ESCHERICHIA COLI O157:H7 IN BEEF CATTLE: PREVALENCE IN GUT CONTENTS AT SLAUGHTER AND THE EFFECT OF NEOMYCIN SUPPLEMENTATION IN FEED ON FECAL SHEDDING IN EXPERIMENTALLY INOCULATED CATTLE

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A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2008

Approved by:

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Abstract

Escherichia coli O157:H7 is a food-borne pathogen that causes hemorrhagic enteritis in humans. Cattle are asymptomatic carriers and their feces are the major source of infection. The objective of the first study was to determine the prevalence of *E. coli* O157:H7 in the gut of cattle at slaughter. Gut contents (rumen, cecum, colon and rectum) were collected from slaughtered cattle (n=815) at a packing plant and prevalence of *E. coli* O157:H7 was determined. The overall prevalence of *E. coli* O157:H7 in cattle was 20.6%. The prevalence (%) in the rumen, cecum, colon, and rectum was 4.9, 9.1, 7.7, and 10.3, respectively. Prevalence in rectal content was positively associated ($P < 0.01$) with that of the rumen or colon and not of the cecum. Pulsed-field gel electrophoresis typing showed that the majority of isolates obtained within the same animal shared a clonal similarity. There was no significant difference in the acid tolerance of ruminal compared to hindgut isolates. It was concluded that hindgut was the major site of prevalence of *E. coli* O157:H7 in cattle at slaughter.

Neomycin, an aminoglycoside, is approved as a feed additive and for use in water to cattle. The objective of the second study was to determine the efficacy of feeding neomycin on fecal shedding of *E. coli* O157:H7 in cattle. Cattle were randomly assigned to control (n=14) or neomycin (n=10) supplemented group and orally inoculated with nalidixic acid-resistant (NalR) *E. coli* O157:H7. Neomycin was fed at 10 mg/0.45 Kg body weight for 15 days. Fecal samples and rectoanal mucosal swab (RAMS) samples were collected day before (d -1), on days 1, 3, 5, 10, 13, 17, 20, 24, 27, 31, 34, 38, 41, 44, and 48, and then approximately weekly through day 111. Fecal shedding of NalR *E. coli* O157:H7 was quantified and prevalence in RAMS was determined. Neomycin significantly reduced prevalence and concentration of *E. coli* O157:H7 compared to the control. Following two weeks of neomycin feeding, concentration and prevalence were similar between the two groups. Short term neomycin feeding before slaughter may reduce the *E. coli* O157:H7 load in cattle.

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Acknowledgements

I would first like to thank Dr. T. G. Nagaraja for serving as my major professor. Through Dr. Nagaraja's guidance I was able to complete my master's degree at Kansas State University. I would also like to thank my committee, Dr. Jan Sargeant, Dr. Mike Sanderson, and Dr. John Shirely, for their guidance.

I would like to thank those that assisted me with my master's projects, Neil Walace, Xiaorong Shi, Amy Hanson, and Trent Fox. Their help is greatly appreciated.

Last I would like to thank my family and friends. My parents Marvin and Karen, and the rest of my family, Erich, Todd, Megan and Cole, for their love and support throughout my educational career.

CHAPTER 1 - A Literature Review

Escherichia coli O157:H7 in cattle: Gastrointestinal Location

Introduction

Escherichia coli O157:H7 is a major food-borne pathogen, first identified in causing illness in association with consumption of contaminated ground beef (Riley et al., 1983). Illnesses caused by *E. coli* O157:H7 range in severity from mild watery diarrhea to more severe bloody diarrhea, hemolytic uremic syndrome (HUS), and in some instances death. Children, the elderly, and those with compromised immune systems are most at risk for developing life threatening complications, contributing to the majority of severe cases. In a review of *E. coli* O157 outbreaks between 1982 and 2002 reported to the Centers for Disease Control (CDC), Rangel et al. (2005) summarized that the pathogen caused 73,000 illnesses in the US annually. The route of transmission for the outbreaks included foodborne (52%), person to person (14%), waterborne (9%), animal contact (3%), laboratory-related (0.3%), and unknown (21%). Among the foodborne outbreaks, 38% was attributed to ground beef and 21% to produce (Rangel et al., 2005). *Escherichia coli* O157:H7 has a low infectious dose unlike other common food borne pathogens (Bell et al., 1994; Tilden et al., 1996; Gansheroff et al., 2000).

Cattle are a major reservoir of *E. coli* O157:H7 because of the ability of the organism to persist in the gastrointestinal tract and shed at varying levels and durations in the feces (Gyles, 2007). Although colonized with *E. coli* O157:H7, cattle and other ruminants remain healthy and appear to have no adverse effect from the organism (Cray and Moon, 1995; Moxley, 2004) Cattle do not have the Shiga toxin receptors needed for the toxins to exert biological activities on cells to cause illness (Pruimboom-Brees et al., 2000). The prevalence of *E. coli* O157:H7 and duration of shedding in cattle feces fluctuates and are affected by a number of factors. The most consistent factor affecting *E. coli* O157:H7 shedding is the season. Cattle typically shed more *E. coli* O157:H7 in the summer months than in the winter months (Chapman et al., 1997; Gansheroff et al., 2000; Hancock et al., 1997; Van Donkersgoed et al., 1999). Edrington et al. (2006) found that day length was correlated with fecal shedding of *E. coli* O157:H7. There is also individual variation in the duration of *E. coli* O157:H7 shed in the feces among animals. Most cattle are categorized as transient shedders of the organism, shedding for a short duration at lower concentrations. The concentration of *E. coli* O157:H7 shed in the feces usually ranges from 10^1 to 10^5 CFU per g of feces (Omisakin et al., 2003). The reason for the different

concentrations in the feces is unknown. A small portion of cattle fecal positive with *E. coli* O157:H7 shed the organism at concentration 10^3 CFU per g of feces or higher, referred to as "super shedders" (Naylor et al., 2003; Matthews et al., 2006; Cobbold et al., 2007). Super shedders despite representing a small portion of the cattle population are problematic in the control of *E. coli* O157:H7. Omisakin et al., (2003) found 44 animals that tested positive for *E. coli* O157:H7 from a total of 589 animals sampled; only 9% of the cattle shed high levels of the bacteria (> 10^4 CFU per g of feces). However, these 4 animals resulted in over 96% of the total *E. coli* O157:H7 produced by all 589 animals tested. Low et al., (2005) noted that the presence of a super shedder in a lot of cattle at a processing facility was significantly correlated with low-level (1 X 10^3 CFU g^{-1}) positive cattle in the same lot. Thirty five out of 267 cattle test were positive for *E. coli* O157. This study also showed that only 3.7% of the cattle were super shedders from the 267 that were sampled. While the researchers made no direct link, these animals are a likely source for inoculating other cattle or contaminating the environment. While on-farm targeting of super shedders is not currently feasible, identifying these cattle may prove to be an important tool to reducing the concentration of bacteria entering slaughtering facilities. Elder et al., (2000) showed a positive correlation between feces and hide prevalence of *E. coli* O157:H7 and carcass prevalence. Omisakin et al. (2003) observed that presence of a high-shedding animal (>10^4 CFU g^{-1}) at slaughter increased the risk of meat contamination during the slaughtering process. McEvoy et al. (2003) concluded that carcass contamination with *E. coli* O157:H7 can occur during the hide removal and bung tying processes and that the contamination can remain on the carcass throughout processing.

A rise in *E. coli* O157:H7 illness linked to contaminated water or direct cattle contact emphasizes a need for on farm level implement strategies (Rangel et al., 2005). Targeting the live animal will help reduce the *E. coli* O157:H7 present at the time of slaughter, lowering the risk for cross contamination of carcasses. For on-farm interventions to be successful, it is important to identify the primary site(s) of *E. coli* O157:H7 in the gastrointestinal tract of cattle and the factors involved in the bacterium's ability to persist in that location.

Gut Location

Fecal shedding of *E. coli* O157 in cattle is related to the organism's ability to persist in the gastrointestinal tract. It is important to know the major site of persistence of *E. coli* O157 in

the gastrointestinal tract because it provides insight into the ecology of the organism and the site can then be targeted for intervention strategies. Understanding where, why and how *E. coli* O157 inhabits the gastrointestinal tract will lead to more effective methods of controlling colonization and shedding of *E. coli* O157:H7. Previous studies have shown that the gallbladder may be a site of persistence and a source for fecal shedding of certain enteric food-borne pathogens, such as *Salmonella* or *Campylobacter* (Buchwald and Blaser, 1984; Ertas et al., 2003). Stoffregen et al. (2004) demonstrated that when calves were immunosuppressed and experimentally-inoculated, *E. coli* O157:H7 localized in the gallbladder. They speculated that the gallbladder may be a site and source of gastrointestinal *E. coli* O157:H7 and contamination of meat at slaughter. Also, Jeong et al., (2006) reported isolation of *E. coli* O157:H7 in gall bladders of cattle and suggested that the organism can reside at a low level in the gall bladder of cattle. Recently, Reinstein et al. (2007) collected gallbladders and rectal contents from cattle (n=933) at slaughter to determine whether the gallbladder harbors *Escherichia coli* O157:H7. Both gallbladder mucosal swabs and homogenized mucosal tissues were used for isolation. Only 5 gallbladders (0.54%) were positive for *E. coli* O157:H7. Fecal prevalence averaged 7.1%, however, none of the cattle that had *E. coli* O157:H7 in the gall bladder was positive for feces. Therefore, the study concluded that gallbladder does not appear to be a common site of colonization for *E. coli* O157:H7 in beef cattle.

There has been much debate over the primary location of *E. coli* O157:H7 in the gastrointestinal tract of ruminants. Generally, two approaches have been used by researchers to determine the location of prevalence or persistence of *E. coli* O157:H7 in cattle or sheep. One approach is to experimentally inoculate cattle and after a period of fecal sampling euthanize and necropsy the animals to collect contents from different gut locations. The second approach is to sample gut contents of cattle immediately after slaughter to determine prevalence *E. coli* O157:H7.

Experimentally Infected

Cray and Moon (1995) experimentally inoculated 17 calves and 22 adult cattle with 10^{10} CFU of Shiga toxin-producing *E. coli* O157:H7 strain 3081, that was resistant to kanamycin and ampicillin. Cattle were housed individually and fecal samples were obtained post inoculation. All cattle were feces positive for *E. coli* O157:H7 strain 3081 post inoculation. One adult animal

each was euthanized on days 2, 3, and 4 days, and 9 calves were euthanized on days 3 (n= 3), 14 (n= 4) and 18 (n= 2) post inoculation, and tissue samples and contents were collected from the rumen, reticulum, abomasum, jejunum, ileum, cecum, and colon. A final fecal sample was also collected at the time of necropsy. The three adult cattle were positive for *E. coli* O157:H7 in the ruminal tissue, cecal tissue, and in the feces. Two of the 3 adult cattle sampled were positive in the abomasum and colon; with only 1 adult was positive in the reticulum. None of the tissue samples collected from the jejunum or ileum of the adult cattle were positive. The highest concentration of *E. coli* O157:H7 was found in the feces averaging 1.0×10^5. Higher concentrations were found in the cecum and colon than in the rumen. The reticulum and abomasum had an average concentration of less than $2.0 \times 10^{1.}$ Three calves were euthanized and sampled on day 3 were positive for *E. coli* O157:H7 in the rumen, reticulum, ileum, cecum, colon, and feces. Two of the 3 calves euthanized and sampled on day 3 were positive in the abomasum. All six calves euthanized between days 14-18 post inoculation tested positive for *E. coli* O157:H7 in the feces. Five of the 6 calves were positive in the cecum and colon, and 3 of 4 animals with rumen samples were positive in the rumen. One calf was positive in the reticulum tissue from the 4 tested and 1 calf was positive in the ileal tissue from the 6 tested. None of the 4 animals tested, sampled 14-18 days post inoculation, were positive in the abomasum tissue. None of the 9 calves sampled, 3 days post inoculation or 14-18 days post inoculation, were positive in the jejunum tissue samples. The concentration of *E. coli* O157:H7 was highest in the feces, colon and cecum. Tissue samples were tested for *E. coli* O157:H7 from other locations; tonsil, liver, bile, spleen, and kidneys. These tissues were all negative for *E. coli* O157:H7 in the adult cattle sampled and only the tonsil tissue was sporadically positive in calves, primarily found in calves 3 days post inoculation. The results from this study indicated that the rumen was the most prevalent site for *E. coli* O157:H7.

Brown et al. (1997) experimentally inoculated 9 calves, 8 weeks of age, with 10^{10} CFU of a 5-strain mixture of *E. coli* O157:H7 adapted to nalidixic acid (50 µg/ml). Calves were housed in individual climate controlled and concrete surfaced BL-2 rooms. Fecal samples were collected once during a two week conditioning period prior to the start of the study to confirm that the calves were negative for *E. coli* O157:H7 prior to inoculation. Calves were inoculated via oral-gastric intubation with the mixture of *E. coli* O157:H7 after withholding feed for 36 hours. Feces (10 g) was collected daily from the rectum of each calf and *E. coli* O157:H7 was

isolated by plating on MacConkey Sorbitol Agar containing 50 µg of naladixic Acid. All 9 calves were fecal positive for *E. coli* O157:H7 post inoculation with concentrations highest during the first 14 days post inoculation. Rumen fluid was sampled from 5 calves on day 7 through day 19 by gastric intubation. The samples were enumerated for *E. coli* O157:H7. The concentration of *E. coli* O157:H7 decreased with time in all 5 calves sampled. Calves were euthanized between 13 and 28 days post inoculation. The gastrointestinal tracts, clamped at the esophagus and rectum, were removed intact. Tissue samples were taken from the following locations in the gastrointestinal tract: rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, distal cecum, proximal cecum, ascending colon, spiral colon, transverse colon, and descending colon. Contents were sampled from the rumen, reticulum, omasum and abomasum. In the nine calves sampled, the highest prevalence was found in the rumen, omasum, reticulum, distal cecum, and spiral colon with 9, 9, 7 and 7 calves testing positive for *E. coli* O157:H7, respectively. Six calves were positive for *E. coli* O157:H7 in the proximal colon and 5 calves were positive in the ascending colon, transverse colon, and descending colon. Four or fewer calves were positive for *E. coli* O157:H7 in the duodenum, jejunum, and ileum with no calves positive in the abomasum. Higher concentrations of *E. coli* O157:H7 were found in the contents of the rumen, reticulum, omasum and spiral colon than in other locations sampled. The authors of this study concluded that the forestomach was a major site of prevalence for *E. coli* O157:H7. The organism was consistently isolated form the rumen fluid of the calves, and was present in the rumen and omasum of all 9 calves at the time of necropsy. The organism appeared to be associated with the contents in the gastrointestinal tract rather than colonized to the mucosal surface. Concentrations of *E. coli* O157:H7 were higher in the contents of the sample site than the associated tissue in all calves tested. Overall, the conclusion was that the rumen and colon are reservoirs for *E. coli* O157:H7 shed in the feces of cattle.

Rasmussen et al. (1999) investigated the role of the rumen and the complex interaction of the microbial population involved in rumen fermentation and its affect on *E. coli* O157:H7 presence in cattle. The conditions in the rumen of individual animals may dictate the survival of *E. coli* O157:H7. The concentration of volatile fatty acids in the rumen could impact the organism. Volatile fatty acids are toxic to *E. coli* O157:H7, thus as concentrations increase potentially less *E. coli* O157:H7 will survive passage through the rumen. Rasmussen et al. (1993; 1999) demonstrated that VFA concentrations decline in animals withheld from feed for

24-48 hours. Cattle withheld from feed have an increased concentration of *E. coli* O157:H7 compared to well-fed cattle (Rasmussen et al. 1993; Brown et al 1997). Rasmussen et. al (1993) stated that fasted calves were susceptible to *E. coli* O157:H7 at a lower dose, 10^7CFU, and shed more *E. coli* O157:H7 than calves maintained on a normal feed regime.

Grauke et al. (2002) experimentally inoculated 29 sheep with *E. coli* O157:H7 (ATCC 43894 or 43895) obtained from a human outbreak. The 12 ewes and 12 rams, 6 to 7 months in age, were housed as a group in a cement floor pen. Five ewes, 1 year of age, which had tested fecal positive for *E. coli* O157:H7 from a previous study were housed in raised grated-floor pens. Sheep were sacrificed weekly to determine gut location of *E. coli* O157:H7. Tissue and digestive contents were obtained from the rumen, abomasum, duodenum, lower ileum, cecum, ascending colon, descending colon, and rectum. *Escherichia coli* O157:H7 were inconsistently cultured from the gut tissue and only during the first week post inoculation. One of 4 sheep was positive for *E. coli* O157:H7 in the rumen tissue at the first week of sampling. Two of the 4 sheep sampled were tissue positive in the abomasum, duodenum, lower ileum, cecum, and ascending colon. The fecal samples were the most prevalent for *E. coli* O157:H7 with 14 of 23 sheep positive. There was a downward trend in positive fecal samples with 4 of 4 on week 1, 3 of 4 on week 2, 2 of 4 on weeks 3 through 5 and 1 of 3 on week 6. Four of 23 cecum samples were positive, but only during week 1 and 2. Three of 23 content samples from the ascending colon were *E. coli* O157:H7 positive, but only during the first 2 weeks. The rumen contents were positive in only 1 of the 23 sheep sampled on week 2. One sheep was positive for *E. coli* O157:H7 in the descending colon on week 6. None of the contents sampled from the abomasum or the duodenum were positive. The results indicated an increase in *E. coli* O157:H7 prevalence in the lower gut. To confirm their findings in sheep, 4 dually-cannulated Angus steers were experimentally inoculated with *E. coli* O157:H7 (ATCC 43894 or 43895) directly into the rumen. Cannulas were surgically fitted in the rumen and duodenum of the steers 1 year prior to the study. Digestive contents were sampled at hours 1, 2, 4 and 6, and then sampled on days 1, 2, and 3 followed by four day interval sampling. The rumen and duodenum contents were positive during the first few hours post inoculation and then only sporadically by enrichment. The feces remained positive through day 7 and then *E. coli* O157:H7 were detected only periodically through enrichment. The length of time a steer was positive for *E. coli* O157:H7 in the rumen or duodenum contents did not predict the length of time the steer would shed the

organism in its feces. Two cannulated steers were reinoculated with *E. coli* O157:H7 to determine if a previous exposure to the bacteria would alter the gut location of the bacteria. In both steers, the results were similar to the observations of the first inoculations in the 4 cannulated steers. The rumen and duodenum were rapidly cleared of the bacteria with only isolated positives from enriched samples 1 day post inoculation. The feces of 2 steers were positive several days after the bacteria cleared the forestomach. The result of the cannulated steers supported earlier findings in the sheep that the hindgut was a more prevalent location for *E. coli* O157:H7 colonization. Reinoculation of *E. coli* O157:H7 didn't appear to affect the prevalence of *E. coli* O157:H7 in the gut or feces of cattle.

Naturally Infected

Most studies conducted with slaughtered cattle in abattoirs have found approximately 7.5 % of the feces were positive for *E. coli* O157:H7 (Omisakin et al., 2003; Van Donkersgoed et al., 1999). The prevalence in feedlot cattle has been reported to range from 0.3 (Galland et al., 2001) to 35.8 % (Elder et al., 2000). The estimated total prevalence in U.S. cattle ranges from 2% to 28% (Hancock et al., 1994; Garber et al., 1995; Zhao et al., 1995; Faith et al., 1996; Rahn et al., 1997; Elder et al., 2000).

Laven et al. (2003) sampled slaughtered cattle to determine the location in the GI tract in which *E. coli* O157:H7 colonizes in naturally shedding cattle. Cattle (n=270) were tested from three processing plants in southern England from August 1999 through May 2000. At least 10 cattle were tested per sampling day with 6 samples per animal. Samples were taken from the dorsal and ventral rumen wall, rumen contents, colon wall, colon contents, and feces. Overall, 8.5% of the cattle sampled were positive for *E. coli* O157:H7 with no significant difference found among the 3 slaughtering facilities. The colon and rectum were the most prevalent sites with 4.6% and 3.2% of the cattle positive for *E. coli* O157:H7, respectively. Less than 2% of the tissues from the rumen or colon were positive for *E. coli* O157:H7. Only one rumen content sample was positive from the 170 cattle sampled. The prevalence for all the sites sampled with in a location (contents, and tissue), were 0.7%, 1.8%, and 2.7% for the rumen, colon, and feces, respectively. The highest concentration of *E. coli* O157:H7 was found in fecal samples, followed by the colon contents and colon wall. While there was significantly higher prevalence of *E. coli* O157:H7 associated with the rumen wall than in the rumen contents, the concentration

in rumen contents was higher than the concentration of *E. coli* O157:H7 associated with the rumen wall. The results confirmed research findings in experimentally inoculated cattle that the hindgut was the apparent location for colonization of *E. coli* O157:H7.

McEvoy et al. (2003) examined the prevalence of *E. coli* O157:H7 in feces and rumen contents of slaughtered cattle over a 12-month study. A total of 132 steers, 75 heifers, and 42 cows were sampled from a single processing plant in Ireland. Samples were collected immediately after cattle were slaughtered. Fecal samples were transported on ice and processed within 2 hours of collection. Rumen contents were strained prior to testing for *E. coli* O157:H7. The liquid fraction was stored overnight at 0^{o}C prior to enrichment for *E. coli* O157:H7 detection. *Escherichia coli* O157:H7 were isolated from 2.4% of feces and 0.8% of the rumen contents sampled. During the 12-month study, there was an increase in positive samples in spring and summer months. The prevalence was highest in the feces sampled. This study supported previous findings which indicated that the hindgut was the main site of *E. coli* O157:H7 colonization (Van Donkersgoed et al., 1999; Grauke et al., 2002).

Rectoanal Mucosal Junction

Naylor et al. (2003) experimentally inoculated calves with 1 of 3 strains *E. coli* O157:H7, 2 strains were isolated from a human illness with cattle linked as the source of the outbreak and the third was isolated from a milk-borne human outbreak. Calves were housed individually in BL-2 rooms. Fecal samples were taken from each calf on two separate occasions to assure the all calves were *E. coli* O157:H7 negative prior to the trial. Fecal samples were collected from each animal prior to euthanasia and enumerated for *E. coli* O157:H7. If feasible the feces collected was split into core and surface components. At necropsy, the rectum and anus were removed as a single piece. Tissue samples visibly free of feces were obtained from several location of the rectum in relation to the rectoanal junction (RAJ). Content from the rectum, 20-30 cm proximal to the RAJ (midrectal), was sampled. Content and tissue samples were also taken from the rumen, ileum, Peyer's patches, cecum, and colon. The feces (antemortem) from 13 of the 15 calves had significantly higher concentrations of *E. coli* O157:H7 than the midrectal content sampled at necropsy. In 11 of the 15 calves, the fecal samples were significantly higher in *E. coli* O157:H7 concentration than the colon or midrectal content. Among the 15 calves

sampled, *E. coli* O157:H7 was not recovered from the contents of 6 rumen, 13 ileum, 7 colon, and 2 midrectal samples. There was no significant difference in the concentration of *E. coli* O157:H7 recovered from the colon and midrectal samples. However, significantly higher *E. coli* O157:H7 counts were found in the feces than the either the colon or midrectal contents. The tissue samples were significantly higher in *E. coli* O157:H7 counts from terminal 5 cm of the rectum than other location sampled. The tissues from the rumen, ileum, Peyer's patches, colon, and 20-30 cm proximal to the RAJ were negative. Of the calves sampled, 5 fecal samples were able to be dissected into core and surface components with significantly higher concentration of *E. coli* O157:H7 on the surface than in the core section. The conclusion of Naylor et al. (2003) was that the lymphoid follicle-dense mucosa at the terminal rectum is the primary location for *E. coli* O157:H7 colonization.

Following the finding of Naylor et al. (2003) that the terminal rectum is the primary site of *E. coli* O157:H7 in cattle, Rice et al. (2003) developed a technique for sampling this area detection *E. coli* O157:H7. The mucosal surface of the rectoanal junction was swabbed with a foam tipped applicator and the method was referred to as the rectoanal mucosal swab (RAMS) technique. The RAMS method was compared to the fecal sampling method for detecting *E. coli* O157:H7 in cattle experimentally inoculated with *E. coli* O157:H7 (n = 16), cattle experimentally exposed to *E. coli* O157:H7 (n = 15) and cattle naturally positive for *E. coli* O157:H7 (n = 40). The results indicated that RAMS method was better at predicting long term prevalence of *E. coli* O157:H7 in cattle.

Sheng et al. (2004) compared oral inoculation of *E. coli* O157:H7 to rectal swab administration of *E. coli* O157:H7 in cattle to determine if the rectal inoculation will result in long term colonization. Cattle (n = 87) were used to compare the two challenge methods. There was more consistent colonization in cattle administered *E. coli* O157:H7 with the rectal swab than cattle orally inoculated with the bacteria.

Low et al. (2005) obtained 267 intact rectums from 267 cattle from 24 separate lots to determine if the terminal rectum is a primary location for *E. coli* O157:H7 to colonize in naturally infected cattle. Rectums were obtained immediately after slaughter, fecal material and mucosal surfaces were cultured for *E. coli* O157:H7 by direct and enrichment techniques. The two rectal sites tested were 1 cm and 15 cm proximal to the RAJ. Among 267 animals sampled 35 animals were positive for *E. coli* O157:H7 in one or both locations. The prevalence of *E. coli*

O157:H7 was highest in samples from 1 cm proximal to the RAJ. This finding confirmed previous challenge results form Naylor et al. (2003) where samples closer to the RAJ were higher in concentration of *E. coli* O157:H7. High levels of *E. coli* O157:H7 ($\geq 10^3$ cfu g^{-1} content or tissue) were observed in 3.7% of the positive cattle. The researchers concluded that the terminal rectum was the primary site for *E. coli* O157:H7 prevalence and that high-level shedding of *E. coli* O157:H7 resulted from colonization by the bacteria.

Greenquist et al., (2005) compared the RAMS method a fecal culture method for detecting natural prevalence of *E. coli* O157:H7 in feedlot cattle. The two methods were compared in 747-crossbred steers, by first swabbing the rectoanal junction and then collecting a fecal sample. The RAMS technique was more sensitive than the fecal culturing in detecting prevalence of *E. coli* O157:H7. Pulse-field gel electrophoresis data showed that there were genetic similarities among isolates cultured from the RAMS method and those cultured from feces of the steers.

Davis et al. (2006) compared the fecal culture method to the RAMS culture method for isolation *E. coli* O157:H7. They sampled dairy heifers from two university dairy herds. There were 20 heifers sampled at each location on the first day of sampling with additional heifers added each visit until the herd reach 40 heifers. The heifers were sampled over 1 yr with no more than 15 d between sampling times. They concluded that RAMS was as sensitive as fecal sampling method in determining prevalence of *E. coli* O157:H7.

Lim et al., (2007) selected long duration culture positive cattle from a larger population to further investigate the rectaoanal junction in *E. coli* O157:H7 positive cattle. Three Holstein steers were euthanized and the rectoanal junction and gastrointestinal tract were examined. *Escherichia coli* O157:H7 was only isolated from the rectoanal junction of these cattle. Lim et al. (2007) concluded that the presence of lymphoid tissue in this region was not the reason for the tropism of this region.

Cobbold et al. (2007) found that rectoanal junction was more prevalent for *E. coli* O157:H7 than the feces in feedlot cattle. Based on the ratios of *E. coli* O157:H7 to other *E. coli,* the rectoanal junction was the preferred site by the O157:H7 serotype. The results showed a correlation between persistence at the rectoanal junction and fecal excretion of *E. coli* O157:H7 in feedlot cattle. Cobbold et al. (2007) identified five super shedders based on concentration and persistence of *E. coli* O157:H7. Cattle were defined as super shedders on the basis of both high

mean RAJ concentrations (>10^4 CFU/RAMS) and persistent RAJ colonization (>4 consecutive positive RAMS samples). Cattle co-penned with a super shedder had higher pen prevalence of *E. coli* O157:H7.

Acid Resistance

Escherichia coli O157:H7 that colonizes and grows in the hindgut of cattle and other ruminants must pass through the acidic pH of the abomasums. The ability to survive low pH has initiated research to determine the acid resistance of *E. coli* O157:H7. This research is critical in understanding the pathogen's ability to survive the acidic conditions in the human stomach. The ability of *E. coli* O157:H7 to tolerate acidic condition is also problematic to the process of preventing this bacterium from entering into the food chain. Products that utilize low pH to kill potential pathogens may not eliminate all *E. coli* O157:H7 from the product. An additional concern for *E. coli* O157:H7's ability to survive acidic environments is the increase prevalence of the bacteria in acidic foods, such as apple juice, apple cider, and yogurt. The acid tolerance is a common characteristic of many enteric bacteria (Park et al., 1999). However, *E. coli* O157:H7 is more acid tolerant than other strains of *E. coli* (Diez-Gonzalez et al., 1997) and is more tolerant to low pH in the presence of oxygen (Diez-Gonzalez et al., 1997). *E. coli* O157:H7 has been shown to be most tolerant to acidic condition when the organism is in the stationary phase of growth (Lange and Hengge-Aronis, 1991). Lin et al., (1995) demonstrated that most *E. coli* O157:H7 strains tested in the stationary growth stage survived in synthetic gastric fluid (pH 1.5) in excess of 3 hours, the average time needed for a meal to clear the stomach. The small percentages of *E. coli* O157:H7 that survive the acid barrier in the human digestive tract have been shown sufficient to cause illness. Miller and Kaspar, (1994) showed that *E. coli* O157:H7 survived better in acidic conditions, pH 2.0, at 4^o C than at 25^o C. This illustrates the organism's ability to maintain its acid resistant properties during refrigeration. Acid tolerance of *E. coli* O157:H7 increases in medium with glucose as a substrate. *Escherichia coli* O157:H7 exposed to pH 4 to 5 produced by fermentation of glucose helped prepare the pathogen for acid shock (pH 2.0; Grauke et al., 2003). Exposure to low pH lead to the bacteria's ability to become more acid tolerant in cattle fed diets containing more easily fermentable substrates, such as high concentrate diets. Cell density has been shown to affect acid survival at

exposure to pH 2.0 *in vit*ro. Grauke et al., (2003) showed significant increase in tolerance to pH 2.0 when cultures were diluted 1:100 to 10^7 from 10^9. The ability of some *E. coli* O157:H7 cell to tolerate the acidic condition of the human stomach is believed to be a factor of the low infectious dose required to cause illness (Park et al., 1999). Hovde et al. (1999) and Grauke et al. (2003) showed no significant diet influence in acid tolerance of *E. coli* O157 among grain-fed and hay-fed animals. The *E. coli* O157:H7 isolates showed no influence in their ability to survive in the rumen, pass through the abomasums or survive the in vitro acid shock of culturing isolates, obtained from the rumen, duodenum or rectum, in media of pH 2.0 for 1 hour. However, there was a negative effect on growth of non *E. coli* O157 when animals were fed higher concentrate diets. Reducing non-pathogenic *E. coli* many increase the pathogenic *E. coli* through a reduction in competitive exclusion of this species. Hovde et al. (1999) showed that while there was not a difference among diets there were differences among the eight Holsteins used in the study. The survival of *E. coli* O157 in cattle fed hay or grain diets ranged from 100% to 15% survival and 82% to 10% survival respectively.

Neomycin

Neomycin, an aminoglycoside antibiotic, interferes with bacterial protein synthesis, resulting in bacterial death. Neomycin is commonly used to treat respiratory infection and scours in calves. Neomycin is approved for use in cattle and has a 24 hour withdrawal. *Escherichia coli* O157:H7 are not typically resistant to a large number of antibiotics (LeJeune and Wetzel, 2007). *Escherichia coli* O157:H7 are susceptible to neomycin sulfate (Galland et al., 2001; Stephan and Schumacher, 2001; Mora et al., 2005). Neomycin sulfate in the feed has decreased fecal shedding of *E. coli* O157:H7 in cattle (Elder et al., 2002; Woerner et al., 2006). Elder et al. (2002) orally administrated of neomycin for 2 days at the same dose that was used in our study (10 mg/0.45 Kg B. W.) significantly reduced generic *E. coli* and *E. coli* O157 shedding in the feces of cattle when sampled a day after the last dose. However, in day 5 following neomycin withdrawal, generic *E. coli* populations had returned to preadministration levels, but *E. coli* O157 remained undetectable. Loneragan and Brashears (2005) reported unpublished data on the efficacy of neomycin in a review of preharvest interventions to reduce carriage of *E. coli* O157 in harvest ready cattle. Neomycin sulfate was included in the drinking water for 2 days in

cattle ready to be shipped for slaughter in a commercial feedlot study. In the control and neomycin-treated cattle, *E. coli* O157 was isolated from 22.1% and 0.4% of feces and 50.0% and 2.5% of hides, respectively Alali et al. (2004) studied the effects of antibiotics in milk replacer on fecal shedding of *E. coli* O157:H7 in 1 week old Holstein calves orally inoculated with naladixic resistant *E. coli* O157:H7. Alali et al. (2004) fed calves milk replacer with or without antibiotics (2 mg oxytetracycline/kg BW/d and 4 mg neomycin/kg BW/d). Calves were fed their respected milk replacer 2 weeks prior to the challenge and for eight weeks post oral inoculation of *E. coli* O157:H7. More calves shed the naladixic resistant *E. coli* in the feces in the antibiotic-fed group than in the control group early in the study (d6 and d10). There were no differences in concentration of *E. coli* O157:H7 in the feces between treatment and control calves. Also, the duration of shedding did not differ between the treated and untreated calves. Alali et al. (2004) concluded that milk replacer supplemented with antibiotics increases probability of shedding *E. coli* O157:H7, but not the magnitude or duration of shedding. Woerner et al. (2006) tested the effect of neomycin to reduce *E. coli* O157:H7 in cattle 4 days prior to harvest. They administered neomycin (10 mg/0.45 Kg B.W.) for 3 days with a 24 hour withdrawal prior to slaughter. Neomycin-treated cattle had 0 and 8.5% compared to 45.8 and 40.3% in control cattle in feces and hides, respectively. Loneragan and Brashears (2005) reported unpublished data on the efficacy of neomycin in a review of preharvest interventions to reduce carriage of *E. coli* O157 in harvest ready cattle. Neomycin sulfate was included in the drinking water for 2 days in cattle ready to be shipped for slaughter in a commercial feedlot study. In the control and neomycin-treated cattle, *E. coli* O157 was isolated from 22.1% and 0.4% of feces and 50.0% and 2.5% of hides, respectively. The difference accounted for 98.2% and 95.0% reduction in fecal and hide prevalence in neomycin-treated cattle.

Conclusion

Despite improvements in the detection and surveillance of *E. coli* O157:H7 coupled with stringent monitoring of food production, the serotype is still a major food-borne pathogen of concern. *Escherichia coli* O157:H7 is among the top five most prevalent causes of foodborne illness in the US. Identifying the location where *E. coli* O157:H7 are most commonly present in the gastrointestinal tract of cattle will help on-farm preventive strategies to target

specific regions of in the gut in attempts to be more precise in controlling this pathogen. Determining the preferred sites of *E. coli* O157:H7 in the bovine host can lead to further discovery of the conditions in the gut that allow *E. coli* O157:H7 to persist. Determination of the location(s) within the gastrointestinal tract of ruminants that provide a favorable environment for *E. coli* O157:H7 growth and replication is important knowledge in developing any preventive techniques for reducing the bacteria.

Understanding the mechanisms whereby *E. coli* O157:H7 become acid tolerant will help to manage cattle in a manner that will reduce the conditions that lead to the acid tolerance. If acid resistance can be reduced or eliminated in the rumen and the hindgut of cattle more of the bacteria will be susceptible to control measures taken to prevent the bacteria from contaminating food products. Additionally, fewer *E. coli* O157:H7 will survive in the human stomach, thus reducing colonization and illness.

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Table 1.1 Prevalence of *E. coli* O157:H7 in cattle.

Author	Prevalence, % Rumen	Colon	Feces
Van Donkersgoed et al., 1999	0.8		7.5
Laven et al., 2003	0.7	1.8	2.7
McEvoy et al., 2003	0.8		2.4

Table 1.2 Gastrointestinal location of Escherichia coli O157:H7 in experimentally challenged cattle.

	Prevalence, %				
Author	Rumen	Cecum	Colon	Rectum	RAJ
Cray et al., 1995	90.0	91.7	83.3	100.0	NS
Brown et al., 1997	100	72.2	48.9	NS	NS
Grauke et al., 2002	4.3	17.4	8.7	60.9	NS
Naylor et al., 2003	60.0	25.0	53.3	100.0	53.3
Rice et al., 2003	NS	NS	NS	48.9	70.3
Van Baale et al., 2004	0	25.0	42.7	33.3	NS

CHAPTER 2 - Prevalence of *Escherichia coli* O157 in Gut Contents of Beef Cattle at Slaughter

Abstract

Fecal shedding of *Escherichia coli* O157:H7, a food-borne human pathogen, in cattle is because of the ability of the organism to prevail in the gut. The site of prevalence in the gut is important to understand mechanisms and factors affecting gut persistence and fecal shedding, and as a potential target for intervention. The objective was to determine the prevalence of *E. coli* O157:H7 in the gut. Gut contents (rumen, cecum, colon and rectum) were collected from slaughtered cattle (n=815) at a packing plant. Isolation of *E. coli* O157:H7 was by selective enrichment followed by immunomagnetic separation and identification of *E. coli* O157:H7 was based on indole production, positive agglutination for O157:H7 antigen, and presence of virulence genes. The overall prevalence of *E. coli* O157:H7 in cattle sampled was 20.6%. The prevalence (%) in the rumen, cecum, colon, and rectum was 4.9, 9.1, 7.7, and 10.3, respectively. *Escherichia coli* O157:H7 in rectal content was positively associated ($P < 0.01$) with presence in the rumen or colon and not in the cecum. Pulsed-field gel electrophoresis was performed to compare clonal similarity of isolates obtained from the rectum with other three regions of the gut (rumen, cecum, or colon) within cattle ($n = 77$, 144 isolates). The majority (79 to 90 %) of isolates obtained within the same animal shared a common PFGE type based on 95% Dice similarity. There were no significant differences in PFGE type between positive samples from the rectum and from other locations within the same animal. The acid tolerance for cattle with positive rumen (pre gastric) isolates and at least one other positive hindgut (post gastric) isolate within the same animal was determined. There was no significant difference between gut locations in the log reduction following acid challenge. It was concluded that hindgut was the major site of prevalence of *E. coli* O157:H7 in cattle at slaughter, majority of the isolates were clonally similar, and acid tolerance was not affected by gut location.

Introduction

Escherichia coli O157 is a major food-borne pathogen that causes enteritis in humans ranging in severity from mild to bloody diarrhea, hemolytic uremic syndrome and even death (Park et al., 1999; Rangel et al., 2002). Although *E. coli* O157 occurs in many domestic and wild animals, cattle have the highest prevalence and are a major reservoir (Gansheroff and

O'Brien, 2000; Bach et al., 2002). Fecal shedding of *E. coli* O157 in cattle is related to the organism's ability to persist in the gastrointestinal tract. The concentration and duration of shedding are highly variable, often intermittent with some shedding a few days only, while others for an extended period, up to a year or longer. Eliminating or reducing the pathogen load in cattle entering the slaughter house is a well recognized strategy to reduce carcass contamination (Hancock et al., 2001). Direct contamination of the carcass in slaughter house from gut contents containing *E. coli* O157 is an obvious hazard. Hide has been shown to be an immediate source of carcass contamination (Hancock et al., 2001; Elder et al., 2000) and feces is the most likely source of hide contamination is feces. Therefore, it is important to know the major site of persistence of *E. coli* O157:H7 in the gastrointestinal tract because it provides insight into the ecology of the organism and the site can then be targeted for intervention strategies.

Previous studies have conflicting data on the major site of persistence in the gastrointestinal tract of cattle. Brown et al. (1997) reported that the fore stomach was the main site in calves orally challenged with *E. coli* O157. Laven et al. (2003) sampled digestive tracts of cattle after slaughter and found a higher prevalence of *E. coli* O157 in the colon and the feces than in the rumen. Grauke et al. (2002) found that the hindgut, not the rumen, was the primary site of persistence in steers challenged with *E. coli* O157. The purpose of this study was to determine the main site of persistence in the gastrointestinal tract of *E. coli* O157:H7 and determine association between site of prevalence and fecal shedding. We used pulsed-field gel electrophoresis (PFGE) to genetically compare fecal isolates to isolates from different gut locations. Also, we compared the acid tolerance of ruminal (before abomasum) to isolates from hindgut sites (after abomasum).

Methods and Materials

Gut content samples were collected from slaughtered cattle at a Midwest packing plant during the summer months (May through August) over two consecutive years. Cattle (n = 823), originating from eleven cattle feeding operations, were sampled on different days (Table 1). Cattle were fed to meet the guidelines of natural-branded beef (9 groups) or organic beef production systems (2 groups) and their diets consisted primarily of corn grain and corn silage.

The origin of the cattle and major components of feed are shown in table 1. Contents were collected immediately post slaughter from four gastrointestinal sites: rumen, cecum, colon and rectum. Contents were placed in sterile Whirl-pak bags (Nasco, Ft. Atkinson, WI). The bags were transported on ice to the laboratory and held in a cold storage over night and processed the following day.

Isolation of E. coli O157

One gram of gut content sample was placed in a tube of 9 ml of gram-negative broth (GN; Becton Dickinson Co., Franklin Lakes, NJ) containing 0.05 mg/L of cefixime, 0.2 mg/L of cefsulodin, and 8 mg/L of vancomycin (GNccv; Greenquist et al., 2005). The tubes were vortexed and placed in an incubator for 6 h at 37^{o} C. After incubation, 1 ml of the sample was pipetted into a sterile tube containing immunomagnetc beads (Dyna Beads; Dynal Biotech ASA Oslo, Norway) specific for O157 and processed according to manufacture's protocol. After the immunomagnetc separation procedure, 50 µl of sample was spread plated onto sorbitol MacConkey agar (SMAC, Difco Laboratories, Detroit MI) containing 50 µl (0.05 mg/L) of cefixime and 50 µl of potassium tellurite (2.5 mg/L) (CT-SMAC). The plates were incubated over night at 37^{o}C. Six non-sorbitol fermenting colonies were selected and transferred onto blood agar plates Remel, Lenexa, KS) and incubated over night at 37^{o} C. Identification of *E. coli* O157 was based on indole production, positive agglutination for O157 antigen (Oxoid Limited, Basingstoke, Hampshire, England) and species confirmation with an API strip (Rapid 20E; bioMérieux, INC, Durham, NC). All confirmed isolates were stored on protect beads (CryoCare™, Key Scientific Products, Round Rock, TX) and frozen at -70 C for PFGE analysis and acid tolerance determinations

PCR for virulence genes

Isolates were tested for virulence genes, intimin (*eae),* Shiga toxins (*stx_1 and stx_2),* hemolysin (*hlyA),* and the H7 flagella gene (*fliC).* The procedure for PCR amplification of *eae, stx_1, stx_2,* and *hlyA* was according to Fagan et al. (1999). In brief, stored isolates were streaked onto blood agar. For the DNA sample preparation, a single colony from the blood agar plate was transferred into 1.5 ml microcentrifuge tube (Fisher Scientific, Pittsburgh, PA) containing 1 ml distilled water. The suspension was boiled for 10 min, then centrifuged at 10,000 rpm for 5 min

in a Micromax microcentrifuge (IEC, Needham Heights, MA) and supernatant was used as the template DNA for PCR. In a 0.2 ml PCR tube (Fisher Scientific), 19 µl of the PCR Master Mix (Promega, Madison, WI) and 1 µl of the sample supernatant were combined. Commercially manufactured oligonucleotide primers (Bio-Synthesis, Lewisville, TX) were added at 2 µM concentrations. The PCR amplification was preformed in a TP600 thermal cycler (Takara Bio Inc., Shiga, Japan). Running conditions for the multiplex PCR were as follows: initial 95°C denaturation for 3 min, followed by 35 cycles at 95°C for 20 sec, 58°C for 40 sec, and 72°C for 90 sec. A 72°C incubation step for 5 min occurred after the final cycle. The gene for H7 flagella, *fliC,* was detected by PCR as described Gannon et al. (1997). The amplified DNA fragments were electrophoresed using a 2% (w/v) agarose gel (Fisher Scientific). The gels were stained with ethidium bromide (0.5 µg/ml). The gels were visualized with UV light and imaged with a Gel-Doc 2000 fluorescent imager (Bio-Rad, Hercules, CA)..

PFGE analysis

Only isolates from multiple gastrointestinal tract sites were analyzed by PFGE. The procedure followed that described by PulseNet (CDC, http://www.cdc.gov/pulsenet/; Greenquist et al. 2005). At the time PFGE was done, the recommended n standard was *E. coli* O157 G5422. Briefly, isolates from protect beads were streaked onto blood agar plates and grown overnight at 37°C. Tris-EDTA buffer (100 mM Tris:100 mM EDTA, pH 8.0) was used to prepare the bacterial suspension to equal an absorbance of 1.3 to 1.4 at 610 nm. Two-hundred micro-liters of each suspension were mixed with 10 µl of Proteinase K (20 mg/ml; Fisher, Houston, TX) and 200 µl of 60° C 1% SeaKem Gold Agarose (BioWhittaker Molecular Applications, Rockland, MN) in TE buffer (10 mM Tris: 1 mM EDTA, pH 8.0) containing 1% sodium dodecyl sulfate. The mixture was then pipetted into disposable plug molds (Bio-Rad, Hercules, CA). Following solidification, plugs were placed into 1.5 ml of lysis buffer (50 mM Tris: 50 mM EDTA, pH 8.0 + 1% Sarcosine) containing 40 µl of proteinase K and the lysing procedure (2 to 4 h) was performed at 54°C in a water bath shaking at 75 rpm. The lysis buffer was then removed and plugs were washed six times (two washes in 10 ml of distilled water and four washes in 10 ml TE buffer. Washes were performed in a shaking water bath at 50°C for 15 min per wash. A 2-mm slice of each plug was digested (37°C, 2 to 5 h) using the restriction endonuclease, XbaI (Promega Corporation, Madison, WI). Plugs were loaded onto one percent SeaKem Gold

Agarose gel and run with *E. coli* O157:H7 strain G5244 (CDC) as the standard. The CHEF II system (Bio-Rad) was used for PFGE with the following run parameters: switch time of 2.2 and 54.2 s; angle of 120°; voltage 200 v; temperature of 14°C; and a run time of 21 h. Gels were stained in 400 ml of distilled water containing 40 µl of 1% ethidium bromide solution for 30 min and then destained by washing three times in distilled water (20 min per wash). Images from each gel were captured with a Gel Doc 2000 system (Bio-Rad), and BioNumerics software (Applied Maths, Inc., Austin, Texas) were used to analyze and compare band patterns. Band-based Dice similarity coefficient and the unweighted pair group method for clustering were used with a position tolerance of 1.5% for optimization and band comparison. Isolates were grouped into subtypes and types based on banding pattern similarities (Davis et al., 2003). Pulsed field gel electrophoresis types were defined as isolates having fingerprint patterns of >95% Dice similarity (only one or two band differences).

Determination of acid tolerance

A total of 73 isolates representing paired matching between the rumen and any one of the other three postruminal locations (cecum, colon, or rectum) were used. Isolates were grown in tryptic soy (TSB) broth for 6 hours (approx. 10^8 CFU/ml). The culture was diluted 1:5 in acidified (pH 2.0) TSB, vortexed, and incubated at 37^{o}C for one hour. Following incubation, the culture was neutralized with phosphate buffer saline. An ATCC strain (ATTC 43890) of *E. coli* O157:H7, originally isolated from a human outbreak of hemorrhagic colitis and determined to be acid sensitive (Grauke et al., 2003), was used as a standard. Bacterial concentrations before and after incubation were obtained by spread plating on SMAC plates..

Statistical Analyses

Overall prevalence in cattle was calculated as the proportion of cattle with at least one positive sample. Prevalence of *E. coli* O157:H7 in each gut location was calculated as the proportion of cattle testing positive in that location divided by the total number of samples collected in that gut location. Differences in prevalence of *E. coli* O157:H7 among samples from gut locations were determined using logistic regression in PROC GENMOD of SAS (SAS Institute, Cary, NC) and accounted for the effect of multiple samples per animal. The outcome was a binary variable corresponding to the presence of absence of *E. coli* O157:H7 in the sample.

Gut location was tested for significance as a fixed effect. The repeated measure was sample within animal. Logistic regression was used to test whether the presence of *E. coli* O157:H7 in gut sites was associated with fecal shedding. The data were aggregated to one observation per animal. The outcome was the presence or absence of *E. coli* O157:H7 in the fecal sample. The independent variables consisted of a yes / no variable for each of the other gut sites (rumen, colon, and cecum). Odds ratios were used to describe the strength of association between each gut location and fecal shedding. For animals with positive samples in the rectum and at least one other site, the probability that pairs of isolates within animals had the same PFGE pattern based on 95% or 100% homogeneity was compared using a chi-square test for homogeneity of proportions.

The difference in bacterial concentrations pre-and post acid treatment was compared between isolates obtained from different sites within animal for cattle with ruminal isolate and at least one other positive hindgut isolate. The difference between pre and post-acid challenge was calculated and a $\log_{10}$ transformation was performed on these values to quantify the outcome. To account for the lack of independence between measurements within each animal, repeated measures analysis of variance was used (PROC MIXED, SAS 8.0, SAS Inst. Inc., Cary, NC). The gut location was included as the independent variable, with gut location within animal as the repeated measure.

Results

Prevalence of E. coli O157

A total of 823 cattle were sampled. The ratio of concentrate to roughage ranged from 4:1 to 12:1 and the number of days on feed ranged from 120 to 300. Overall, 20.3% of cattle had *E. coli* O157:H7 isolated from at least one gut location. The prevalence (%) in the rumen, cecum, colon, and rectum was 4.9, 9.9, 7.6, and 11.1, respectively (Fig. 1). Prevalence in the rectum was significantly higher ($P < 0.05$) than in colon or rumen but similar to that in the cecum. Prevalence in the rumen was significantly lower than in the cecum, colon, or rectum. In the 167 animals that tested culture positive for *E. coli* O157:H7, the gut locations and number of locations that were positive varied (Table 2). Among 167 cattle that were culture positive for *E. coli* O157:H7 at least in one gut location (rumen, cecum, colon or rectum), only 90 cattle

(53.8%) had a positive rectal content (feces) sample. Majority of cattle (156 of 167; 93.4%) was culture positive for *E. coli* O157:H7 in the hindgut (cecum, colon or rectum). Among cattle that were positive in only one gut location, rectum had the highest prevalence (47 of 167 cattle; 28.1%; Fig. 2). Among cattle that were positive in multiple gut locations, prevalence in cecum, colon and rectum (15 of 167; 9%) was higher than in other combinations. Interestingly, only 5 of 167 (3%) cattle were positive for *E. coli* O157:H7 in all four gut locations sampled (Table 2).

The presence of *E. coli* O157:H7 in each of the gut location (rumen, cecum or colon) was significantly associated with rectal sample being positive. If *E. coli* O157:H7 was present in the rumen, it was 4.9 times more likely to be present in the feces (95% CI = 2.3, 10.4), if present in the cecum it was 2.2 times more likely to be present in the feces (95% CI = 1.1, 4.5), and if present in the colon it was 5.1 times more likely to be present in the feces (95% CI = 2.3, 10.4).

PCR

Analysis of the virulence genes by PCR revealed the presence of *eae*, *fliC*, *hlyA* in all *E. coli* O157:H7 isolates (100%). The majority of the isolates (85%) was positive for *stx2* and only 56% of the isolates was positive for *stx1* (Table 3). Of the total isolated examined, 13 isolates (4.9%) were negative for both Shiga toxins.

PFGE Analysis

Pulsed-field gel electrophoresis was performed on *E. coli* O157:H7 isolates to compare clonal similarity of isolates obtained from the rectum with other three regions of the gut (rumen, cecum, or colon) within cattle (n = 77, 144 isolates). The proportion of isolate pairs within cattle having clonal similarity (> 95 or >100% dice similarities) are shown in Table 4. The majority (79 to 90 %) of *E. coli* O157:H7 isolates obtained within the same animal shared a common PFGE type based on 95% Dice similarity. Based on 100% Dice similarity, 60 to 62% of isolates from the rectum were clonally similar to isolates from rumen, cecum, or colon. There were no significant differences in PFGE type between positive samples from the rectum and from other locations within the same animal based on 95% homogeneity (P = 0.54) or 100% homogeneity (P = 0.95) (Table 4).

Acid Resistance

The reduction in colony counts following acid challenge for cattle with positive rumen (pre gastric) isolates and at least one other positive hindgut (post gastric) isolate within the same animal is shown in table 5. The extent of reduction to 1 hour acid exposure ranged from 1 to 5 log cell concentrations. The ATCC strain used as a positive control (acid sensitive) exhibited 4 to 5 log reduction. There was no significant difference between gut locations in the log reduction following acid challenge. In comparison to the ATCC strain, 6 of 29 ruminal isolates (20%) and 10 of 46 hindgut (cecum, colon, or rectum) isolates (22%) showed 4 or 5 log reduction in colony counts (Fig. 3).

Discussion

In this study, *E. coli* O157:H7 was isolated from samples collected from four different fermentative compartments (rumen, cecum, colon, and rectum) of slaughtered cattle. The overall prevalence of *E. coli* O157:H7 in cattle (positive at least at one location) at slaughter was 20.6%, which was higher than prevalence in any one location, even the rectum. This suggests that prevalence estimation based on fecal sample or contents from one gut location may in fact under represent the actual prevalence of *E. coli* O157:H7 in cattle. Among gut locations, the prevalence was higher in hindgut compartments (cecum, colon and rectum) than in the rumen. Fecal *E. coli* O157:H7 is reflective of the ability of the organism to persist or colonize the gastrointestinal tract. Brown et al. (1997) reported that rumen and omasum were the primary sites of *E. coli* O157:H7 localization and proliferation experimentally inoculated calves. In contrast, Cray and Moon (1997) reported that in experimentally inoculated calves and adult cattle *E. coli* O157:H7 was found in the rumen and the upper gut, but its concentration was higher in the lower gut. Dean-Nystrom et al. (1999) and Buchko et al. (2000) reported that *E. coli* O157:H7 was rapidly eliminated from the rumen of the animals but persisted in the feces of some animals, suggesting that the hindgut may be the site of *E. coli* O157:H7 persistence. Also, Buchko et al. (2000) cultured *E. coli* O157:H7 from the ruminal digesta once, and was present in the feces and the saliva on many occasions, suggesting that the growth and maintenance of the organism was most likely to occur in the hindgut as opposed to the rumen. Grauke et al. (2002) reported a higher prevalence in the lower gut particularly the cecum, colon, and feces than in the rumen in sheep orally inoculated with *E. coli* O157:H7. Grauke et al. (2002) in challenged

steers, dual-cannulated in the rumen and duodenum, rarely isolated *E. coli* O157:H7 from the rumen or duodenum at 48 and 72 hours post inoculation, and that a positive in either of these sites did not predict length *E. coli* O157:H7 was shed in the feces. Laven et al. (2003) sampled cattle at the time of slaughter in three gut locations (rumen, colon, and rectum) and showed that the prevalence was higher in colon and feces than in the rumen. In another study of steers inoculated with *E. coli* O157:H7, reported by Grauke et al., (2003), the organism colonized the most distal region of the gastrointestinal tract and was not consistently cultured from the rumen or the duodenum. Naylor et al. (2003) have provided evidence that the primary site of *E. coli* O157:H7 colonization in cattle was the rectoanal junction in the terminal rectum. In experimentally inoculated cattle, Van Baale et al. (2004) observed that fecal shedding of *E. coli* O157:H7 continued long after it was undetectable in the rumen and data collected from necropsied cattle in that study indicated that none of the ruminal digesta sampled had any *E. coli* O157:H7; however, *E. coli* O157:H7 was detected more often in the cecum and colon than other locations in the gut. In our study, the cecum was the most prevalent hindgut location followed by rectum and colon. It appears that *E. coli* O157:H7 colonizes the hindgut region of the bovine gastrointestinal tract and the rumen perhaps is a transient site. The reason for the persistence of *E. coli* O157:H7 in the cecum and colon as opposed to the rumen is not known. Possibly, the conditions in the cecum and colon (higher pH, lower VFA concentrations, absence of ciliated protozoa, slower rate of passage of digesta, etc., compared to the rumen) are more favorable to the survival and growth of *E. coli* O157:H7 (Fox et al., 2007).

In our study, only 5 of the 823 animals sampled were positive in all four sites sampled, while 39 animals were positive in three locations. It seems that *E. coli* O157:H7 may not be continuously present in all locations of the gastrointestinal tract of cattle. The most prevalent three site combination was the cecum, colon and rectum (feces). Cattle with a culture positive rectal sample were more associated with a positive sample in the contents of the colon or rumen of the animal. Although the overall presence was higher in the cecum than in the rumen it did not predict a positive rectal sample. The animal's ability to shed *E. coli* O157:H7 includes physiological and biochemical conditions in the gastrointestinal tract, but the factors are largely undetermined. The virulence gene profiles of isolates from all gut locations were similar to the profiles of fecal isolates from beef cattle (Galland et al., 2001; Renter et al., 2002; Sargeant et al., 2006).

Pulsed-field gel electrophoresis has been used to compare genetic types of *E. coli* O157:H7 among colonized cattle (Avery et al., 2004;Barkocy-Gallagher et al., 2001; Davis et at., 2003; Lahti et al., 2003; LeJeune et al., 2004; ; Rice et al, 1999; Sanderson et al., 2006). Of the 823 cattle sampled, 67 had *E. coli* O157:H7 isolates in two or more locations (rumen, cecum, colon, and rectum). We were interested in cattle that were positive for *E. coli* O157:H7 in the feces and one or multiple other sites. Pulsed-field gel electrophoresis of Xbal1 digested DNA was used to compare these isolates within animals to determine if the isolates were genetically similar within that animal. There was a significant percentage of isolates that were clonally similar >95% Dice similarity. The genetic similarity among isolates cultured from the same animal shows that the predominance of an *E. coli* O157:H7 genotype in different gut locations (rumen, cecum, colon, and rectum).

Because of low infectious dose (< 700 cells; Padhey and Doyle, 1992), an important feature of *E. coli* O157:H7 contributing to its pathogenesis is its ability to survive acid as it passes through the stomach of humans (Gordon and Small, 1993) or in the environment (apple cider). In cattle, the organism has to survive the abomasal pH of 2.0 to 2.5 before reaching the hindgut. Interest on acid resistance was sparked by the report that grain-fed cattle shed more acid resistant *E. coli* because of exposure to higher VFA concentration than hay fed cattle (Diez-Gonzales et al., 1998; Fu et al., 2003). However, this conclusion was based on generic *E. coli* and was discounted in a study by Hovde et al. (1999) that showed acid resistance of *E. coli* O157:H7 shed in the feces was similar between grain-fed and hay-fed animals. This was in contrast to the observation of Tkalic et al. (2001) that ruminal fluid from steers fed a high-grain diet rapidly induced acid resistance in *E. coli* O157:H7. Grauke et al. (2003) showed that grain-feeding or hay-feeding did not affect the ability of *E. coli* O157:H7 to pass through the abomasal acidity (pH 2.0 to 2.5) to the lower gut. In this study, we compared isolates from ruminal contents to isolates from the hindgut region (cecum, colon, feces) in tolerance to acidic condition. Our hypothesis was that the hindgut isolates would be more tolerant of the acid shock procedure than those isolates cultured from ruminal contents. The results showed that acid susceptibility was similar between ruminal and hindgut isolates. While the procedure used mimics that of the change in pH condition from the rumen to the abomasums, the low tolerance may also be a result of the drastic change in pH used in testing the isolates.

Conclusion

The study confirms that *E. coli* O157:H7 is more often in the distal region than the proximal region of the gastrointestinal tract in cattle. The organism was isolated more often from the rectum than other regions of the hindgut. In analyzing the animals with two or more *E. coli* O157:H7 positive sites, it was concluded that fecal shedding was positively associated with persistence in the rumen or colon but not the cecum. There were clonal similarities between isolates from multiple locations of the gut. There were no significant differences in acid tolerance among isolates from the rumen and those from the hindgut.

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Figure 2.1 Prevalence of *Escherichia coli* O157 in gut locations in beef cattle sampled at slaughter. Bars not sharing the same subscript differ at p value < 0.05.

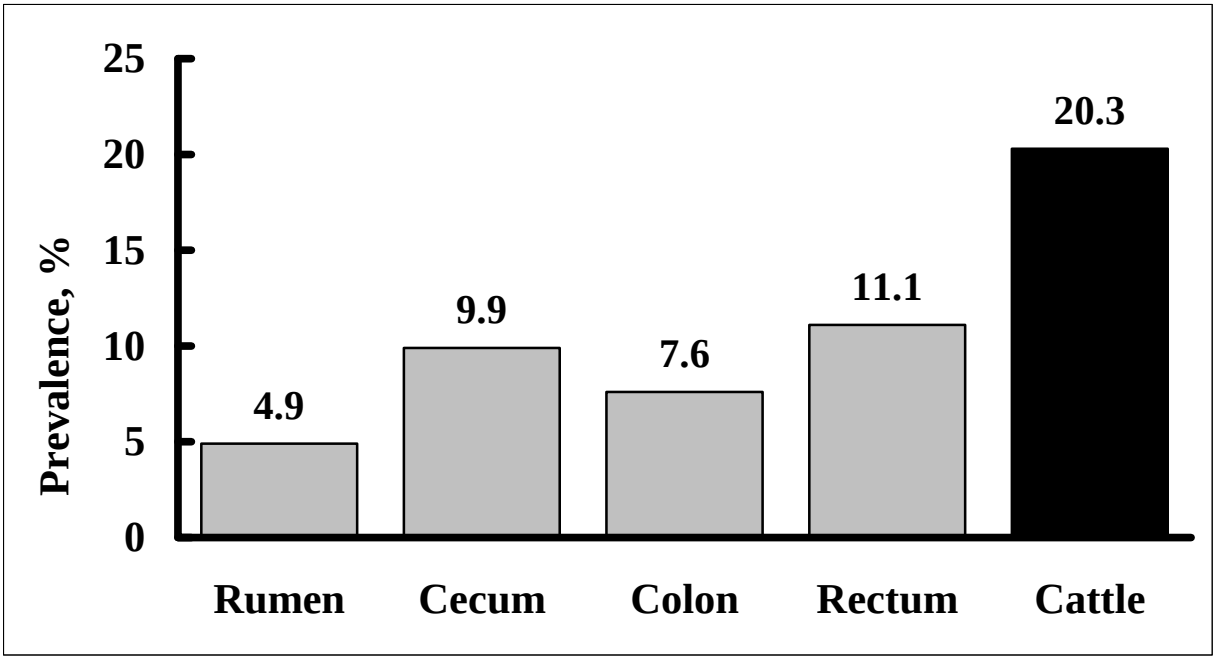

Figure 2.2 Prevalence in rumen, cecum, colon or rectum only in slaughtered cattle culture positive for *Escherichia coli* O157 at least in one gut location.

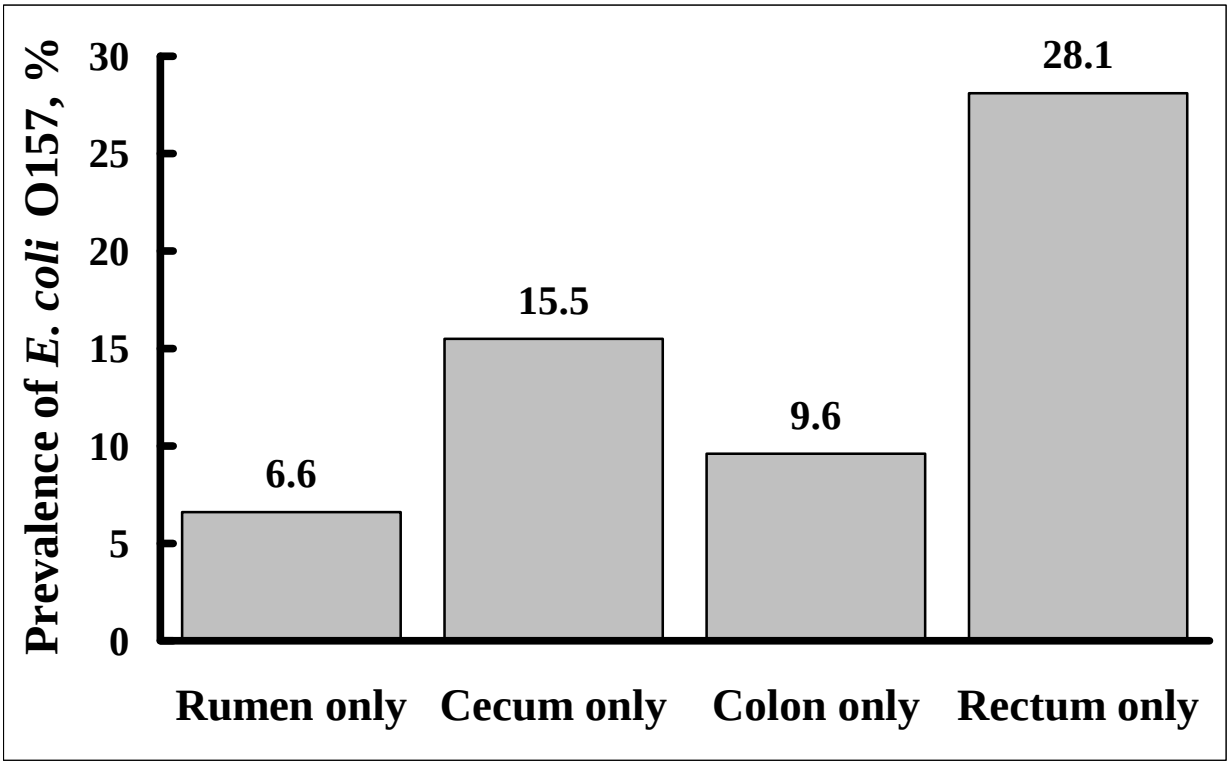

Figure 2.3 Percentage of *Escherichia coli* isolates from the rumen and the hindgut (cecum, colon, and rectum) sensitive (< 3 log10 reduction) to acid pH (2.5).

Table 2.1 Source of cattle and major feed components

Feedlot #	State	Diet	Number Sampled
1	Montana	High moisture corn, Alfalfa hay	262
2	Iowa	Corn, Alfalfa hay	131
3	Minnesota	Dry corn, High moisture corn, Corn silage, Alfalfa hay	79
4	Minnesota	Corn, Alfalfa	73
5	Montana	Corn, Alfalfa hay (Organic)	54
6	South Dakota	Cracked corn, Alfalfa hay	43
7	Minnesota	Cracked corn, Alfalfa hay	42
8	Nebraska	High moisture corn, Corn silage, Alfalfa hay	42
9	Iowa	High moisture corn, Corn silage, Alfalfa hay silage	40
10	Minnesota	Cracked corn, Alfalfa hay	34
11	Virginia	Whole corn, Pelleted alfalfa (Organic)	23

Table 2.2 Gut locations and combinations of gut locations culture positive for *Escherichia coli* O157 in 167 cattle with at least one positive sample.

Gut location	Cattle positive for *E. coli* O157	
	Number of cattle	Percent of cattle
Rumen only	11	6.6
Cecum only	26	15.5
Colon only	16	9.6
Rectum only	47	28.1
Rumen and cecum only	4	2.4
Rumen and colon only	2	1.2
Rumen and rectum only	9	5.4
Cecum and colon only	12	7.2
Cecum and rectum only	6	3.6
Colon and rectum only	6	3.6
Rumen, cecum, and colon only	4	2.4
Rumen, cecum, and rectum only	2	1.2
Rumen, colon, and rectum only	2	1.2
Cecum, colon, and rectum only	15	9.0
All four locations	5	3.0

Table 2.3 Virulence gene profiles of Escherichia coli O157:H7 isolates from gut locations of slaughtered cattle

Location	No. of isolates	No. of isolates positive for (%)				
		eae	*stx1*	*stx2*	*fliC*	*hlyA*
Rumen	39	39 (100)	19 (49)	35 (90)	39 (100)	39 (100)
Cecum	74	74 (100)	45 (61)	66 (89)	74 (100)	74 (100)
Colon	61	61 (100)	34 (58)	51 (84)	61 (100)	61 (100)
Rectum	92	92 (100)	52 (57)	74 (80)	92 (100)	92 (100)
Total	266	266 (100)	150 (56)	226 (85)	266 (100)	266 (100)

Table 2.4 Genomic finger print analyses of *Escherichia coli* O157 isolates from two different gut locations of cattle as determined by pulsed-field gel electrophoresis.

Gut locations	Number of cattle positive in both locations	PFGE patterns with ≥ 95% homogeneity[a]		PFGE patterns with 100% homogeneity[b]	
		No. of cattle	Percent	No. of cattle	Percent
Rectum vs. Rumen	20	18	90	12	60
Rectum vs. Cecum	28	22	79	18	64
Rectum vs. Colon	29	23	79	18	62

[a] $p = 0.54$
[b] $p = 0.95$

Table 2.5 **Reduction in colony counts of ruminal and hindgut isolates of *Escherichia coli* O157 after exposure to acid pH**

Location	# of isolates	# of isolates showing reduction in colony counts					
		0 Log	1 Log	2 Log	3 Log	4 Log	5 Log
Rumen	29	1	5	12	5	6	0
Cecum	15	1	3	5	2	3	1
Colon	13	1	1	8	0	1	2
Rectum	18	3	3	4	5	1	2
Hindgut (Cecum+Colon+Rectum)	46	5	7	17	7	5	5
ATTC 43890 (Control)		0	0	0	0	1	2

CHAPTER 3 - Effect of Neomycin supplementation in Feed on Fecal Shedding of *Escherichia coli* O157 in Experimentally Inoculated Cattle

Abstract

Neomycin, an aminoglycoside, is an antibiotic approved as a feed additive and for use in water to cattle with a 24 h withdrawal time before slaughter. Neomycin, with a bactericidal activity against gram negative bacteria, is most often used for the control or treatment of colibacillosis in neonatal calves. Our objective was to determine the efficacy of feed supplementation of neomycin on fecal shedding of *Escherichia coli* O157 in cattle. Twenty-four calves were randomly assigned to 1 of 2 treatment groups: control (n=14) and neomycin (n=10). Cattle were orally inoculated with 4.0 X 10^9 CFU per animal of a mixture of three strains of nalidixic acid-resistant (*Nal*^{R}*) E. coli* O157. Neomycin was fed at a concentration of 10 mg/0.45 Kg body weight for 15 days starting on the day prior to and 14 days post oral inoculation. Fecal samples were collected one day before inoculation (d -1), and fecal samples and rectoanal mucosal swab (RAMS) samples were collected on three days during week 1 of oral inoculation (d 1, 3, and 5), twice weekly from week 2 through week 7 (d 10, 13, 17, 20, 24, 27, 31, 34, 38, 41, 44, and 48), and then approximately weekly through d 111 (d 52, 59, 68, 75, 81, 89, 96, and 111). Fecal shedding of *Nal*^{R} *E. coli* O157 was quantified and prevalence in feces and RAMS was detected by selective enrichment and plating on selective medium. Neomycin feeding significantly (P =0.05) reduced prevalence and concentration of *E. coli* O157 compared to the control. However, following two weeks of neomycin feeding, concentration and prevalence were similar between the two groups. Short term neomycin feeding before slaughter may be a preharvest intervention strategy to reduce the fecal prevalence and concentration of *E. coli* O157 in cattle.

Keywords: Cattle, *E. coli* O157, Neomycin, Fecal shedding.

Introduction

Escherichia coli O157 is a major bacterial foodborne pathogen that causes illness in humans ranging in severity, from mild watery diarrhea to bloody diarrhea, and in children and the elderly the enteritis could lead to complication, such as hemolytic uremic syndrome (Rangel et al., 2005). The pathogen was first recognized as a major foodborne pathogen in association with a

multi-state outbreak in 1982 (Riley et al., 1983). Cattle are a major reservoir of this organism and adulterated ground beef was the initial source for transmission to humans (Bell et al., 1994). More recently, outbreaks have been associated with drinking contaminated water or consumption of contaminated produce (CDC, 2006; Rangel et al., 2005). In cattle, the organism resides in the gastrointestinal tract and is shed in the feces (Gansheroff and O'Brien, 2000; Grauke et al., 2002; Van Baale et al., 2004). Cattle feces are the major source of human illnesses. The terminal rectum, specifically the rectoanal junction (RAJ), has been proposed as the primary site of colonization of *E. coli* O157 in cattle (Naylor et al., 2003; Low et al., 2005). The swabbing of the region, 1 to 5 cm proximal to the rectoanal junction, with a foam-tipped applicator (Rectoanal Mucosal Swab or RAMS) has been shown to be a more sensitive sampling method for detecting prevalence of *E. coli* O157 in cattle (Davis et al., 2006; Fox et al., 2008; Greenquist et al., 2005; Rice et al., 2003). The mucosal carriage of *E. coli* O157 at the rectoanal junction is associated with long-duration fecal shedding and high level of fecal excretion (Cobbold et al., 2007; Davis et al., 2006; Lim et al., 2007; Low et al., 2005).

Intervention strategies are used at the preharvest (Callaway et al., 2002; LeJeune and Wetzel, 2007; Loneragan and Brashears, 2005) and post harvest stages (Koohmaraie et al., 2005) to reduce the pathogen load in cattle and to reduce the carcass contamination, thus lowering the risk of the pathogen entering the food chain. Preharvest intervention strategies have a distinct advantage over post harvest in reducing potential transfer of the organism by direct contact or a source of contamination of the environment, including water and produce.

Oral administration of neomycin sulfate to cattle has been shown to decrease fecal concentration and shedding of *E. coli* O157 (Elder et al., 2002; Woerner et al., 2006). The previous research evaluating the effects of neomycin on fecal shedding of *E. coli* O157:H7 have not determined if supplementing with the antibiotic will prevent *E. coli* O157:H7 shedding for an extended time after neomycin is withdrawn. The objective of this study was to determine the effect of feeding neomycin on rectal mucosal and fecal prevalence of *E. coli* O157 in experimentally inoculated cattle.

Material and Methods

Cattle

Cattle (Holstein or Holstein-cross), approximately 200 ± 50 Kg body weight, housed in dry feedlot pens, were prescreened for fecal shedding of *E. coli* O157 prior to the start of the study. Cattle were pen fed a dry-rolled corn based high grain diet (70% concentrate and 30% alfalfa hay) with monensin (Rumensin 80, 15 g/ton; Elanco Animal Health, Greenfield, IN). Cattle that tested negative for *E. coli* O157 were selected for use in the study and randomly assigned to two treatment groups, control (n=14) or neomycin (n=10), and moved to a biosafety-level 2 facility with the neomycin group housed separately from the control group. Neomycin (Neomycin sulfate 100^{R}[100 g/450 Kg]; Agland Coop., Oakland, NE) was incorporated in the feed daily, as a top dress, and antibiotic feeding was initiated one day prior to and administered 14 days (total of 15 days) following oral inoculation of *E. coli* O157. The dosage was calculated to provide 10 mg/0.45 kg B. W. per animal daily.

Oral inoculation with E. coli O157

Cattle were orally inoculated with a mixture of three strains of *E. coli* O157 of bovine origin, FRIK920, FRIK1123 and FRIK2000 (Supplied by Andrew Benson, University of Nebraska at Lincoln, NE), adapted to be resistant to nalidixic acid (Nal^{R}) at a concentration of 50 µg per ml. The strains were stored in protect beads (CryoCare™, Key Scientific Products, Round Rock, TX) at -80°C. The major virulence genes (*eae, fliC, hlyA, stx1* and *stx2)* and the genetic relatedness of the five isolates were determined by PCR (Fagan et al., 1999; Gannon et al., 1997) and pulsed-field gel electrophoresis (Greenquist et al., 2005), respectively.

To prepare the inoculum, isolates of each strain from protect beads were streaked onto blood agar plates and incubated for approximately 16 h at 37°C. An individual colony of each strain was picked and inoculated into 10-ml tryptic soy broth (TSB; Becton Dickinson and Co., Sparks, MD) and incubated overnight (16 to 18 h). From the tube, one ml of the culture was inoculated into 100 ml of TSB and incubated for 8 h. After incubation, cultures from each bottle were pooled and stirred on a magnetic stirrer to obtain the 3-strain mixture of Nal^{R} *E. coli* O157 for oral inoculation. An aliquot of the pooled mixture was serially diluted in buffered peptone water (Sigma-Aldrich, St. Louis, MO) and spread plated on blood agar to determine the concentration of the inoculum. Ten ml of the pooled mixture of Nal^{R} *E. coli* O157 was

inoculated into each animal via a gastric tube. The dose was estimated to provide 10^9 CFU per animal. The actual concentration inoculated to each calf was 4.0 X 10^9 CFU.

Sample collection

Cattle fecal samples were collected one day before oral inoculation (d -1), fecal and rectoanal mucosal swab (RAMS) samples were collected on three days during week 1 of oral inoculation (d 1, 3, and 5), twice weekly from week 2 through week 7 (d 10, 13, 17, 20, 24, 27, 31, 34, 38, 41, 44, and 48), and then approximately weekly through d 111 (d 52, 59, 68, 75, 81, 89, 96, and 111). Fecal samples were collected rectally and placed in whirlpack bags (Nasco, Ft. Atkinson, WI). Rectoanal mucosal samples were collected, according to Rice et al. (2004), using a sterile, foam-tipped applicator (VWR International, Buffalo Grove, IL), which was inserted approximately 2 to 5 cm into the anus of each calf and used to gently scrape the epithelium of the rectoanal junction. The swabs were placed in screw top tubes containing 3 ml of Gram Negative broth (GN; Becton Dickinson Co., Franklin Lakes, NJ) containing 0.05 mg/L of cefixime, 0.2 mg/L of cefsulodin, and 8 mg/L of vancomycin (GNccv; Greenquist et al., 2005). Whirl-Pak bags and GN tubes were held on ice and transported to the Kansas State University Prehavest Food Safety Laboratory, Manhattan, Kansas. All samples were kept at 4^o C and were processed within 24 h for quantification and detection of *Nal^R E. coli* O157.

Detection of E. coli O157 (Screening fecal and pre-inoculation fecal or RAMS samples)

Approximately 1 g of feces was placed in a tube containing 9 ml of GNccv broth. The fecal samples and RAMS samples contained in 3 ml GNccv broth were vortexed for 1 min and incubated for 6 h at 37˚C. They were then subjected to immunomagnetic separation (IMS; Dynal, Inc. New Hyde Park, NY) and spread-plated onto sorbitol MacConkey agar supplemented with 50 µg/ml cefixime and 2.5 µg/ml potassium tellurite (CT-SMAC). Plates were incubated overnight (16 to 18 h) and up to six sorbitol-negative colonies for each sample were streaked onto blood agar plates (Remel, Lenexa, KS) and incubated for 12 to 18 h at 37˚C. Growth on blood agar plates were tested for indole production, O157 antigen using latex

agglutination (Oxoid Limited, Basingstoke, Hampshire, England), and species confirmation was performed using API strips (Rapid 20E; Biomerieux, Inc., Hazelwood, MO).

Detection and quantification of NalR E. coli O157 (Post-inoculation RAMS or fecal samples)

Approximately 1 g of feces was added to a pre-weighed test tube containing 9 ml of GNccv broth. The tube was reweighed to determine the amount of fecal sample. The RAMS (in 3 ml of GNccv) and feces tubes were vortexed for 1 min and 1 ml of samples (fecal suspension or RAMS GNccv broth) was pipetted into a 96-well (2.4 ml well capacity) assay block (Corning Inc., Corning, NY). Samples were serially diluted (ten-fold) in buffered peptone water (Sigma-Aldrich) and 100 µl of appropriate dilutions (typically 10^0 to 10^{-4} dilutions) were spread plated onto CT-SMAC supplemented with 50 µg/ml of nalidixic acid (CTN-SMAC). The plates were incubated 16 to 18 h at 37^{o}C to determine the fecal concentration *Nal*^{R} *E. coli* O157 (CFU/g). Colonies on plates inoculated with RAMS samples were counted and used for detection only and not for quantification. Non-sorbitol fermenting colonies (up to three per sample) were picked from the CTN- SMAC plates and transferred onto blood agar plates and incubated overnight at 37^{o}C. Identification of *Nal*^{R} *E. coli* O157 was based on indole production, and positive agglutination for O157 antigen.

Samples that were negative for *Nal*^{R} *E. coli* O157 by direct plating (detection limit 10^2 per g or ml) were further processed by an enrichment procedure to detect low numbers of organisms. The tubes containing fecal suspension (1 g and 9 ml of GNccv) or RAMS (swab in 3 ml GNccv broth) minus aliquots taken for direct plating were incubated for 6 h at 37˚C. After incubation, 1.0 ml of inoculum was transferred to another 9.0 ml GNccv broth and incubated for an additional 18-24 h at 37˚C. After incubation, 100 µl was spread onto CTN-SMAC and incubated for 24 h at 37˚C. Following incubation, a maximum of 3 non-sorbitol fermenting colonies per sample were plated onto blood agar, incubated for 24 h at 37˚C and tested for indole production and for the O157 antigen by latex agglutination.

Statistical analyses

Fecal concentration of *Nal*^{R} *E. coli* O157 (log_{10} transformed) was analyzed for each treatment (neomycin or control), with animal as the experimental unit, using repeated measures (days) MIXED procedure (SAS Inst. Inc., Cary, NC). Also, the proportion of calves culture positive for *Nal*^{R} *E. coli* O157 in feces, in rectoanal mucosa (RAMS) or either was expressed as a binomial response (positive or negative) for each treatment, with animal as the experimental unit and was analyzed using repeated measures in GENMOD procedure (SAS Inst. Inc., Cary, NC).

Cattle in each treatment (control or neomycin-fed) were in two separate pens and were fed as a group. Therefore, the experimental unit for each treatment should have been the pen, which was one per treatment. In order to allow us to apply statistics to look for neomycin effect, we have chosen to use the animal ass the experimental unit.

Results

All three strains used for inoculation were positive for *eae, fliC, hly, stx1* and *stx2* genes. Based on PFGE analysis of *Xba*1 digested genomic DNA, all three strains were clonally different (Dice similarity < 95%). Interestingly, adaptation to nalidixic acid did not alter the banding patterns between parent and nalidixic acid-adapted strains (Figure 1).

In the control calves, oral inoculation with the cocktail of three strains of *Nal*^{R} *E. coli* O157 resulted in mean fecal concentration of 4 to 5 log per g of feces in the first three days. Subsequently, the fecal shedding began to decline gradually and by day 15 the concentration was less than log 2. Dietary supplementation of neomycin at 10 mg/0.45 Kg body weight caused a significant reduction (Figure 2; $P < 0.001$). There was a significant day effect and treatment by day interaction. Once the feeding of neomycin was stopped (day 14) the fecal concentration gradually increased. Analysis of fecal concentration of the control and neomycin-supplemented cattle from days 17 to 111 were similar ($P > 0.60$; Figure 3). In the neomycin-fed group, the proportion of calves culture positive in the feces (Figure 4), rectoanal mucosa (RAMS sample; Figure 5), or by either of the two methods (Figure 6) were lower ($P < 0.01$) than the calves in the control group. Again, after neomycin was withdrawn in the treatment group, there was no

difference between the two groups (data not shown) in the number of calves culture positive by feces, RAMS or either of the two methods.

Comparison of the two sampling methods for detection of cattle culture positive for *E. coli* O157 revealed that fecal samples were more sensitive than the RAMS technique in the detection of *E. coli* O157 ($P < 0.001$; Figure 7). More calves tested positive in the feces than by the RAMS method in 18 of the 22 sampling days.

Discussion

We used an experimental challenge study with nalidixic acid-resistant *E. coli* O157 to determine the efficacy of neomycin. The challenge model allowed us to quantify fecal concentration and also a fecal enrichment procedure was used to detect *E. coli* O157 positive cattle shedding below the detection limit of quantification. Additionally, we swabbed the rectoanal mucosal region to increase sensitivity of detection calves culture positive for *E. coli* O157 (Greenquist et al., 2005; Fox et al., 2008). The rectoanal junction, specifically 3 to 5 cm proximal to it, in the bovine rectum is considered to be the primary site for colonization of *E. coli* O157 (Low et al., 2005; Naylor et al., 2003). The frequency of isolation and the numbers of *E. coli* O157 are higher at the site (1 to 5 cm) proximal to the rectoanal junction than the other areas of the rectum (Low et al., 2005; Naylor et al., 2003). The finding is also supported by necropsy analysis of the gastrointestinal tract of sheep and cattle experimentally inoculated with *E. coli* O157 (Alali et al., 2004; Grauke et al., 2003; Van Baale et al., 2004). Localization at this site is in contrast to other *E. coli* serotypes, which are present throughout the hindgut (Cobbold et al., 2007). The reason for this tissue tropism is not known. The rectoanal region is rich in lymphoid tissue, which apparently has no relationship to the tropism (Lim et al., 2007). Rectal swab administration of *E. coli* O157 has been shown to result in consistent, long-term colonization in cattle (Sheng et al., 2004). There is some evidence that mucosal carriage at the terminal rectum is also associated with high concentration of fecal shedding (Low et al., 2005). The RAMS technique was designed to sample the mucosal surface of the rectoanal region (Rice et al., 2003). Studies comparing RAMS to traditional fecal culture for detection of *E. coli* O157 in live cattle, found that in nearly all cases, the RAMS was as sensitive, or more sensitive than fecal culture of either 1 or 10 g (Davis et al., 2006; Fox et al., 2008; Greenquist et al., 2005; Rice et al., 2003).

The increased sensitivity of detection of *E. coli* O157 is generally attributed to direct sampling of the colonized site and fewer competing organisms in the sample (Fox et al., 2008; Greenquist et al., 2005; Rice at al., 2003). In our study, RAMS did not detect any more positive calves than fecal samples analyses. We found fewer positive samples with the RAMS method than by culturing the feces, which could be attributed to fewer animals colonized at the rectoanal mucosal region with *E. coli* O157:H7.

Neomycin is an antibiotic approved as a feed additive and for use in water for cattle with a 24 h withdrawal time before slaughter. Neomycin is an aminoglycoside antibiotic with a bactericidal activity against aerobic, gram negative bacteria and because of toxicity, the use is most often limited to oral use or local applications, such as to wounds, skin infections and for mastitis (Prescott and Baggot, 1993). The antibiotic is also used in milk replacers for the control or treatment of colibacillosis in neonatal calves (Shull et al., 1978). Most isolates of Shiga-toxin producing *E. coli* O157 or non O157 are susceptible to neomycin (Galland et al., 2001; Stephan and Schumaker, 2001; Mora et al., 2005).

Elder et al. (2002) were the first to report that oral administration of neomycin for 2 days at the same dose that was used in our study (10 mg/0.45 Kg B. W.) significantly reduced generic *E. coli* and *E. coli* O157 shedding in the feces of cattle when sampled a day after the last dose. However, on day 5 following neomycin withdrawal, generic *E. coli* populations had returned to preadministration levels, but *E. coli* O157 remained undetectable. In our study, *E. coli* O157:H7 populations were below the detectable concentration ($< 10^2$ per g of feces) in 2 of 5 samples taken during the 15 days of neomycin feeding and on day 4 following withdrawal. However, on day 10 in the feces, and on days 2 and 3 in RAMS samples, none of the neomycin-fed calves was positive. Subsequent to neomycin withdrawal, calves remained positive by either quantification method or enrichment method for up to day 111. Our results suggest that neomycin feeding can reduce *E. coli* O157:H7, but does not eliminate the organism from the gastrointestinal tract of cattle. Also, neomycin does not appear to have a long term effect on fecal shedding of E. *coli* O157:H7.

Alali et al. (2004) compared the concentration and duration of fecal shedding of Nal^R *E. coli* O157 between calves fed milk replacer with or without antibiotics (oxytetracycline and neomycin) supplementation. The percentage of calves shedding Nal^R *E. coli* O157 in the feces of the antibiotic treated group was higher initially but the fecal concentration was not different.

Also, there was no significant difference in the duration of fecal shedding between calves fed milk replacer with antibiotics than milk replacer without antibiotics. The apparent difference between the study reported by Alali et al. (2004) and our study may be related to the dose of neomycin (4 mg/ kg BW) included in the milk replacer and also the strain of Nal^R *E. coli* O157 used the study was resistant (MIC > 40 μg/ml) to neomycin. Woerner et al. (2006) administered neomycin (10 mg/0.45 Kg B.W.) for 3 days with a 24 hour withdrawal in feedlot cattle before slaughter. Neomycin-treated cattle had 0 and 8.5% compared to 45.8 and 40.3% in control cattle in feces and hides, respectively. Loneragan and Brashears (2005) reported unpublished data on the efficacy of neomycin in a review of preharvest interventions to reduce carriage of *E. coli* O157 in harvest ready cattle. Neomycin sulfate was included in the drinking water for 2 days in cattle ready to be shipped for slaughter in a commercial feedlot study. In the control and neomycin-treated cattle, *E. coli* O157 was isolated from 22.1% and 0.4% of feces and 50.0% and 2.5% of hides, respectively. The difference accounted for 98.2% and 95.0% reduction in fecal and hide prevalence in neomycin-treated cattle. Apparently, two day administration was effective in significantly reducing the pathogen prevalence in cattle ready for slaughter but did not eliminate the organism from all cattle.

The results of our study indicate that feeding of neomycin for up to 15 days will reduce fecal concentration and shedding, but did not eliminate *E. coli* O157 in calves. The reduction in fecal concentration suggests that cattle fed neomycin for a short term (2 to 15 days) before harvest will reach the slaughter house with a lower *E. coli* O157 load in the gut than the cattle that were not fed neomycin. There is some evidence that cattle shedding at high concentrations transmit *E. coli* O157 to a greater number of cohorts than those that shed intermittently or at low concentrations, therefore, increasing the probability of carcass contamination at slaughter (Omisakin et al., 2003; Mathews et al., 2006).

Feeding neomycin may lead to *E. coli* O157 developing a resistance to the antibiotic, which could have lead to more calves testing positive during the final sampling days of this trial. In this study, *E. coli* O157:H7 isolates were not tested for their resistance to neomycin... However, *E. coli* O157 resistance to neomycin is low (Alali et al., 2004; Alam and Zurek, 2004; Callaway et al., 2004; Woerner et al., 2006).

Neomycin appears to be an effective antibiotic for reducing *E. coli* O157:H7 and is an ideal candidate because of its approved use in feed and in drinking water, requires only

short term administration, and has only a 24-hour withdrawal before slaughter. However, the use of an antibiotic in feed or water as a potential preharvest intervention strategy is a controversial issue. Neomycin has limited use in human medicine and is generally restricted to topical applications. However, the antibiotic belongs to the aminoglycoside family, which includes other antibiotics (Gentamycin, Kanamycin, and Streptomycin) widely used to treat human infections. Therefore, resistance and cross-resistance must be considered before widespread implementation of neomycin inclusion in feed or water. Currently, neomycin has no label claim for use to reduce or eliminate *E. coli* O157 in cattle. Because of the increased concern on the use of antimicrobial feed additives in animal agriculture, it is highly unlikely that neomycin will receive such a claim.

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Figure 3.1. Dendrogram generated to show the relationship between the parent strains (FRIK 2000, FRIK 920, and FRIK 1123) and nalidixic acid-adapted strains (FRIK 2000 Nal-R, FRIK 920 Nal-R, and FRIK 1123 Nal-R) of Escherichia coli O157 of bovine origin. The scale at the top of the dendrogram indicates the levels of similarity between the isolates.

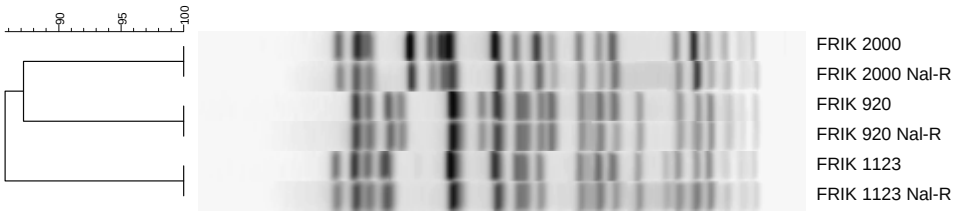

Figure 3.2. Concentration of nalidixic acid-resistant (*Nal*^{R}) *Escherichia coli* O157:H7 in feces of control or neomycin-supplemented calves. Neomycin (10 mg/0.45 Kg body weight) was administered in the feed from days -1 through 14 (Horizontal arrow).

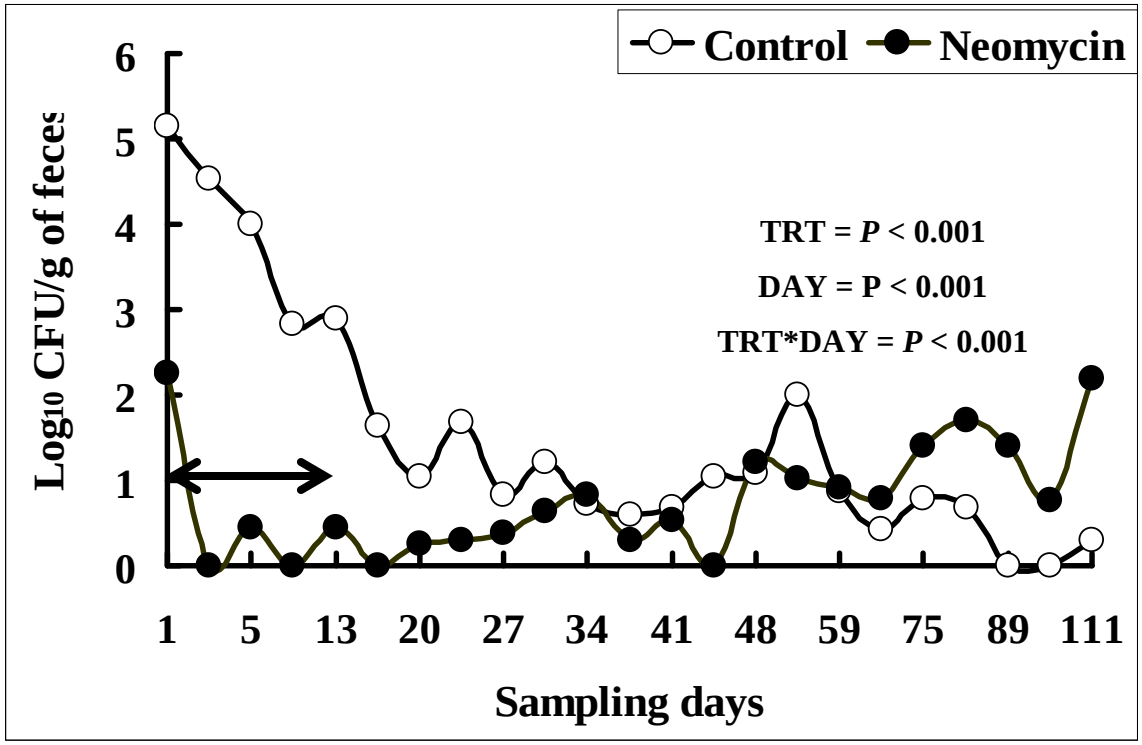

Figure 3.3. Concentration of nalidixic acid-resistant (NalR) Escherichia coli O157:H7 from days 17 to 111 in feces of control or calves that were supplemented with neomycin (days -1 to 14).

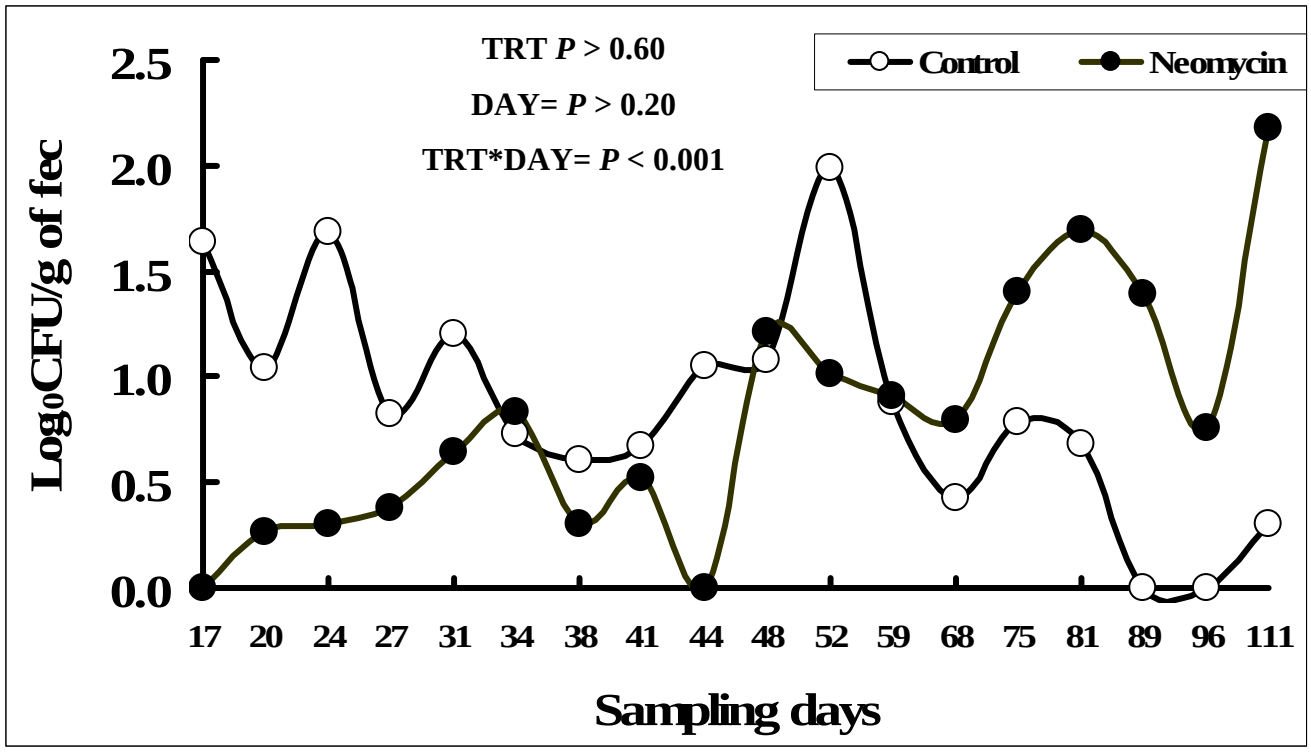

Figure 3.4. Percentage of calves culture positive for fecal nalidixic acid-resistant (NalR) Escherichia coli O157:H7 in control or neomycin-supplemented group. Neomycin (10 mg/0.45 Kg body weight) was administered in the feed from days -1 through 14 (Horizontal arrow).

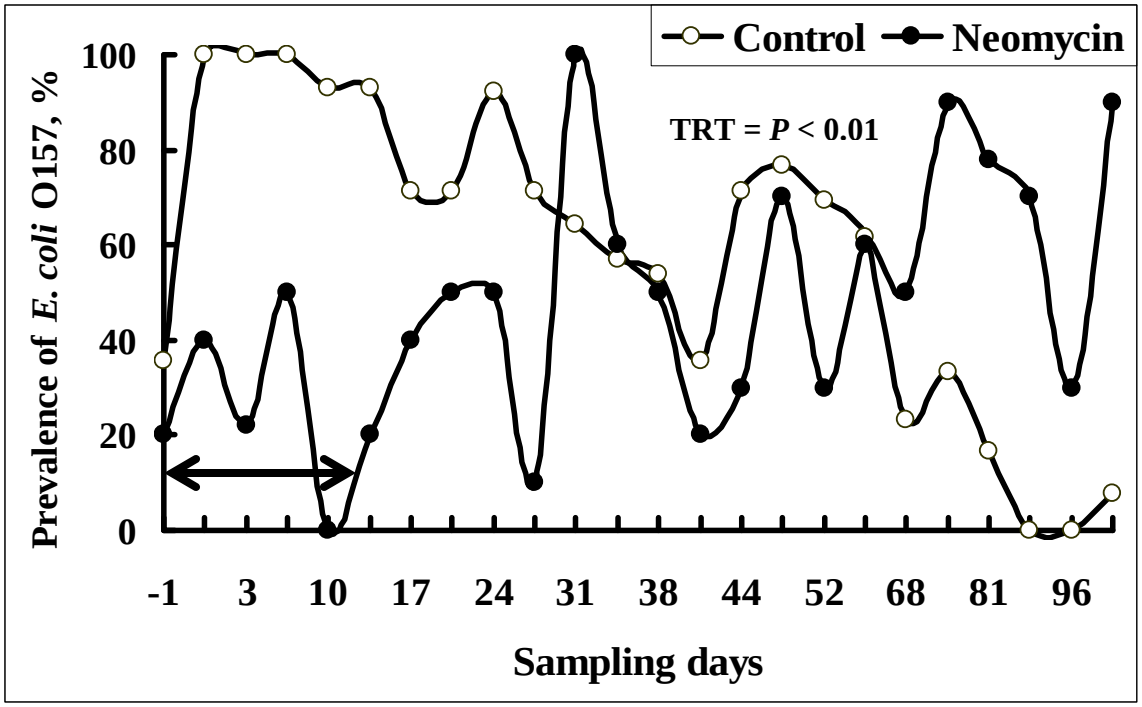

Figure 3.5. Percentage of calves culture positive for nalidixic acid-resistant (NalR) Escherichia coli O157:H7 based on sampling by recotoanal mucosal swab technique in control or neomycin-supplemented group. Neomycin (10 mg/0.45 Kg body weight) was administered in the feed from days -1 through 14.

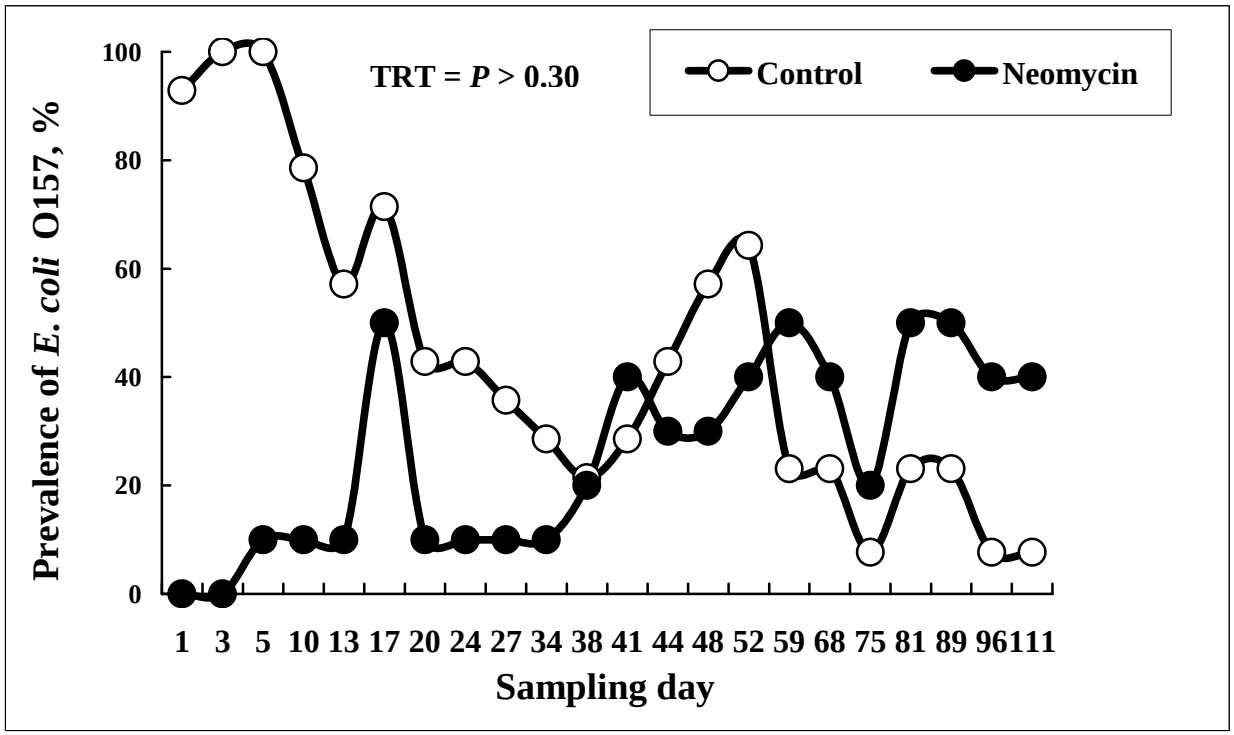

Figure 3.6. The number of cattle culture positive for nalidixic acid-resistant (NalR) Escherichia coli O157:H7 as determined by either fecal or rectoanal mucosal swab analysis in control or neomycin-supplemented calves. Neomycin (10 mg/0.45 Kg body weight) was administered in the feed from days 1 through 14 (Horizontal arrow).

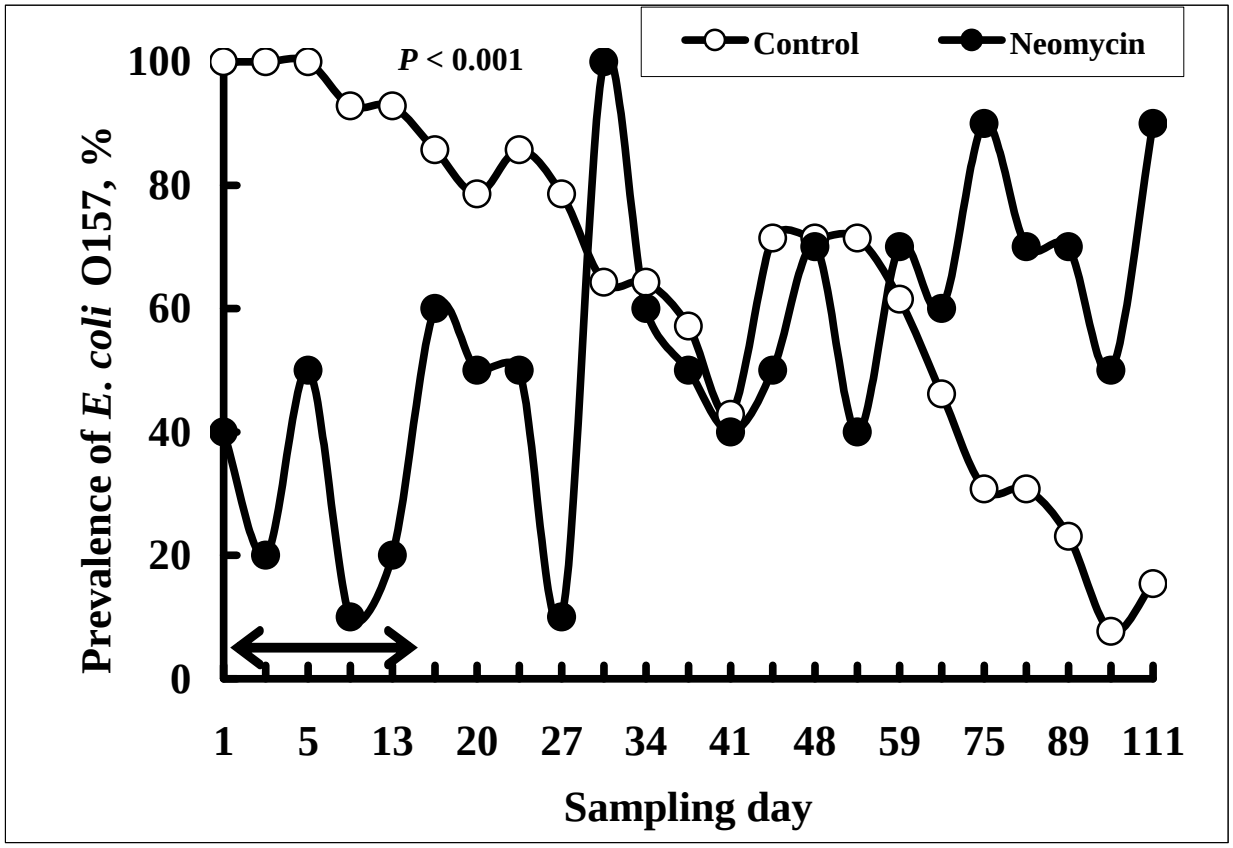

Figure 3.7. Prevalence of nalidixic acid-resistant (NalR) Escherichia coli O157:H7 in calves based on sampling methods, feces or rectoanal mucosal swab.