PREVALENCE OF SHIGA TOXIN PRODUCING *ESCHERICHIA COLI* IN RANGE-GRAZING CATTLE LOCATED IN NORTHERN CALIFORNIA

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B.S., California State University, Sacramento 1997

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PREVALENCE OF SHIGA TOXIN PRODUCING *ESHERICHIA COLI* IN RANGE-GRAZING CATTLE LOCATED IN NORTHERN CALIFORNIA

A Thesis

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Department of Biological Sciences
Abstract

of

PREVALENCE OF SHIGA TOXIN PRODUCING ESCHERICHIA COLI IN RANGE-GRAZING CATTLE LOCATED IN NORTHERN CALIFORNIA

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Michelle L. Hopkins

Shiga toxin-producing *Escherichia coli* (STEC) are one of the leading causes of human gastrointestinal disease in the United States and other developed countries worldwide. Illnesses caused by STEC range from mild non-bloody diarrhea to hemorrhagic colitis that can lead to life threatening renal failure, a condition known as hemolytic-uremic syndrome (HUS).

The most common reservoir of STEC is cattle. Therefore, the majority of human outbreaks reported have been associated with the consumption of undercooked ground beef and other beef products (Arthur et al. 2002; Gyles 2007; Hussein et al. 2003).

There are two main groups of cattle that are used in the beef manufacturing industry, feedlot cattle and adult range-grazing cattle. Earlier investigations of STEC prevalence have focused primarily on the O157:H7 *Escherichia coli* serotype in cattle on feedlots. This is problematic, as over 100 other serotypes have been identified as having pathogenic potential in humans (Gioffre et al. 2002; Gyles et al. 1998; Law 2000), and not all cattle that enter the food chain spend time on feedlots. Adult range-grazing cattle are removed from their herd once past their reproductive prime, and sent directly to meat
processing. They are not grain fed on a feedlot like younger cattle. Research that uses feedlot cattle for their investigations of STEC prevalence should not be applied to non-feedlot cattle. The two groups of cattle are managed differently and should be investigated separately.

The aim of this study was to determine the prevalence levels of all types of STEC in healthy adult range-grazing cattle from Northern California, specifically Sacramento County and neighboring counties. Our hypothesis was that healthy adult range-grazing cattle would have a prevalence of STEC lower than what previous studies utilizing feedlot cattle have reported. In this study, we also assessed the effects of precipitation and temperature on the prevalence of STEC in one isolated cattle herd over a sixteen-month time period, and then analyzed our data using linear regression analysis.

To test our hypothesis, fresh fecal samples from 152 healthy range-grazing cattle were collected and evaluated for the presence of the Shiga toxin genes by PCR. It was determined that 71.7% of the samples tested positive for the Shiga toxins genes. It was also determined that no statistically significant relationship existed between the cattle herd’s STEC prevalence and the herd’s size or density. Finally, the results from our seasonal prevalence study suggested that precipitation and temperature did not have a statistically significant relationship on the prevalence of STEC in adult range-grazing cattle. Taken together, our findings suggest that other environmental factors besides temperature and precipitation are contributing to the prevalence of STEC in range-
grazing cattle, and that the percent of STEC in range-grazing cattle is much higher than originally hypothesized.

_______________________, Committee Chair
Susanne Lindgren, Ph.D.

_______________________
Date
DEDICATION

I dedicate this to my children, Madalynn and Mason. I hope this serves as an example, that anything is possible through dedication, hard work and faith. You can truly achieve anything you put your heart and mind to. I love you both.
ACKNOWLEDGMENTS

Thank you to all my friends and family that supported me and helped me with this project. Without your help, this thesis never would have been completed. Thank you Dr. Lindgren for all of your time and energy you invested in this project and in me. I will never forget it. Thank you to the ranchers that were kind enough to allow me to sample their herds, you made this research possible. Finally, to my husband, thank you for being so understanding and patiently standing by me throughout this long process. Babe, we are finally done. I love you!
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Chapter 1
INTRODUCTION

Seventy-thousand people a year in the United States are diagnosed with infections caused by Shiga toxin-producing *Escherichia coli* (STEC) (CDC. 2008). STEC has emerged as a leading food-borne pathogen responsible for human gastrointestinal disease. STEC infections cause a range of outcomes such as asymptomatic infections, mild diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (Karmali, et al. 1998). In 1982, STEC strain O157:H7 was recognized for the first time as a causative agent of human illness after a hemorrhagic colitis outbreak in the United States (Ludwig et al. 2002; Wells et al. 1983). To date, over 100 different serotypes of STEC have been identified to cause human disease (Gioffre’ et al. 2002; Gyles et al. 1998; Law, 2000). Several of these have caused recent outbreaks including STEC O104:H21, O26:H11, O103:H2, 111, and O114:H21 (CDC. 1995; Fey et al. 2000; Karmali et al. 2003). Although, O157:H7 is believed to be the predominant outbreak causing strain in the United States and is therefore the focus of most investigations, several studies have suggested that all STEC bacteria could be pathogenic because of the pathogenic effect of the Shiga toxin it produces (Arthur et al. 2002; Gyles et al. 1998; Hussein et al. 2003; Paton et al. 1999).

STEC outbreaks are an increasingly global problem. Outbreaks have occurred in six continents and in over 14 countries, initiated in households, day-care centers, schools, restaurants, nursing homes, outdoor events on pasture land, and prisons (Karmali, 2004).
Outbreaks have been associated with the consumption of beef and other food that may have come in contact with bovine fecal material such as, lettuce, spinach, un-pasteurized juices and milk (Beutin, 2006). Young children under the age of five and the elderly are more likely to become ill with life threatening infections of STEC (Gyles, 2007). In North America, the rate of infection has been estimated to be two to three out of 100,000 children under the age of five (Philpott and Ebel, 2003).

There are two different types of toxins STEC produces, type 1 (Stx1) and type 2 (Stx2). Stx1 is almost identical to *Shigella dysenteriae* type I toxin at the amino acid sequence level. Stx2 shares 60% homology at the DNA and amino acid level with Stx1 (Arthur et al. 2002; Boerlin et al. 1999; Law, 2000). STEC isolates have been identified that produce either one or both of these toxins. Recent studies suggest that strains carrying Stx2 are more virulent than strains carrying Stx1 or strains carrying both Stx1 and Stx2 (Arthur et al. 2002; Boerlin et al. 1999). One such study by V. Tesh, using a murine model, showed that Stx2 had a lethal dose 400 times lower than that of Stx1 when injected into mice (Tesh et al. 1993).

The Shiga toxin molecules are heterodimers consisting of two subunits, A and B. Subunit B binds to a glycolipid host cell receptor called Gb3. Once bound to the cell, the holo-toxin is internalized by receptor mediated endocytosis. Next, subunit A is enzymatically activated. Having RNA N-glycosidase activity, subunit A then cleaves a specific bond on a single adenosine residue, removing one adenine from the 28S rRNA which prevents binding of amino acyl-tRNA to the ribosome (Paton, et al. 1998; Sandvig, 2001). This inhibits peptide chain elongation, shutting down protein synthesis. Once
protein synthesis is halted, cell death is inevitable (Law, 2000; Paton, et al. 1998; Philpott and Ebel. 2003; Sandvig, 2001).

Two other genes contributing to the virulence of STEC are eaeA and ehxA. The eaeA gene codes for the surface protein intimin, which allows the bacteria to attach to and efface to the host intestinal epithelial cells that line the lumen of the large intestine. The ehxA gene codes for hemolysin and has been observed to cause enterocyte damage. Both eaeA and ehxA genes are found in most human pathogenic STEC strains but not all. Therefore, it is believed that they can increase STEC virulence but are not required to cause severe disease (Arthur et al. 2002; Gyles et al. 1998; Hussein and Bollinger 2005; Paton et al. 1999).

Clinical features of STEC infection can vary. Previous research indicates STEC has a low infectious dose, less than 100 bacteria are capable of causing disease (Tilden et al. 1996; Tuttle et al. 1999). Most cases begin with a three to five day incubation period followed by a short period of abdominal cramps and non-bloody diarrhea. For most healthy people, these symptoms are dismissed as having a touch of the stomach flu and the bacterial infection is self-limiting. However, for some individuals, symptoms progress to hemorrhagic colitis and 2-7% of these cases will progress into hemolytic uremic syndrome (HUS). Clinical features of HUS are acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia (Beutin, 2006; Karmali, 2004). The HUS mortality rate is 3-5%. Of the individuals that STEC infection progresses to HUS, up to 40% will have renal dysfunction (Phipott and Ebel 2003; CDC.
There is no direct cure for STEC infections, only supportive therapy and currently no vaccines are available.

Cattle are major reservoirs of STEC (Arthur et al. 2002; Gyles, 2007; Hussein et al. 2003). *Escherichia coli* occur naturally in the intestinal tract of ruminant animals and usually are nonpathogenic to them. In a symbiotic relationship, *E. coli* are just one of the many microflora that digest the cellulose that the ruminant consumes (Highfall, 2005). During meat processing, if any of the meat comes in contact with bacteria from the intestines, the entire lot of meat could potentially become contaminated. This is especially a concern for meat that is ground together from multiple sources, such as ground beef.

The US beef and cattle industry is a 76 billion dollar industry. In the US, over 27.3 billion pounds of beef were consumed in 2008. In January 2009, the cattle inventory for the US was approximately 94.5 million head of cattle. Twenty percent of these were “culled” cattle (USDA. 2009). Culled cattle typically are older cows and bulls that are kept for reproductive purposes but are now past their reproductive prime. They are counted separately from the young cattle (steers and heifers) because culled cattle are older and are not “finished” by sending them to a feedlot for grain feeding, but they are usually sent straight for slaughter and meat processing. Because culled cattle are not finished, the cuts of meat that they are used for is also different from grain fed, feedlot cattle. Over 50% of the carcasses from culled cattle are used for ground beef (USDA, 2006).
From the meat processing plant to the grocery store, STEC finds its way into our food supply, unfortunately more commonly than one would like to think. Several studies have investigated the prevalence of STEC in ground beef in the U.S. The prevalence of STEC vary from 2% to 23% (Fantelli and Stephan 2000; Samadpour, 2001). One local study done on retail ground beef from Sacramento, California found STEC levels greater than 10% (Oliver, 2000). Taken together, all of these studies implicate STEC as a major health concern with regard to the safety of consuming ground meat.

Cattle are the source of STEC isolated in beef products. The prevalence of STEC in cattle has been well documented. A summary of several recent studies is found in Table 1. Most studies tested cattle located at processing plants/slaughterhouses and do not make a distinction between young heifers and steers from the older unfinished culled cattle. This makes it very difficult to draw any conclusions with regard to the prevalence of STEC in healthy range-grazing cattle that do not go to crowded feedlots and are not grain finished.

It is difficult to locate research dedicated to culled cattle. One study by Hussein et al. sampled specifically range-grazing culled beef cattle from Reno, Nevada. The prevalence of STEC in 82 range-grazing cattle was 11% (Hussein et al. 2003). There has not been a study such as this for cattle in California. With over five million cattle in the state and cash revenue of over a billion dollars, the prevalence of STEC in cattle from California is important to know, especially in cattle that will be culled, and used predominately for ground beef (California Resource Directory 2009).
Knowing the STEC prevalence in cattle before processing and comparing it to an average percent of STEC in ground meat and other beef products, will help determine if more preventative steps are needed during and after beef processing. As food production increases to meet demands, there will continue to be STEC in the food supply and a risk of infection to consumers. Investigations such as this are vital in protecting consumers and maintaining a healthy beef supply.
Table 1. Previous investigations determining the percent prevalence of STEC in cattle

<table>
<thead>
<tr>
<th>Animal Type Tested</th>
<th>Location</th>
<th>Prevalence of STEC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy, Feedlot, Range</td>
<td>WA, USA</td>
<td>7.0%</td>
<td>Cobbold, R. (2004)</td>
</tr>
<tr>
<td>SH (non-specified)</td>
<td>WA, USA</td>
<td>18.4%</td>
<td>Samadpour, M. (2001)</td>
</tr>
<tr>
<td>Steers/ SH</td>
<td>Argentina</td>
<td>8.0%</td>
<td>Gioffre', A. (2002)</td>
</tr>
<tr>
<td>Steer/SH</td>
<td>Argentina</td>
<td>69.0%</td>
<td>Meichtri, L. (2004)</td>
</tr>
<tr>
<td>Multiple Herds</td>
<td>Australia</td>
<td>9.5%</td>
<td>Fagan, P. (1998)</td>
</tr>
<tr>
<td>SH (non-specified)</td>
<td>Canada</td>
<td>42.6%</td>
<td>Van Donkersgoed, J. (1999)</td>
</tr>
<tr>
<td>SH (non-specified)</td>
<td>France</td>
<td>70.0%</td>
<td>Pradel, N. (2000)</td>
</tr>
<tr>
<td>Dairy Calves</td>
<td>Germany</td>
<td>18.2%</td>
<td>Wieler, L. (2007)</td>
</tr>
<tr>
<td>Bulls and Dairy Cows/ SH</td>
<td>Germany</td>
<td>10.8%</td>
<td>Montenegro, M. (1990)</td>
</tr>
<tr>
<td>Cow and Calves at Farm</td>
<td>Spain</td>
<td>36.0%</td>
<td>Blanco, M. (1997)</td>
</tr>
</tbody>
</table>

*SH = slaughterhouse
Chapter 2

MATERIALS AND METHODS

Cattle Fecal Sample Collection

The cattle used for this research were healthy adult, grazing, breeding stock from ranches in northern California that raise cattle for beef production. Ranches were chosen based on ease of access to the herd, location, size of the herd, and being able to obtain verbal permission by the owner or an owner representative. All ranches were kept anonymous and samples were obtained over a sixteen-month period. One hundred and fifty two fecal samples were collected from eleven apparently healthy cattle herds located in Sacramento County and neighboring counties (Figure 1). Approximately 100 g of fresh fecal material was collected in Ziploc™ sandwich bags. The fecal samples were at room temperature for approximately 24 hrs or less until they were brought to the laboratory for analysis. After reviewing a study by Kudva, demonstrating manure and manure slurry infected with STEC remained positive after being exposed to environment factors for over 47 days, it was felt that the time between the actual collection of the samples and getting the samples to the laboratory for testing would not be a significant factor for this study (Kudva et al. 1998).
Figure 1. Map of California, indicating location of El Dorado, Sacramento and Sutter counties where samples were collected.
Purification/Isolation of DNA

DNA was purified from enriched overnight cultures of cow manure using an InstaGene matrix™ from Bio-Rad as follows. Fecal broth cultures were prepared by inoculating 0.2 g of each fecal sample into sterile 50 ml Falcon™ collection tubes with 50 ml of sterile LB broth. The mixture was incubated at 37°C with shaking for 18 to 24 h. Next, 15 μl was taken off the top of the enrichment and added to 1 ml of sterile dH₂O which was then centrifuged at 11,000 rpm for 1 min and then the supernatant was discarded (Fagan et al. 1990). Following manufacturer’s instructions, 200 μl of InstaGene matrix™ was added to the remaining pellet and incubated at 56°C for 30 min. Next, the samples were vortexed for 10 s and placed in a 100°C heat block for 8 min. Finally, the samples were vortexed for 10 s and then centrifuged at 11,000 rpm for 3 min prior to the removal of the DNA template for multiplex PCR.

Determination of Fecal Broth Dilutions for Enrichment

To determine the optimal amount of fecal sample to use in the overnight culture for the purpose of enrichment, sample amounts of 0.2 g, 0.5 g, and 1.0 g of a fecal material were enriched overnight in 50 ml of LB broth at 37°C. After enrichment, 15 μl of each of the fecal broths was removed to isolate bacterial DNA using InstaGene matrix™ following manufacturer’s instructions. Next, multiplex PCR and visualization were done as described in materials and methods. The sample amount of fecal sample in
LB broth that gave the most consistent internal control results was utilized for this research.

**Multiplex PCR**

Each multiplex PCR was done in a 50 µl volume, which included oligonucleotide primers for Stx1 and Stx2 or *E. coli* 16s rRNA pairs. The primers used for this study were purchased through Sigma laboratories. The primer sequences and predicted product sizes are listed in Table 2. Each PCR assay consisted of 19.5 µl of dH₂O, 5.0 µl of 10X Taq buffer with MgSO₄, 1.0 µl of each primer: Stx1-F primer, Stx1-R primer, and Stx2-F primer, Stx2-R primer, 1.0 µl of dNTP (200 µM of each), 20 µl of isolated DNA template, and 0.5 µl Taq polymerase (2.5 U). For a positive control, 5 µl of an overnight enriched culture of EHEC ATCC 43894 (CDC EDL 932) and 32.5 µl of dH₂O were added to the PCR assay in place of the DNA and water amounts previously mentioned. EHEC ATCC 43894 is an O157:H7 strain that produces both Stx1 and Stx2 toxins (Wells et al. 1983). The internal control PCR assay consisted of 21.5 µl of dH₂O and 1 µl each of the primers for 16s ribosomal rRNA, 1.0 µl of dNTP (200 µM of each), 20 µl of isolated DNA template, and 0.5 µl Taq polymerase (2.5 U). For the negative control, 37.5 µl dH₂O and no DNA template was included in the PCR assay. An overlay of 100 µl of sterile mineral oil was placed on the top of the reactants prior to being placed in the thermocycler to prevent the PCR assays from evaporating during the PCR process. A MJ Thermal Cycler 480 was used for the PCR reactions. The parameters used for all of the
PCR reactions per cycle were first to denature the DNA at 94°C for 2 min. Next, annealing took place at 55°C for 1 min. Then primer extension took place at 72°C for 1 min. The cycle was repeated for a total of 30 cycles. Final elongation was completed at 72°C for 7 min. Upon completion of the PCR, samples were stored at 4°C until they were resolved using agarose gel electrophoresis. Additional PCR was done as described above using oligonucleotide primers for the *eaeA* gene. The primer sequences and the predicted product size are listed in Table 2.

**Agrose Gel Electrophoresis**

A 2% agarose gel was used for the agarose gel electrophoresis. From each PCR sample, 20 µl was loaded onto the agarose gel. The gel was placed in the electrophoresis chamber where it ran for 45-70 min. at 90 V. Next, the gel was stained with ethidium bromide for eight min and rinsed using dH₂O for 5-10 min. Finally, the agarose gels were illuminated and imaged using an Alphaimager™ 2200 program.

**Sensitivity of Methods**

Sensitivity studies were performed to determine the lowest colony forming units of STEC per gram of sample that could be detected using this protocol. A series of 10-fold dilutions of a positive overnight culture were made using EHEC ATCC 43894 and added to previously determined negative bovine fecal samples. In addition, each dilution was directly plated onto LB agar to enumerate the total number of bacteria in the original overnight culture. Following the methods previously described, all spiked samples were
enriched in LB broth overnight and then 15 μl was removed and used for bacterial DNA isolation using the InstaGene Matrix ™ following the manufacturer’s instructions. Isolated DNA templates were used in the PCR assays and upon completion of the PCR, were visualized using gel electrophoresis.

**Statistical Analysis**

Regression analysis and Spearman’s rank correlation tests were used to analyze data to determine if statistically significant relationships existed using SPSS software.
Table 2. Primer sequences and predicted PCR amplification product lengths

<table>
<thead>
<tr>
<th>Primer</th>
<th>Direction</th>
<th>Primer Sequence 5'-3'</th>
<th>PCR Product Size (bases)</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td>Stx-I</td>
<td>forward</td>
<td>5'-TGTAACTGGAAGGTGGAGTATACA-3'</td>
<td>210</td>
<td>Meng et al. 1997</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5'-GCTATTCTGAGTCAACGAAAAATAAC3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stx-II</td>
<td>forward</td>
<td>5'-GTTTTTCTTCGGTATCTATTCC-3'</td>
<td>484</td>
<td>Meng et al. 1997</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5'-GATGCATCTCTGGTCATTGTATTAC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S</td>
<td>forward</td>
<td>5'-AGAGTTTGAATCAGCAGCTCAG-3'</td>
<td>798</td>
<td>Osek et al. 2003</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5'-GGACTACCAGGGTATCTAAT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eaeA</td>
<td>forward</td>
<td>5'-GTGGCGAATACTGGCGAGACT-3'</td>
<td>890</td>
<td>Fagen et al. 1998</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5'-CCCCATTCTTTTTCACCGTCG-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 3

RESULTS

**Determination of Fecal Broth Dilutions for Enrichment.** In order to determine the prevalence of STEC in bovine fecal specimens, we first needed to develop a method for DNA isolation to remove any fecal products that would act as inhibitors in the molecular PCR assay method. From our initial set of trials and from discussing our application with a BioRad representative, it was determined that a high concentration of fecal material in the aliquot used for the DNA isolation procedure, could interfere with the PCR due to so many PCR contaminants normally present in fecal material. Thus, even though we thought a large fecal sample size would be better for our screen, we found the less we used, the more consistent we were able to get viable results. To determine the amount of fecal material to use for each enrichment step, different fecal amounts were tested using the procedure described in materials and methods. It was determined that when using 1 g or more of fecal sample, the PCR results were not consistent, and many of results from these samples could not be included due to a negative 16S ribosomal internal control result. After multiple trials, it was determine that 0.2 g of fresh fecal material when added to 50 ml of LB broth, provided an aliquot to use in the DNA isolation procedure that gave the best consistent positive internal control results.

**Sensitivity of Methods.** After we were able to obtain consistent result with our 16S control, we then needed to establish the sensitivity of the new PCR assay. Sensitivity studies were done to determine the lowest colony forming unit of STEC per gram of
sample that could be detected using the protocol described for this research. From the
direct plate dilutions, it was determined that the overnight culture had a starting amount
was $4.4 \times 10^8$ CFU per ml. PCR results where visualized showing successful detected of
both stx$_1$ and stx$_2$ down to $10^{-7}$ of the overnight culture, equaling a level of detection of
44 CFU per gram of sample (Figure 2).
Figure 2. PCR results for the sensitivity study. Lane 1 equals 44 CFU per gram of sample. Lane 2 equals 440 CFU per gram of sample. Lane 3 equals 4,400 CFU per gram of sample. Lane 4 equals 44,000 CFU per gram of sample. Lane 5 equals 440,000 CFU per gram of sample and lane 6 is a 100 base pair ladder.
The detection of STEC in fecal samples from multiple ranches. To identify the presence of STEC in the cattle fecal samples collected, PCR was performed on samples using the primers specific for the \textit{stx}_1 and \textit{stx}_2 genes (Table 2). Next, the PCR products were resolved using agarose gel electrophoresis, stained with ethidium bromide and then visualized on a UV transillumination (Figure 3). Out of 152 fecal samples collected from eleven different cattle herds, 138 of the PCR results were considered valid by means of a positive internal PCR control, the 16S ribosomal primers (Figure 4). Either one or both Shiga toxins genes were detected in 99 (71.7\%) of the 138 samples collected. Out of the positive 99 samples, 30 (30.3\%) fecal samples tested positive for only \textit{stx}_1, 25 (25.3\%) samples tested positive only for \textit{stx}_2, and forty-four (44.4\%) samples tested positive for having both \textit{stx}_1 and \textit{stx}_2 genes (Table 3). Samples positive for \textit{stx}_1 and \textit{stx}_2 suggest that they contained either one isolate of STEC that produced both toxins or multiple STEC bacteria present that produced either one or both toxins. The individual ranches varied greatly with regard to their individual percent prevalence of STEC (Table 4). Taken together, all of these results indicate that a much greater proportion of cattle, specifically adult range-grazing cattle, are carrying STEC than originally hypothesized.

Determination of the presence of \textit{eaeA} gene in positive samples. The \textit{eaeA} gene codes for the protein intimin which allows the bacteria to attach and efface to colonic epithelial cells. Intimin has been characterized as an important factor in the virulence of EHEC and many STEC (Ethelerg, S. et al. 2004). After determining which of our samples were STEC positive, we wanted to determine if any of these were also positive for the \textit{eaeA}
gene. DNA isolation, PCR, agarose gel electrophoresis, and visualization were all done as previous described in the materials and methods. Unfortunately the results were inconclusive as multiple sized DNA fragments were amplified besides the DNA fragment coinciding with the predicted molecular size for the eaeA primer set. Often, after amplification, our resulting PCR DNA fragments appeared so close together, it was difficult to distinguish between the fragments. We hypothesized that the particular eaeA primer set used in this research was not specific enough when used with fecal sample enrichments. Perhaps, either PCR amplification of suspected colonies from direct plating of the fecal samples, or designing a more specific primer set to use with the fecal enrichment, would be more successful for detection of the eaeA gene to use with our methods designed for this research.
Figure 3. Example of multiplex PCR products from eleven fecal samples collected from herd “G”. Lane 1: 100 base pair ladder; Lane 2: negative control; Lane 3: positive control EHEC ATCC 43894; Lane 4 through 14: samples from the herd.

* Identifies positive samples.
Figure 4. Example of the PCR products for the positive *E. coli* 16s internal control from eleven fecal samples collected from herd “G”. Lane 1: 100 base pair ladder; Lane 2: negative control with no template; Lane 3: positive control EHEC ATCC 43894; Lane 4 through 14 samples collected from the herd.
Table 3. Summary of fecal samples collected from eleven range-grazing cattle herds from Northern California.

<table>
<thead>
<tr>
<th>TOTAL SAMPLES TESTED&lt;sup&gt;a&lt;/sup&gt;</th>
<th>stx&lt;sub&gt;1&lt;/sub&gt; POSITIVE</th>
<th>stx&lt;sub&gt;2&lt;/sub&gt; POSITIVE</th>
<th>stx&lt;sub&gt;1&lt;/sub&gt; and stx&lt;sub&gt;2&lt;/sub&gt; POSITIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>138</td>
<td>30&lt;sup&gt;b&lt;/sup&gt; (21.7%)</td>
<td>25 (18.1%)</td>
<td>44 (31.9%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> of the 152 total fecal samples tested, only 138 fecal samples were positive for *E. coli* 16s ribosomal DNA, detected by PCR.

<sup>b</sup> the number of positive fecal samples.
Table 4. PCR data from eleven range-grazing cattle herds.

<table>
<thead>
<tr>
<th>Herd</th>
<th>Total Tested</th>
<th>( stx_1 ) positive</th>
<th>( stx_2 ) positive</th>
<th>Positive for both toxin genes</th>
<th>Total positives</th>
<th>% Total Samples Testing Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>18</td>
<td>4</td>
<td>6</td>
<td>4</td>
<td>14</td>
<td>77.8</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>6</td>
<td>60.0</td>
</tr>
<tr>
<td>C</td>
<td>12</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>66.7</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>6</td>
<td>60.0</td>
</tr>
<tr>
<td>E</td>
<td>10</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>7</td>
<td>70.0</td>
</tr>
<tr>
<td>F</td>
<td>12</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>12</td>
<td>100.0</td>
</tr>
<tr>
<td>G</td>
<td>10</td>
<td>4</td>
<td>0</td>
<td>6</td>
<td>10</td>
<td>100.0</td>
</tr>
<tr>
<td>H</td>
<td>10</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>40.0</td>
</tr>
<tr>
<td>I</td>
<td>11</td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>72.7</td>
</tr>
<tr>
<td>J</td>
<td>30</td>
<td>3</td>
<td>3</td>
<td>13</td>
<td>19</td>
<td>63.3</td>
</tr>
<tr>
<td>K</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>100.0</td>
</tr>
<tr>
<td>Total</td>
<td>138</td>
<td>30</td>
<td>25</td>
<td>44</td>
<td>99</td>
<td>71.7</td>
</tr>
</tbody>
</table>
Comparing Herd Size to Percent Prevalence of STEC. The bovine fecal samples were collected from eleven different range-grazing cattle herds that varied in size considerably, ranging from 15 to 1000 animals. Due to the great diversity in sizes between ranches, we wanted to evaluate if the population size of the herd had any effect on the prevalence of STEC determined for each herd. After testing the samples from all 11 herds, our results indicated that the individual herds varied greatly in their percent prevalence. The largest herd (herd “J”) of 1000 cattle had a percent prevalence of 63.30% (Table 5). All three ranches that had 100% prevalence of STEC ranged in size from 15, the smallest herd (herd “K”) to 30 cattle, yet other herds of a similar size had some of the lowest percent prevalence. Regression analysis revealed that there was no positive correlation between herd size and percent prevalence \[ P = 0.33 \] (Figure 5). This held true even when regression analysis was repeated on the data omitting the herds with 100% prevalence.
Table 5. STEC prevalence in eleven range-grazing cattle herds organized by herd size.

<table>
<thead>
<tr>
<th>Herd&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Herd Size</th>
<th>STEC Prevalence&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>15</td>
<td>100.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>E</td>
<td>20</td>
<td>70.0</td>
</tr>
<tr>
<td>H</td>
<td>20</td>
<td>40.0</td>
</tr>
<tr>
<td>I</td>
<td>21</td>
<td>72.7</td>
</tr>
<tr>
<td>F</td>
<td>31</td>
<td>100.0</td>
</tr>
<tr>
<td>G</td>
<td>31</td>
<td>100.0</td>
</tr>
<tr>
<td>D</td>
<td>50</td>
<td>60.0</td>
</tr>
<tr>
<td>B</td>
<td>80</td>
<td>60.0</td>
</tr>
<tr>
<td>C</td>
<td>80</td>
<td>66.7</td>
</tr>
<tr>
<td>A</td>
<td>140</td>
<td>78.8</td>
</tr>
<tr>
<td>J</td>
<td>1000</td>
<td>63.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Herds and their corresponding STEC prevalence are listed in ascending order based on individual herd size.

<sup>b</sup> Values are in percentages.
Figure 5. Relationship between prevalence of STEC and the herd size of range-grazing cattle.

\[ a \text{ Herd “J” with a herd size of 1000 cattle and a prevalence of 63.3% was not included in data set.} \]
Effects of herd density on percent prevalence of STEC. Often the density of a population can affect the impact of an environmental factor, in our case the presence of STEC within the cattle herd. Usually, if the factor is density dependent, then the higher the population density, the stronger the effect felt within the population. We wanted to determine if the prevalence of STEC was a density dependent factor. To calculate the density of each herd, the total grazing acreage provided to each herd was obtained from each cattle herd owner or owner representative. Each acreage amount was converted into square kilometers. The number of cattle per square kilometer was then calculated for each herd and compared to that particular herd’s prevalence for STEC. As can be seen by the results in Table 6, the number of cattle per square kilometer varied greatly between the individual herds. Herd “J” had the highest density of 412 head of cattle per square kilometer, and herd B had the lowest density of 20 heads of cattle per square kilometer. However, the cattle density did not seem to have an effect on the percent prevalence (Table 6). For example, even though Herd “J” had the highest cattle density, it had one of the lowest percents (63.3%) of STEC. While herds “F”, “G”, and “K” all had a STEC prevalence of 100% yet they had densities of 38, 78, and 300 head of cattle/km², respectively (Table 6). Using regression analysis confirmed that there was not a statistically significant relationship between the number of cattle per square kilometer and the prevalence of STEC [$P = 0.77$] (Figure 6).
Table 6. Summary of the herd density from eleven cattle herds.

<table>
<thead>
<tr>
<th>Herd&lt;sup&gt;a&lt;/sup&gt;</th>
<th>cattle/km&lt;sup&gt;2&lt;/sup&gt;</th>
<th>STEC Prevalence&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>20.0</td>
<td>60.0</td>
</tr>
<tr>
<td>E</td>
<td>22.5</td>
<td>70.0</td>
</tr>
<tr>
<td>A</td>
<td>23.1</td>
<td>77.8</td>
</tr>
<tr>
<td>C</td>
<td>24.7</td>
<td>66.7</td>
</tr>
<tr>
<td>D</td>
<td>30.9</td>
<td>60.0</td>
</tr>
<tr>
<td>F</td>
<td>38.3</td>
<td>100.0</td>
</tr>
<tr>
<td>G</td>
<td>77.5</td>
<td>100.0</td>
</tr>
<tr>
<td>I</td>
<td>105.0</td>
<td>72.7</td>
</tr>
<tr>
<td>H</td>
<td>250.0</td>
<td>40.0</td>
</tr>
<tr>
<td>K</td>
<td>300.0</td>
<td>100.0</td>
</tr>
<tr>
<td>J</td>
<td>411.5</td>
<td>63.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Herds are listed in ascending order based on their cattle densities.

<sup>b</sup> Values are in percentages.
Figure 6. Relationship between prevalence of STEC and cattle herd densities.
Seasonal percent prevalence following one herd over a fifteen month time period.

Temperature and precipitation have been implicated as having an effect on the prevalence of STEC in cattle (Barkocy-Gallager, G. et al. 2003; Cobbold, R. et al. 2004; Fremaux, B. et al 2008; Gagliardi, J. and J. Karns, 2000; Van Donkersgoed et al. 1999). However, much of the research available has presented conflicting results. We wanted to determine over a 15 month period, if there was a statistically significant relationship between temperature and the prevalence of STEC or precipitation and the prevalence of STEC in one isolated herd. To do this, samples were collected every three months, over a 15 month period, starting April 2007 and concluded July 2008. Over this time period, no new cattle were introduced to the herd, the herd was managed on the same property, and the management processes remained constant (vaccinations, weaning, sterilization). The cattle were maintained on irrigated grass pasture for the majority of the time of this investigation. Alfalfa hay was fed twice daily from November to March, when conditions were poor for grass growth. Approximately half the herd was tested at every sampling. Specifically, an average of ten fresh fecal samples were collected from a total of 20 cattle in the herd. Each fecal sample was collected from a different cow. All samples were screened for both \textit{stx}_1 and \textit{stx}_2 by PCR analysis to identify the presence of STEC. Samples collected in the spring repeatedly showed the lowest levels of STEC: 40% in April 2007, and 36.4% in April 2008. Interestingly, sampling from early winter, (January 2008) and mid-summer, (July 2008), showed the highest levels of STEC: 70% and 90.9% respectively. A summary of the data collected is found in Table 7.
Data was also collected on the precipitation amounts and the mean average temperatures from each month the fecal samples were collected. The data was then compared to the prevalence of STEC determined from each sampling (Figure 7 and Figure 8). Using Spearman’s rank correlation test, there was no significant relationship between the percent of STEC found in the cattle and average monthly temperature for each month collect \([r = 0.37, P = 0.47]\) (Figure 9). Additionally, no statistically significant relationship was found between the percent of STEC and average monthly precipitation \([r = 0.00, P = 1.00]\).
Table 7. Prevalence of STEC in cattle feces collected from one herd over a 16 month period.

<table>
<thead>
<tr>
<th>Date Collected</th>
<th>No. of Samples Collected</th>
<th>stx₁ positive</th>
<th>stx₂ Positive</th>
<th>Both Toxins</th>
<th>Percent Total Positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>4/10/2007</td>
<td>10</td>
<td>1(^a) (10)</td>
<td>2 (20)(^b)</td>
<td>1(10)</td>
<td>40.0</td>
</tr>
<tr>
<td>6/17/2007</td>
<td>9</td>
<td>3 (33.3)</td>
<td>0 (0)</td>
<td>1 (11.1)</td>
<td>44.4</td>
</tr>
<tr>
<td>10/28/2007</td>
<td>9</td>
<td>0 (0)</td>
<td>2 (22.2)</td>
<td>3 (33.3)</td>
<td>55.6</td>
</tr>
<tr>
<td>1/17/2008</td>
<td>10</td>
<td>1 (10)</td>
<td>3 (30)</td>
<td>3 (30)</td>
<td>70.00</td>
</tr>
<tr>
<td>4/16/2008</td>
<td>11</td>
<td>1(9.1)</td>
<td>2 (18.2)</td>
<td>1 (9.1)</td>
<td>36.4</td>
</tr>
<tr>
<td>7/22/2008</td>
<td>11</td>
<td>4 (36.4)</td>
<td>4 (36.4)</td>
<td>2 (18.2)</td>
<td>90.9</td>
</tr>
</tbody>
</table>

\(^a\) Number of positive fecal specimens.  
\(^b\) Values in parenthesis are given in percentages.
Figure 7. Comparison of the STEC prevalence to the average mean temperature for the day the sample was collected.
Figure 8. Comparison between STEC prevalence for the days sampled and the average precipitation for the month sample was collected.
Figure 9. Comparison between STEC prevalence in one range-grazing cattle herd to the mean average temperature of the month the sample was collected.
Due to the fact that many other previous studies have reported their highest percent of STEC prevalence in warmer months, statistical analysis was repeated but without the seemingly unusually high STEC results from January 2008 (Table 7) (Barkocy-Gallagher, G. et al. 2003; Cobbold, R. et al. 2004; Van Donkersgoed, J. et al. 1999). Using Spearman’s rank correlation test, a significant positive relationship was found between the temperature and the percent STEC in the herd \( r = 0.9 \) and \( P = 0.037 \) (Figure 10).

Soil moisture content has been linked to the long-term survival of STEC isolated out of soil. Specifically, saturated soil conditions that occur during rainfall events may increase the spread and enhance the growth of STEC (Fremaux, B. et al. 2008; Garliardi, J. and J. Karns. 2000). Therefore, to be certain that the unusual high STEC prevalence for January 2008 was not affecting the statistical results comparing precipitation and prevalence, statistical analysis was repeated omitting the data from January’s 2008. However, there was still no significant relationship between prevalence of STEC and precipitation for the months the samples were collected \( r = -0.32 \) and \( P = 0.60 \).
Figure 10. Comparison between STEC prevalence in one range-grazing cattle herd to the mean average temperature of the month the sample was collect.

January 2008 (STEC prevalence of 70%) not included in data set.
Chapter 4
DISCUSSION

For this research, we focused on healthy adult range-grazing cattle. This is a cohort that’s been largely ignored in previous research. Most adult cattle will be “culled” once they are past their reproductive prime, meaning they will be removed from the herd. Once removed from the herd, culled cattle are usually sent straight for processing. These animals are not “finished” with a grain diet and therefore do not spend time on a feedlot, and do not go through the usual production system that other younger cattle would. Large portions of the carcasses from cattle that are culled, are used for ground beef. Ground beef has been implicated in many reported outbreaks of STEC (Samadpour, 2001). Therefore, knowing the prevalence of STEC in cattle that are largely used for ground beef is extremely valuable. By knowing a baseline prevalence of STEC from the cattle coming into the meat processing plant and comparing it to the prevalence of STEC found in ground beef and other meat products leaving production, one can make assessments on the quality of the beef products. For this reason, only healthy adult range-grazing cattle, specifically those that would be considered acceptable for meat production, were sampled for this research.

In this study, we collected fecal specimens from 152 head of cattle that belonged to eleven different herds in the greater Sacramento region. After collecting the fecal samples, and performing PCR analysis for STEC by screening for the \( stx_1 \) and \( stx_2 \) genes, an average prevalence of 71.7% was determined. Our results clearly indicate that STEC is highly prevalent in healthy adult range-grazing cattle in Northern California.
The data obtained from this investigation does not support our original hypothesis that adult range-grazing cattle would have a lower prevalence of STEC than feedlot cattle (Table 1). Range-grazing cattle are much less confined than feedlot cattle or cattle in pens waiting to be processed. Therefore, it seems likely that there would be fewer opportunities for cross-contamination in range-grazing cattle and hence, they would have a lower prevalence of STEC. That was not what the results of our study indicated. In this investigation, STEC levels were as high if not higher than those previously reported (Table 1).

A possible explanation for why other studies have reported lower prevalence of STEC than this study may be the differences in methodology. In this study, samples were enriched in LB broth for at least 18 hours, at 37°C. This long enrichment step provided any STEC bacteria in the fecal sample a sufficient amount of time to replicate to detectable amounts. Furthermore, following the enrichment step for this study, the DNA from the bacteria was isolated using InstaGene matrix™, and then placed directly into the PCR assay with the primer sets (as described in Materials and Methods). Most other studies do not do an enrichment step, but rather plate the fecal samples onto solid media first, and then pick colonies out to use in the PCR assay. This is an effective method for removing the fecal inhibitors that are known to interfere with the PCR (Gioffre’ et al. 2002) but it reduces the sensitivity of the assay, as it is unlikely that every possible colony that could be STEC, is picked and placed in a PCR reaction. It would be like “finding a needle in a hay stack”.
Another method we used to assure the quality of each sample tested was the use of an internal control. For our internal control, we chose to use an oligonucleotide primer set specific for *E. coli* 16s ribosomal DNA. Like humans, cattle carry *E. coli* as part of their normal flora in their intestines. Thus, regardless of whether STEC was present or not, all DNA preps that could be amplified by PCR should have an amplified 16s ribosomal DNA band for *E. coli*. By using this internal control throughout our study, we were able to confirm the integrity of our DNA samples. In fact, if it were not for the use of this internal control, some of our specimens would have been deemed “negative” for STEC, when in fact there was actually a problem with our DNA sample.

In addition to using the *E. coli* 16s ribosomal DNA as an internal control for our DNA prep and PCR, we also used it to determine the best dilution of fecal sample into enrichment media that would give the most reliable results. It was determined that a dilution of 0.2 g of fecal sample to 50ml of LB broth, for the overnight enrichment prior to DNA isolation, gave consistent results while having good sensitivity. By using this valuable control, we discovered the importance of diluting the overnight enrichment of fecal sample to remove the many fecal inhibitors that interfere with PCR.

An additional finding from our assay development study, that was extremely relevant to the sensitivity of our detection method was that the sensitivity went down 10-fold when the *E. coli* 16s ribosomal primers were included as part of a multiplex PCR. Sensitivity was optimal when running the 16s primer set separately for each sample. Therefore, for each sample, we ran this crucial control in parallel to the other PCR assay specifically for STEC. By running the *E. coli* 16s internal control separately from the
Stx1 and Stx2 primer sets, we were able to obtain good sensitivity following our methods for this research. From direct plate dilutions and PCR, sensitivity results were visualized for both stx₁ and stx₂ down to $10^{-7}$ of the overnight culture equaling a level of detection of 44 CFU per gram of sample (Figure 2).

Although we developed a highly sensitive assay, which may account in part for the high prevalence results we obtained, there may be other explanations as to why our results were higher than other studies that evaluated feedlot cattle rather than free range-grazing cattle (Table 1). One key factor is based on the different diet fed to range-grazing cattle. As mentioned earlier, culled adult cattle are not grain fed while most other young cattle are switched to feed with a high grain ratio right before meat processing. Grain finishing causes the animal to increase weight, as well as add desirable characteristics of tenderness and juiciness to the meat for the consumer. This difference in diet can cause dramatic changes in the bowels of the animal where the bacteria live, particularly in the intestinal tract where E. coli thrive in cattle fed high-grain rations (Callaway et al. 2003). A lot of attention has been given to the characteristics associated with changing the diet and the affects on the concentration of bacteria in the animal, but many of the results are conflicting. Research done by Diez-Gonzalez showed that cattle, fed good quality hay contained 100-fold less E. coli than cattle fed a feedlot-type ration of grain (Diez-Gonzalez et al. 1998). However, other studies using experimentally infected sheep found that sheep fed a low quality forage diet increased their shedding of E. coli O157:H7 (Kudva, et al. 1995). Unfortunately, the research in this area varies greatly in methodology, so making direct comparisons is very difficult.
In our study, comparisons between the individual herd’s prevalence of STEC were made. Samples were collected from March 2007 through June 2008. The individual prevalence of STEC did vary between the herds, as one would expect (Table 4). High levels of STEC were consistently observed from all 11 herds that samples were collected, the lowest being 40% and the highest was 100% (Table 7). The 11 different cattle herds tested were owned by six different ranches. It is unlikely that animal husbandry practices by all six ranches were identical. As one would expect, there were variances between the herds in factors such as, time of year the cows calved, time of year the calves were weaned, types of forage in the pastures, and whether the herd was supplemented with hay when environmental conditions limited pasture grass growth. Despite all of the different variables that could be affecting the individual herds, it is apparent from our research that STEC is very common in healthy range-grazing cattle that have not been to a feedlot or finished with grain for meat processing.

In our investigation, we wanted to determine if herd density, (number of head of cattle per square kilometer) and herd size had a significant effect on the STEC prevalence determined for each herd. We concluded that neither herd density nor herd size had a statistically significant effect on the individual herd’s prevalence of STEC. The individual herd densities varied greatly from herd to herd but did not significantly have an effect on the percent of STEC determined for each herd. This was surprising because it seems reasonable to hypothesize that the more cattle sharing a common space, the greater the likelihood of multiple cows becoming contaminated. Our results however, disproved this hypothesis. For example, one herd (herd “A”) consisted of only 140 cattle,
that grazed on over six kilometers squared, which is approximately 1500 acres equaling a density of approximately 23 cows/ km². This herd had an average STEC prevalence of 78% STEC. Yet another herd (herd “J”) with 1000 cattle on 600 acres (~411 km), equaling a density of 412 cows per km², had a STEC prevalence of only 63.3% (Table 6). One would think that herd “J”, with a high density of 412 cows per km², would have a higher prevalence compared to the less dense herds. This is the opposite of what we found. What’s more, herd “J” sample’s were collected during the warmer months of the year for this region, a time when many studies have reported STEC prevalence at its highest. Therefore, our results cannot be explained by the fact that the samples were collected at different times of the year (Cobbold, et al. 2004; Van Donkersgoed, et al. 1999). If anything, herd “J” should have had a higher prevalence due to these previously reported seasonal trends. It is more probable that our results are due to cattle behavior. Demographically, cattle follow a clustered pattern of dispersion and despite having more areas to forage and graze, they will usually stay within close proximity to the other cattle, making cross-contamination highly probable. Cattle are also creatures of habit, meaning they often bed down in the same places day after day, follow the same trail to their water source, share the same water sources, and graze the same areas often at the same times of day. It has been shown that cattle water sources can be infected at high levels of STEC and remain infected for up to 40 days at 21°C and 70 days and 5°C. (Fremaux et al. 2008; Rice et al. 1992). Fecal material left behind by an infected animal could easily contaminate other cattle that return to the same location, as well as re-infecting the same cow. Studies have shown that manure with STEC can remain STEC positive for up to
231 days when left out in the environment (Fremaux et al. 2008; Kudva et al. 1998). This would help explain why the amount of land available for grazing is not as much of a factor with regard to the prevalence of STEC in a herd as one may think.

The seasonal study was the second major piece in this research. We wanted to evaluate the effect of temperature and precipitation on the STEC prevalence in one herd over an extended amount of time. The cattle herd used for this part of our research was fairly isolated, grazed on the same acreage all year, was maintained on a consistent diet, and was owned by one small ranch. Approximately 50% of the herd was sampled every three to four months. For every sampling, the prevalence of STEC was determined, and used to ascertain if there was a correlation between the prevalence of STEC to temperature and precipitation.

Interestingly, STEC was detected at every sampling interval from this small herd (19 cows and 1 bull). Our study did not suggest a significant relationship between temperature and precipitation and STEC prevalence. The prevalence of STEC did fluctuate throughout the year. It peaked in July, one of the warmest and driest months, and then again in January, one of the cooler and wettest months of the year. April repeatedly had the lowest percent prevalence (Table 7). Because other previous research has suggested that there is a seasonal relationship between STEC and temperature, mainly that the STEC prevalence is higher in warmer months, we repeated our statistics but omitted the January 2008 data. Interestingly, when January’s data is left out, there is a statistically significant relationship between STEC prevalence and temperature. Warmer temperatures did have higher prevalence levels of STEC.
As to why January 2008 had such a high level of STEC is yet to be determined. For comparison purposes, herd “J” from the first part of our study was also sampled twice, once in December 2007, and then again in July 2008. Interestingly, the percent prevalence of STEC was also very high in December 2007, with 94% of the fecal samples testing positive for the Shiga toxin genes. When herd “J” was tested again in July of 2008, the percent of STEC positive samples was down to 64%. Taken together, our data suggest that the herd involved in the seasonal study was not unique in the seasonal pattern observed, and that other environmental factors, may play a role in STEC levels in cattle.

One factor we took into consideration was the average temperature and precipitation of the month prior to the sample being collected. According to the California Department of Water Resources, November 2007, had low amounts of precipitation (3.1 cm) and was fairly warm (mean average of 13°C) which perhaps contributed to the high prevalence of STEC (94%) in herd “J” in December 2007. However, this incident of warm temperatures and dry conditions the month prior to collecting the samples with high levels of STEC, as in herd “J”, is not observed in the seasonal study herd for the samples collected in January 2008. In December 2007, the average mean temperature was cool (5°C) with some precipitation (15.8 cm). Yet, despite the cooler and wetter weather the following month’s sampling (January 2008) had a high prevalence of STEC (70%). Since only two of the herds were re-sampled, it is difficult to draw any conclusions as to why there was such a high percent of STEC during December and January, which are relatively cool months for the region. However, it
does not seem that temperature and precipitation of the month prior to the samples being collect, is a factor.

The survival and longevity of STEC in cattle herds is a great concern, particularly concerning animal husbandry practices. As cattle make their way from the field to the market as beef, it is important to know if all cattle, at all times of the year, carry STEC. An important observation that our research demonstrated, was that the STEC prevalence in the seasonal study never went down to zero throughout the duration of our research. The lowest prevalence of STEC was 36.4% for the month of April 2008 (Table 7).

There are several possible explanations as to why STEC persisted for 15 months in the cattle herd for the seasonal study. One possible explanation as to why the STEC persisted in this herd is that competition in the bowel mucosa of the cattle selects for STEC. Cattle and other ruminants have a four chambered stomach which includes the reticulum, rumen, omasum, and the abomasums. The rumen is where most bacterial fermentation takes place, and it contains a very diverse combination of bacteria, protozoa and fungi that function as part of a symbiotic relationship with ruminate animals (Highfill, 2005). Over 150 microorganisms have been identified in the rumen of cattle (Highfill, 2005). These bacteria partially breakdown complex dietary forage that the ruminant consumes, making the nutrients more readily available to the animal.

Some research has suggested that protozoa help control bacteria populations in the rumen (Ozutsumi et al. 2008). Additionally, research by Steinberg et al. using Tetrahymena, suggest that in the presence of the bactivorous protozoans, Escherichia coli with the stx-encoding prophage had considerable increased fitness compared to E.coli
that did not carry the prophage (Steinberg et al. 2007). Although the fecal samples we collected, were not analyzed for *Tetrahymena* it is a likely explanation for why STEC persisted in this herd, as ciliated protozoa are commonly found in the rumen of cattle (Highfill, 2005; Steinberg et al. 2007).

Another explanation as to why STEC persisted in the herd for our seasonal study comes from the other animals that share the pastureland with the cattle herd. The property that the cattle herd grazes on, is also commonly inhabited by geese and deer. Research has shown that like other ruminants, deer are a natural reservoir for STEC (Caprioli et al. 2005). Birds on the other hand, are not considered true reservoirs of STEC, but act more as vectors, dispersing the bacteria (Hussong, D. et al. 1979; Synge et al. 2003). One particular study found STEC shedding significantly higher in grazing cattle herds where wild geese were also present (Synge et al. 2003). The natural wildlife that share the grazing land with the cattle could potentially be shedding STEC along with the cattle, contributing to the STEC infection within the herd. Companion animals, such as dogs and horses are also in close proximity to the herd tested, and could potentially contribute to the continuous levels of STEC isolated from these cattle.

Another factor to consider concerning the persistence of STEC in the seasonal study is the water source for the cattle and the ground they graze. It is quite possible that the cattle drinking water plays a major role in the persistence of STEC in this herd. As mentioned earlier, cattle drinking water is known to be a reservoir for STEC (Fremaux et al. 2008; Rice et al. 1992). All of the different wild animals in the area, as well as companion animals, and the cattle in the herd used for this study, share a large pond as
their water source. With so many different animals using the pond, it is quite possible that the shared available water contributes to infections and re-infections of STEC in the cattle herd. Also mentioned earlier, is the long-term survival of STEC in feces and in farm waste (Fremaux et al. 2008, Kudva et al. 1998). Since the herd for the seasonal study was always maintained on the same property, and their fecal matter was not discarded, the long-term survival of STEC in their feces would be a significant factor and a source of STEC infections and re-infections as well.

With all the different factors involved in this one small herd, it is really not that surprising that the seasonal herd’s prevalence never went to zero. With so many potential STEC shedders interacting with the herd, it highlights the important of clean, sanitary farm management. Currently there is a lot of research being done that is identifying farm management practices that reduce STEC in cattle. Some of these practices include the use of probiotics, vaccinations, and antimicrobial pharmaceuticals (Karmali, et al. 2000)

There are many implications of this study. STEC prevalence is high in range-grazing cattle tested in Sacramento and neighboring counties. Most adult range-grazing cattle will be culled once they are past their reproductive prime. At this point, they will enter the food chain mostly as ground beef. It was previously determined in our lab that at least 10% of the ground beef in supermarkets carries STEC (Oliver, 2000). Other studies have reported similar results, some as high as 30% (Hussein and Bollinger 2005). Our research can serve as a baseline for the amount of STEC present in cattle before the cattle even leave their herds and go to slaughter to be processed into ground beef and
other beef products. By having a baseline for the prevalence of STEC, it makes it possible to analyze the effectiveness of pre and post processing measures used to control STEC as beef enters the food supply.

Every year thousands of individuals are exposed to STEC and many become seriously ill and even die. Most professionals in the field say that many STEC cases are not reported, and that the number of cases is actually much higher than what the CDC claims. A recent study done by M. Werminski, from our laboratory, determined that out of 200 human stool samples that were brought to a local hospital lab and tested negative by culture, 27% of the stool samples were later determined to be STEC positive by PCR. (Werminski, M. 2008). In this one isolated study, 54 individuals actually had been infected with STEC and never received a STEC diagnosis. Getting a correct diagnosis is crucial to treating the patient, something that these 54 patients never received. One can’t help but wonder how often this happens in other clinical laboratories across the United States despite the mounting plethora of evidence that STEC strain O157:H7 is not the only potentially life threatening strain of STEC. Our study, along with the findings of M. Werminski, clearly underscore the need for STEC screening in the agricultural laboratories as well as clinical laboratories, rather than limiting the detection method to just O157:H7 strains.

With the rise in O157:H7 and other STEC cases reported and the many others that remain unreported, it is important to pay close attention to all possible sources of contamination in the food industry. Cattle are undeniably a major reservoir for STEC entering our food supply. Consumers often do not fully realize the potential for STEC
infections associated with food products and are lax in their food handling. If more individuals had a more realistic idea of how common STEC infections can be, then perhaps more care would be taken during food preparation and general hygiene practices, such as hand washing.

It is critical to inform consumers of the very real risk of STEC in beef products to protect them. It is equally important to monitor every step of beef production, the meat and the meat source. Having a baseline for the percent prevalence of STEC in cattle before they are sent for processing, will help cattle ranchers strive to keep high standards of animal husbandry. Our research demonstrates the over 71% of the cattle that could potentially enter the food chain as ground beef are carrying STEC. Our lab has also determined at least 10% of the retail ground beef in the Sacramento area is STEC positive (J. Oliver 2000). Others have reported STEC prevalence levels from 2% to as high as 30% in ground meet (Hussein and Bollinger 2005). This research supports that high standards of pre and post harvesting measures are necessary and are currently being implemented effectively.
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