PREVALENCE OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN THE SACRAMENTO VALLEY EQUINE POPULATION

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THESIS

Submitted in partial satisfaction of
the requirements for the degree of

MASTER OF SCIENCE

in

BIOLOGICAL SCIENCES
(Molecular and Cellular Biology)

at

CALIFORNIA STATE UNIVERSITY, SACRAMENTO

FALL
2009

PREVALENCE OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN THE SACRAMENTO VALLEY EQUINE POPULATION

A Thesis

by

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Abstract

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Prevalence of Shiga Toxin-Producing *Escherichia coli* in the Sacramento Valley Equine Population

by

Donica G. Larson

Illnesses such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) caused by Shiga toxin-producing *Escherichia coli* (STEC) remain a health concern in the United States and throughout the world. Although the majority of STEC cases have been linked to undercooked ground beef and contaminated fruits and vegetables, companion animals can also be a source of transmission, specifically dogs and horses (21). This is of particular concern to immunocompromised individuals, elderly and children. Cattle, and other ruminant animals, are known carriers of STEC (4). To date, most STEC research has focused on cattle due to their link to numerous outbreaks as a result of contaminated ground beef, water, fruits and vegetables. Non-ruminant animals such as horses, dogs and birds have also been implicated as carriers of STEC, but at a lower prevalence than ruminant animals (7). There is currently no information in the literature on the prevalence of STEC in horses. The purpose of this study was to develop an assay system for the evaluation of pathogenic bacteria in horse feces and on horse hides and then to evaluate the prevalence of STEC in the equine population in the Sacramento Valley by evaluating both fecal and hide-swab samples. Methods were successfully developed to sample equine feces and hide samples and to isolate the DNA from each sample type to determine the presence of the Shiga toxin genes stx_1 and stx_2 as a measure of STEC prevalence. The horses sampled in this study were placed in one of two groups: those that interact with ruminant animals and those with no ruminant interaction. One hundred fifty-six horses were sampled in the greater Sacramento Valley between June 2008 and September 2009: 78 ruminant-interacting and 78 non ruminant-interacting. Of the 156 horses sampled, 4 (2.6%) were positive for stx_2, all of which interacted with ruminants. Therefore, of the ruminant-interacting group, 4 of 78 (5.1%) were positive for stx_2 and of the non- ruminant-interacting group, 0 of 78 (0.0%) were positive for either stx_1 or stx_2. None of the 156 horses sampled were positive for stx_1.

_____ Committee Chair

Susanne W. Lindgren, Ph.D.

ACKNOWLEDGEMENTS

I owe the successful completion of my thesis to my friends and family. Without their help sampling horses all over the Sacramento Valley in the heat and rain, the hours sweating in the lab until well into the night and, of course, their love and support, this achievement would not have been possible. You have my undying gratitude, each and every one of you.

I would also like to thank my professor, Dr. Susanne Lindgren, and my committee members, Dr. Enid Gonzalez and Dr. Thomas Landerholm for their input into my project and their willingness to rush to meet deadlines. Thank you.

Finally, I want to thank my daughter, Breanna who has patiently waited since the day she was born for her mommy to be done with school. I love you so much, Breanna. Thank you for being so understanding.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

Introduction

For over two decades, *Escherichia coli* O157:H7 has been in the forefront of the popular press due to its implication in numerous outbreaks. The most commonly reported source for these outbreaks has been associated with cattle, particularly undercooked ground beef (4), with contaminated spinach and lettuce also being implicated recently in the headlines (12). However, the O157:H7 serotype is not the only Shiga toxin-producing *E. coli* that is responsible for human disease. Other types of Shiga toxin-producing *Escherichia coli* (STEC), known as serotypes, have also been isolated from humans exhibiting symptoms of STEC infection. Although the most prevalent serotypes implicated in human disease have been O157:H7 and O157:H-, other serotypes such as O111:H-, O26:H11, O26:H-, O103:H2, O111:H8, and O145:H- have also been attributed to STEC outbreaks (8, 26). In addition to the multiple STEC serotypes that play a role in human disease, livestock sources other than cattle have also been found to be problematic. Multiple studies have been conducted that have confirmed isolation of O157 from sheep, goats and pigs (47, 49), free-ranging deer (43) and interestingly, from companion animals, specifically dogs (50) and horses (13, 50). Our concern is with transmission of STEC from companion animals, specifically horses, to people. Horses are often in close physical contact with people, and if infected with STEC, the horses pose a potential risk to their owners and riders, as well as to those involved in horse-related recreation and leisure such as vacation riders and petting zoo patrons.

STEC has been associated with uncomplicated diarrhea in humans (40) and has been isolated from healthy individuals (20, 27), however, it is frequently found to cause

severe disease, requiring hospitalization of infected individuals. One such disease is hemorrhagic colitis, which is characterized by bloody diarrhea. Another disease, that is a sequelae to hemorrhagic colitis is hemolytic uremic syndrome (HUS). HUS is a triad of the ailments including hemolytic anemia, thrombocytopenia, and acute renal failure (5, 8). Diarrhea-associated HUS primarily affects children from 7 months to 6 years of age and occurs in approximately 10% of children infected with *Escherichia coli* O157:H7. Although 65% to 85% of HUS patients make a full recovery, up to 5% of cases can be fatal, and many of those that survive often have long-term persistent renal abnormalities (5). However, recent research has suggested that non-O157 strains of STEC may exceed that of O157 strains among patients with diarrhea and HUS, specifically in Germany, Belgium, Finland, the Czech republic, and Italy (25).

As the name indicates, STEC is known for its production of Shiga toxins. These toxins are responsible for the more serious complications associated with STEC, such as HUS and hemorrhagic colitis (33). The research detailed in this thesis focuses on the two Shiga toxins: Shiga toxin 1, encoded by stx_1 and Shiga toxin 2, encoded by stx_2. The amino acid sequence of Stx1 differs from the Shiga toxin of *Shigella dysenteriae* by only one amino acid, and therefore the two toxins are serologically indistinguishable. The Stx2 family of toxins is more heterogeneous and less related to *Shigella dysenteriae* and stx_1. Thus, it cannot be neutralized by antibodies to Stx1 or the Shiga toxin of *S. dysenteriae* (8, 37). It is important to note that a given STEC isolate can carry the genes for one or both of the Shiga toxins. Interestingly when STEC is implicated in human

disease, the STEC that produces only Shiga toxin 2, compared to producing only Shiga toxin 1, is more likely to cause serious complications such as HUS (41).

STEC has been isolated from many different types of animals, but has been found to have a greater prevalence and persistence in ruminant animals, animals which have a four chambered stomach consisting of the rumen, reticulum, omasum and abomasum. Examples of ruminant animals include cattle, goats, sheep, deer, and llamas (1, 14). Petting zoos typically include farm animals, many of which are ruminants, which interact closely with one another. Although it is cattle that are primarily implicated in STEC carriage, one study by Beutin *et al*. examined the prevalence of STEC in healthy domestic animals. These investigators found STEC in cattle, sheep, pigs, goats, cats and dogs, and demonstrated a significantly higher carriage rate in sheep (66.6%) than in cattle (21.1%) (7). These data emphasize the importance of ruminant animals other than cattle in the carriage of this pathogenic bacterium in domestic animals.

One source of *E. coli* O157:H7 outbreaks, more common to pre-school aged and school-aged children, has been from petting zoos (3). From 2000 to 2005, numerous *E. coli* outbreaks have been reported from petting zoos and petting farms from suburban county and state fairs across the United States (3, 18) Investigation into each of these outbreaks has demonstrated frightening similarities in the lack of preventative and/or basic hygiene facilities for humans around farm animals. Petting zoos are frequently found at fairs and food festivals where patrons are able to eat inside the petting zoo area or able to purchase food concessions nearby without opportunity for effective hand washing prior to eating. Visitors in many of these outbreaks were able to touch cattle,

sheep, goats, llamas, chickens and/or pigs without proper emphasis on the importance of hand washing following handling of these animals (18). Between Fall 2000 and Fall 2004, petting zoo outbreaks of O157: H7 *E. coli* led to more than 300 illnesses, primarily in children, in Pennsylvania, Ohio, Wisconsin, Oregon, and North Carolina (3). All of these petting zoo outbreaks were directly linked to infected ruminant animals (3). These petting zoo outbreaks demonstrate the risk of infection through direct contact with farm animals, and highlight the risk associated with casual and brief contact with these animals.

Although there is published research reporting the detection of Shiga toxin-producing *E. coli* in horses, dogs, cats and other domestic animals, there is little to no published research evaluating the actual prevalence of STEC in these companion animals (21, 48). This is especially the case for horses where virtually no studies have been published on the subject. The horse industry in the United States, however, boasts more than 9.2 million horses, almost 700,000 of which are in California. These horses are used for racing, rodeos, recreation, ranch work, and more. All of these activities involve physical contact between the horses and their owners or riders (2) which highlights the possibility of transmission of STEC from horse to rider.

A unique characteristic of horses used for work and as companion animals is that in both cases, horses may have interaction with ruminant animals. Working horses, such as those on cattle ranches, are in contact with cattle in particular, but may also have contact with other ruminants present on the ranch. Deer, another ruminant known to carry STEC (43) were found to frequent some of the ranches and the ranch resort tested

in this study and came into contact with the companion and recreation and leisure horses present on the properties. Companion horses that are housed on ranches and farms may also be in contact with ruminant animals. Interestingly, according to the horse owners in this study, goats and/or sheep are often kept as companions for the horses. As companions, the horses and goats or sheep interact closely and frequently, thus increasing the opportunity for transmission of STEC from the ruminant companion to the horse.

Numerous STEC outbreaks have occurred worldwide and have been the cause of severe disease. Children are not only more affected by serious ailments such as HUS, after infection with STEC, they are less likely than adults to effectively wash their hands following direct contact with animals, therefore increasing the possibility of STEC transmission from animals. The enormity of the horse industry, and the risk to children associated with companion animals, support the need for more research in STEC carriage and transmission in the equine population.

The purpose of this study was two-fold: 1) to develop a sensitive molecular assay system for the evaluation of pathogenic bacteria in horse feces and hides and 2) to evaluate the prevalence of STEC in fecal and hide samples in the equine population in the Sacramento Valley using this assay system. Because of the known prevalence of STEC in ruminant animals, two sample groups were selected. One sample group consisted of horses that interact with ruminant animals. The second group consisted of horses that have no interaction with ruminant animals. I hypothesized that the group that associated with ruminant animals would have a higher prevalence of STEC than the group of horses with no interaction with ruminant animals.

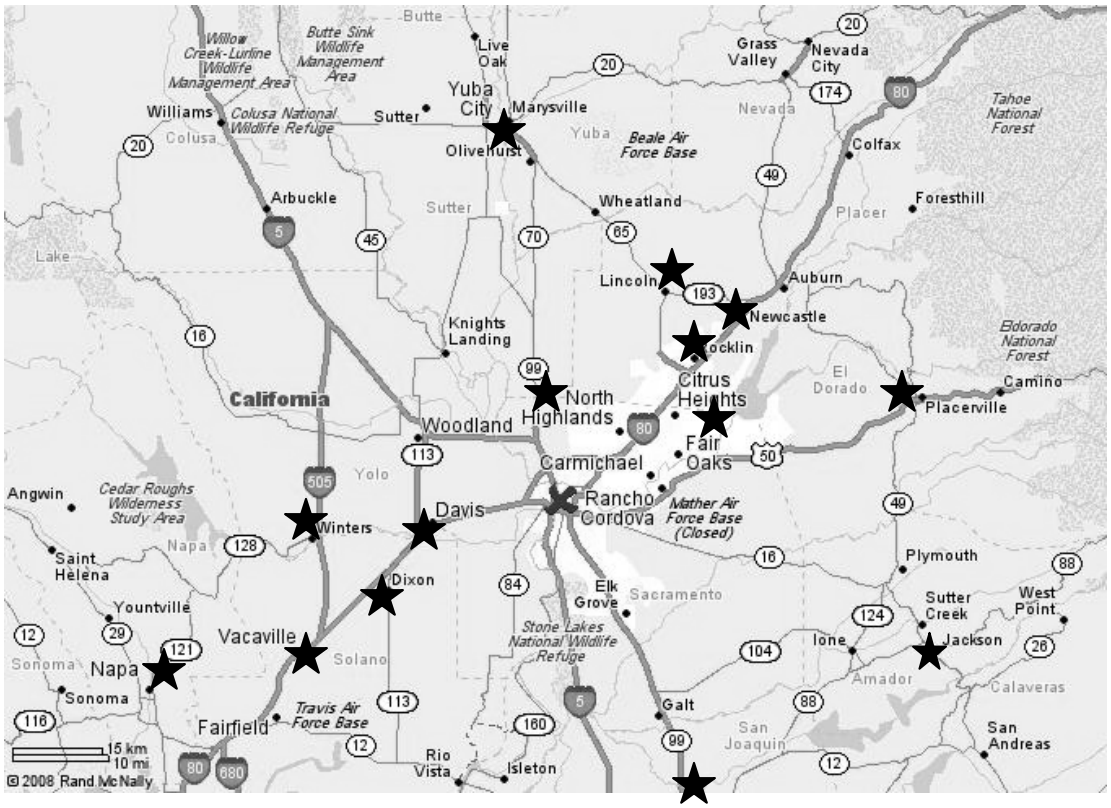
Materials and Methods

Sample Selection

Horses of various ages and breeds including Lipizzaner, Friesian, Quarter Horse, Thoroughbred, Clydesdale, Appaloosa, American Paint Horse, Shetland pony and mixed breeds were examined for carrying Shiga toxin-producing *Escherichia coli* using fecal and hide-swab sampling. Horses were selected at random, without regard to health history or diet.

A fecal sample and two hide-swab samples were obtained from each of 156 horses. The horses that were tested lived in a variety of different conditions. Horses were housed in equestrian centers, private residences, ranches and petting farms within the Sacramento Valley (Figure 1). The sampling was done from June 2008 through September 2009. Approximately half of the horses selected had at least some interaction with ruminant animals. These horses interacted with cattle, goats, deer, llamas and alpacas. Interaction was defined as coming into contact with a ruminant animal or ruminant animal feces. There was no distinction made between those with frequent interaction and those that had sporadic or a singular interaction with ruminant animals. The other half of the horses selected for this study had no documented interaction with ruminant animals. The number of horses sampled per location was based on the sample availability. To determine if STEC is present in the horse's digestive tract or present on the horse hide, fecal and hide-swab samples were collected concurrently, with one exception due to sampling availability of the horse.

Figure 1: Equine sampling locations in Sacramento Valley



Black stars indicate the geographical locations of the 19 participating ranches in the Sacramento Valley where horses were tested for STEC.

Fecal sampling and DNA extraction

A small sample (approximately 10 – 20 grams) of fresh horse feces was collected in a clean, new Ziplock® bag. Samples were transported to the laboratory at room temperature. 85% (132 samples) of the samples were processed the same day they were collected. The remaining samples were collected and held at room temperature until they were processed. These samples were as follows: four samples from Ranch H and I were processed within 24 hours, one sample from Ranch Q was processed on day two, eight samples from Ranch G were processed on day three, and 11 samples from Ranch T were

processed on day four. All of these samples that were not processed the same day were held due to scheduling conflicts. Previous studies on the viability of STEC has demonstrated that *E. coli* O157:H7 can survive in bovine feces and surrounding soil for up to 99 days (9). Therefore, holding the samples at room temperature for up to four days should not impact any of the results.

Each fecal specimen was enriched for bacterial growth by culturing 0.5 g feces in 30 ml Luria Broth (LB) overnight in a sterile 50ml conical tube. Samples were vortexed for approximately 5 seconds on high speed and incubated overnight in a 37°C waterbath, shaking at approximately 35 strokes per minute. After 18 – 24 hours of incubation, the fecal overnight cultures were removed from the 37°C shaking water bath. Samples were then vortexed for approximately 5 seconds on high speed, and DNA was extracted from 0.25 ml of the overnight enrichment culture using the UltraClean™ Fecal DNA Kit (MO BIO Laboratories, Inc., Carlsbad, CA), following the manufacturer's instructions.

Hide sampling and DNA extraction

Sterile Whirl-Pak® Speci-Sponges® (Nasco, Fort Atkinson, WI) were used for sampling the horse hides. Prior to swabbing, each sponge was wet with approximately 15 – 20 ml of sterile water. Two separate sponges were used for the neck and the belly of the horse (Figure 2). An area of approximately 500 cm^2 of the horse was then sampled using 10 passes of the sponge over the hide (5 vertical, 5 horizontal – flipping the sponge between the vertical and horizontal passes). The sponges were returned to the sterile Whirl-Pak® bag and transported to the laboratory at room temperature. Most samples

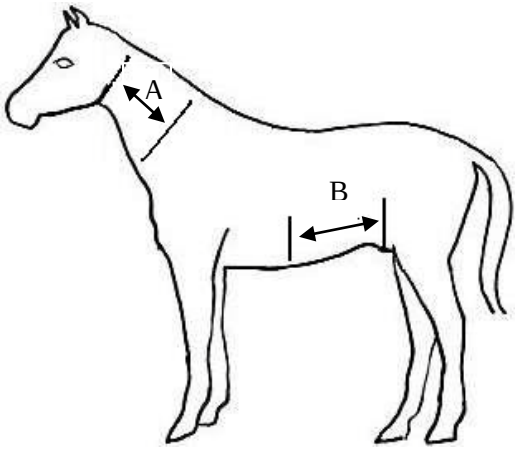
were processed the same day samples were collected. Eight samples from Ranches A, H and I were processed within 24 hours. Samples from Ranch Q were processed on day two, eight samples from Ranch G were processed on day three, and 11 samples from Ranch T were processed on day four. All samples that were not processed the same day they were sampled were held at room temperature until enrichment was able to be done.

Sponge samples were enriched for bacterial growth in the Whirl-Pak® sample bags. Prior to overnight enrichment, 25 ml of Dey-Engley neutralizing broth (D/E broth) (Cole-Parmer Instrument Company, Vernon Hills, IL) at a 2X concentration was added to each Speci-Sponge®, massaged into the sponge through the bag by hand, and allowed to sit for two to 15 minutes in order to neutralize any inhibitory compounds that may have been on the horse's coat. Approximately 75 ml of Trypticase Soy Broth (TSB) was then added to each bag. The bag was placed in a Seward Stomacher® 80 BioMaster Lab System (Brinkmann, Canada) for 30 seconds on normal speed. The sponges were then incubated in the Whirl-Pak® bags overnight in a 37°C incubator.

After overnight incubation, sponge cultures were removed from the 37°C incubator and mixed thoroughly in the bag by hand. Approximately 3 ml of sample was removed from each bag and placed in individual 5 ml sterile snap cap tubes for DNA extraction. Additionally, a loopful of each sponge sample was streaked for isolation on Trypticase Soy Agar (TSA) media and incubated overnight at 37ºC to confirm bacterial growth. For each sample, 5 µl of the belly sponge sample, 5 µl of the neck sponge sample and 10 µl of *Staphylococcus aureus* control were each added together in one Eppendorf tube with1 ml of sterile water. DNA was extracted from this sample using

InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions.

Figure 2 – Horse hide swabbing locations



A: Neck; B: Abdomen and flank (referred to as "belly")

Multiplex PCR (50µl reaction)

Multiplex PCR was used to amplify more than one gene concurrently in a single 50 µl reaction. Specifically, a multiplex reaction was used on both fecal and sponge samples that amplified both *stx*$_1$ and *stx*$_2$ genes. Controls were run along side of each sample tested to confirm successful DNA extraction and PCR amplification. The fecal controls targeted the 16s rRNA gene specific for *E. coli* while the sponge samples targeted the *tuf* gene specific to the *Staphylococcus* genus. Each 50 µl Multiplex PCR was composed of the following: 25 µl 2X Master Mix (Fermentas Life Sciences, Hanover, MD) that included: *Taq* DNA Polymerase (recombinant) in reaction buffer (0.05 units/µl), 4 mM $MgCl_2$ and 0.4 mM of each dNTPs (dATP, dCTP, dGTP, dTTP); 0.1 µM (each) *stx*$_1$ and *stx*$_2$ forward primers and 0.1 µM (each) *stx*$_1$ and *stx*$_2$ reverse

primers; 5 µl template DNA; and dH_2O to 50 µl volume. The resultant PCR product was visualized by agarose gel electrophoresis using a 2% agarose gel which was subsequently stained with ethidium bromide. Each gel ran for approximately 60 minutes at 90V.

Table 1 – PCR primers used in this study

Gene / Primer	Product size	Primer Sequence	Reference
***stx*$_1$ (F)**	210 bp	5'TGTAACTGGAAAGGTGGAGTATACA	(36)
***stx*$_1$ (R)**		5'GCTATTCTGAGTCAACGAAAAATAAC	
***stx*$_2$ (F)**	484 bp	5'GTTTTTCTTCGGTATCCTATTCC	(36)
***stx*$_2$ (R)**		5'GATGCATCTCTGGTCATTGTATTAC	
16s (F)	798 bp	5'AGAGTTTGATCATGGCTCAG	(39)
16s (R)		5'GGACTACCAGGGTATCTAAT	
TStaG422Â	370 bp	5'GGCCGTGTTGAACGTGGTCAAATCA	(34)
TStag765Â		5'TIACCATTTCAGTACCTTCTGGTAA	

Statistical Analysis

The final objective of this research was to determine the prevalence of Shiga toxin-producing *Escherichia coli* (STEC) in horse feces and hide samples from 150 horses in the Sacramento Valley. The horses were divided into two groups: horses that interact with ruminant animals and horses that have no interaction with ruminant animals. The chi-square analysis was used to test for the statistical significance of the difference between the two sample groups (Minitab Inc., chi-square test). The prevalence of STEC in the equine population was evaluated by comparing the number of positive STEC samples with the total number of horses sampled. The relationship between the fecal and sponge samples was also differentiated between samples positive for *stx*$_1$ and/or *stx*$_2$ or neither gene.

Results

Fecal assay development

To evaluate the feces of horses to determine the prevalence of STEC in the equine digestive tract, we first needed to develop an effective and sensitive assay method. To our knowledge, molecular methods have not been developed to extract bacterial DNA from horse feces for the detection of Shiga toxin genes. Methods had previously been developed in our lab for extracting bacterial DNA from bovine and human feces for the detection of Shiga toxin genes *stx*$_1$ and *stx*$_2$ with PCR (23, 51). To apply PCR methods to equine feces, the removal of molecular enzyme inhibitors through DNA purification was required. Therefore, we developed a new assay system specifically for the evaluation of equine feces.

The horse digestive tract consists of two main components: the foregut and the hindgut. Digestively, the horse is classified as a non-ruminant herbivore. Due to the nature of the horse's diet and digestion, horse manure is high in hay and other forage content (22). The unique nature of this manure led to some novel challenges in developing an assay capable of detecting pathogenic bacteria in horse feces. Therefore a new method needed to be developed for PCR analysis from horse fecal samples.

For the development of our assay, we used fresh horse feces suspended in Luria Broth and seeded with EHEC O157:H7, ATCC strain 43894, a strain obtained from an outbreak of hemorrhagic colitis in Michigan in 1982 (44). ATCC strain 43894 contains both the *stx*$_1$ and *stx*$_2$ genes. A successful fecal assay trial was determined by the recovery of the 16s rRNA gene for *Escherichia coli* and Shiga toxin genes *stx*$_1$ and *stx*$_2$

after amplification with PCR. Recovery of all three target genes indicated DNA was successfully isolated and PCR inhibitors were sufficiently removed.

Most aspects of the fecal assay underwent multiple variations until success was achieved (see Table 2 for overview). The culture concentration began with 5 g of feces added to 25 ml of Luria Broth (LB) in a 50 ml conical tube, enriched overnight in a 37ºC shaking waterbath, shaking at 35 strokes per minute. The resulting culture was difficult to pipette due to the high amount of fecal debris present. The culture concentration was adjusted to minimize fecal debris and reduce the amount of PCR inhibitors present in the sample (Table 2). A total of three DNA extraction kits were evaluated: DNeasy DNA Extraction Kit (QIAGEN Inc., Valencia, CA), ExtractMaster™ Fecal DNA Extraction Kit (EPICENTRE Biotechnologies, Madison, WI) and UltraClean™ Fecal DNA Kit (MO BIO Laboratories, Inc.), each with varying results (Table 2). The volume of enrichment culture used in DNA extraction and the amount of DNA template used in the PCR reaction were also variables that were adjusted during the fecal assay development (data not shown). PCR was then done using 2.5 µl of the resulting DNA in a 50 µl PCR reaction.

Upon discussion with our lab group, others had success recovering Shiga toxin genes from fecal slurries after reducing the fecal concentration even further (23, 51). The reduced amount of fecal matter in the culture reduces the amount of inhibitors, allowing for more successful DNA extraction and gene amplification by PCR. With that in mind, the overnight culture concentration was reduced and concurrent trials were attempted with 0.5 g of feces in 10 ml LB and 0.05 g in 10 ml LB, each prepared separately in 15

ml conical tubes. Using the UltraClean™ Fecal DNA Kit (MO BIO Laboratories, Inc.), DNA was extracted from 0.25 ml of each overnight culture. The amount of DNA template was reduced back to 2.5 µl in a 50 µl PCR reaction. These trials resulted in recovery of the *E. coli* 16s rRNA gene, but neither of the two Shiga toxin genes (Table2).

Research by Hopkins *et al.* in our lab group had demonstrated a dramatic improvement in DNA isolation and PCR amplification when fecal enrichment was done at a 1:60 ratio of fecal material to LB in a 50 ml conical (21). Therefore, the next overnight culture concentration was prepared with 0.5 g of feces in 30 ml of LB, prepared in a 50 ml conical tube. This concentration provided an enrichment culture with minimal fecal debris. The DNA was extracted with 0.25 ml of overnight culture using the UltraClean™ Fecal DNA Kit (MO BIO Laboratories, Inc.). The amount of DNA template used remained at 2.5 µl in a 50 µl PCR reaction. The trial was a success, recovering the 16s rRNA gene for *E. coli* and both stx_1 and stx_2 (Table 2). The only protocol change made after this attempt was to increase the amount of DNA template used to 5.0 µl in a 50 µl PCR reaction. This was done to streamline the PCR work so that all PCR reactions had the same template amount.

An effective molecular assay that allowed consistent detection of stx_1 and stx_2 in equine feces was then established in our lab to be as follows: 0.5 g of horse feces in 30 ml of Luria Broth, enriched overnight at 37ºC in a shaking waterbath at 35 strokes per minute. The DNA extraction was done using 0.25 ml of the overnight enrichment culture with the UltraClean™ Fecal DNA Kit (MO BIO Laboratories, Inc.). PCR was then done

using 5.0 µl of the resulting DNA for each 50 µl PCR reaction using the primer sets detailed in Table 1.

It should also be mentioned that throughout the assay development process, attempts were made to extract the DNA directly from the fecal sample without enriching the cultures overnight (Table 2). This was done so that the fecal samples could be processed the same day they were collected, and to reduce the sample processing cost by reducing the amount of reagents and supplies required. For this purpose, we used both the ExtractMaster™ Fecal DNA Extraction Kit (EPICENTRE Biotechnologies) and the UltraClean™ Fecal DNA Kit (MO BIO Laboratories, Inc.). Although a few attempts resulted in recovery of the *E. coli* 16s rRNA gene, none of the attempts resulted in recovery of *stx*$_1$ or *stx*$_2$. This suggests that for our assay, enrichment of the equine feces is required for DNA extraction. Additionally, based upon previous results from other researchers in the laboratory, we knew that the assay sensitivity would be improved by enriching the culture overnight prior to DNA extraction, and as we were wanting the most sensitive assay possible for this study, we chose sensitivity over assay time (23, 51).

Table 2 - Fecal assay method development

Trial	Enrichment culture		O157:H7 ATCC 43894 (µL)	DNA extraction kit	*stx*$_1$	*stx*$_2$	*E. coli* 16s (control)
	feces (g)	LB (mL)					
1	5	25	5	DNeasy DNA Extraction Kit	-[1]	-	+[2]
2	1	9	10	ExtractMaster™ Fecal DNA Extraction Kit	-	-	-
	no enrichment		10		-	-	+
3	1	9	50	UltraClean™ Fecal DNA Kit	-	-	+
	no enrichment		25		-	-	-
	no enrichment		50		-	-	-
4	1	9	50	UltraClean™ Fecal DNA Kit	-	-	-
	no enrichment		50		-	-	-
	no enrichment		100		-	-	-
5	0.05	10	1	UltraClean™ Fecal DNA Kit	-	-	++[3]
			10		-	-	++
			100		-	-	++
	0.5	10	1		-	-	++
			10		-	-	++
			100		+	+	++
	no enrichment		1		-	-	-
	no enrichment		10		-	-	-
	no enrichment		100		-	-	-
6	0.5	30	1	UltraClean™ Fecal DNA Kit	++	++	++
			10		++	++	++
			100		++	++	++

[1](-) Negative result; [2] (+) Weak positive result; [3] (++) Positive result

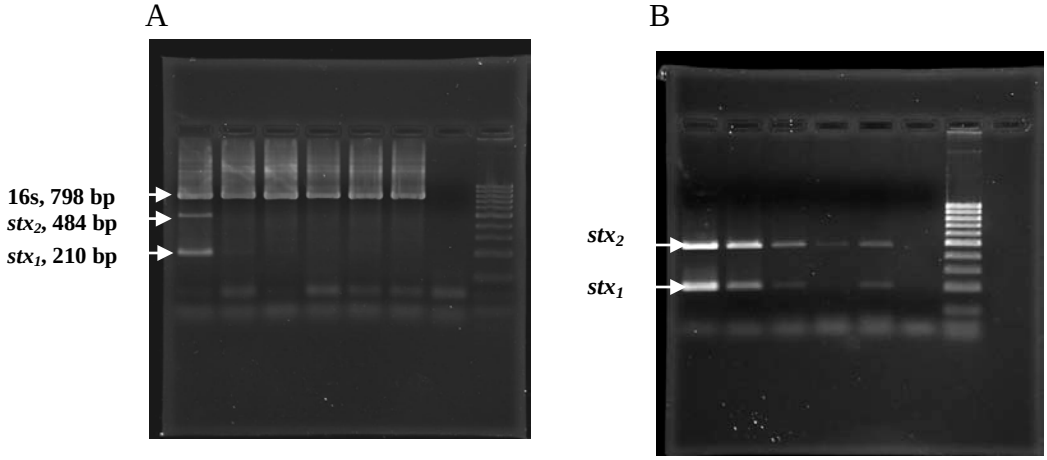
To determine the sensitivity level of the new molecular assay that was developed, six fecal enrichments were prepared by adding 0.5 g of freshly collected horse feces to 30 ml LB for each preparation. Each fecal enrichment was then spiked with 0157:H7 ATCC strain 43894 at varying concentrations from 10-10^6 CFU and incubated overnight in a 37°C shaking waterbath set at 35 strokes per minute. Following incubation, the DNA extraction, PCR, and product visualization were performed as detailed above and in the Materials and Methods section. The last dilution to have bands present for both *stx*$_1$ and

stx$_2$ determined the level of sensitivity of the new assay. The actual CFU/ml required for detection was determined using colony counts that were obtained after plating each dilution prior to seeding the fecal sample. In the first fecal sensitivity trial, Multiplex PCR included the 16s rRNA gene for *E. coli*, *stx*$_1$ and *stx*$_2$. Bands for both *stx*$_1$ and *stx*$_2$ were detected from the fecal culture inoculated with $5x10^3$ CFU and a band for *stx*$_1$ only was detected from the fecal culture inoculated with $5x10^2$ CFU. A band was present for the *E. coli* 16s rRNA gene at every dilution. Thus, the initial assay sensitivity for *stx*$_1$ was calculated to be approximately $1x10^4$ CFU/g of horse feces and $1x10^5$ CFU/g of horse feces for detecting both *stx*$_1$ and *stx*$_2$. This is the amount required to be in the fecal sample prior to overnight enrichment. The second and third sensitivity trials were conducted exactly as the first. In the second trial, bands for both *stx*$_1$ and *stx*$_2$ were detected from the fecal culture inoculated with $2.45x10^3$ CFU and a band for *stx*$_1$ only was detected from the fecal culture inoculated with $2.45x10^2$ CFU. As before, the *E. coli* 16s rRNA gene was detected at every dilution. Thus, trial two resulted in an assay sensitivity for *stx*$_1$ of approximately $4.9x10^2$ CFU/g of horse feces and $4.9x10^3$ CFU/g of horse feces for detecting both *stx*$_1$ and stx$_2$. The third trial resulted in an assay sensitivity slightly higher than the second trial with a sensitivity for *stx*$_1$ of approximately $1.3x10^3$ CFU/g of horse feces and $1.3x10^4$ CFU/g of horse feces for detecting both *stx*$_1$ and *stx*$_2$. Taken together, our results indicate that the level of sensitivity for the new assay was an average of $7x10^3$ CFU/g for detecting both *stx*$_1$ and *stx*$_2$ in horse feces.

The results of all of the fecal sensitivity trials were variable from trial to trial. Sensitivity was between $1x10^3$ to $1x10^5$ CFU/g of horse feces. Based upon the findings

of Oliver *et al*. (38) and other research groups that also enrich the sample overnight prior to PCR, we were surprised at the relatively low sensitivity of the assay. Recent studies in our research group, however, had found that removing the *E. coli* 16s rRNA gene from the Multiplex PCR reaction improved the assay sensitivity (23, 51). We wanted to have a more sensitive assay, so we removed the *E. coli* 16s rRNA gene and performed a fourth sensitivity exactly as the first three, but with two Multiplex PCR reactions: one reaction omitted the *E. coli* 16s rRNA gene from the Multiplex PCR reaction, the other ran the *E. coli* 16s rRNA gene, stx_1 and stx_2 as previously conducted. In the multiplex reaction for all three genes, bands for stx_1 and stx_2 and *E. coli* 16s rRNA were detected from the fecal culture at a level $1.19x10^5$ CFU EHEC/g of horse feces. In comparison, in the reaction that omitted the *E. coli* 16s rRNA gene, bands for both stx_1 and stx_2 were detected from the fecal culture at a dramatically higher sensitivity level of approximately 119 CFU/g of horse feces for detecting both stx_1 and stx_2 (Figure 3).

Figure 3: Fecal sensitivity multiplex PCR with and without *E. coli* 16s rRNA gene



A: With *E. coli* 16s rRNA gene, left to right: 10^{-2} dilution ($6x10^5$ CFU/0.1ml) to 10^{-6} dilution (60 CFU/0.1ml), negative control, 100bp ladder; B: Without *E. coli* 16s rRNA gene, left to right: 10^{-2} dilution ($6x10^5$ CFU/0.1ml) to 10^{-7} dilution (6 CFU/0.1ml), 100bp ladder (exACTGene, Fisher Scientific International Inc., Canada)

Each sensitivity trial utilized a new fecal sample from a different horse, and all had previously been determined to be absent of *stx*$_1$ and *stx*$_2$. Although some variability may be attributed to laboratory technique, the wide variability in sensitivity from trial to trial is most likely attributed to variability in the fecal samples. Although equine fecal specimens are always high in roughage, horses that are boarded frequently have different diets which may lead to differences in fecal content. The molecular inhibitors present in the feces may vary depending on the horse's diet. Therefore, the sensitivity of this fecal assay must be considered variable throughout the study, but certainly detects STEC at a level equal to or greater than $1x10^5$ CFU/gram feces and may detect to levels as low as $1x10^2$ CFU/g of feces.

To ensure the accuracy of our results using the molecular detection assay, two PCR controls, a negative control and a positive control, were run concurrent with each set of fecal test samples. The negative control had sterile water in place of the DNA template, and ensured that any bands observed in the gel were from the sample and not from a contaminated reagent or primer-dimers. The positive control was run to ensure the DNA extraction and PCR reaction were successful, and was a set of *E. coli* 16s rRNA primers specific for *E. coli* (Table 1). As stated above, this control was run in a separate multiplex tube from the *stx*$_1$/*stx*$_2$ reaction, and was run on each fecal sample (39) and validated the results. When no 798 bp band was present for the *E. coli* 16s rRNA fecal control, the DNA extraction was repeated using a new overnight culture, by adding 1 ml of the previous fecal overnight culture to 30 ml of LB and was processed as indicated in

the Materials and Methods section. Approximately 24% of the samples tested required a second DNA extraction.

Equine hide assay development

We wanted to investigate horse hides for the presence of Shiga toxin producing *Escherichia coli* in order to determine if there was a risk of transmission to horse owners or riders through contact with their horses. As horses are often companion animals, people tend to be in close contact with their horses, usually rubbing the horse's neck and face when giving them attention. Because people are touching their horses while out in a pasture or barn or while riding, usually hand washing is not an immediate option, increasing the risk of transmission from the horse coat to the person. In fact, in an article from the United Kingdom, an 84-year-old farmer contracted an infection with *E. coli* O157 after cleaning feces from two horses with diarrhea. *E. coli* O157 producing Stx_2 was isolated from both the farmer and one of the horses with diarrhea. The two isolates were indistinguishable by pulse field gel electrophoresis and restriction fragment length polymorphism analysis. The farmer did not have any contact with the cattle on his farm, confirming he either contracted the infection from the horse, or both were infected by a common source (13).

In order to closely mimic the locations touched by people and where horses have contact with the ground that may be contaminated with fecal material, we evaluated the presence of STEC on both the neck and belly of each horse. The neck area was chosen because this is the area most often and continuously touched by owners and riders. The

abdomen and flank of the horse was chosen because of the high likelihood of this region coming into contact with contaminated soil or feces. In order to conduct this study, we first had to develop a sensitive assay method capable of detecting the bacteria from the horse hide. We wanted to use a method similar to our fecal assay, but to our knowledge, a method had not previously been developed to extract bacterial DNA from horse hide swabs for PCR. However, there have been published studies that have evaluated cow hides for the detection of *E. coli* O157 (4) using this molecular method. Therefore, we used this published method as a foundation for the development of our assay using horse hides.

Similar to our equine fecal assay, the initial method development work was geared towards creating a rapid, inexpensive method to sample a horse hide, extract the DNA and amplify the target genes through PCR. During all the development work and research sampling, sterile Whirl-Pak® Speci-Sponges® (Nasco) were used for sampling. Swabbing was performed as discussed above in the Materials and Methods section. Each sponge was seeded with EHEC 0157:H7, ATCC strain 43894 just prior to overnight incubation. All enrichment media was added straight to the sponge inside the Whirl-Pak® bag. The media and sponge were mixed by placing in a stomacher on normal speed for 30 seconds. The entire bag was then placed in a 37ºC incubator overnight. The variables evaluated in the development of the molecular assay were the swab wetting agent, the use of a neutralizer, the specific enrichment media, and the DNA extraction method used (Table 3).

In the first hide-sponging assay development trial, we moistened the sponges with sterile water and ran two separate media enrichment trials concurrently: one with 30 ml of Trypticase Soy Broth (TSB), the other with 30 ml of Brilliant Green Bile Broth (BGBB). The TSB and BGBB enrichment media were chosen based on their success in studies by Arthur *et al*. (4) and Elder *et al.* (15), respectively. In this first trial, we attempted to perform PCR without an initial DNA extraction step, using 2.5 µl of the overnight enrichment in a 50 µl PCR reaction. One of the three samples enriched in TSB resulted in detection of all three target genes; *E. coli* 16s rRNA, stx_1 and stx_2, however no bands were detected for the other samples (Table 3). The conclusion to this trial was that there was some success recovering the target genes using TSB for the enrichment media, but most likely a DNA extraction step would be required. Therefore, in the next sponge assay trial, we discontinued the use of BGBB, enriched instead with TSB and added a DNA extraction step. In an effort to create an "easy" and affordable assay, we steered away from using an extraction kit. Following the research of Kuske *et al.*, we attempted to extract the DNA using heat freeze cycles, minus the hot detergent step (32). Three different trials were run side-by-side; two utilizing a DNA extraction step and one with no DNA extraction. For the two DNA extraction trials, one utilized a 70ºC heat treatment; the other had no heat treatment. The samples were then held at -20ºC for five minutes followed by 100 ºC for five minutes and the freeze-heat cycle was repeated three times. All three treatments were performed using three sponge samples. Two sponge samples were from the belly and neck of the horse respectively. The third sample was a sponge that had not had horse contact. This control was included to determine if there

was something in the horse sample that might inhibit recovery of the target genes. Recovery of the *E. coli* 16s rRNA, *stx*$_1$ and stx$_2$ genes were all unsuccessful for sponge samples that had horse contact, regardless of the DNA extraction method. The sponge sample that had no horse contact successfully recovered all three target genes for all DNA extraction methods (Table 3). This was a strong indication that something was present on the horse hide that was inhibiting recovery of the target genes.

Horses are often treated topically with a spray to protect the horses from fly infestations that can cause allergic reactions, skin damage, blood loss and transmission of disease agents (46). Horses that are used in competition are also often treated with a coat shine such as ShowSheen® to improve the horse's appearance. Both fly spray and coat shine products can be bought commercially or be homemade and can contain vinegar, dish soap, baby oil, hair conditioners and natural oils, such as citronella, lavender or tea tree oils (8, 19) .

Bosilevac *et al.* conducted a study testing the potential of an antimicrobial agent, cetylpyridinium chloride (CPC), to reduce hide contamination in cattle (10). In his hide sampling procedure, he used the addition of Dey-Engley (D/E) neutralizing broth at a 2x concentration to neutralize the inhibitory compounds on the cattle hides prior to any DNA isolation and analysis (10). The main components in D/E broth that act as neutralizing components are sodium thioglycollate, sodium thiosulphate, sodium bisulphate, soya lecithin and polysorbate 80. D/E broth has been demonstrated to be effective in neutralizing a broad spectrum of disinfectants and antiseptics which most likely accounts for its effectiveness on cow hide samples (16).

In an attempt to neutralize any inhibitory compounds that may be present on the horse hides in our study, we added 25 ml of D/E broth at a 2x concentration prior to addition of the enrichment media and prior to overnight enrichment. The enrichment media remained TSB, but the volume was increased to 75 ml, per the Bosilevac *et al.* method (10). The DNA extraction was performed using the same two freeze-heat treatments as performed in trial two, including the trial with no DNA extraction. This trial was successful in recovering *stx*$_1$ and *stx*$_2$ from the two heat-freeze methods as well as the method that did not undergo any DNA extraction method. Based on the results of these experiments, the effectiveness of the use of a neutralizer was confirmed, and it was established that the most reliable DNA extraction method was the freeze-heat method with an initial 70ºC heat treatment.

Table 3: Hide assay method development

Area of horse sponged	Neutralizer (25mL)	Enrichment Media		Extraction Method	*stx*$_1$	*stx*$_2$	*E. coli* 16s (control)
		Type	Amount (mL)				
flank to tail head	N/A	TSB	30	no treatment	+[1]	+	-[2]
		BGBB	30		-	-	-
belly	N/A	TSB	30	heat treat, freeze/heat x3	-	-	-
				freeze/heat x3	-	-	-
				no treatment	-	-	-
neck				heat treat, freeze/heat x3	-	-	-
				freeze/heat x3	-	-	-
				no treatment	-	-	-
no horse				heat treat, freeze/heat x3	+	+	+
				freeze/heat x3	+	+	+
				no treatment	+	+	+
belly	2X D/E broth	TSB	75	heat treat, freeze/heat x3	+	+	+
				freeze/heat x3	+	+	-
				no treatment	+	+	+
neck				heat treat, freeze/heat x3	+	+	+
				freeze/heat x3	+	+	+
				no treatment	+	+	-
no horse				heat treat, freeze/heat x3	+	+	+
				freeze/heat x3	+	+	+
				no treatment	+	+	+

[1](+) Positive result; [2] (-) Negative result

With the optimized equine hide assay in hand, we next needed to determine the sensitivity for identification of STEC with this assay. For this, five separate locations were swabbed from a single horse per the sponging procedure detailed above and in the Materials and Methods section. Each sponge sample was then spiked with 0157:H7 ATCC strain 43894 at varying concentrations from 10-10^6 CFU and incubated in the Whirl-Pak® bag overnight in a 37°C incubator. Following incubation, the DNA extraction was done using the freeze-heat method with initial 70ºC heat treatment. PCR and product visualization were performed as detailed above and in the Materials and

Methods section. PCR was done with and without the *E. coli* 16s rRNA gene to determine if it would have an impact on the sensitivity. The results were the same for the PCR reaction with the *E. coli* 16s rRNA gene and without the *E. coli* 16s rRNA gene. All three target genes, *E. coli* 16s rRNA, stx_1 and stx_2 were detected from the sponge inoculated with 2.35×10^4 CFU of STEC. It was interesting that unlike the fecal assay, the removal of the *E. coli* 16s rRNA primers did not have an impact on the sensitivity. However, the fecal samples have completely different flora than the hide samples. Most notably, the *E. coli* concentration in the fecal samples would most likely be considerably higher than that in the hide swab samples

Because of the many steps required in the equine hide protocol, it was imperative that a control be included to confirm successful DNA extraction and PCR amplification. Although in the first trial *E. coli* was detected in two of the five hide swab samples, unlike the fecal samples, *E. coli* is not guaranteed to be in every hide sample and therefore is not an appropriate positive control for this assay. Therefore, another organism needed to be selected for this assay. Similar to humans, staphylococci are part of the normal equine skin flora (30, 35). Previous investigators have developed a PCR-based assay capable of detecting all Staphylococcus species by targeting the *tuf* gene (34). We therefore chose to have staphylococci as our positive control instead of *E. coli*, and used the *Staphylococcus* genus primers published by Martineau *et al.* (34). Ironically, although we confirmed that staphylococci are part of the normal equine skin flora in our assay system, we found that it was present in only 50% (2/4) of the hide samples tested (data not shown). Therefore, to ensure that a positive control would be

present in every hide sample for the DNA extraction and PCR portions of the protocol, each overnight sponge enrichment sample was spiked with 10 µl of a *Staphylococcus aureus* overnight culture (approximately 1 x 10^7 CFU) immediately prior to DNA extraction. The volume of *S. aureus* added to each overnight sponge enrichment sample was determined after running trials adding 1 µl, 5 µl, 10 µl and 100 µl of *S. aureus*. The addition of 10 µl yielded the most consistent results. The overnight *S. aureus* culture was prepared fresh for each trial. Even though the melting and annealing temperatures and PCR product size for the *Staphylococcus* genus primers were compatible in a multiplex PCR reaction with the *stx*$_1$ and *stx*$_2$ primers, it was found that the *Staphylococcus* primers were more effective when run independent of the *stx*$_1$ and *stx*$_2$ primers (Data not shown).

Although the staphylococci control worked well for DNA isolation and PCR verification, we still wanted to be certain that the overnight enrichment was not detrimental to bacterial growth. Therefore, to ensure bacteria were being recovered from the equine hide, a bacterial growth assessment was performed by subculturing a loopful from each sponge overnight enrichment sample onto Trypticase Soy Agar, a non-selective media, prior to the addition of the *S. aureus* control. Any bacterial growth on the media was considered a positive result for bacterial growth and recovery.

In addition to the *S. aureus* positive control discussed previously, a negative control was also prepared to confirm bands observed in the post-PCR gel electrophoresis were from the sample and not from contamination or primer-dimers. The negative control was prepared during the PCR amplification step by using sterile water in place of the DNA template. This negative control was run with every sample set.

Now that all of the controls were in place, additional sensitivity testing needed to be done to confirm that the sensitivity would remain the same when multiple horses were sampled in our prevalence study. Therefore, hide swab samples were collected, enriched and seeded, however, rather than all of the sponges being collected from a single horse, three samples were collected from each of two horses in order to account for sample variability between horses. The results of this trial were better with a detection of both *stx*$_1$ and *stx*$_2$ from the sponge inoculated with $6.8x10^3$ CFU and *stx*$_1$ detected from the sponge inoculated with 68 CFU. Thus, these results indicated that our equine hide assay has a sensitivity of 6750 CFU of STEC required to be on the sponge prior to overnight enrichment in order to detect both *stx*$_1$ and *stx*$_2$.

In order to improve consistency of results and sensitivity of the assay, the decision was made at this point to use InstaGene™ matrix (Bio-Rad Laboratories) for the DNA extraction. Another member of our lab group had had success using InstaGene™ matrix for DNA extraction from bovine fecal samples (23). Because we were able to extract the DNA from the sponge samples using only temperature variation, we predicted InstaGene™ matrix, would not only be effective in extracting the DNA, but would also provide more consistent results than the heat/freeze method. We conducted another sensitivity test using a total of six sponges from three different horses. As in the previous sensitivity studies, one sponge was used for each dilution. Each sponge sample was spiked with 0157:H7 ATCC strain 43894 at varying concentrations from 10-10^6 CFU and incubated in the Whirl-Pak® bag overnight in a 37°C incubator. Following incubation, four 1 ml aliquots of each sponge sample were added to 1 ml sterile water for InstaGene™

DNA extraction. Additionally, different amounts of *Staphylococcus aureus* were added separately to each of the four tubes to determine the ideal amount of *S. aureus* to add. DNA extraction was performed using InstaGene™ matrix, following the manufacturer's instructions. PCR was done with 5 µl of resulting DNA in a 50 µl PCR reaction. The results were highly variable. In all instances, the *S. aureus* control and Shiga toxin genes were detected at varying dilutions. After a discussion with our lab group, others had had success using less overnight enrichment for the DNA extraction (23). Therefore, the DNA extraction was repeated with 10 µl and 100 µl of overnight enrichment and 10 µl of *S. aureus* control. The results were more consistent using10 µl of overnight enrichment and the *S. aureus* control and both Shiga toxin genes were detected at the lowest dilution tested. However, there were still two dilutions (10^{-1} and 10^{-4}) that did not detect either of the Shiga toxin genes. These results were unsettling, and suggested that there may be variability between the horse samples, whether from different areas sampled on a horse, or between different horses. To eliminate this variability for the sensitivity test, the next trial was conducted using a sample from a single horse. This entire study was performed in duplicate using a sponge sample from the horse's belly and another from the same horse's neck. The results were identical for both sponge samples. The *S. aureus* control and both Shiga toxin genes were detected at the lowest dilution tested, corresponding to a sensitivity level of 3 CFU of STEC required to be on the sponge prior to overnight enrichment. Because the results were the same for both sponge samples, there is probably little variability among different locations on the same horse. This does not address, however, the potential variability between horses.

Prevalence

With the fecal and hide methods established, horses throughout the Sacramento Valley were sampled to investigate the second objective of this thesis: to evaluate the prevalence of STEC in fecal and hide samples in the equine population in the Sacramento Valley. Between June 2008 and September 2009, 156 horses were sampled from various locations around the greater Sacramento Valley (Figure 1). The experiment was designed so that half of the 156 horses tested had some interaction with ruminant animals while the other half of the horses tested had no known interaction with ruminant animals. Of the 78 ruminant-interacting horses, a total of 4 (5.1%) fecal samples were positive for *stx*$_2$ (Table 4). None of the samples were positive for *stx*$_1$ (Table 4). None of the 78 non-ruminant-interacting horses were positive for *stx*$_1$ or *stx*$_2$ (Table 4). All of the samples tested had good negative and positive controls.

Along with the fecal data we collected, we also sampled the neck and belly region of each horse to determine if STEC was present on the horse hide. None of the sponge samples were positive for *stx*$_1$ or *stx*$_2$ for all 156 horses (Data not shown).

Significance

In order to determine the statistical significance of the findings in our study, a chi-square analysis was performed using Minitab (Minitab Inc.) comparing the difference between the proportion of STEC-positive horses that interacted with ruminant animals and the proportion of STEC-positive horses that did not interact with ruminant animals.

Four out of 78 (5.1%) ruminant-interacting horses were positive for STEC. Zero out of 78 (0.0%) non ruminant-interacting horses were positive for STEC. Using the chi-square test, it was found that there is a statistically significant difference between the two groups ($P < 0.05$), indicating a correlation between ruminant-interaction and no ruminant-interaction in the presence of STEC in horses. Overall, four of 156 (2.6%) horses were positive for Shiga toxin-producing *E. coli*.

Table 4 – Shiga toxin detection from horse feces sampled in Sacramento Valley equine population

Month / Year Sampled	Ranch ID	**Interacts with ruminant animals**			**Does NOT interact with ruminant animals**			Total Samples	*stx* pos	% *stx* pos	Type of ruminant interaction
		Number of Samples	*stx* pos	% *stx* pos	Number of Samples	*stx* pos	% *stx* pos				
JUN 08	B	*	*	*	3	0	0.0	3	0	0.0	N/A
JUL 08	E	*	*	*	5	0	0.0	5	0	0.0	N/A
JUL 08	D	*	*	*	4	0	0.0	4	0	0.0	N/A
NOV 08	F	6	1	16.7	*	*	*	6	1	16.7	goats, alpacas, llamas
FEB 09	G	8	0	0.0	*	*	*	8	0	0.0	goats, sheep
FEB / APR 09	A	4	0	0.0	*	*	*	4	0	0.0	cattle, deer
MAR 09	H	*	*	*	1	0	0.0	1	0	0.0	N/A
MAR 09	I	2	0	0.0	1	0	0.0	3	0	0.0	cattle, goats
APR 09	J	*	*	*	10	0	0.0	10	0	0.0	N/A
APR 09	K	*	*	*	6	0	0.0	6	0	0.0	N/A
APR 09	L	*	*	*	6	0	0.0	6	0	0.0	N/A
APR 09	M	2	1	50.0	12	0	0.0	14	1	7.1	cattle
JUN 09	N	4	0	0.0	2	0	0.0	6	0	0.0	cattle
JUL 09	O	17	1	5.9	*	*	*	17	1	5.9	deer
JUL 09	P	10	0	0.0	12	0	0.0	22	0	0.0	goats
AUG 09	Q	1	0	0.0	*	*	*	1	0	0.0	goats, sheep
AUG 09	R	20	0	0.0	*	*	*	20	0	0.0	cattle
SEP 09	L	*	*	*	1	0	0.0	1	0	0.0	N/A
SEP 09	S	3	0	0.0	4	0	0.0	7	0	0.0	cattle
SEP 09	T	*	*	*	11	0	0.0	11	0	0.0	N/A
SEP 09	A	1	1	100.0	*	*	*	1	1	100.0	cattle, deer
Totals:		78	4	5.1	78	0	0.0	156	4	2.6	

* = None sampled

Discussion

The purpose of this study was to evaluate the prevalence of Shiga toxin-producing *Escherichia coli* in the Sacramento Valley equine population. Specifically, we wanted to assess the risk that these companion animals might have to people. To do this, we wanted to develop an assay that was sensitive, specific, rapid, and relatively inexpensive. We chose to sample both equine feces and equine hides in order to determine if STEC was present in the horse's digestive tract or on their hide. At the outset of our project, we hoped to achieve a sensitivity level in our detection method that was similar to other studies utilizing multiplex PCR (17) and within the reported infectious dose of some STEC strains, which has been estimated to be between 10 and 100 CFU for humans (41). However, we quickly learned that PCR analysis of both equine fecal material and hides is highly problematic due to the consistency and number of inhibitors found in horse manure as well as the variety of treatments and solutions used on horse hides. Despite these unique challenges, we were able to develop an assay for detection of stx_1 and stx_2 genes in the equine fecal material at a sensitivity level, ranging from 119 CFU/g to $1x10^5$ CFU/g of horse feces. The method we developed for sampling and testing horse hides was capable of detecting bacteria from a horse's coat after overnight enrichment at a sensitivity level as low as 3 CFU per sponge; however the results were not always consistent. Both Shiga toxins were detected at the lowest tested dilution, but in two of our trials, were not detected at higher the dilutions, thus making it difficult to determine a sensitivity range.

Between June 2008 and September 2009, horse hide and fecal samples were collected from 156 horses throughout the greater Sacramento Valley. The overall prevalence for STEC in the equine population in the Sacramento Valley was 2.6%. Of even greater significance was a prevalence of 5.1% for horses interacting with ruminant animals, compared to 0.0% of horses that had no reported interaction with ruminant animals. All positive horse fecal samples were positive for *stx*$_2$ only.

The first positive sample, collected in November 2008, was obtained from a horse at a private residence in Loomis, California where all of the horses interacted with dogs, goats, pigs, alpacas and llamas. The owner had six horses (3 mares and 3 geldings) ranging in age from 5 to 22 years. All six horses shared a common living area and thus, individual fecal samples were unable to be determined and the specific STEC positive horse was not identified amongst the six horses. As horses may defecate every two to three hours (46), we examined the entire area and collected the six freshest fecal samples. This would give us the greatest likelihood of obtaining samples from all six horses. The horse owner had mentioned one horse sporadically having bouts of diarrhea. She supposed it was due to the horse consuming large amounts of acorns. There was no way to verify if the *stx*$_2$ positive sample was from this horse. None of the other five horses were positive for *stx*$_1$ or *stx*$_2$. The owner has since moved from that location and was unable to be reached for further follow-up sampling.

The second positive sample was collected in April 2009 from boarding stables in Lodi, California. The stables were home to approximately 25 horses ranging in age from one year to 30 years old. The *stx*$_2$ positive horse identified at this stable was a six year

old male that shared a living area with a pony. There were cattle present in an adjacent pasture, but it was initially thought that the horse and pony had no interaction with the nearby cattle. However, when searching for a fresh fecal sample, bovine feces were discovered inside the horse and pony's living area. The STEC-positive horse did not show any signs of ailment. The co-housed pony was not positive for either *stx*$_1$ or *stx*$_2$. The horse has since been moved to another location and is boarded with a different horse. Follow-up sampling was conducted in September 2009 to see if the STEC-positive horse was still positive and if the pen-mate was positive for STEC. Results indicated that at this time, neither horse is positive for either *stx*$_1$ or *stx*$_2$. This was an interesting finding and brings up the question of how long horses may carry the STEC and whether or not the horses are symptomatic. In the case of the farmer that contracted O157 from his horse, the farmer became symptomatic after cleaning horse feces from two horses with diarrhea. One of the horses tested positive for *stx*$_2$ two weeks after the farmer became symptomatic. However, no follow-up studies were conducted to evaluate how long the horse remained positive (13).

The third *stx*$_2$ positive sample was collected in July 2009 at a resort ranch in Napa, California that houses approximately 100 horses on the property that range in age from 7 to 32 years old. An interview with the stable manager claimed deer were often present on the property, interacting with the horses. Although all horses on the property had separate stalls, they interacted with one another during exercise and trail riding. The STEC-positive horse we identified in this study was a 29-year-old mare that showed no signs of ailment at the time of testing, but died within a month. The death was reported

to be due to colic, according to the stable manager. None of the other 17 horses sampled on the property were positive for either *stx*$_1$ or *stx*$_2$.

The fourth and final horse that tested positive for the Shiga toxin 2 gene in this study was collected from a private residence in Placerville, California. The owner of this property had five horses ranging in age from 8 to 22 years of age. Four of the horses were sampled in February 2009 for prevalence in feces and a follow-up was done on this farm with the same four horses for hide sampling in April 2009. These four horses had only casual contact with the cattle on the property and all tested negative for both *stx*$_1$ and *stx*$_2$ in hide and fecal samples. The fifth horse on the farm, an eight year old mare, was sampled in September 2009 and tested positive for *stx*$_2$ in the fecal sample. The sponge sample of the hide was negative for either Shiga toxin. This horse interacts with deer and geese and also grazes on the same field with cattle that were recently confirmed to be positive for Shiga toxin-producing *E. coli* by another investigator in our research group (23). The mare showed no signs of ailment at the time of sampling.

One of the most interesting findings in this study was the detection of only *stx*$_2$ from the STEC-positive horses. As previously stated, this is significant because the STEC that produces only Shiga toxin 2, compared to producing only Shiga toxin 1, appears to be more commonly responsible for serious complications such as HUS (41). Additionally, in the United Kingdom study mentioned previously, the only STEC-positive horse identified in the scientific literature, also was positive for *stx*$_2$ only (13). In our efforts to develop and validate an assay for STEC in equine fecal material, and determine the sensitivity of our new method, both *stx*$_1$ and *stx*$_2$ were able to be identified

in the STEC-spiked samples. In fact, *stx*$_1$ was consistently identified at a 10-fold higher sensitivity level than *stx*$_2$ and therefore should have been detected if present in the samples. During the method validation, the fecal samples were seeded with STEC prior to overnight enrichment and were subsequently able to be identified after DNA extraction and PCR amplification. This indicates that the absence of *stx*$_1$ is not a flaw of the study, but rather a true finding. That *stx*$_1$ is indeed absent in the equine fecal samples we tested and is therefore not being shed by the horse. It would be interesting to determine if the incidence of *stx*$_2$ is higher than that of *stx*$_1$ in non-ruminant versus ruminant animals.

The fourth horse that tested positive for STEC had direct interaction with cows that have tested positive for *stx*$_1$ only, *stx*$_2$ only, and both *stx*$_1$ and *stx*$_2$. The cattle herd in that study was monitored six times over a 16-month period from April 2007 to July 2008 (23). In evaluating the data from that study, the prevalence of *stx*$_1$ (cows testing positive for *stx*$_1$ only or both *stx*$_1$ and *stx*$_2$) ranged from 18.2% to 54.5%, with the highest *stx*$_1$ percentage occurring in July 2008. The prevalence of *stx*$_2$ (cows testing positive for *stx*$_2$ only or both *stx*$_1$ and *stx*$_2$) ranged from 11.1% to 60.0% with the highest *stx*$_2$ percentage occurring in January 2008. The last herd sampling, the one closest to the horse sampling in my study, was done in July 2008, and had an equal prevalence of 54.5% for both *stx*$_1$ and *stx*$_2$ (23). The STEC shed by the cows fluctuated within the herd. Although the horse was exposed to a herd that was positive for STEC containing *stx*$_1$, *stx*$_2$, or both genes, it does not indicate the horse interacted with a cow or cow feces that had only *stx*$_1$, *stx*$_2$, or both genes. As the STEC makeup of the herd varies, there is no way to be sure the affected horse was not merely exposed to STEC that contains only the *stx*$_2$ gene.

The horse's digestive system is quite different than that of cattle. With no rumen, it is the large intestine that ferments the fiber-rich plant material (46). This leads to a very different fecal composition and consistency between cattle and horses. Gamage *et al.* suggested that lytic infection by Shiga toxin-encoding phages could be influenced by the composition of intestinal flora (19). The equine gastrointestinal tract contains numerous parasites, including protozoa (46). In the review by Rasmussen and Casey, it is suggested that some bacteria, including members of the *Escherichia* genus, may be able to resist degradation by surviving in the vacuoles of protozoa (42). Perhaps the unique composition of horse feces, including protozoa, may offer a selective advantage to the Shiga toxin-producing *E. coli* that encodes for only stx_2 over those that encode for only stx_1 or both stx_1 and stx_2.

Cattle are considered to be a natural reservoir for STEC and they are most commonly implicated in STEC transmission. Although two of the four STEC-positive horses in this study had interaction with cattle, the ruminant-interaction for the other two STEC-positive horses was limited to deer, goats, llamas and alpacas. The fourth STEC-positive horse had interaction with deer in addition to the cattle. It is important to note that deer have been implicated as the source of transmission in *E. coli* O157:H7 outbreaks. Specifically, *E. coli* O157:H7 was recovered from deer fecal pellets and venison jerky (28, 43). Other reports have demonstrated a prevalence of *E. coli* O157:H7 in free-ranging deer from 0.25 - 2.4% (43, 45). In our study, it is possible that the STEC-positive horses that had interacted with deer may have had contact with deer feces. The

deer were wild and most likely did not have close contact with the horses, indicating contact with their feces as a more likely source of transmission.

Because STEC is shed in the feces of STEC-positive animals, another possible vehicle of transmission between these animals is from flies. A study evaluating sources of *Escherichia coli* O157 in the Northwestern US discovered a prevalence of 3.3% from pooled fly samples (21). Another study demonstrated fruit flies transmitting *E. coli* O157 to wounded apples where the bacteria freely proliferate (24). Research has also shown that flies do not just simply transmit the bacteria through mechanical transmission via contact with contaminated legs and mouthparts. Rather, *E. coli* O157 can colonize the alimentary canal and proliferate in mouthparts of flies, thus increasing the potential to disseminate the bacteria through a bioenhanced transmission (31). According to the horse owners I spoke with, when cattle are present near horses, it is the "cow flies" that land on and stay near the horses. Although cattle and horses do not tend to have close physical interaction with one another, the flies go between both of these animals and their feces. The STEC-positive horses may have acquired the stx_2 directly from the ruminant animals, but it is possible there was another vehicle of transmission, such as flies. It seems more likely that contact with the feces of an STEC-positive animal, as opposed to contact with the animal itself, will lead to transmission of the STEC. In fact, as mentioned previously, the horse identified in April 2009, was found in a space that had obvious cow fecal material present, but no known close interaction with the cattle themselves, thus supporting this hypothesis. Additional research could be conducted in which flies that are near cow fecal material are captured and tested for STEC.

Further work should be conducted with the four *stx*$_2$ positive samples. After isolating the STEC from each of the positive samples, the samples can be further characterized to determine serotype or O serogroups. The O111 and O157 serogroups specifically have been associated with significant human disease, and are the most common outbreak strains of STEC in the U.S. (41). The four equine fecal samples in this study are positive for the Shiga toxin gene *stx*$_2$, but cytotoxicity assays could be performed using HeLa and Vero cells to confirm protein expression from the Shiga toxin genes identified by PCR (6). An additional study that would address the lack of *stx*$_1$ detection in this thesis would be to evaluate the survival of the *stx*$_1$ phage in horse fecal material. The *stx*$_2$ genes in the strains we isolated could also be further studied to determine if *stx*$_2$ is located on a phage that can become lytic or not. Finally, another key virulence factor to look for in these isolates is the presence of intimin, encoded by the *eae* gene, an outer membrane protein required for intimate attachment (33) of the bacterium to human intestinal epithelial cells.

Interestingly, none of the sponge samples tested in this study were positive for *stx*$_1$ or *stx*$_2$. There could be many reasons for this. Throughout the assay development and sensitivity studies for the hide sampling, the results were quite variable. The Shiga toxin genes were detected from various dilutions, being detected when inoculated with 223 CFU and 2.23×10^4 CFU, but not when inoculated with 2.23×10^3 CFU or 2.23×10^6 CFU. During these sensitivity trials, some of the cases where the Shiga toxin genes should have been detected, such as when inoculated with 2.23×10^3 CFU in the example listed above, the positive control was also not recovered, whereas in other cases the

positive control was recovered. We concluded that this was due to variability between the horse-hide samples and decided to conduct the sensitivity study from a single horse sample. However, this did not adequately address the possibility of false negative results. Additionally, the inconsistent results occurred when using the heat/thaw DNA extraction method and before we had concluded on an appropriate DNA extraction volume for the overnight enrichment culture using the InstaGene™ matrix. Once we switched to the InstaGene™ matrix and determined an appropriate DNA extraction volume, the sensitivity assay was only performed one more time, with only one horse. I would recommend that any future work on horse hides evaluate this further and repeat the sensitivity trials using multiple horses and continuing the use of InstaGene™ matrix for the DNA extraction. In every sponge sample, the *Staphylococcus* control was positive, indicating the PCR was successful, however, there is the possibility that my results are underreported for the hide samples and positive samples may have been present been undetected. Furthermore, it is possible that adding the *Staphylococcus* control after the overnight enrichment step is problematic, as it does not control for the viability of STEC in the overnight enrichment process. Further analysis could be done, seeding the sponge sample with various concentrations of STEC prior to overnight enrichment to see if there are viability issues of STEC culture in the presence of the sponge or D/E media.

One additional possibility for the lack of Shiga toxin-producing *E. coli* detected on any of the equine coat samples goes back to the products used on many horses. One of the many ingredients that make up the coat shine products and fly spray solutions is “essential oils”. Some research has shown that some of these natural oils have

antibacterial properties, including antimicrobial effectiveness against *E. coli* and even *E. coli* O157:H7 (29) *Melaleuca alternifolia*, or tee tree oil is one of the oils used in some fly spray solutions and has shown antimicrobial effectiveness against *E. coli* (11). An interesting additional study would be to evaluate the viability of STEC in the various horse coat treatment solutions, or evaluate individual horse hides by paying particular attention to specific products used on each horse coat tested. Some of that information was gathered in this study for the purpose of evaluating the need for a neutralizer. I was not able to obtain specific information regarding products used, however, for each horse.

In conclusion, this study determined that STEC was present at a low level in the Sacramento Valley equine population. Because the only horses that tested positive for STEC had interaction with ruminant animals, our research indicates that horses are more likely to acquire STEC from another animal, specifically a ruminant. Although the overall prevalence of STEC in the equine population we tested was relatively low (2.6%), it was actually higher than the prevalence reported for deer (0.25% - 2.4%) (43, 45). Our research on horses also includes ponies which are often present in petting zoos. We sampled 11 ponies on three ranches, and although all of the ponies sampled were negative for STEC, there is still a possible risk of ponies contracting STEC from their ruminant pen-mates, if positive for STEC. Due to the findings of our research and that of others (13), it is clear that horse owners and petting zoo patrons alike should be aware of the risk when handling horse feces and good hand washing practices should be encouraged. Finally, our study clearly indicates that horses that have recent interaction

with ruminant animals are at a higher risk of acquiring Shiga toxin-producing *Escherichia coli*.

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