12 beef cattle before and after grazing indicate that cattle grazing decreases fecal shedding of sorbitol-fermenting *E. coli*.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) are foodborne pathogens that threaten public health worldwide. EHEC are the major causative agents of hemorrhagic diarrhea and post-diarrheal hemolytic uremic syndrome (HUS). In EHEC strains, the serotype O157:H7 is particularly virulent and is a major etiological strain associated with hemorrhagic diarrhea and HUS cases. Severe O157:H7 infections occur at a higher frequency than infections caused by the other known EHEC serotypes (Gyles, 2007). Further, the incidence of non-O157 EHEC infections have reportedly increased, unlike that of O157:H7 infections, according to a recent survey (Gould et al., 2013). Therefore, continuous monitoring will help us to prevent the incidence of EHEC infections and to understand the prevalence of both O157 and non-O157 EHEC strains.

EHEC asymptomatically propagate on the surface of the bovine terminal rectum and are shed in feces (Naylor et al., 2003). Although the correlation between diet and EHEC survival varies among experimental conditions, feeding regimen is thought to affect fecal shedding of EHEC (Kudva et al., 1995; Diez-Gonzalez et al., 1998; Looper et al., 2006; Fraser et al., 2013). Moreover, although EHEC shedding is assumed to be affected by management systems including grazing on pasturage, little is known about the effects of grazing on the fecal shedding of EHEC or on the frequency of virulence-associated *E. coli* genes. In this study, we examined the effect of grazing on fecal shedding of *E. coli* and on the fate and

occurrence of virulence-associated EHEC genes in cattle.

Materials and Methods

Fecal samples

To monitor fecal shedding of *E. coli* and virulence-associated EHEC serotypes before and after grazing in Rokkaku paddock of Kawatabi Field Center (Osaki, Miyagi, Japan), rectal feces were obtained from healthy 12 beef cattle. In brief, rectal feces were sampled from the cattle fed in a free-stall barn before grazing, and they were defined as the pre-grazing sample. After 60 days of summer to autumn grazing, rectal feces were obtained from the same cattle and defined as the after-grazing sample. The feeding regimens were shifted from high-grain based diets to high grass-based diets before and after grazing.

Isolation and enumeration of *E. coli* cells in fecal samples

Fecal samples were diluted with sterilized water in a 1:20 ratio, and 10 serial dilutions were spread on Chromocult agar (CCA; Merck, NJ, USA) and sorbitol MacConkey agar (Merck) containing cefixime and tellurite (CT-SMAC). For the CCA, violet and red colonies were identified and enumerated as *E. coli* and all coliform bacteria, respectively. For the CT-SMAC, pinkish and whitish colonies were identified and enumerated as sorbitol-fermenting (SF) and non-sorbitol-fermenting (NSF) *E. coli*, respectively. Subsequently, multiplex PCR was performed as described below for further detection of virulence-associated genes. The cell counts were assessed by performing Student's *t* test.

Detection of virulence genes in E. coli isolates

To determine the presence of *E. coli* O157:H7 and the virulence-associated genes, 10 each of SF and NSF colonies grown on CT-SMAC plates were isolated and further analyzed with a multiplex PCR assay (Table 1 lists the oligonucleotide primers used in this study). In brief, *rfbE*, encoding perosamine synthetase, and *fliC*, encoding flagellar (H) antigen, were targeted to determine the presence of the O157 and H7 antigens in the isolates. The virulence genes *slt-I*, *slt-II*, and *eaeA* encoding shiga-like toxin type I (SLT-I) and II (SLT-II) and intimin, respectively, were targeted. The *E. coli* O157:H7 strain EDL933 was used as a positive control for all experiments. PCR conditions and protocols were described in previous studies (Hu et al., 1998; Awais et al., 2007). In brief, PCR was performed in a volume of 100 µl containing 200 µM each of dNTPs, 2.5 mM of Mg^{2+}, 2.5 units of KOD DNA polymerase (Toyobo, Osaka, Japan). The ideal concentration of each primer pair that yielded five distinct bands were 60, 75, 100 and 200 nM for $FLIC_{h7}$-F/$FLIC_{h7}$-R, IntF/IntR, RfbF/RfbR and SLT-IF/SLT-IR and SLT-IIF/SLT-IIR, respectively. Each bacterial colony was picked up with a sterilized toothpick and directly transferred to the PCR tube as DNA templates. Each PCR cycle consisted of 30 s at 94°C, followed by 60 s at 59°C and 60 s at 72°C in a PCR thermal cycler (Takara Bio, Shiga, Japan). In total 35 cycles were performed for each reaction. Amplified DNA fragments were resolved by gel electrophoresis using 2% agarose and stained with ethidium bromide.

Table 1. Primer sequences used in the multiplex PCR and the expected sizes of the products

Target	Gene	Size (bp)	Primer	Sequence
O157	*rfbE*	292	RfbF	5′-GTGTCCATTTATACGGACATCCATG-3′
			RfbR	5′-CCTATAACGTCATGCCAATATTGCC-3′
H7	*fliC*	625	$FLIC_{h7}$-F	5′-GCGCTGTCGAGTTCTATCGAGC-3′
			$FLIC_{h7}$-R	5′-CAACGGTGACTTATCGCCATTCC-3′
Intimin	*eaeA*	368	Int F	5′-GACTGTCGATGCATCAGGCAAAG-3′
			Int R	5′-TTGGAGTATTAACATTAACCCCAGG-3′
SLT-I	*slt-I*	210	SLT- IF	5′-TGTAACTGGAAAGGTGGAGTATAC-3′
			SLT- IR	5′-GCTATTCTGAGTCAACGAAAAATAAC-3′
SLT-II	*slt-II*	484	SLT-IIF	5′-GTTTTTCTTCGGTATCCTATTCCG-3′
			SLT-IIR	5′-GATGCATCTCTGGTCATTGTATTAC-3′

Results and Discussion

Table 2 summarizes the comparison of *E. coli* cell numbers grown on each media. Using CCA plates, 5 of 11 cattle demonstrated decreased fecal shedding of *E. coli* after grazing. In two cattle, the total number of coliform bacteria was in the range of 10^5 to 10^7 colony-forming units (CFU)/g, and this number did not change after grazing ($P = 0.115$). We detected SF colonies using CT-SMAC; fecal shedding of bacteria decreased after grazing for all the cattle ($P = 0.036$), excluding two unmeasurable samples. Unlike the changes in number of SF colonies, NSF colonies insignificantly remained unchanged between pre-grazing and after grazing sample ($P = 0.128$).

To determine existence of pathogenic *E. coli* in isolates, multiplex PCR assay was performed. As demonstrated in a previous study (Awais et al., 2007), 5 genes were distinctly detected when the multiplex PCR was performed with *E. coli* O157:H7 strain EDL933. Then, multiplex PCR was performed with the *E. coli* isolates grown on CT-SMAC plates. Table 3 summarizes the multiplex PCR results of the *E. coli* isolates from pre-grazing and after grazing cattle fecal samples. We did not detect *rfbE* and *fliC* in any of the *E. coli* isolates, indicating that the beef cattle using in this study do not carry serotype O157:H7. In pre-grazing samples, each SF and NSF isolate had the gene encoding SLT-I and SLT-II, respectively, and four cattle shed non-O157 EHEC. In the after-grazing samples, three isolates had either the gene encoding intimin or SLT-I, and of these, one individual shed an equal number of SLT-I-positive *E. coli* before and after grazing. Although there was no statistically significant difference in distribution of virulent-gene associated *E. coli*, the shedding of non-O157 EHEC might be affected by the grazing in three of the four EHEC-shedding cattle. It may result from dietary changes, from high grain feeding to high roughage feeding. High grain feeding is known to increase ruminal volatile fatty acid (VFA) production and decrease colon pH following an increase in the number of acid-resistant *E. coli* colonies in ruminants (Diez-Gonzalez et al., 1998). Diets with low nutrient composition and high fiber content are thought to inhibit ruminal VFA production and lead to increased colon pH, with eliminating bacterial colonization (Kudva et al., 1995; Diez-Gonzalez et al., 1998). These results may also reflect the fact that populations of breeding cattle in free stall housing systems are more concentrated than cattle grazing on pastures, and a decreased occurrence of contagions may prevent bacterial dissemination from cattle to cattle. Although types of grasses, endophyte infections, and grazing periods are thought to influence shedding of *E. coli* during grazing, the experimental infection of O157:H7 revealed no correlation between these conditions and bacterial shedding (Looper et al., 2006). Thus, dietary changes and breeding systems may each be important predic-

Table 2. Comparison of *E. coli* cell counts by the Chromocult agar and Sorbitol MacConkey agar containing cefixime and tellurite.

Selective media	Pre-grazing*	After-grazing*	*P* value
CCA	$1.3 \times 10^8 \pm 1.2 \times 10^8$	$2.3 \times 10^8 \pm 2.2 \times 10^8$	0.115
CT-SMAC			
SF	$1.2 \times 10^7 \pm 1.8 \times 10^7$	$8.6 \times 10^5 \pm 1.0 \times 10^6$	0.036
NSF	$2.4 \times 10^7 \pm 3.4 \times 10^7$	$1.3 \times 10^6 \pm 1.6 \times 10^6$	0.128

*Results are shown as mean ± SD.

[a] CCA: Chromocult agar.

[b] CT-SMAC: Sorbitol MacConkey agar containing cefixime and tellurite.

[c] SF: Sorbitol fermenting *E. coli* cells grown on CT-SMAC. SF was counted by total 11 cattle before and after grazing. Each of one unmeasurable sample in both before and after grazing cattle sample was excluded.

[d] NSF: Non-sorbitol fermenting *E. coli* cells grown on CT-SMAC. NSF was counted by limited number of cattle before and after grazing.

Table 3. Prevalence of virulence-associated genes in *E. coli* isolates from pre-grazing and after grazing cattle fecal samples.

Target	Gene	Pre-grazing*	After-grazing*
O157	*rfbE*	0/12	0/12
H7	*fliC*	0/12	0/12
Intimin	*eaeA*	0/12	1/12
SLT-I	*slt-I*	3/12	2/12
SLT-II	*slt-II*	1/12	0/12

*Results are represented as positive number of cattle / total number of cattle.

tors for the fecal shedding of *E. coli*. Further, almost pathogenic *E. coli* excluding serotype O157 is known to be grown on SMAC agar as SF colonies (March & Ratnam, 1986). After grazing, the decrease numbers of SF colonies reveal that cattle grazing might inhibit colonization of sorbitol-fermentative *E. coli* such as non-O157 EHEC in bovine intestinal tracts. In this study, we observed the occurrence of culture-based *E. coli* and PCR-based virulence-associated EHEC genes in 12 beef cattle before and after grazing, and determined that fecal shedding of SF and the frequency of virulence genes were affected by the grazing.

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