Identification of Bovine Reservoirs of Human Pathogenic non-O157 Shiga Toxin-producing *E. coli.*

Final report

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Objectives: To determine the prevalence, level, and types of O157 and non-O157 Enterohemorrhagic *E. coli* (EHEC) in cattle based on analysis of rectal anal mucosa swabs (RAMS) collected at harvest from cattle originating from different production systems.

Conclusions: Overall**,** EHEC-O26 was most frequent in eastern dairy cattle (16.8%), while EHEC-O45 was more widely spread in central fed cattle (21.2%), eastern fed (25.2%) and cull dairy cattle (28.1%), and northern fed (22.3%) and cull beef cattle (28.7%). EHEC-O103 was most common in northern fed cattle (32.2%), but also frequent in central (22.3%), western (21.7%) and southern (19.5%) fed cattle. EHEC-O111 was most common in southern cull beef (3.4%) and fed (2.0%) cattle and eastern cull beef (2.4%) and cull dairy (2.3%) cattle. Finally EHEC-O145 was most commonly identified in northern cull beef cattle (12.0%). EHEC-O121 was the least frequent (0.3%) but identified in all regions except the north and identified in all cattle types at least once. Only samples that were culture confirmed to contain an EHEC were enumerated. Across all regions, the fed cattle had the highest percentage of EHEC super shedding, $>10^5$ CFUs/RAMS, followed by cull dairy and then cull beef cattle. Within each log range of CFUs/RAMS (i.e.: 10^5,10^6, 10^7 *etc*…) the proportion of shedding was similar amongst the production groups. The most commonly identified EHEC shed at high levels were EHEC-O157 (23.9%), EHEC of non-top-7 O-groups (19.7%), EHEC-O103 (18.5%), and EHEC-O26 (12.6%), while other EHEC-O111, O145, O45 and O121 were identified as being shed less often, 5.9%, 2.9%, 0.8%, and 0.4% respectively.

Deliverable: The results will assist the meat industry, regulators and public health officers by identifying the particular EHEC serogroups that are more commonly associated with different types of cattle, allowing more targeted and efficient testing and intervention use. As well as offer information on the levels of EHEC shed by different groups of cattle.

Technical Abstract

Ruminants, and especially cattle, are considered the primary reservoir of Shiga-toxin producing Escherichia coli that cause enterohemorrhagic disease (EHEC) and contaminated beef products are considered one vehicle of transmission to humans. However, cattle entering the beef harvest process originate from very different production systems: feedlots, dairies, and beef breeding herds. The objective of this study was to determine whether inherent differences in production system may affect the prevalence, levels shed, and serotypes present of EHEC in US cattle. Feces was collected in the form of rectal anal mucosal swabs (RAMS) from 1,042 fed cattle, 1,062 cull dairy cattle and 1,019 cull beef cattle at harvest in five regions of the US. Prevalence of the *stx* gene in feces ranged from 91% in cull dairy and cull beef cattle to 99% in fed cattle ($P<0.01$). When two additional factors predictive of EHEC (*eae*, and the Roka EHEC target) were considered, the predicted prevalence of EHEC was different ($P<0.0001$) between all 3 groups of cattle (fed = 77.2%, cull dairy = 47.7%, and cull beef = 38.7%). The presence of the top 6 non-O157 EHEC serotypes (O26, O45, O103, O111, O121 and O145) was determined using NeoSEEK molecular analysis as well as physical culture isolation. NeoSEEK analysis identified 33.9%, 20.4% and 15.0% and culture isolation confirmed 7.8%, 6.4%, and 5.1% of fed, diary and beef cattle feces to contain a top 6 EHEC, respectively. The most common serotypes identified using NeoSEEK analysis were EHEC-O26 in 16.8% of eastern dairy cattle and EHEC-O103 in 32.2% of northern fed cattle. EHEC-O45 was more widely spread in about 20% of central fed cattle, eastern fed and cull dairy cattle, and northern fed and cull beef cattle. The culture confirmed samples had the level of pre-culture enrichment EHEC enumerated across a detectable range of $>10^9$ to 10^3 CFUs/RAMS, with 10^5 CFUs/RAMS being the most common concentration shed. Amongst the cattle production groups the top 6 non-O157 EHEC serotypes, O103, O26, and O111 were identified as those most highly shed in 18.5%, 12.6% and 5.9% on the enumerated samples respectively.

Introduction (Goals/Objectives)

Shiga toxin–producing *Escherichia coli* (STEC) are associated with human disease ranging from mild diarrhea to severe enterohemorrhagic diseases such as hemorrhagic colitis, and hemolytic-uremic syndrome (HUS). More than 100 non-O157 Shiga toxin producing *E. coli* serotypes have been implicated in cases of human disease but a more limited number of serotypes are responsible for enterohemorrhagic disease. The non-O157 enterohemorrhagic *E. coli* (EHEC) of serogroups O26, O45, O103, O111, O121 and O145 have been increasingly isolated from clinical cases and outbreaks as well as from meat animals and environmental sources (Brooks 2005). The CDC has estimated that non-O157 STEC cause up to 37,000 illnesses each year in the U.S. For this reason the FSIS has recently declared the six listed serotypes also be considered adulterants in certain beef products.

Ruminants, are considered the primary reservoir of Shiga-toxin producing *Escherichia coli* (Beutin 1993; Karmali 2010), and contaminated beef products are considered one vehicle of transmission to humans. However, cattle entering the beef harvest process originate from very different production systems and are destined for different beef products. Feedlot cattle are young animals that originate primarily from densely populated confined animal feeding operations and 60% of a fed beef carcass is fabricated into whole muscle beef cuts with the remaining 40% destined for ground beef. Culled dairy and beef cattle are significantly older when harvested and are primarily destined for ground beef products. Cull beef cattle are typically pasture-fed and maintained at a lower density of animals than dairy cattle.

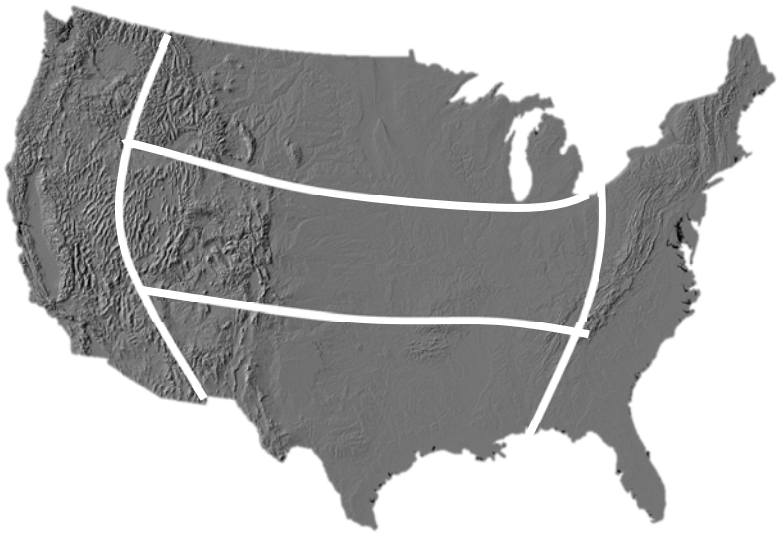
The feces prevalence rates, globally, of non-O157 STEC are reported to range from 4.6 to 55.9% in feedlot cattle and 4.7 to 44.8% in grazing cattle (Hussein, 2005). Studies of non-O157 STEC prevalence in feces of US dairy cattle have reported rates of 0 to 19% (Cray 1996; Thran, 2001; Wachsmuth 1991; Wells 1991) while the range of prevalence of non-O157 STEC in feces of US fed beef cattle has been reported to be 19 to 30% (Barkocy-Gallagher 2003; Renter 2005). Some STEC and EHEC serotypes appear to be more frequently found than others, and the effects of production system (e.g. feedlot, dairy, beef herd) may play an unrecognized role in their prevalence in beef products.

A thorough evaluation of cattle from different production systems, however, has not been performed. Since cattle entering the beef chain from the feedlot, dairy and beef herds are destined for different types of beef products, it becomes essential to determine if any particular population of cattle is more likely to harbor pathogenic EHEC, especially of the top six non-O157 serogroups. The studies available of STEC in feces of cattle from various environments are difficult to compare since different methods of detection and isolation were used. Many of the studies focused on a regionally limited sample set and often the category STEC is used to refer to EHEC or no distinction is made between these two groups of *E. coli*.

It has been shown that cattle shedding *E. coli* O157:H7 can shed the organism at varying levels. Some animals are only found positive using sensitive culture methods and are considered colonized while other animals may have high numbers of enumerable *E. coli* O157:H7 in their feces that can be cultured without enrichment steps. The levels of *E. coli* O157:H7 in high shedders can exceed 10^7 CFU/g or swab and cattle shedding in excess of 10^4 CFU/g or swab have been classified as super-shedders (Arthur, 2009, Cobbold 2007). Super-shedding cattle have been proposed to be the primary source of contamination in beef production environments (Arthur 2007, 2009). Data on the levels of non-O157 EHEC shed by cattle are limited but are thought to vary in a fashion similar to *E. coli* O157:H7.

This report describes the prevalence and levels of STEC, *E. coli* O157:H7 and EHEC of the six most frequent non-O157 serotypes in feces samples collected as rectal anal mucosa swabs (RAMS) from fed, cull dairy and cull beef cattle during harvest at multiple locations across the U.S. This was done using molecular markers that indicated the presence of STEC and EHEC (Shiga-toxin genes, intimin genes, Roka Biosciences EHEC marker, and NeoSEEK analysis panel) and culture isolation. Additionally, a quantitative PCR method was developed to estimate the levels of EHEC (O157 and non-O157) shed by cattle found positive by the culture methods.

Figure 1. Map of the US showing the regional divisions[a] where processing plants[b] were sampled in this study.



[a]Western, eastern, northern, southern and central US are self explanatory.
[b]Samples were collected from numerous production lots of cattle at two processing plants in the western, eastern, central and southern regions and one processing plant in the northern region.

Materials and Methods

Experimental Design.

A minimum of 1,000 cattle of each production type (fed, dairy and beef) were targeted for collection at nine regionally diverse beef processing plants located across the United States. The region boundaries are shown in Figure 1, and generally represent the northern, southern, eastern, western and central US. Samples were analyzed for EHEC levels before enrichment and STEC and EHEC prevalence after enrichment by molecular screening methods and confirmatory culture isolation. The analyses followed in this order: *stx* and *eae* positive samples, examined for the Roka EHEC target (RET), followed by NeoSEEK molecular top6 (and O157) EHEC confirmation. The samples identified in the final group were then taken into culture confirmation. Every sample that was culture confirmed then had its pre-enrichment DNA extract used in the enumeration assay.

Sample collection.

Samples of feces were collected from various lots of cattle during harvest using RAMS prepared in 15mL conical tubes containing 5mL chilled 5-10C modified tryptic soy broth (mTSB). RAMS were collected from hide-on carcasses after stunning and exsanguination at a point prior to any hide-on carcass washing, if present. When the sample was taken, the processing plant identification number and lot number of the carcasses were recorded as was the type of cattle (fed, cull dairy, and cull beef) and gender (cow, bull, steer, heifer). Processing plant slaughter management records were later consulted to ensure proper classification of cattle by lots and sources. RAMS were placed in ice chests and transported or shipped via overnight courier to the USMARC laboratory for processing.

Sample processing and handling.

When samples were received in the laboratory, each RAMS in 15mL tube was vortexed at high speed for 10 s and any debris was allowed to settle for 5 min then a 20uL aliquot was removed for an enumeration DNA extract prepared in 200uL of BAX lysis buffer (DuPont) according to manufacturers protocol. The enumeration DNA extract was placed at -20C until results of culture isolation were known. The RAMS were left in the tubes of mTSB and incubated at 42C for 8 h followed by a 4C hold in a programmable incubator until further processed. The time at 4C ranged from 4 to 8 hours. Further processing included a 10 s vortexing followed by removal of one 1mL aliquot for prevalence DNA preparation according to the FSIS MLG 5B, two 1mL aliquots for addition of 0.5 mL 50% sterile glycerol for freezer storage at -20C and one 1.2 mL aliquot for addition to Roka Atlas tubes.

Prevalence of STEC and EHEC

The prevalence DNA extracts that were prepared from enriched RAMS were sent to Neogen NeoSEEK laboratory for pathogenic Shiga toxin-producing *E. coli* confirmation service which included identification of *stx* and *eae* genes as well as top 6 non-O157 EHEC and EHEC-O157 detection based on *eae* subtypes and proprietary O group single nucleotide polymorphisms (snp). Roka Atlas tubes were shipped to Roka BioSciences laboratory for EHEC detection testing. Raw data from both NeoSEEK and Roka was returned to USMARC for interpretation. STEC prevalence was determined by presence of *stx*. EHEC presence was determined by presence of *stx*, *eae* and RET. Top 6 non-O157 EHEC and/or EHEC-O157 presence was determined by presence of *stx*, *eae*, RTE and identified as containing a STEC O group snp marker that correlated with EHEC *eae* subtype as follows. EHEC-O26: STEC O26 and *eae*-Beta; EHEC-

O45, O103, and O121 and *eae*-Epsilon; EHEC-O111 and *eae*-Theta (Gamma2); and EHEC-O145 and O157 and *eae*-Gamma (Gamma1). All samples identified as containing a top 6 non-O157 EHEC or EHEC-O157 were taken forward for attempted confirmation by culture isolation.

STEC isolation.

For each sample that was identified as prevalence positive for an EHEC as described above, one of the 1mL glycerol aliquots was thawed, a 500uL portion was mixed with 500uL PBS-tween and 20uL of serotype specific immuno-magnetic beads. Anti-O157 Dyna-beads (Invitrogen) were used first if applicable, then the appropriate non-O157 IMS beads (SDIx) were used sequentially, as some samples were identified as prevalence positive for more than one EHEC. The sample and bead mixtures were placed on a rotary plate shaker for 15 m and then the bead-bacteria complexes were captured using a Thermo KingFisher IMS robot. If more than one EHEC-O group was indicated to be present by molecular testing, then at this point the next O-group specific IMS bead was added and the process repeated. IMS beads were serially diluted to 1:100 and 1:1000. 50 uL on the 1:1000 dilution were plated using a spiral plater (Spiral technologies) onto washed sheeps blood agar plates containing mitomycin C (WBAM, Sugiyama), and 50 uL on the 1:100 dilution was spiral plated onto STEC differentiation agar (SDA, Kalchayanand). The suspect phenotypes on these media are enterohemolytic colonies on WBAM and serotype specific colony colors of blue to green to indigo on SDA. Representative colonies of the suspect phenotypes were picked to 96 well plates of tryptic soy broth, grown overnight at 37C, then screened by multiplex PCR to identify O-group and EHEC virulence factors: *stx, eae, nleB, nleF, espK and subA*. The *nle* virulence factors are non-locus of enterocyte effacement effector products located in different EHEC pathogenicity islands. *espK* is another type III secreted effector of EHEC, while *subA* is the prototype subtilase cytotoxin associated with STEC that lack *eae*.

Enumeration of EHEC.

Samples collected from high EHEC shedding and EHEC super-shedding cattle were identified using the enumeration DNA preparations of pre-enrichment samples. Only samples that had been confirmed to contain E. coil O157:H7 and/or non-O157 top 6 EHEC by culture isolation were used as template in multiplexed quantitative PCR (qPCR) reactions that used proprietary RET primers and FAM labeled probe supplied by Roka BioSciences and *eae* primers and VIC labeled probe as described in the MLG 5B. The sample loading volume was optimized, to prevent qPCR inhibition, by comparing CT values from a set amount of top 6 EHEC gDNA spiked into various volumes of BAX lysis buffer and pooled DNA extracts that were found to be negative for EHEC. RET and *eae* primers and probes were used in singleplex reactions to determine the occurrence of competitive inhibition, based on CT values, in the multiplexed reactions. Samples and standards were run concurrently using an ABI Model 7500 Fast thermal cycler (Applied Biosystems) in duplicate 25 uL reactions (12.5 uL of PerfeCTa® qPCR ToughMix® with low ROX™ master mix (Quanta Biosciences), 2 uL of RET primer probe mix, 1 µM of each *eae* primer, 200 nM of *eae* probe, and 8 uL of DNA). Cycling conditions were an initial denaturation at 95C for 10 minutes then 45 cycles of 95C for 15 seconds and 50C for 60 seconds. Standard curves were created on each run for the RET and the *eae* target using dilutions from $4.8x10^1$ to $4.8x10^5$ CFU equivalents per 8 uL of an EHEC strain. A pooled approach was used to generate a global standard curve for each target, which was then used for the interpolation of CFUs per sample reaction based on the Ct values for RET and *eae* targets. Ct values were converted to CFUs/RAMS.

Data analysis.

All data on prevalence of STEC and the prevalence of pathogenic STEC isolates were analyzed using functions in GraphPad Prism 6 with differences of $P < 0.01$ considered significant. $P < 0.01$ was considered significant because $P < 0.05$ is arguably too permissive for complex systems in the absence of massive amounts of data collected over very long time frames. Effects examined in the prevalence of highly shed STEC, prevalence of STEC and prevalence of pathogenic STEC isolates were compared across cattle types (fed, cull dairy and cull beef) and across regions for each group and all groups combined.

Results and Discussion

The goal of this work was to determine the prevalence, levels, and types of non-O157 EHEC in cattle from different production systems at harvest in order to determine if production system or regional variations in non-O157 EHEC serogroups exist. To accomplish this goal, sample collections were made at numerous beef processing plants located across the United States. Large plants were visited that drew cattle from diverse areas in order to have the greatest variability of cattle represented. Three of the processing plants only harvested fed cattle, while the others harvested mixtures of culled dairy and culled beef cows and bulls as well as periodic lots of fed steers and fed heifers. Table 1 shows the number of samples of each cattle type collected in each region. In total there were 1,032 fed cattle, 1,062 cull dairy cattle and 1,019 cull beef cattle sampled for analysis. Although collections were made in short time spans of one or two days at a location, slaughter management records were used to ensure multiple production lots of cattle from a variety of production locations were sampled. For example, the 131 fed cattle sampled in the eastern US represented 12 different production lots, while the 352 cull dairy and 204 cull beef cattle sampled in that region represented 41 and 22 different production lots respectively. The only region and cattle type that was not represented by 100 or more samples is cull dairy cows from the central US. This is in part due to the fact that there are fewer central US dairies compared to the other regions of the US. Likewise, fewer fed cattle were available for sampling in the Eastern US.

Table 1. Total number of RAMS samples collected from cattle of each production type in the designated regions of the US harvested during August through October.

	Total Collected, *n*		
	Fed	**Dairy**	**Beef**
Central	269	28	260
Northern	202	182	108
Southern	200	299	149
Western	240	201	298
Eastern	131	352	204

Our sample collection consisted of RAMS rather than grab samples or feces removed from colons. Sample collection using RAMS allowed simple and efficient sample collections from numerous cattle at processing line speeds. The use of RAMS was chosen over other feces

collection methods because it was recently shown that non-O157 STEC adhered to bovine recto-anal junction squamous epithelial cells in a similar fashion as that of *E. coli* O157:H7 (Kudva 2013) therefore allowing RAMS to be effective samples for the monitoring of non-O157 EHEC. Further, in comparisons between RAMS and other feces sampling methods it was shown that there was no significant difference in the prevalence of pathogens present in samples of each type (Rice 2003, Cobbold 2007, Arthur unpublished data). The identification of high shedding and super shedding cattle using RAMS as opposed to direct fecal samples was also shown to not be different (Cobbold 2007, Arthur, unpublished data).

The prevalence of Shiga toxin genes *stx1* and *stx2* was determined by NeoSEEK sample analysis. Often numerous *stx* alleles including *stx1, stx2* and *stx2c* were present in the same sample. Our analysis grouped all Shiga toxin genes together to provide an indication of STEC prevalence in cattle feces. Fed cattle had the highest rate of *stx* (99.0%) while cull dairy and cull beef cattle had lower rates of 91.9 and 91.6 % respectively (Table 2). Based on *stx,* the prevalence of STEC was not different (P>0.01) between the cull dairy and cull beef groups of cattle, but STEC prevalence was significantly higher (P<0.01) in the fed cattle group. The *stx* gene is not an absolute indicator of STEC presence as other organisms have been shown to also possess *stx* (Schmidt 1993, Haque 1996) but the occurrence of these other organisms is a rare event and unlikely to have a significant impact on the STEC prevalence values.

Many methods that identify EHEC containing samples initially screen for *stx* and *eae* since the adulterant EHEC, as well as other EHEC, contain both of these two virulence factors. We again turned to the NeoSEEK analysis to determine the presence of *eae*. The analysis provides both a universal *eae* marker as well as the identification of *eae* subtype(s) present. Like *stx*, when positive, many samples contained multiple *eae* subtypes. Ninety-five percent of fed cattle samples were positive for *stx* and *eae*, while 76.3% of cull dairy and 60.1% of cull beef cattle were positive for *stx* and *eae*. The number of samples identified as *stx* and *eae* positive in each group of cattle was different (P<0.01).

Table 2. Percentages of feces samples from cattle of different production types positive following each screening assay that was performed[a].

Screening Assay - Analysis	Fed (*n*=1042)	Dairy (*n*=1062)	Beef (*n*=1019)	Combined (*n*=3123)
stx$^+$	99.0A	91.9B	91.6B	94.2
stx$^+$ *eae*$^+$	95.0A	76.3B	60.1C	77.2
stx$^+$ *eae*$^+$ RET^{+b}	77.2A	47.7B	38.7C	54.6
NeoSEEK	37.9A	23.5B	17.1C	26.2
culture$^+$	8.3A	6.4AB	5.1B	6.6

[a]Values within a row followed by the same letter are not different (P>0.01) for the three cattle types sampled.
[b]RET = Roka BioSciences EHEC Target.

When analyzing enriched complex microbiological samples for *stx* and *eae* it is impossible to determine if the genes are present in the same organism, or present in two separate organisms such as an *stx* positive STEC and *eae* positive Enteropathogenic *E. coli* (EPEC). To resolve this issue we utilized the molecular target identified by Roka Biosciences that has been show to be present only in lineages of *E. coli* that contain both *stx* and *eae*. The specificity of

RET for *E. coli* that lack either *stx* or *eae* in our sample set was determined to be less than 1% for either virulence factor (Kristin Livezey personal communication). The addition of RET to our analysis identified EHEC in 77.2% of fed, 47.7% of cull dairy and 38.7% of cull beef cattle RAMS samples that were positive for *stx* and *eae*. All differences in EHEC prevalence were different ($P<0.01$) between the production groups.

The most common EHEC isolated from cattle is EHEC-O157. The top six non-O157 EHEC of serogroups O26, O45, O103, O111, O121 and O145 make up approximately 70% of human infections (Brooks 2005) and a similar fraction of EHEC isolated from beef (Bosilevac 2011). Thus we anticipated that there would be some portion of our samples that had been identified as positive for EHEC (*stx*$^+$, *eae*$^+$, RET$^+$) that would not contain an adulterant EHEC of these top seven serogroups. For this determination the multiple molecular targets of the NeoSEEK analysis were examined. Approximately one-half (49% fed and cull dairy and 44% cull beef) of the samples considered positive for an EHEC were found to contain the proper combination of NeoSEEK molecular markers that identified the presence of a top seven EHEC. Overall, top seven EHEC were identified in 37.9% of fed cattle samples, 23.5% of cull dairy cattle samples and 17.1% of cull beef cattle samples. The greatest percentage of EHEC that were identified as EHEC-O157 was in fed cattle, 4.0% EHEC-O157 and 33.9% non-O157 EHEC, while the percentages of EHEC-O157 in cull dairy and cull beef were less, 3.1% EHEC-O157 with 20.4% non-O157 EHEC and 2.1% EHEC-O157 with 15.0% non-O157 EHEC respectively. However, since fewer EHEC were identified in cull dairy and cull beef cattle overall, the prevalences of EHEC-O157 were not different ($P>0.01$) between the three production groups.

Next, all of the samples that had been identified as positive for a top seven EHEC were taken forward for confirmation by culture isolation. A top seven EHEC was confirmed by culture isolation in 8.3% of fed cattle RAMS samples, 6.4% of cull dairy RAMS samples and 5.1% of cull beef RAMS samples. The greatest difference in samples confirmed positive by culture isolation was between the fed and cull beef cattle samples, which was not different ($P=0.015$) using the less permissive statistical cutoffs established for this experiment. A number of samples in each group contained multiple EHEC isolates such that there were more isolates than positive samples (Table 3). The most common non-O157 EHEC serotypes isolated were O26, and O103. Even though not targeted in the IMS steps, a large number of non-top seven EHEC were isolated as well. When using culture methods similar to those described here but without specific IMS capture, STEC were isolated from beef at a rate of about 30% (Bosilevac 2010). In this study, 22% of RAMS samples from fed cattle identified as containing an EHEC were culture confirmed. The rates of culture confirming the EHEC containing RAMS samples collected from cull dairy and cull beef cattle were 27% and 30% respectively. This is due in part to the sensitivity of molecular screening methods over the culture isolation methods. Based on the data in this study, it is best to consider the percentage of samples confirmed by culture to be the minimum prevalence of EHEC in cattle from each production group, and to consider the molecular predictions of EHEC to represent the maximum prevalence.

Table 3. Number isolates and the distribution of top seven serotypes of EHEC isolated from RAMS samples collected from cattle of different production types..

	Fed	Dairy	Beef	Total
Isolates[a]	92	80	61	238
O26	4	20	11	35
O45	2	2	0	4
O103	21	15	16	52
O111	1	4	10	15
O121	0	0	1	1
O145	2	5	4	11
O157	32	22	13	67
OX[b]	30	12	6	53

[a]A number of samples were confirmed to contain multiple EHEC isolates of different serogroups. 28 samples contained EHEC of 2 different serogroups, and 2 samples contained EHEC of 3 different serogroups, therefore numbers presented will not equal calculated numbers based on percentages presented in Table 2 and the text.
[b]OX indicates an EHEC of O-group that was determine to be not of the seven O groups identified.

Since we only had a limited number of culture confirmed samples, we used the molecular analysis data to compare EHEC prevalence by cattle type and region for each top 7 EHEC (Table 4). There are some obvious differences between the culture confirmation data and the molecular screening data. For instance, EHEC-O103 and O26 were the EHEC most often confirmed by culture, but the molecular profiles identified three times as many EHEC-O103 positive samples (11.7% of total samples) than EHEC-O26 positive samples (3.7 of the total samples). This may be due in part to culture growth conditions and isolation techniques that favored EHEC-O26. Another notable difference between culture confirmation and molecular profiles is that of EHEC-O45. EHEC-O45 was identified as the most common EHEC present when using the molecular profiles (12.6% of total samples), but was only confirmed by culture in 4 samples. In this case the EHEC-O45 genetic information that is used in the molecular profiling and sample classification may not be as specific as that used for other EHEC. However the ranges of EHEC-O45 prevalence using the molecular profiling method by region, varied from 6.0 % in the South to 22.4% in the East, and the highest prevalence was determined to be 28.7% in northern cull beef cattle and the lowest in central cull dairy cattle (0%). If there was a general weakness in the molecular markers for EHEC-O45 one would expect more uniform results from the cross reacting background bacteria. Therefore, the lack of successful EHEC-O45 isolations may also have a cultural component as well.

Based on the molecular profiling of the RAMS samples, there are notable differences ($P<0.01$) in the prevalences of particular EHEC by region. When pooled across all cattle types, samples collected in the eastern region, had a higher prevalence for EHEC-O26, O45, and O157. Samples collected in the eastern and southern regions had the highest prevalence of EHEC-O111, and samples collected eastern and northern region had the highest prevalence of EHEC-O145. No

region had consistently lower prevalence of EHEC and each EHEC was identified in every region with the exception of EHEC-O26 in the central US and EHEC-O121 in the northern US. EHEC-O26 was most frequent in eastern dairy cattle, while EHEC-O45 was more widely spread and was in about 20% of central fed cattle, eastern fed and cull dairy cattle, and northern fed and cull beef cattle. EHEC-O103 was most common in northern fed cattle, but also frequent in central, western and southern fed cattle. EHEC-O111 was most common in southern beef and fed cattle and eastern cull beef and dairy cattle. Finally EHEC-O145 was most commonly identified in northern cull beef cattle.

The prevalence levels of EHEC-O121 were very low across all regions and cattle types. The overall prevalence of this EHEC was just 0.3%. The low numbers of samples identified makes drawing conclusion on this group of EHEC difficult. EHEC-O121 was identified in all regions at least once except the north where it was absent. It was also identified in all cattle types, but only once in cull dairy cattle. The average prevalence of EHEC-O157 in our study was 5.2%, and ranged from 2.8% in the samples collected from the southern US to 8.3% in samples collected from the eastern US. No direct correlations or relationships between the prevalence of EHEC-O157 and the non-O157 EHEC could be drawn from the data.

EHEC and STEC have been previously reported in US cattle herds and beef processing environments as well as some beef products. Generally, studies on farms report lower prevalence rates than studies of beef processing environments, most likely due to contamination events occurring between cattle in the pre-slaughter lairage environment (Arthur 2007). Also most on farm studies include animals in a different phase of the production cycle and not cull or harvest ready animals. Prevalence of STEC within central US cattle production operations has been reported to range from about 5-33% and EHEC were reported in 1.5% of fed cattle, 1.2% of dairy cattle and 3.3% of beef cows and calves (Renter 2005). Renter *et al* identified EHEC-O26, O111, O145 and O157 but did not describe in which group of cattle these were found.

In a study of dairy farms in Minnesota, Cho *et al* (2006) reported fecal prevalence of STEC based on *stx* PCR to be 3.2%. Nineteen Serogroups of STEC were identified, including EHEC-O26, O103, O111, O145 and O157. A comparison of STEC prevalences in dairy, feedlot and beef herds in Washington state (Cobbold 2004) reported 3.8% of fed cattle, 10.3% of dairy cattle and 11.8% of beef cattle were positive for STEC. EHEC were isolated but only at rates of 0.4, 1.4 and 2.4 % for fed, dairy and beef cattle respectively. During the 1991-1992 Dairy Heifer Evaluation Project performed by USDA APHIS NAHMS 1,305 *E. coli* isolates were randomly selected for analysis and 5.9% were STEC. Among the STEC were EHEC-O26, O45, O103, O111, O145 and O157 (Cray 1996).

In another report (Monaghan 2011) *stx* frequency in feces samples collected from dairy and beef cattle on farms in Ireland was shown to range from 20-65% over the course of the calendar year. Overall, 40% of feces samples were positive for *stx*, and the culture-based prevalence for STEC was shown to be 1.9%. Of the 90 STEC isolates recovered from feces, only 14 were top 6 EHEC serotypes (O26 and O145). The results of the previous reports point out that EHEC have been found in a number of separate populations of cattle however our data is the first to examine STEC and EHEC in cattle presented for slaughter from large geographic areas of the US. Also, making direct comparison or drawing conclusions from the data with previous studies should be done with caution because methods used in the previous reports to identify STEC and EHEC varied considerably.

Table 4. Percentages of feces samples collected from cattle of different production types containing EHEC of the top 7 serotypes[a] by region.

Cattle Type and Region	*n*	EHEC O group						
		O26	O45	O103	O111	O121	O145	O157
Fed Total	1042	2.1	18.0	22.0	0.5	0.4	4.2	4.6
Central	269	0.0	21.2	22.3	0.0	0.0	1.9	5.9
East	131	6.9	25.2	9.9	0.0	0.8	9.9	5.3
North	202	3.0	22.3	32.2	0.5	0.0	7.9	7.4
West	240	1.3	12.5	21.7	0.0	0.4	2.5	2.1
South	200	2.0	11.5	19.5	2.0	1.0	2.0	2.5
Dairy Total	1062	6.4	11.7	7.3	1.5	0.1	4.5	6.8
Central	28	0.0	0.0	0.0	0.0	0.0	7.1	7.1
East	352	16.8	28.1	12.5	2.3	0.3	7.1	11.1
North	182	1.6	5.5	4.9	1.6	0.0	3.8	3.3
West	201	0.5	3.5	5.0	0.5	0.0	0.5	7.5
South	299	1.7	2.7	4.7	1.3	0.0	4.3	3.3
Beef Total	1019	2.5	8.0	6.0	1.3	0.5	5.3	4.2
Central	260	0.0	4.2	4.6	0.8	0.8	3.1	3.1
East	204	6.3	10.7	7.3	2.4	0.5	5.9	5.4
North	108	0.0	28.7	18.5	0.0	0.0	12.0	5.6
West	298	1.7	3.4	1.7	0.3	0.7	4.4	5.0
South	149	4.7	5.4	6.0	3.4	0.0	5.4	2.0
All Total[b]	3124	3.7	12.6	11.7	1.1	0.3	4.7	5.2
Central	557	0.0	12.2	12.9	0.4	0.4	2.7	4.7
East	688	11.8	22.4	10.5	1.9	0.4	7.3	8.3
North	492	1.8	17.5	19.1	0.8	0.0	7.3	5.5
West	739	1.2	6.4	9.1	0.3	0.4	2.7	4.7
South	648	2.5	6.0	9.6	2.0	0.3	3.9	2.8

[a]Prevalence values presented are based on molecular profiles of samples to include *stx*, *eae*, RET (Roka BioSciences EHEC Target), and specific NeoSEEK EHEC markers.
[b]All total represents pooled data across all cattle production types.

In addition to determining the prevalence of EHEC in cattle from different production systems and in different regions of the US, our project addressed the levels of EHEC shed by these groups of cattle. To this end, each sample that was culture confirmed to contain one or more EHEC had its pre-enrichment DNA lysate evaluated to quantitate the level of EHEC present. This selection process biased results towards the identification of high shedding cattle since EHEC culture results have been shown to be increased in samples that harbored increased numbers of enumerable EHEC (Arthur 2009, 2010). The detection limit in our study was approximately 6.9 x 10^2 CFUs/RAMS. This biased selection was evident when the samples were analyzed and 88.4% of the samples had enumerable levels of EHEC, which is significantly greater than the typical values of 5 to 20 percent of samples that are enumerable with EHEC-O157 (Arthur 2009). Further quantitative PCR analysis of the remaining samples that were not culture confirmed will likely identify few additional shedding cattle. This additional analysis is warranted though, in order to accurately determine the percentages of cattle within each production type that are EHEC shedders.

Cattle shedding at levels greater than 10^4 CFU/RAMS have been classified as super-shedding cattle by others (Arthur, 2009, Cobbold 2007). In our selected samples the most common level observed for shedding was at 10^5 CFU/RAMS (24.8%%), with lesser and greater, up to 10^9 CFU/RAMS in a few cases identified. The percentage of cattle identified as high and super shedding at different levels was not different between the production system groups given the biasly selected samples (Table 5). Across all of the cattle production groups EHEC-O157, EHEC-non-O Group, EHEC-103, and EHEC-O26 were the most prevalent in association with super shedding (Table 6).

The concentration at which EHEC-O157 is shed in feces has been shown to vary from animal to animal and range from 10^2 to 10^5 CFU/g (Zhao et al., 1995). It is important to note that high levels of fecal shedding of EHEC-O157 was determined to be the greatest factor that contributed to increased prevalence and in turn, increased risk of beef contamination and human disease (Arthur , Omisakin et al., 2003; Ogden et al., 2004). Therefore the EHEC shedders identified here also pose a similar increased risk to public health through the beef supply.

In conclusion, animal management practices used in modern cattle production have been suspected to be associated with the emergence EHEC (Armstrong, 1996). There exists significant differences in production practices between fed cattle, cull dairy cattle and cull beef cattle that are presented for slaughter in the US which may influence the prevalence and shedding levels of EHEC. This work does not address the specific differences in practices, but instead identified fed cattle as having the highest prevalence of EHEC followed by dairy and beef respectively. EHEC-O26 was most frequently observed in eastern dairy cattle, while EHEC-O45 was more widely spread in about 25% of central fed cattle, eastern fed and cull dairy cattle, and northern fed and cull beef cattle. EHEC-O103 was most common in northern fed cattle, but also frequent in central, western and southern fed cattle. EHEC-O111 was most common in southern beef and fed cattle and eastern cull beef and cull dairy cattle. Finally, EHEC-O145 was most commonly identified in northern cull beef cattle. Examining a selected set of samples which were culture positive showed cattle from all production areas were shedders of EHEC. The majority of cattle shed EHEC at >10^5 CFU/RAMS. The most common EHEC shed at high levels were EHEC-O157, EHEC-Non Top 7 O group, EHEC-O103, and EHEC-O26. The identification of bovine production systems populated by specific EHEC serogroups, as well as the identification of the regional variations in serogroup prevalence and shedding level may offer opportunities to be exploited to manage the risk from these EHEC.

Table 5. Distribution and percentage of enumerable CFUs/RAM from cattle of different production types containing isolated and PCR confirmed EHEC[a] (*n*=207).

Log_{10} range CFU/RAMS	Fed	Dairy	Beef	Total
> 9.0	0 (0)	2 (2.9)	0 (0)	2 (1.0)
8.0-8.9	4 (4.6)	2 (2.9)	2 (3.8)	8 (3.9)
7.0-7.9	8 (9.2)	6 (8.8)	6 (11.5)	20 9.7)
6.0-6.9	22 (25.3)	12 (17.6)	9 (17.3)	43 (20.8)
5.0-5.9	22 (25.3)	18 (26.5)	11 (21.2)	51 (24.8)
4.0-4.9	22 (25.3)	15 (22.1)	12 (23.1)	49 (23.7)
2.8-3.9	7 (8.0)	3 (4.4)	0 (0)	10 (4.8)
< 2.8[c]	2 (2.3)	10 (14.7)	12 (23.1)	24 (11.6)

[a]Quantitative PCR was perform on pre-enrichment DNA from RAMS that contained a culturable EHEC isolate that was confirmed by end point PCR.
[b]Values represent the number and percentage in parenthesis of the culture confirmed samples examined within each group of cattle that had enumerable EHEC within the specified ranges.
[c]The limit of detection of the enumeration method was 2.8 log CFU/RAMS.

Table 6. Distribution and percentage of enumerable and non-enumerable EHEC[a] by O-Group across all cattle production types where an EHEC was isolated and confirmed by PCR[b].

Log_{10} range CFU/RAMS	EHEC O group								
	O26	O45	O103	O111	O121	O145	O157	OX[c]	Total
> 9.0	1 (0.4)[d]	0 (0)	1 (0.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (0.8)
8.0-8.9	0 (0)	0 (0)	1 (0.4)	0 (0)	0 (0)	0 (0)	4 (1.7)	3 (1.3)	8 (3.4)
7.0-7.9	2 (0.8)	0 (0)	7 (2.9)	1 (0.4)	1 (0.4)	1 (0.4)	5 (2.1)	6 (2.5)	23 (9.7)
6.0-6.9	5 (2.1)	0 (0)	14 (5.9)	5 (2.1)	0 (0)	3 (1.3)	16 (6.7)	8 (3.4)	51 (25.2)
5.0-5.9	10 (4.2)	1 (0.4)	10 (4.2)	6 (2.5)	0 (0)	0 (0)	18 (7.6)	15 (6.3)	60 (24.4)
4.0-4.9	12 (5.0)	1 (0.4)	11 (4.6)	2 (0.8)	0 (0)	3 (1.3)	14 (5.9)	15 (6.3)	58 (24.4)
2.8-3.9	2 (0.8)	1 (0.4)	1 (0.4)	0 (0)	0 (0)	0 (0)	4 (1.7)	3 (1.3)	11 (4.6)
< 2.8[e]	3 (1.3)	1 (0.4)	6 (2.5)	1 (0.4)	0 (0)	4 (1.7)	8 (3.4)	2 (0.8)	25 (10.5)
Total	35 (14.7)	4 (1.7)	51 (21.4)	15 (6.3)	1 (0.4)	11 (4.6)	69 (28.9)	52 (21.9)	238

[a]Quantitative PCR was perform on pre-enrichment DNA from RAMS that contained a culturable EHEC isolate that was confirmed by end point PCR.
[b]In some instances two or more EHEC were isolated per RAMS and were classified according to total CFUs/RAMS therefore calculated totals exceed the number of positive samples.
[c]OX indicates an EHEC of O-group that was determine to be not of the seven O groups identified.
[d]Values represent the number and percentage in parenthesis of the selected samples examined that have enumerable EHEC within the range given.
[e]The limit of detection of the enumeration method was 2.8 log CFU/RAMS.

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Conclusions

Animal management practices used in modern cattle production have been suspected to be associated with the emergence EHEC (Armstrong, 1996). There exists significant differences in production practices between fed cattle, cull dairy cattle and cull beef cattle that are presented for slaughter in the US which may influence the prevalence and shedding levels of EHEC. This work does not address the specific differences in practices, but instead identified fed cattle as having the highest prevalence of EHEC followed by dairy and beef respectively. EHEC-O26 was most frequently observed in eastern dairy cattle, while EHEC-O45 was more widely spread in about 25% of central fed cattle, eastern fed and cull dairy cattle, and northern fed and cull beef cattle. EHEC-O103 was most common in northern fed cattle, but also frequent in central, western and southern fed cattle. EHEC-O111 was most common in southern beef and fed cattle and eastern cull beef and cull dairy cattle. Finally, EHEC-O145 was most commonly identified in northern cull beef cattle. Examining a selected set of samples which were culture positive showed cattle from all production areas were shedders of EHEC. The majority of cattle shed EHEC at $>10^5$ CFU/RAMS. The most common EHEC shed at high levels were EHEC-O157, EHEC-Non Top 7 O group, EHEC-O103, and EHEC-O26. The identification of bovine production systems populated by specific EHEC serogroups, as well as the identification of the regional variations in serogroup prevalence and shedding level may offer opportunities to be exploited to manage the risk from these EHEC.

Recommendations for Future Research

Future research directly related to this project includes the enumeration of all EHEC containing RAMS samples. This will allow a more accurate estimate of shedding and super shedding of the non-O157 EHEC to be made. The 53 EHEC of non-top seven serotypes that were isolated in this study should be serotyped for comparisons to other known EHEC recognized by the CDC. Further, a large number of RAMS were identified that contain a non-top seven EHEC. These samples should have culture confirmation attempts made in order to confirm the validity of the screening methods as well as identify what EHEC are making up this large pool of positives. Other future work should compare the top 6 EHEC from this study and others to similar isolates from human disease in order to determine genetic relatedness (PFGE) as well as survival characteristics that allow potential transmission through the beef chain.

Presentations and Publications

Two peer reviewed papers are anticipated from this work.

1. Bosilevac et al. Identification of Bovine Reservoirs of non-O157 Enterohemorrhagic *E. coli.*
2. Luedtke et al. Method for enumeration of non-O157 Enterohemorrhagic *E. coli* in samples from beef production environments.