A COMPARISON OF DIAGNOSTIC TECHNIQUES FOR DETECTING *SALMONELLA* SPP. IN EQUINE FECAL SAMPLES USING CULTURE METHODS, GEL-BASED PCR, AND REAL-TIME PCR ASSAYS

A Thesis

by

SHELLE ANN SMITH

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2006

Major Subject: Veterinary Microbiology

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Approved by:

Chair of Committee,	R. Bruce Simpson
Committee Members,	Loyd Sneed
	Anton Hoffman
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ABSTRACT

A Comparison of Diagnostic Techniques for Detecting *Salmonella* spp. in Equine Fecal Samples Using Culture Methods, Gel-based PCR, and Real-time PCR Assays.

(May 2006)

Shelle Ann Smith, B.S., Texas A&M University-Commerce

Chair of Advisory Committee: Dr. Russell Bruce Simpson

Salmonellae are enteric bacteria infecting animals and humans. Large animal clinics and Veterinary Teaching Hospitals are greatly affected by *Salmonella* outbreaks and nosocomial infection. The risk of environmental contamination and spread of infection is increased when animals are confined in close contact with each other and subjected to increased stress factors. This study was designed to compare double-enrichment culture techniques with Gel-based and Real-time PCR assays in the quest for improved diagnostic methods for detecting *Salmonella* in equine fecal samples. 120 fecal samples submitted to the Clinical Microbiology Laboratory of the Veterinary Medical Teaching Hospital at Texas A&M University (CML, VMTH, TAMU) were tested for *Salmonella* using all three techniques. Double-enrichment bacterial culture detected 29 positive results (24%), Real-time PCR detected 33 positive results (27.5%), and Gel-based PCR detected 73 positives results (60.8%). While culture and real-time PCR methods had similar results, the gel-based PCR method detected many more positive results, indicating probable amplicon contamination. Real-time PCR can be completed as soon as the day after submission while culture techniques may take 2 to 5 days to complete. However, viable bacterial cells are needed for antimicrobial susceptibility

testing and serotyping: both important for epidemiological studies. Therefore, double-enrichment bacterial culture performed concurrently with real-time PCR methods could be efficient in clinical settings where both accurate and expedient results are required.

ACKNOWLEDGMENTS

I give great thanks to my committee chair, Dr. R.B. Simpson, and my committee members, Dr. Loyd Sneed, and Dr. Anton Hoffman, for helping me achieve this goal.

I would like to thank Texas Veterinary Medical Laboratory (TVMDL) for allowing me to complete my molecular work using their equipment. Thanks especially to Feng Sun and Jennifer Meier for their guidance. Thank you Kim DuBose for getting me started, and to everyone in clinical microbiology for all your help.

Thanks to my husband for being patient and supportive.

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CHAPTER I

INTRODUCTION: SALMONELLOSIS - THE INFECTION

Salmonellosis is a health concern for both humans and animals. *Salmonellae* are gram negative bacilli belonging to the family *Enterobacteriaceae*. *Salmonella enteritidis*, the species responsible for 99% of gastroenteritis infections in animals and humans (Rubin and Weinstein, 1977; Kim et al., 2001), consists of more than 2400 serotypes (Quinn et al., 2002). *Salmonellae* are facultatively intracellular organisms that have the ability to proliferate inside macrophages and, therefore; evade destruction by the host immune system. The entry of *Salmonella* organisms into the epithelial cells that line the gastrointestinal tract may cause severe illness, intestinal damage, and death. *Salmonella* spp. are mesophilic (grow best at temperatures between 15 and 40^{o}C), and are resistant to drying, heat, and cold. The ability to adapt to changing environmental conditions allows *Salmonellae* to persist in the environment and spread via the fecal-oral route, creating the potential for environmental contamination and nosocomial infection (Guthrie, 1992). The primary goal of this study was to validate our double-enrichment culture technique by comparing the rate of *Salmonella* detection using bacterial culture to the rate of *Salmonella* detection using molecular methods. The secondary goal of this study was to find a Polymerase Chain Reaction (PCR) assay that could be used concurrently or, in some cases, in place of culture methods in order to reduce detection time.

This thesis follows the style of Veterinary Microbiology.

Equine *Salmonella* infections are of significant concern among large equine breeding farms and Veterinary hospitals, as horses in confinement are at greater risk of contracting Salmonellosis (Cohen et al., 1994; Murray 1996; Amavisit et al., 2001; Kurowski et al., 2002). At least 40 serovars of *Salmonella enteritidis* have been isolated from the horse (Collett and Mogg, 2004). Many cases of nosocomial *Salmonella* outbreaks have been documented in the literature and in some cases have caused the temporary closure of veterinary hospitals (Schott et al., 2001; Alinov et al., 2003; Ernst et al., 2004; Smith, 2004). Horses that become infected with *Salmonella* spp. intermittently shed the organism into the environment thru the feces for 30 to 300 days. This environmental shedding creates a potential for infection of multiple animals. It is thus important to detect an animal that is shedding *Salmonella* as soon as possible to prevent further contamination and spread of infection (Cohen et al., 1994, 1996; Murray, 1996; Amavisit et al., 2001; Kurowski et al., 2002). A rapid response to a possible outbreak is required to minimize exposure and control the infection (Schott et al., 2001; Hyatt and Weese, 2004).

Increased risk factors for Salmonellosis in horses include stress, seasonal changes, increase in barn temperature, change in diet, large colon impaction, nasogastric intubation, surgery, anesthesia, concurrent disease, and treatment with antimicrobials (Cohen et al., 1995; House et al., 1999; Kim et al., 2001; Burgess et al., 2004; Ward et al., 2005). An increase in stress may activate clinical *Salmonella* disease and shedding in asymptomatic carrier animals. For example, an increase in barn temperature by ten degrees may double the risk of shedding (Burgess et al., 2004; Ward et al., 2005). One study found that only 1.65% of normal non-hospitalized horses shed *Salmonella* in their

feces, while 23.8% of hospitalized horses shed *Salmonella* and up to 30% of clinically normal foals may shed *Salmonella* in their feces (Collett and Mogg, 2004). Several studies also claim that the isolation of *Salmonella* in equine feces is increased by 10-40 fold for animals receiving antimicrobial therapy (Hird et al., 1984; Hird et al., 1986; House et al., 1999; Collett and Mogg, 2004).

Salmonellosis is considered a zoonotic disease which peaks in the summer months and has a reported 60% transmission rate upon consumption of contaminated food or water (Rubin and Weinstein, 1977). Infection with *Salmonella* spp. does not always induce clinical signs, however; as several factors affect the pathogenesity of the infection. Four important factors of pathogenesis include 1) risk of contact with the organism, 2) the number of viable organisms ingested, 3) the level of susceptibility of the exposed individual, and 4) characteristics of the *Salmonella* serotype.

Although many *Salmonella* serotypes are found in horses, the most common serotype found in both clinically infected and asymptomatic carrier horses is *S. typhimurium*. One source reports that approximately 10^6 to 10^9 organisms are required to cause disease in 50% of individuals and organism load per infection varies with serotype (Rubin and Weinstein, 1977). A recent publication stated that an infected foal can shed as much as 3×10^5 organisms per gram of feces (Collett and Mogg, 2004), and in another study, performed by Burgess and colleagues, their results stated that 75% of environmental *Salmonella* isolates from the veterinary teaching hospital matched the phenotypes of isolates that were obtained from animals admitted to that veterinary teaching hospital in the previous month (Burgess et al., 2004). This study, along with a

multitude of others, document the need for proper monitoring and detection of *Salmonella* shedding of horses, especially in veterinary hospitals.

CHAPTER II

THE PROBLEM: *SALMONELLA* DETECTION

This study compared three different techniques for detecting *Salmonella* spp. in equine fecal samples. A double enrichment microbiological culture technique, gel-based PCR, and real-time PCR assays were performed on fecal specimens submitted to the Clinical Microbiology Laboratory of the Veterinary Medical Teaching Hospital at Texas A&M University (CML,VMTH,TAMU), for *Salmonella* testing. The general purpose of this study was to compare these different techniques for detecting *Salmonella* spp. in equine fecal samples to determine if our current method is adequate, or if other techniques should be implemented.

The gold standard for *Salmonella* detection is currently bacterial culture. However, *Salmonella* isolation techniques are not internationally standardized and vary greatly among laboratories (Hyatt and Weese, 2004). Because of intermittent shedding, submission and testing of five consecutive samples containing 5 to 25g of feces is recommended (Hyatt and Weese, 2004). In the Clinical Microbiology Laboratory, VMTH, TAMU, the current method of *Salmonella* detection in equine fecal samples from the Large Animal Clinic is a double enrichment bacterial culture with tertiary plating. Five negative cultures of five consecutive fecal samples are required.

It has been shown that bacterial culture of equine fecal samples may test negative for *Salmonella* because of dilution, loss of viability, intermittent shedding, treatment with antimicrobials, sample storage, subclinical infection, and carrier state animals (Amavisit et al., 2001). However, preliminary comparisons performed at Texas Veterinary Medical Diagnostic Laboratory, College Station, TX (TVMDL) between their traditional culture

methods (Tergitol and XLT) and the double enrichment technique have shown approximately a 27% increase in *Salmonella* detection in feces using multiple serotypes (personal conversation with Sonja Lingsweiler, 09/2005). Rostagno and colleagues reported 94% sensitivity and 100% specificity using a similar double enrichment technique with Tetrathionate broth and Rappaport Vassiliadis broth in swine, compared with various other methods (Rostagno, et al., 2005).

Polymerase Chain Reaction (PCR) was first described by Kleepe and colleagues in 1971, but was not demonstrated until 1985 by Saiki and colleagues (Edwards et al., 2004). Many advances have been made since then and PCR assays have become much more common and easier to use. Due to rapid technological advances, many laboratories now use PCR methods routinely. PCR amplification provides millions of copies of identical DNA from very few copies of target sequence (Edwards et al., 2004). Many studies have shown that PCR techniques are significantly more sensitive than microbiological culture techniques, especially when the organism is present in low numbers or is not viable (Cohen et al., 1994, 1995, 1996; Amavisit et al., 2001). However, more recent reports also claim that end-point PCR may not be as sensitive as previously thought, due in part to false positives, and may not be appropriate for *Salmonella* identification in clinical settings (Amavisit et al., 2001; Ewart et al., 2001; Alinovi et al., 2003). Studies have also shown that PCR positive results are obtained much more quickly than culture positive results (Stone et al.1994), but according to current literature, there are large discrepancies between culture and PCR results (Ewart et al., 2001; Alinovi et al., 2003; Hyatt and Weese, 2004). Since DNA is detected by PCR, a positive result from feces indicates that a horse is shedding *Salmonella*, but does not

determine viable from non-viable organisms (Hyatt and Weese, 2004; Collett and Mogg, 2004). To date, studies have not established the significance of PCR positive but culture negative results for *Salmonella* in horses with regard to environmental contamination and spread of infection (Amavisit et al 2001, Collett and Mogg 2004).

Technological advances in PCR methods now allows real-time detection of genus specific DNA by fluorescence labeling. Real-time PCR was first demonstrated by Higuchi and colleagues in 1992, and the first commercial platform was released by Applied Biosystems in 1996 (Edwards et al., 2004). Real-time PCR is considered quantitative whereas gel-based or end-point PCR is considered qualitative (Edwards et al., 2004). Real-time PCR software collects data throughout the process of amplification and detects the target DNA by correlating PCR product concentration with fluorescence intensity. This rapid detection needs no post amplification processing thus decreasing the risk of contamination of the sample, especially in a clinical microbiological laboratory (Uyttendaele et al., 2003; Edwards et al., 2004). Because of the closed tube technique, false positives and amplicon contamination are not as common as with gel-based methods but cross-contamination may still occur.

One of the advantages of PCR is the rapid detection of possible *Salmonella* shedding which reduces the risk of environmental contamination and infection of other animals. One of the disadvantages of PCR is the inability to perform antimicrobial testing or serotyping, which could affect epidemiological studies and the identification of nosocomial infections. Also, real-time PCR equipment is quite expensive and requires highly trained technicians to interpret results.

CHAPTER III

MATERIALS AND METHODS

SAMPLE COLLECTION AND STUDY DESIGN

All horses admitted to the Large Animal Hospital at Texas A&M University that exhibit diarrheal symptoms are culture tested for *Salmonella* infection by submitting fecal samples to the Clinical Microbiology Laboratory. Negative results from 5 consecutive fecal samples are required to report an animal that is exhibiting diarrhea as *Salmonella* negative. Only one positive result is needed to report an animal *Salmonella* positive. Any animal reported *Salmonella* positive is required to be placed in the isolation ward until 5 consecutive negative cultures are obtained or until the animal is discharged.

Equine fecal samples used in this study were collected from those samples submitted to the Clinical Microbiology Laboratory at Texas A&M University Veterinary Teaching Hospital. All samples submitted were cultured for *Salmonella* by lab technicians using a double enrichment protocol on the day they were submitted. The culture procedure took 2 to 5 days to complete. On day one, primary plates were cultured directly from the fecal sample; on day two, secondary plates were cultured from tetrathionate broth inoculated with 1 gram of fecal material; on day three, tertiary plates were cultured from Rappaport Vassiliadis R10 broth inoculated with material from the initial tetrathionate broth/fecal mixture. Each series of cultures was then incubated overnight at 37^{o}C. Suspicious colonies were isolated and identified using a commercially available computer system (VITEK, Biomerieux, Durham, NC). Cases that were identified as *Salmonella* spp. were then serogrouped and sent to the National Veterinary

Services Laboratory (NVSL) in Ames, IA for serotyping. Original samples were stored in airtight containers at 4^{o}C for up to two weeks.

A second set of cultures were performed on days 2-14 using the same double enrichment microbiological culture procedure. Suspicious colonies were tested biochemically and those with positive reactions for *Salmonella* were serogrouped. All fecal samples were also subsequently enriched and prepared for DNA extraction in order to perform gel-based polymerase chain reaction (PCR), and real-time PCR assays developed to detect *Salmonella* spp. DNA was extracted and purified from a mixture of 1 gram of fecal sample added to 10mL of Tetrathionate selective enrichment broth. DNA extraction occurred on day 2 after 24 hr incubation at 37^{o}C. The same DNA was used for both PCR procedures.

BACTERIAL CULTURE

The culture technique performed was a double enrichment technique developed by Sonia Lingsweiler, Texas Veterinary Medical Diagnostic Laboratory (TVMDL), College Station, TX.

Day 1: Primary plates were prepared directly from the submitted fecal samples using a sterile swab to streak MacConkey Agar (MAC) (BD Diagnostics, Becton, Dickinson and Company, Franklin Lakes, New Jersey) and Xylose Lysine Tergitol 4 plates (XLT4) (BD Diagnostics, Franklin Lakes, NJ, prepared by the Clin Micro Lab), and a sterile loop was used to streak for colony isolation. One gram of fecal material was then weighed and added to 10 mL of Tetrathionate broth (TTH) [BD Diagnostics, Franklin Lakes, NJ, prepared by the Department of Veterinary Pathobiology (VTPB)

media kitchen] with 5 drops of iodine solution added. The MacConkey Agar plates, XLT4 plates, and inoculated Tetrathionate broth were incubated for 24 hrs at 37^{o}C.

Day 2: After 24 hr incubation, 1.5mL of undisturbed supernatant from the Tetrathionate broth/feces (10/1) mixture was removed and placed in a 1.7mL microcentrifuge tube for DNA extraction. The primary plates of MAC and XLT4 were inspected for *Salmonella* suspicious colonies. On MAC, suspicious colonies were lactose negative, tan to brown in color, and suspicious colonies on XLT4 were red to pink in color with a black center. Secondary plates were prepared using a sterile swab to culture fresh MAC and XLT4 plates from the Tetrathionate broth/feces tube after thorough mixing. After streaking the MAC and XLT4 plates for isolation, 10mL of Rappaport-Vassiliadis R10 broth (RV) [BD Diagnostics, Franklin Lakes, NJ, prepared by the Department of Veterinary Pathobiology (VTPB) media kitchen] was then inoculated with solution from the Tetrathionate broth mixture using a sterile swab. The MAC plates, XLT4 plates, and inoculated RV broth were then incubated for 24 hrs at 37^{o}C.

Day 3: Primary and secondary plates of MAC and XLT4 were inspected for suspicious colonies after 24 hr incubation at 37^{o}C. Using a sterile swab, tertiary plates of MAC and XLT4 were cultured from the RV broth inoculate.

Day 4: Primary, secondary, and tertiary plates were examined for suspicious colonies.

Suspicious colonies: Any suspicious colonies from 1^{o}, 2^{o}, and 3^{o} plates were subjected to further biochemical tests. Lactose negative (tan or brown) colonies on MAC or red colonies with black centers on XLT4 were used to inoculate Triple Sugar Iron (TSI), and Lysine Iron Agar (LIA) slants [BD Diagnostics, Franklin Lakes, NJ, prepared

by the Department of Veterinary Pathobiology (VTPB) media kitchen]. The inoculated TSI and LIA slants were then incubated at 37^{o}C for 24 hrs. Results considered positive for *Salmonella* on TSI were K/AG, H2S (an alkaline slant with an acidic butt having hydrogen sulfide and gas production), positive results on LIA were P/P, H2S (decarboxylase positive with or without hydrogen sulfide production). These results were presumed *Salmonella* positive. Positive samples were inoculated on to tryptose slants and stored for later serogrouping to define somatic 'O' antigens and verify positive *Salmonella* identification.

SEROGROUPING

Presumptive *Salmonella* positive isolates were tested with *Salmonella* polyvalent groups A – I antisera (BD Salmonella O Grouping Antisera Kit, BD Diagnostics, Franklin Lakes, NJ). A tryptose slant that had been inoculated with the *Salmonella* isolate and grown overnight at 37^{o}C was then killed by adding approximately 1-2 mL of phenolized saline to make a killed *Salmonella* suspension. A small drop of polyvalent antisera and 1 drop of organism was added to a slide and mixed with a clean toothpick to make an even suspension. The slide was then gently rocked back and forth and observed for clumping or agglutination. If the polyvalent antisera gave a positive reaction, then the process was repeated with monovalent antisera A, B, C1, C2, D,E,F,G,H, and I (BD Salmonella O Grouping Antisera Kit, BD Diagnostics, Franklin Lakes, NJ). All Salmonella isolates were forwarded to the National Veterinary Services Laboratory (NVSL) in Ames, Iowa for serotyping using the Kaufmann, White schema identifying Somatic (O) and Flagellar (H) antigens.

DNA EXTRACTION

DNA extraction was achieved using a commercially available kit for isolation of genomic DNA. The DNeasy Tissue Kit (QIAGEN Inc., Valencia, CA) was used, and the protocol for DNA isolation from bacteria and tissues was followed. The same DNA was used for both PCR assays.

Day 1: 1 gram of fecal material from the submitted sample was weighed and added to 10 mL of Tetrathionate selective enrichment broth for 24 hr incubation at 37^{o}C.

Day 2: After 24 hr incubation, 1.5 ml of supernatant from the inoculated TTH broth was added to a 1.7 ml microcentrifuge tube and cells were harvested by centrifuging for 10 min ant 7500 rpm. Supernatant was then discarded and the cell pellet was resuspended in 180 microliters of Buffer ATL and 20 microliters of Proteinase K. The suspension was mixed by vortexing and incubated in a water bath at 55^{o}C for 1-3 hrs, or until the cells are completely lysed. 200 microliters of Buffer AL was then added to the sample with additional incubation at 70^{o}C for 10 min. After cell lysis, 200 µL of ethanol was added to the sample and mixed by vortex. The mixture was then pipetted into the provided DNeasy mini column filter in a 2 mL collection tube. The mini column was centrifuged at 8000 rpm for 1 min, after which, the flow-through and the collection tube was discarded. The DNeasy mini column was then placed in a new collection tube and 500µL of Buffer AW1 was added and centrifuged for 1 min at 8000 rpm. Flow-through and collection tube was again discarded and the mini column filter was placed in a new 2 ml collection tube. 500µL of Buffer AW2 was then pipetted directly onto the filter and centrifuged for 3 min at 8000 rpm to dry the DNeasy membrane. Flow-through and collection tube was once again discarded and the DNeasy mini column was placed in

a clean 1.7 ml microcentrifuge tube, and 200µL of Buffer AE was pipetted directly onto the DNeasy membrane and then centrifuged for 1 min to elute. After elution, the DNeasy filter was removed and the DNA sample was stored at -80 ^{o}C until ready for amplification.

DNA AMPLIFICATION USING GEL-BASED PCR

The polymerase chain reaction assay used oligonucleotide primers of 25 base pairs that define the amplified region of a 496 base pair, highly conserved segment of the histidine transport operon gene of *Salmonella typhimurium* (Cohen et al., 1994,1995,1996). The genus specific oligonucleotide primers consist of an upper strand: 5' ATG TTG TCC TGC CCC TGG GAG A 3', and a lower strand: 5'ACT GGC GTT ATC CCT TTC TCT GGT C 3' (Integrated DNA Technologies, Coralville, Iowa) (see Table 1). Using 1.7 ml microcentrifuge tubes, each assay required a water blank, one positive control (*Salmonella typhimurium* ATCC 14028), one negative control (*Escherichia coli* ATCC 25922), and one tube for each reaction which contained 25µL of TAQ ready mix, 23µL of master mix and 2µL of DNA. A PCR Master Mix was prepared in a 1.7 ml microcentrifuge tube using 21µL of PCR water (Sigma, St. Louis, Missouri), 1µL upper strand primer, and 1µL lower strand primer for each reaction. The water blank was prepared before each assay using 25µL of TAQ ready mix (Jumpstart Readymix REDTaq DNA polymerase, Sigma, St. Louis, Missouri), 1µL upper strand primer and 1µL lower strand primer, and 23µL of PCR water. Each reaction tube contained 25µL of TAQ ready mix, 23µL of master mix and 2µL of DNA. The reactions were run in a thermocycler for 10 min at 95^{o}C followed by 40 cycles of 30 sec at 94 ^{o}C, 40 cycles of 60 sec at 36 ^{o}C, and 40 cycles of 60 sec at 72 ^{o}C followed by a final

extension phase of 10 min at 72 ^{o}C. Products of the PCR underwent electrophoreses on a 2 % agarose gel and were then visualized by a UV transilluminator and photographed.

Table 1
Sequence of PCR primers and probe

Gel-PCR		
	Upper primer	5'ATGTTGTCCTGCCCCTGGGAG3'
	Lower primer	5'ACTGGCGTTATCCCTTTCTCTGGTC3'
Real-time PCR		
	Forward primer	SAL1203F: 5'TCCTGGCCTGGCGAAAG3'
	Reverse primer	SAL 1203R: 5'CAGCTCGGAAGCATCAACCA3'
	Probe	6FAM CACCATTGCGGGCCGTGTGAAT TAMRA

GEL ELECTROPHORESIS

Gel electrophoresis was used to detect *Salmonella* DNA in the amplified PCR product. The gel matrix was prepared using 2 grams of agarose (Bio-Rad, Hercules, California), 98 ml of 1X Tris/Acetic Acid/EDTA (TAE) buffer (Bio-Rad, Hercules, California), and 3µL ethidium bromide (Bio-Rad, Hercules, California) to produce a 2% agarose gel. The gel was placed in an electrophoresis apparatus and covered with 1X TAE buffer. Wells were loaded with 10µL of the PCR samples, 10µL each of the positive control, negative control, and water blank, and 5µL of the 100 base pair (bp) DNA ladder (Bio-Rad, Hercules, California). Eighty-five volts of electricity were passed through the gel for 45 minutes. The gel was then exposed to ultraviolet transillumination and photographed. A positive result for *Salmonella* revealed a distinct band at 496 bp (see Figure 1).

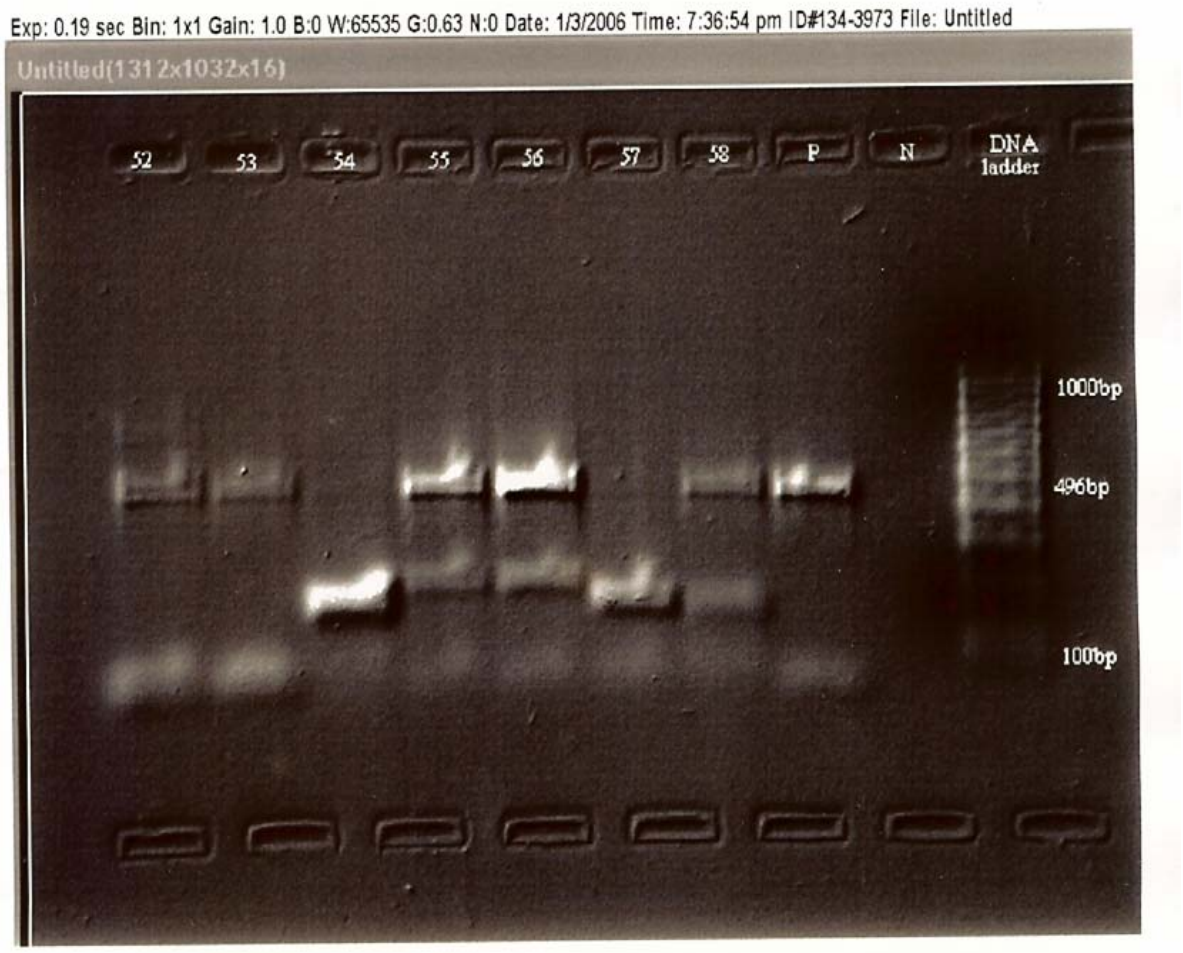

Figure 1. Photograph of UV transillumination of gel electrophoresis with a *Salmonella* positive result appearing at 486 base pairs. The DNA 100 bp ladder is in lane 10; the negative control is in lane 9; the positive control is in lane 8; and positive results are shown in lanes 1, 2, 4, 5, and 7.

DNA AMPLIFICATION AND DETECTION USING REAL-TIME PCR

The real-time polymerase chain reaction assay was used to both amplify and detect the presence of a specific DNA sequence. A *Salmonella* specific primer and fluorescence labeled probe [developed by Dr Loyd Sneed and Feng Sun, TVMDL,

College Station, TX using Primer Express ABI Software, Applied Biosystems (ABI), Foster City, CA] was used. The undefined genetic target was located in a highly conserved region approximately 100 bp in length. The genus specific oligonucleotide primers consisted of a forward strand (primer one) SAL 1203F: 5' TCC TGG CCT GGC GAA AG 3', and a reverse strand (primer two) SAL 1203R: 5'CAG ATC GGA AGC ATC AAC CA 3' (ABI, Foster City, CA) (see Table 1). The fluorescence labeled probe for *Salmonella* used the sequence: 6FAM CAC CAT TGC GGG CCG TGT GAA T TAMRA (ABI, Foster City, CA) (see Table 1). A PCR Master Mix was prepared (per reaction) using 8.0µL of deionized water, 12µL of 2X Taqman universal mix (ABI, Foster City, CA), 0.5µL primer one, 0.5µL primer two, and 1µL probe for each reaction. 22.5µL of Master Mix was added to each optical PCR tube and 2.5µL of purified sample DNA was added to the master mix. The capped optical tubes (ABI, Foster City, CA) were then placed into the Taqman thermocycler with camera and started using the appropriate program (ABI Prism 7000 SDS Software, ABI, Foster City, CA). The thermocycling conditions used were one cycle at 50 ^{o}C for 2 minutes, one cycle at 95 ^{o}C for 10 minutes, 45 cycles at 95 ^{o}C for 15 seconds, and 45 cycles at 60 ^{o}C for 60 seconds. The PCR product was doubled after each cycle until a plateau was reached. The fluorescence was read automatically after each cycle. Detection of a positive result revealed an increase in fluorescence intensity displayed by a standard curve method plotted in graph form. The real time sequence was tested against 38 different *Salmonella* serovars (see Table 2).

Table 2
Salmonella serogroups and serotypes detected by the real-time primer/probe sequence

serogroup	serotype
V	44:Z4,Z23 (arizona III)
W	45:G,Z51 (arizona IV)
Z	50:K-Z (arizona III)
	9,12: nonmotile
E1	Anatum
Z	50:Z52-Z53 (arizona III)
B	banana
C3	bardo
H	beaudesert
C1	braenderup
B	bredeney
K	cerro
D1	dublin
E1	give
B	heidelberg
C1	infantis
C3	kentucky
B	kiambu
C2	litchfield
L	minnesota
C1	montevideo
C2	muenchen
	multiple serotypes
E2	newington
C2	newport
C1	norwich
C1	oranienburg
F	rubislaw
B	saint-paul
I	saphra
B	schwarzengrund
H	soahanina
H	sundsvall
B	typhimurium
B	typhimurium (copenhagen)
N	urbana
E	taksony
G	poona

STATISTICAL ANALYSIS

Statistical analysis of linear data was interpreted using frequencies and percentages. Comparative data were interpreted via graphical methods. Correlation between culture and PCR results was computed using the Pearson test showing a similarity matrix. Sensitivity and specificity of PCR compared with culture were computed using the McNemar test. A 95% confidence interval was used and statistical methods were computed using SPSS 11.5 for Windows.

CHAPTER IV

RESULTS

Double enrichment culture procedures, real-time PCR procedures, and gel-PCR procedures were tested. Serial dilutions of *Salmonella typhimurium* were created to measure the sensitivity of each procedure. The starting concentration was made using a colorimeter and the .5 McFarland Standard of turbitity which equals10^8 colony forming units per milliliter (cfu/ml). Eleven ten-fold dilutions were made from 10^8 to 10^{-3} cfu/ml. The culture procedure detected bacteria from 10^8 to 10^0 cfu/ml and both PCR procedures detected *Salmonella* DNA from 10^8 to 10^{-2} cfu/ml. Therefore, the culture procedure detected bacteria as low as one colony forming unit per milliliter but PCR was able to detect DNA with a two-log increase in sensitivity. This increase, however; could be attributed to an increase in volume of starting material: 100µl for culture plates compared to 1500µl for DNA extraction.

Fecal samples were collected from horses that were admitted to the Large Animal Clinic at VMTH, TAMU from January 2004 to June 2005. All horses tested were either admitted with diarrhea as the presenting complaint or they developed diarrhea while admitted. Samples were randomly chosen to be part of the study. 120 samples from 55 horses were tested using a double enrichment culture technique, gel-based PCR, and real-time PCR assays. The mean number of samples submitted per horse was 2.2 with the mode being 5.

The first culture procedure was performed the same day the fecal sample was submitted to the Clinical Microbiology Lab of the TAMU, VMTH. The sample was then stored in the walk-in cooler at 4 ^oC for 2 to 14 days. The second culture procedure was

then performed sometime during days 2 thru 14. Each sample was plated on XLT4 and MAC and one gram of feces was inoculated into Tetrathionate broth on the first day. After overnight incubation, a second set of plates was streaked from the tetrathionate inoculate, RV broth was inoculated, and 1.5 ml of the inoculated tetrationate supernate was extracted for DNA purification. After purification, the DNA was stored at -80 ^{o}C until needed for PCR. The same DNA was used for both PCR procedures, which were performed concurrently at TVMDL. A *Salmonella* positive result for gel PCR showed a definite band at 496 base pairs (see Figure 1). A positive result for real-time PCR showed an increased intensity in fluorescence shown in graph form by the computer program.

Results from both cultures and both PCR techniques were compared (see Table 3, Table 4, Figure 2, Figure 3). Of the 120 fecal samples tested, 29 (24.1%) isolates were found positive for *Salmonella* by the first culture, which was performed on the day the sample was submitted to the lab. The second culture, which was performed between days 2 and 14, detected 28 (23.3%) *Salmonella* positive isolates. The real-time PCR procedure detected 33 (27.5) positive results and the gel-based PCR procedure detected 73 (60.8%) positive results (see Table 3, Figure 2). Of the 55 horses culture tested, the first culture detected 15 out of 55 horses (27.3%) as positive for *Salmonella* and the second culture detected 13 out of 55 horses (23.6%) as positive for *Salmonella.* Of the 55 horses PCR tested, real-time PCR detected 18 out of 55 horses (32.7%) as *Salmonella* positive, while gel-based PCR detected 37 out of 55 horses (67.3%) as *Salmonella* positive (see Figure 3). Two horses (5 fecal samples) were found *Salmonella* positive by the first culture and gel-PCR, but were found *Salmonella* negative by the second culture and real-time PCR.

Three horses (10 samples) were found *Salmonella* negative with both cultures, but tested *Salmonella* positive with both PCR procedures. The real-time PCR procedure detected an additional 12 positive fecal samples (from 5 horses) that tested negative with both cultures while gel PCR detected an additional 44 positive samples (from 19 horses) that tested negative with both culture procedures. Gel PCR also detected an additional 31(25.8%) positive samples (from 14 horses) that were considered negative by the other three procedures.

Table 3
Frequency and percent negative and positive for culture and PCR procedures

	Frequency	Percent
1st Culture		
Negative	91	75.8
Positive	29	24.2
Total	120	100.0
2nd Culture		
Negative	92	76.7
Positive	28	23.3
Total	120	100.0
Real-time PCR		
Negative	87	76.5
Positive	33	27.5
Total	120	100.0
Gel-PCR		
Negative	46	39.3
Positive	73	61.7
Total	120	100.0

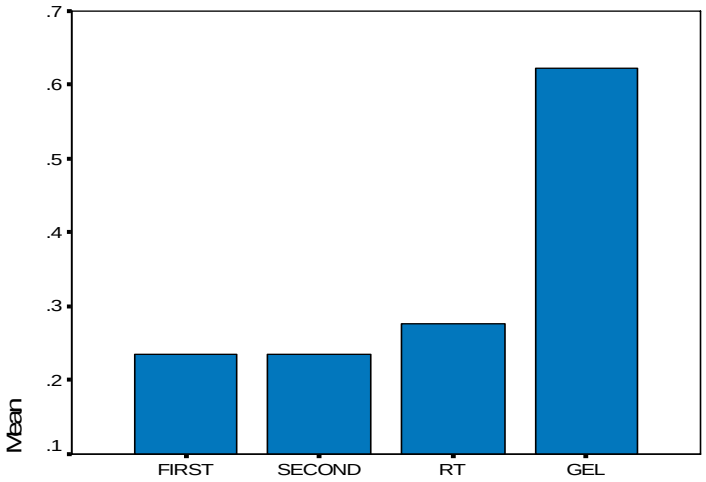

Figure 2. Bar graph showing the percentage of *Salmonella* positive results detected for each procedure 1st Culture – (29/120), 24.1%;2nd Culture – (28/120), 23.3%;Real-time PCR – (33/120), 27.5%; Gel PCR – (73/120) 60.8%

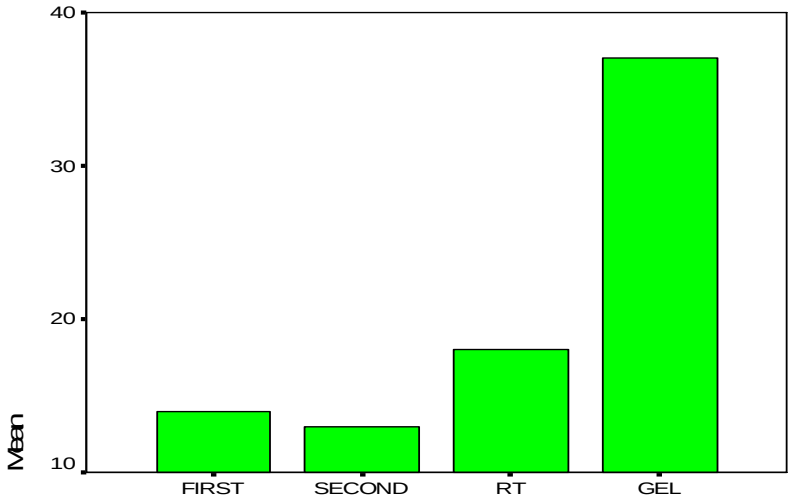

Figure 3. Bar graph showing the number of horses testing *Salmonella* positive in each procedure. 1st Culture – 14 horses; 2nd culture – 13 horses; Real-time PCR -18 horses; Gel PCR – 37 horses.

The fecal samples that tested positive by culture were serogrouped and sent to NVSL in Ames, IA for serotyping (see Figures 4 and 5). Two isolates (from one horse) were serogroup B and serotype *S. typhimurium*. Four isolates (from two horses) were

serogroup C1 and serotype *S infantis*. Fifteen isolates (from six horses) were serogroup C2 with eleven *S. newport*, one *S. litchfield*, and three isolates that were not detected on first culture, but were from a horse that had been serotyped with *S. newport* on three prior occasions. Eight isolates (from five horses) were serogroup E with five *S. anatum*, two *S. taksony*, and one that was serogrouped as E but the serotype information was not received from NVSL. Three isolates (from one horse) were serogroup F and serotype *S. rubislaw*. One isolate (from one horse) was serogroup G and serotype *S. poona*. One horse tested positive for two serotypes (*S. rubislaw* and *S. Litchfield*). The most dominant serogroup was C2 (see Figure 5).

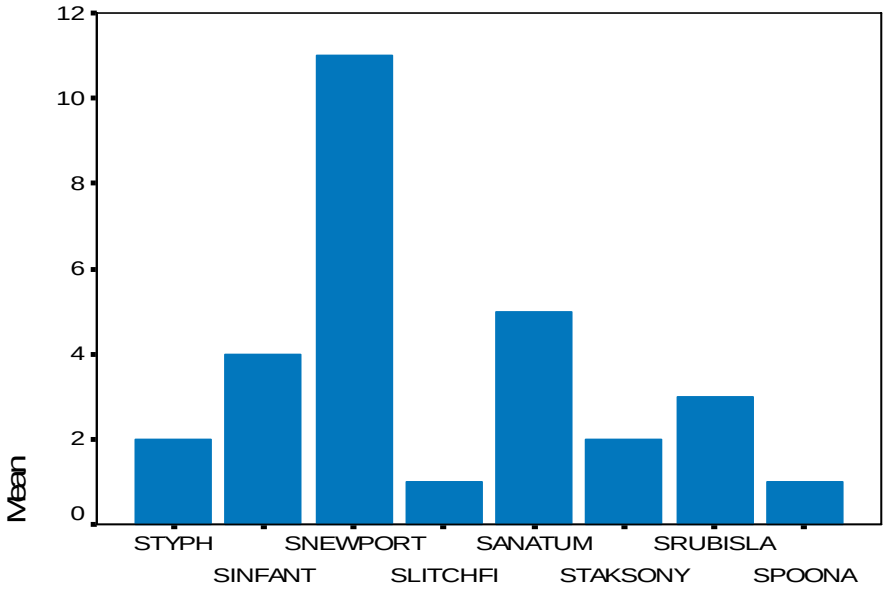

Figure 4. Bar graph showing the serotype distribution.

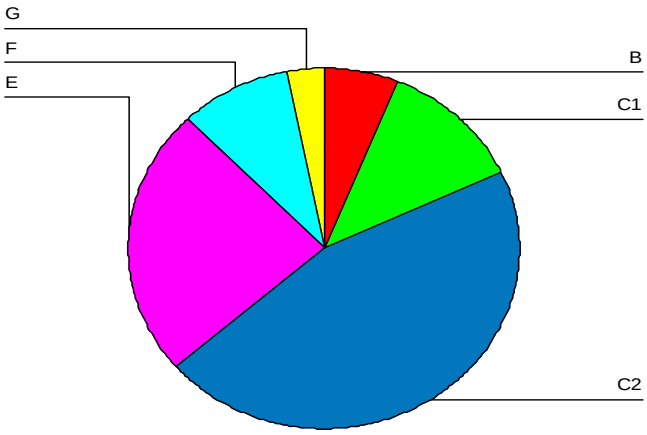

Figure 5. Pie graph of serogroup distribution from culture positive isolates. Serogroup C2 being the most prevalent and serogroup G being the least prevalent.

The horses that tested *Salmonella* positive by culture had a variety of presenting complaints with diarrhea being the most common (see Figure 6). Eleven adult horses and four foals were culture positive with eight male horses and seven female horses. Of the culture positive samples submitted, two horses died; one foal and one adult; both with serogroup E infections (one *S. anatum*, one *S. taksony*), and both with colic as the presenting complaint.

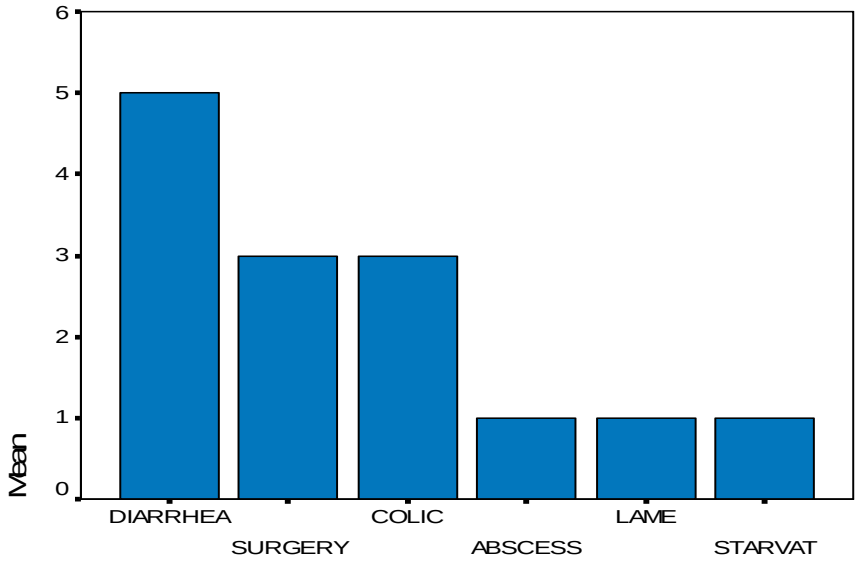

Figure 6. Bar graph showing distribution of presenting complaints. Diarrhea-5; Surgery-3; Colic-3; Abscess, lameness, and starvation-1.

Table 4
Proximity matrix showing correlation using the Pearson test

	Correlation between Vectors of Values			
	FIRST	SECOND	RT	GEL
FIRST	1.000	.839	.524	.325
RT	.524	.587	1.000	.332
GEL	.325	.313	.332	1.000

CHAPTER V

DISCUSSION AND CONCLUSION

Both gel PCR and real-time PCR detected more positive results than culture procedures, as expected from previous studies (Cohen et al., 1994, 1995, 1996; Kurowski et al., 2002). However, results from the two cultures (29 positives, 24.1%; 28 positives, 23.3%) were more compatible with the real-time results (33 positives, 27.5%) than with gel-PCR (73 positives, 60.8%) (see Tables 3 and 4). Because horses shed viable *Salmonella* intermittently, it would not be unreasonable to detect 3.4% more non-viable DNA than viable bacterial cells. While gel PCR detected significantly more positive results than all other procedures, it is possible that many of those positive results could be due to false positives from amplicon contamination.

In a large animal clinic, early detection of *Salmonella* infection is required to prevent environmental contamination and nosocomial outbreaks. Results from real-time PCR with pre-enrichment can be obtained as soon as the day after submission. Culture results can take two to five days to be final. However, it would be very difficult to determine the source of an outbreak without antimicrobial patterns and serovar information.

A larger study including more samples and/or examination of a different population could show different results. However, based on the results of this study, real-time PCR and double-enrichment culture procedures performed concurrently seems to be a logical conclusion. In a clinical setting, gel PCR doesn't appear to be effective because of the possibility of contamination. While many more positive results were detected by gel-based PCR, the meaning of those results is unknown. Using culture as the "gold

standard" to calculate sensitivity and specificity indicates 100% sensitivity and 81.9% specificity for gel-PCR (Kurowski et al. 2002).

Real-time PCR is a simple procedure that requires no post amplification manipulations. This particular primer-probe sequence obtained positive results against 36 *Salmonella* serovars previous to this study and an addition two serovars in this study (see Table 2). While real-time did not detect five serogroup E samples and one serogroup C2 sample, the reason for that is unknown. It appears to be sensitive to a wide range of serovars and sensitivity and specificity were calculated as 91.7% and 95.2% respectively using culture as the "gold standard". The results of this study show that this molecular sequence correlates well with our culture technique and would be a useful addition to our lab procedures.

Future studies should include samples from a larger number of horses to compare outcomes. A more extensive range of equine serovars should also be tested. Real-time PCR use is rapidly growing and becoming more popular in clinical settings. The rapid detection of *Salmonella* positive horses, especially early infections with a low bacterial load, would be beneficial in controlling environmental contamination. While the initial cost of real-time PCR equipment is high, a nosocomial outbreak of *Salmonella* in a large animal clinic would likely also be high.

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