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Acyl-Homoserine-Lactone Autoinducer in the Gastrointesinal Tract of Feedlot Cattle and Correlation to Season, *E. Coli* O157:H7 Prevalence, and Diet

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Abstract

Acyl-homoserine-lactone autoinducer (AHL) produced by nonenterohemorrhagic *Escherichia coli* species in cattle appears to be required for enterohemorrhagic *E. coli* (EHEC) colonization of the gastrointestinal tract (GIT). The current research aimed to examine the effect of season, diet, EHEC shedding, and location within the GIT on AHL prevalence in the ruminant. Luminal content samples were collected from the rumen and rectum of feedlot cattle at slaughter in the spring, summer, fall, and winter for culture of *E. coli* O157:H7 and AHL determination. During the spring collection, samples were additionally collected from the cecum and small intestine, but

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these samples all were AHL negative and therefore not examined again. To assess the influence of diet on AHL prevalence, 14 lambs were fed either 100% forage or 80% concentrate diets and experimentally inoculated with EHEC. At 8 days after infection, all the lambs were killed, and necropsies were taken, with luminal contents collected from the GIT. The collections from the feedlot cattle had AHL in 100% of the rumen content samples from the spring, summer, and fall, but not in any of the winter samples. No other GIT samples from feedlot cattle were AHL positive, and all the samples from the sheep study were AHL negative. The cattle seemed to show a weak correlation between ruminal AHL and EHEC prevalence. This research found AHL only in the rumen and not in the lower GIT of feedlot cattle. However, it is unclear whether this is because the pH of the lower gut destroys the AHL or because a lack of certain bacteria in the lower gut producing AHL.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is responsible for major outbreaks of bloody diarrhea and hemolytic uremic syndrome throughout the world. Each year, EHEC causes an estimated 73,000 illnesses, 2,000 hospitalizations, and 69 deaths in the United States alone [14]. Cattle, the natural reservoirs for this bacterium, typically appear asymptomatic while shedding this pathogen into the environment [1, 12]. Previous research identified a number of EHEC genes required for intestinal colonization of cattle, including genes from the locus of enterocyte effacement necessary for EHEC adhesion to human and cattle intestines [16] as well as *sdiA* encoding the *LuxR* homolog *SdiA* in *E. coli* [4].

Quorum sensing is a mechanism of cell-to-cell signaling that involves hormone-like compounds called autoinducers that the bacteria use to sense both their own populations and those of other bacteria and that subsequently regulate gene expression [21]. The quorum-sensing system in *Vibrio fischeri* consists of two proteins: *LuxI*, which is responsible for the production of the acyl-homoserine-lactone (AHL) autoinducer, and *LuxR*, which is activated by this autoinducer to increase transcription of its target genes [9, 10]. Subsequently, homologues of *LuxR–LuxI* have been identified in other bacteria, and in all of these *LuxR–LuxI* systems, the bacteria produce an AHL, which binds to the *LuxR* protein and regulates the transcription of several genes. *Escherichia coli* and *Salmonella* have a *LuxR* homologue, *SdiA* [23], but do not have a *luxI* gene. Nor do they produce AHL [17, 22].

The precise role of *SdiA* in quorum sensing was previously unknown until reported findings showed that *SdiA* was not sensing an autoinducer produced by *E. coli* itself but rather AHL produced by other bacterial species [17]. However, no targets for *SdiA* regulation are known in EHEC. The fact that a functional *SdiA* is necessary for EHEC to colonize the intestinal tract of cattle successfully is especially intriguing given previous observations that the rumen contains bacteria that produce AHL signals [11].

Recently, it was reported that ruminal extracts from cattle can decrease the gene expression necessary for EHEC colonization, an effect enhanced in the presence of *SdiA* [13]. This, taken with the aforementioned data, suggests that AHL in the rumen of cattle is involved in repressing the expression of genes in EHEC necessary for colonization. Consequently, this research aimed to examine the effect of season and diet on the presence of AHL in the

ruminant and to determine whether a correlation exists between AHL in the rumen of cattle and the fecal shedding of EHEC. Finally, in light of the research suggesting that the rectal–anal junction is the primary site of EHEC colonization in cattle [18, 19], we examined luminal contents collected throughout the gastrointestinal tract (GIT) for AHL.

Materials and Methods

Prevalence of AHL and *E. coli* O157:H7 in Feedlot Cattle at Slaughter

All samples were collected from feedlot cattle at a single slaughterhouse in the southwestern United States. Four collections representing four seasons were made, respectively, in May, August, and October of 2007 and January of 2008. Luminal content samples were aseptically collected on each of the four dates from the rumen (1,000 ml) and rectum (50 ml) of 60 cattle. During the spring collection, samples also were collected from the cecum and small intestine and analyzed. However, these samples were not included in future collections due to the absence of AHL in these GIT segments. The samples were shipped on dry ice to the laboratories in Dallas (AHL analysis) and College Station, TX (*E. coli* O157:H7 culture) for processing the next day, as described later.

Influence of Diet on AHL Prevalence

For this study, 14 crossbred weaned lambs (average body weight, 25 kg) were purchased and transported to the livestock facilities in the USDA laboratory at College Station, Texas. At arrival, the lambs were ear tagged, vaccinated for enterotoxemia, weighed, and randomly assigned to treatment: Their diet consisted of forage (90% grass hay, 10% grain/mineral supplement) or concentrate (90% commercial sheep feed, 10% grass hay).

After a 2-week period of adaptation to the diet, all the sheep were individually penned indoors and inoculated with a cocktail of two *E. coli* O157:H7 strains (EDL 933 and 6058) made resistant to different antibiotics (rifampicin [6058], novobiocin and naladixic acid [933]). All the lambs were administered 10 ml of each strain (933 [$4.9 \times 9\ 10^9$/ml] and 6058 [$5.3 \times 9\ 10^9$/ml]) via oral gavage. Before inoculation with the challenge strains, fecal samples were collected from all the lambs, cultured, and found negative for *E. coli* capable of growth on our antibiotic-supplemented agars. Fecal samples collected daily from each lamb for 7 days were used to quantify each inoculated strain of EHEC.

All the lambs were humanely killed on postinoculation day 8, and necropsies were performed. Luminal contents and tissue were aseptically collected from the rumen, ileum, cecum, spiral colon, and rectum. The luminal contents were serially diluted for quantification of the challenge strains of EHEC and assayed for prevalence and concentrations of AHL. Tissue samples were enriched and plated for qualitative analysis of the challenge strains of EHEC.

Detection of AHL in Ruminal Fluid and Luminal Contents

Acyl-homoserine-lactone was extracted as described previously [11]. Briefly, ruminal fluid and samples of luminal contents were extracted using dichoromethanol and subsequently tested in a reporter strain for their presence. This reporter strain is a strain of *Agrobacterium*

tumefaciens that has a gene regulated by AHL fused to a gene encoding *β*-galactosidase that in the presence of AHL and its substrate (X-Gal) turns blue. The extracted AHL was defined further through thin-layer chromatography (TLC) to identify its chemical composition.

E. coli O157:H7 Isolation and Enumeration

The luminal content samples from feedlot cattle were shipped immediately after collection to our laboratory at College Station, Texas for qualitative and quantitative analysis of *E. coli* O157:H7. Briefly, 10 g of each sample was enriched in 90 ml of tryptic soy broth (TSB) with phosphate (30 g/l TSB, 2.31 g/l KH_2PO4, 12.54 g/l K_2HPO_4, final pH 7.2) and incubated at 25°C for 2 h, at 42°C for 6 h, and then at 25°C overnight until processed the next day. After enrichment, a 1-ml aliquot was removed and mixed with 20 μl of IMS beads (Neogen Corp., Lansing, MI, USA) for 15 min at room temperature with shaking.

Next, the IMS beads were removed from the mixture, washed, and resuspended in phosphate-buffered saline (PBS) Tween 20. After 50 μl had been plated on Chrom-O157 agar (DRG International, Mountainside, NJ, USA) containing 5 mg/l novobiocin and 2.5 mg/l potassium tellurite (ntChrom-O157), the plates were incubated at 37°C for 18 to 20 h. Putative *E. coli* O157 colonies (flat mauve colonies lacking a distinct center) were confirmed as the O157:H7 serotype using the Reveal microbial screening test (Neogen Corp., Lansing, MI, USA) according to the manufacturer's instructions.

Enumeration of *E. coli* O157:H7 was performed by direct plating of the TSB phosphate–feces mixture (before enrichment) onto ntChrom-O157 agar using a commercially available spiral plater (Spiral Biotech Autoplate 4000, Advanced Instruments, Inc., Norwood, MA, USA). The plates were incubated overnight at 42°C, and after one or two colonies had been confirmed as *E. coli* O157:H7, all morphologically similar colonies were counted and their concentration calculated.

For the sheep study, fecal samples and luminal contents (1 g) were serially diluted in sterile PBS (pH 6.5) and plated on MacConkey's agar containing 25 μg/ml rifampicin (strain 6058) or 20 μg/ml novobiocin and 25 μg/ml naladixic acid (strain 933).

Colonies exhibiting typical EHEC morphology were counted manually after incubation. Tissue samples were rinsed with sterile water and enriched with TSB broth for 24 h at 37°C, after which a portion of the enrichment was plated on each of the selective agars described earlier for qualitative culture of the challenge strains.

Statistical Analysis

Data from the feedlot cattle collected at slaughter are presented in terms of prevalence by collection and not analyzed statistically. All data from the sheep study were analyzed using SAS Version 8.02 (SAS Inst. Inc., Cary, NC, USA). Daily fecal shedding data were analyzed using the PROC mixed procedure, with treatment, day, and treatment per day interactions included in the model. The incidence of *E. coli* O157:H7-positive tissues and luminal contents were subjected to chi-square analysis using the Proc Freq procedure. Differences among means were considered significant at a 5% level of significance.

Results and Discussion

Prevalence of AHL and *E. coli* O157:H7 in Feedlot Cattle at Slaughter

The prevalence of AHL and *E. coli* O157:H7 in luminal content samples collected from feedlot cattle are presented in Table 1. *Escherichia coli* O157:H7 was cultured (with enrichment) from at least one luminal content sample each of the rumen, small intestine, cecum, and rectum in the spring collection. Subsequent collections examined only rumen and rectal contents. Only one rumen sample had positive test results for *E. coli* O157:H7 in the summer and fall. However, 8.5% of the winter samples were culture positive after enrichment.

The percentages of rectal contents with positive test results for *E. coli* O157:H7 were 5.5% for the spring collection, 13.3% for the summer collection, 3.3% for the fall collection, and 16.7% for the winter collection. Only four samples contained quantifiable concentrations of *E. coli* O157:H7 via direct plating (1 spring, 1 winter, and 2 summer collections). Therefore, the results are presented as the number of animals with positive results rather than as the concentration in the contents. We found AHL in 100% of the rumen samples collected in the spring, summer, and fall but not in any of the winter samples (50 samples each were tested in the spring and summer, 30 in the fall, and 10 in the winter) or in any of the samples from the small intestine or cecum (10 each, spring collection only).

These results are consistent with the chemistry of AHL because the homoserine ring of this molecule is hydrolyzed in an alkaline pH (as found in the lower GIT), rendering it inactive, although AHL is very stable in an acidic environment such as that of the rumen (particularly in animals fed a high-concentrate diet). Assuming that pH is responsible for AHL presence, we would not expect to see a seasonal affect. Rumen pH in feedlot cattle is primarily affected by diet, which does not change seasonally and should be relatively consistent before slaughter.

We examined the effect of season in the current research based on previously published research investigating our hypothesis that seasonal shedding of *E. coli* O157:H7 in cattle is due to physiologic changes within the animal in response to changing day length [8]. Our data indicated that hormones known to respond to day length (melatonin, tri-iodo-thryonine, thyroxine) were involved in the population dynamics and subsequent fecal shedding of *E. coli* O157:H7 in cattle [6, 7].

Based on these findings, we speculated that AHL may respond similarly to changing day length and may explain, at least in part, the seasonality of this pathogen. In general, our seasonal sampling data appears to support the idea that pH and not season is the primary factor concerning AHL presence. All the spring, summer, and fall samples were AHL positive, and all the winter samples were AHL negative. However, we were able to analyze only 10 samples due to difficulties encountered during the collection. For AHL determination, the majority of the rumen sample must be liquid. The group of cattle sampled for the winter collection had a much higher rumen dry matter content, which interfered with the analysis. Therefore, although the winter data suggests that AHL may be produced seasonally, this may be an artifact of the limited sample number. However, it should be

noted that the greatest prevalence of EHEC was observed in the winter collection, a time when no AHL was detected.

Considering some possible functions of AHL (discussed later in more detail), this observation tends to make more sense. Acyl-homoserine-lactone autoinducer is involved in regulating gene expression, thereby influencing the ability of EHEC to colonize the GIT. Therefore, the higher prevalence of EHEC in the winter rumen samples may be explained by the absence of AHL in these same samples. When AHL was detected in the rumen, EHEC prevalence in the rumen, as expected, was very low. We were unable to quantify AHL concentration or types present from the feedlot cattle samples because these determinations require a significantly greater sample volume than we were able to collect in the slaughterhouse.

Influence of Diet on AHL Prevalence

Daily shedding (Table 2) of the two inoculated *E. coli* O157:H7 strains decreased ($p < 0.05$) over the collection period, but this decrease was not influenced by diet ($p > 0.10$). There tended to be a diet × day interaction ($p = 0.08$) for the inoculated *E. coli* O157:H7 strain 933. Serial dilutions of luminal contents from the rumen, cecum, ileum, colon, and rectum were largely negative. Therefore, data were analyzed as qualitative results (positive or negative) (Table 3).

No differences ($p > 0.10$) were observed in the number of luminal content samples positive for either challenge strain of *E. coli* O157:H7 due to treatment. Numerically, there were more positive tissue samples from the rumen, cecum, and ileum in the forage-fed sheep. However, these differences were not statistically significant. Similarly, no significant treatment differences were observed for the number of rectal tissues that were *E. coli* O157:H7 positive, although the lambs fed the high concentrate diet had a numerically higher number of positive samples (Table 3). Assuming that the magnitude and directions of the differences remained the same, then increasing the number of experimental animals used in the study would likely have resulted in significant differences compared with the treatment trends observed for rumen, cecal, and rectal tissues.

Somewhat surprising was the finding that all the GIT luminal content samples from the sheep tested negative for AHL (data not shown). Perhaps this was simply a function of species and cattle but not sheep, which have AHL in the GIT. Sheep, like cattle, are naturally colonized with EHEC. The EHEC populations are similar to those found in cattle and have been used as an economic experimental ruminant model for EHEC colonization [2, 3, 15]. Therefore, it seems unlikely that cattle and not sheep would have assayable concentrations of AHL. Perhaps the use of experimentally infected rather than naturally colonized animals influenced our ability to assay AHL in sheep.

Previous research with experimentally infected animals using protocols similar to those used in this study demonstrated that fecal concentrations of the inoculated strain or strains generally decrease relatively fast during the first few days to low or undetectable levels [5, 20]. This suggests that the challenge strains of *E. coli* are largely transitory and may not become colonized within the GIT. It seems unlikely that this would significantly influence

AHL prevalence, but differences in diets, pH, sample size, and presumably bacterial populations likely would not have been great enough to explain this finding. Perhaps our sampling methodology, although essentially the same (e.g., volume of sample, GIT segment) as those used in the cattle research, failed to obtain samples containing assayable levels of AHL.

The results of this research may provide insight into EHEC colonization in cattle as well as differences in fecal shedding and GIT populations attributed to diet. The current research appears to show a weak correlation between AHL and EHEC prevalence, particularly in the winter data from feedlot cattle. During the winter collection, we did not detect any AHL. However, at this time, we did observe the highest incidence of EHEC in the rumen contents. Conversely, when AHL was detected in the rumen contents (spring, summer, fall), EHEC prevalence in the rumen was nondetectable or very low.

The results of this research support the idea that AHL is not present in the lower GIT. However, it is unclear whether this is because pH of the lower gut destroys the AHL, because a lack of certain bacteria in the lower gut producing AHL, or both.

Although we did not find AHL in the any of the sheep samples, if we assume that AHL is present but missed in our analysis due to sampling, then this could explain some of the trends we observed in the sheep study. More rumen and fewer rectal tissue samples were positive for the inoculated *E. coli* strain in the forage- versus the grain-fed sheep, as would be expected if AHL was present and its functions in the GIT was similar to that in cattle. Taken together, these findings indicate that AHL and the bacterial species producing these signals may be as important or even more important than the development of acid resistance by EHEC for the colonization of the lower GIT.

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Table 1

Prevalence of *Escherichia coli* O157:H7 (after direct plating [DIR] or enrichment [ENR]) and acyl-homoserine-lactone autoinducer (AHL) in feedlot cattle at slaughter[a]

Season	Method	*E. coli* O157:H7 prevalence				AHL prevalence		
		Rumen	SI	Cecum	Rectum	Rumen	SI	Cecum
Spring	DIR	0/58	1/58	0/58	1/54	50/50	0/10	0/10
	ENR	1/58	2/58	3/58	3/54			
Summer	DIR	0/60	ND	ND	1/60	50/50	ND	ND
	ENR	0/60	ND	ND	8/60			
Fall	DIR	0/60	ND	ND	0/60	30/30	ND	ND
	ENR	1/60	ND	ND	2/60			
Winter	DIR	0/59	ND	ND	1/60	0/10	ND	ND
	ENR	5/59	ND	ND	10/60			
Overall	DIR	0/237	1/58	0/58	3/234	130/140	0/10	0/10
	ENR	7/237	2/58	3/58	23/234			

ND not determined

[a]Luminal content samples were collected seasonally from the rumen, small intestine (SI), cecum, and rectum

Table 2

Daily fecal shedding (colony-forming units [$\log_{10}$)/g feces]) of two *Escherichia coli* O157:H7 strains (EDL strains 6058 and 933) in experimentally inoculated sheep fed either a forage- or grain-based diet

Day	Forage		Grain	
	6058	933	6058	933
1	5.16	5.39	5.97	5.83
2	3.69	3.44	3.68	3.51
3	2.73	2.89	2.78	3.51
4	1.7	2.46	1.76	2.61
5	1.44	3.42	1.97	1.77
6	1	3.02	1	1.88
Average	2.61	3.44	2.86	3.19

Table 3

Tissue and luminal content enrichments positive for *Escherichia coli* O157:H7 (strain 933) in experimentally inoculated sheep fed either a forage- or grain-based diet

Item	Diet		*p* > *F*
	Forage	Grain	
Tissue enrichments (% positive)			
Rumen	50	14.3	0.16
Cecum	66.7	28.6	0.17
Ileum	83.3	57.1	0.31
Colon	50	57.1	0.8
Rectum	16.7	57.1	0.13
Luminal content enrichments (% positive)			
Rumen	100	100	1.0
Cecum	33.3	42.9	0.72
Colon	33.3	28.6	0.85
Ileum	16.7	28.6	0.61
Rectum	16.7	28.6	0.61