DISSERTATION

EPIDEMIOLOGY AND PREVENTON OF *SALMONELLA ENTERICA* IN VETERINARY HOSPITALS

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ABSTRACT

EPIDEMIOLOGY AND PREVENTON OF *SALMONELLA ENTERICA*

IN VETERINARY HOSPITALS

Salmonella enterica is the most commonly reported cause of outbreaks of nosocomial infections in large animal veterinary teaching hospitals and the most common cause of closure of equine hospitals at these facilities. Congregating horses from multiple sources, as is common at breeding farms, racetracks, or equestrian events, is also associated with increased risks for spread of contagious diseases such as *Salmonella*. Significant environmental contamination is inevitably present when *Salmonella* spreads between horses, whether as a cause or effect, and it is well documented that environments in equine facilities that appear clean can still be contaminated with *Salmonella.* Additionally, horses returning home from veterinary hospitals or other facilities can serve as a source of infection for others. Control of *Salmonella* is further complicated by the fact that subclinical infection and shedding are much more common than clinical infections and horses can shed infectious doses of *Salmonella* in the absence of disease. Despite this, we have a limited understanding of its natural history and epidemiology. Much of what is known about *S. enterica* in veterinary populations is derived from experimental infections and data collected from high-risk sub-groups (i.e., horses with colic or colitis) or during periods of epidemic disease. Experimental infection clearly does not reflect what occurs in nature. Consequently, extrapolation to naturally occurring disease is rather limited. While collecting data from high-risk sub-groups and periods of epidemic disease are worthwhile endeavors, it is difficult to uniformly apply these findings to the general animal population or to

times of endemic disease. Effectively managing this organism in animal populations and their environments continues to be a challenge. Veterinarians have an ethical responsibility to take reasonable precautions to reduce risks related to *S. enterica* among animals and their environment. Critical to this effort are improving our understanding of the epidemiology of *Salmonella* and developing more accurate and rapid diagnostic tests. The overarching goal of the studies contained within this dissertation was to build a foundation upon which to practice evidence-based prevention strategies to reduce the transmission risk of *S. enterica* among patients, personnel, and the environment.

Developing methods for point-of-care testing and performing objective comparisons of *Salmonella* detection methodologies were recently identified as critical needs for infection control in equine populations by an international panel of infection control experts. To that end, two experimental studies (Chapters 3 and 4) were undertaken to determine an optimal culture method to be used with commercially available lateral flow immunoassays to detect *S. enterica* within 18-24 hours in veterinary relevant samples (i.e., equine feces [2 methods] and environmental samples [4 methods]); and to assess immunoassay variability in detection of 10 different serotypes (total of 112 randomly selected isolates) commonly detected in a veterinary teaching hospital. One gram fecal samples (n=40 per each method) from a known culture-negative horse herd and environmental samples (n=20 per each method) obtained from a culture negative hospital environment were experimentally inoculated with a known concentration of *S*. Typhimurium ($4x10^0$ – $4x10^4$ cfu/g of feces or cfu/ml). In general, the limit of detection from experimentally inoculated samples was approximately 4 cfu/g. In addition, there was variability in serotype detection among the tests evaluated, however this occurred

most commonly with serotypes derived from cattle – not the target species for the intended use of these lateral flow immunoassays. The lateral flow immunoassays evaluated could reliably detect *S. enterica* within 18 hrs indicating they may be useful for rapid point-of-care testing in equine practice applications. Additional evaluation is needed using samples from naturally-infected patients and the environment to gain an accurate estimate of test sensitivity and specificity and further substantiate the true value of these tests in clinical practice.

During nosocomial *Salmonella* outbreaks in veterinary hospitals there tends to be widespread environmental contamination. Previous work indicates patient isolates can have the same phenotype (i.e., serotype and susceptibility) as environmental isolates, suggesting animals to be a likely source. Factors for animal shedding have been identified however many of these studies focus on a subset of inpatients with results being minimally generalizable to the general hospital population. The objectives of this study (Chapter 5) were, 1) to determine factors associated with fecal shedding of *Salmonella* among large animal inpatients within the general hospital population; 2) do so in comparison to two different groups of patients – a group in which there is high confidence in negative status (having at least 3 negative cultures) and a group with potential for misclassification of shedding status (at least 1 negative culture); and 3) to demonstrate that the choice of comparison group can affect resultant associations. Inpatients included in this case-control study had fecal samples collected and cultured, using standard techniques, as part of long-term infection control efforts. Factors related to patient stress and defense mechanisms were evaluated. Data on factors of interest were collected retrospectively from electronic medical records. Multivariable conditional logistic regression was used to evaluate associations between animal factors and fecal shedding of *S. enterica*.

During the study period, there were approximately 11,061 inpatients of which 5.9% (n=648) were fecal culture-positive for *S. enterica*. The majority of culture-positive inpatients were bovine (72%) and equine (22%) with the remaining being New World camelid and small ruminant. Overall, 69.4% of patient shedding could be attributed to systemic illness (i.e., population attributable fraction) in this study. The findings of this study will provide a better understanding of factors associated with fecal shedding in the general large animal inpatient population, allowing for the implementation of evidence based preventive measures. This information will be integral to risk management related to periods of epidemic as well as endemic disease.

Salmonella enterica can be an important factor in healthcare-associated epidemics and zoonotic disease in veterinary hospitals – with outbreaks of multi-drug resistant (MDR) *Salmonella* among equine patients resulting in high case fatality rates and substantial financial cost. The objectives of this study (Chapter 6) were, 1) to determine factors associated with fecal shedding of MDR-*Salmonella*; and 2) to determine what effect *Salmonella* shedding may have on health outcomes of previously hospitalized horses and their stablemates. Patients eligible for this case-control study included those having fecal cultures for *S. enterica* as part of a surveillance program from January 2011 through December 2012. Data regarding exposures of interest were collected retrospectively from medical records. Information on long-term outcomes was obtained by administering a phone survey to horse owners. Multivariable regression techniques were used to determine factors associated with shedding MDR-*Salmonella* and subsequent health outcomes. Equine patients enrolled in this study included 94 culture-positive (29 MDR and 65 susceptible) and 279 culture-negative (on at least 3 fecal

samples) horses from 199 different farms. Horses experiencing diarrhea during hospitalization were more likely to shed *Salmonella* (OR 1.88; 95% CI 1.02, 3.45) compared to horses without diarrhea; and horses having decreased feed intake during hospitalization were more likely to shed MDR-strains (OR 5.95; 95% CI 1.21, 29.20) compared to horses with normal feed intake. In general, shedding *Salmonella* did not increase the long-term risk for non-survival, colic or abnormal feces after discharge of hospitalized horses nor did it increase the risk for hospitalization or abnormal feces in stablemates. In general, receiving antimicrobial therapy during hospitalization was not associated with shedding *Salmonella*, nor was it associated with shedding of MDR-strains. Despite these findings, in order to mitigate the exposure risk to other horses and personnel, it is still recommended to manage horses shedding *Salmonella* separately from other resident horses and to employ rigorous personal and environmental hygiene.

Epidemics of healthcare-associated infections in veterinary hospitals are commonly attributed to *Salmonella enterica* and characteristically there is widespread environmental contamination identified during these times. The objective of this study (Chapter 7) was to determine risk factors associated with environmental contamination of a veterinary hospital with *S. enterica*; and secondarily to determine a suitable analytic method to model such a complex ecology. Environmental surveillance samples were collected from March 2003 through January 2013, using a commercially available electrostatic wipe, as part of a long-term infection control program. Sampling sites included both floor and hand-contact surfaces throughout the veterinary teaching hospital (VTH). Risk factors evaluated included hospital caseload, hospital use areas, severity of disease, presence of culture-positive inpatients and season. Data on risk factors of interest were collected retrospectively from the VTH electronic

medical records database. Variable cluster analysis and principal components analysis were used to understand the underlying data structure and multicollinearity. Multivariable logistic regression was performed using generalized estimating equations to determine factors associated with environmental contamination with *S. enterica* while controlling for environmental sample clustering by sample date. During the study period a total of 5273 environmental samples were collected on 167 unique sampling dates (approximately 46 samples were collected monthly). Of the samples collected, a total of 8.2% (n=434) were culture-positive for *S. enterica* using standard culture techniques. In general, *Salmonella* was most likely to be detected in environmental samples collected in the livestock hospital and from floor samples. The probability of detecting *Salmonella* in the hospital environment was associated with livestock caseload, patient disease severity, the presence of patients shedding *Salmonella*, and was affected by the types and locations of environmental samples tested. This study demonstrates the complex ecology of *Salmonella* in a veterinary hospital emphasizing the role latent (unmeasured) factors may play in driving endemic contamination to become hospital-wide and ultimately develop into an epidemic. Results of this study suggests that the probability of detecting *Salmonella* in the environment increases as the demand on personnel increases thereby emphasizing the need to remain vigilant in the practice of infection control measures that we know empirically will mitigate the risk for widespread environmental contamination and sustained transmission among patients (i.e., rigorous personal and environmental hygiene).

The relationship between *Salmonella enterica*, patients, and the hospital environment is a very complex ecology – creating considerable gaps in our understanding of its epidemiology.

A deeper appreciation of its natural history in veterinary populations is critical to improving prevention efforts – we cannot manage what we cannot measure.

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1 CHAPTER 1: INTRODUCTION

Salmonella enterica has long been recognized as a cause of disease in both humans and animals. As one of the most common causes of human foodborne illness [1.1] and of epidemic disease among patients in veterinary hospitals [1.2], it has received considerable scientific attention. Despite this, we have a limited understanding of its natural history and epidemiology.

Much of what is known about *S. enterica* in veterinary populations is derived from experimental infections and data collected from high-risk sub-groups (i.e., horses with colic or colitis) or during periods of epidemic disease. Experimental infection clearly does not reflect what occurs in nature. Consequently, extrapolation to naturally occurring disease is rather limited. While collecting data from high-risk sub-groups and periods of epidemic disease are worthwhile endeavors, it is difficult to uniformly apply these findings to the general animal population or to times of endemic disease.

Effectively managing this organism in animal populations and their environments continues to be a challenge – in part due to the limitations of currently available diagnostic tools. Animals with subclinical disease characteristically shed low numbers of organisms in feces and do so intermittently. To combat this we typically employ lengthy enrichment techniques and test multiple samples – thus delaying risk recognition and implementation of prevention strategies.

Veterinarians have an ethical responsibility to take reasonable precautions to reduce risks related to *S. enterica* among animals and their environment. Critical to this effort are

improving our understanding of the epidemiology of *Salmonella* and developing more accurate and rapid diagnostic tests [1.3]. The overarching goal of the studies contained within this dissertation was to build a foundation upon which to practice evidence-based prevention strategies to reduce the transmission risk of *S. enterica* among patients, personnel, and the environment.

We begin with a chapter on equine salmonellosis – written for Veterinary Clinics of North America, Equine Practice – as a basis upon which to apply the findings of the subsequent studies. This chapter was written with the veterinary practitioner in mind – covering details that we thought were pertinent to effectively managing *Salmonella* in equine populations. While the emphasis is on horses the concepts equally apply to all large animal species.

Next we focus on the agent – *Salmonella enterica* – and our ability to rapidly detect its presence in samples relevant to veterinary medicine (i.e., fecal and environmental samples). This is a series of two experimental studies. The first was designed to optimize a culture method that will allow for the use of commercially available rapid tests to detect *Salmonella* in veterinary relevant samples (i.e., fecal and environmental samples) within 24 hours; and the second was to assess the effect *Salmonella* serotype may have on detection rates when using these tests. We hypothesized that commercially available rapid tests would have a greater analytic sensitivity and equivalent specificity for detection of *Salmonella* when compared to traditional culture methods for equine fecal and environmental samples. These rapid tests are in a lateral flow immunoassay (LFI) platform that is easy to use, requires minimal equipment, and can be employed as a point-of-care test by practitioners in the field setting. In addition, these tests represent a significant cost savings compared to traditional detection methods (i.e.,

enriched culture or polymerase chain reaction [PCR]) allowing for many more samples to be tested. In so doing, practitioners can increase the overall sensitivity of the testing strategy. More costly testing (i.e., enriched culture) and isolate characterization (i.e., serotype and antimicrobial susceptibility) would only be pursued on LFI positive samples. While it is enticing to not pursue further isolate characterization – it is imperative to the advancement of our understanding of disease epidemiology and to effectively manage outbreaks associated with this organism.

Then we turn to the host – specifically large animal species – and factors associated with patient shedding and long-term health outcomes. To that end we conducted two different studies – 1) a case-control study to evaluate shedding among hospitalized patients; and 2) a case-control study to evaluate shedding of multi-drug resistant (MDR) strains with a follow-up study to assess health outcomes.

In the first, we had the unique opportunity to use 10 years of fecal surveillance data from long-term infection control efforts at Colorado State University Veterinary Teaching Hospital (CSU-VTH). What makes this data set so unique is its all-encompassing nature – namely surveillance was conducted on the entire large animal inpatient population while tracking the occurrence of healthcare-associated infections (HCAIs) and disease epidemics associated with *S. enterica*. This afforded us the opportunity to develop an understanding of those factors that may contribute to patient shedding and ultimately to the occurrence of epidemic disease. We hypothesized that large animal patients which have experienced increased stress (i.e., severe illness or lengthy hospitalization) or that have decreased defense mechanisms (i.e., severe illness, use of gastroprotectants, and antibiotic therapy) would be

more likely to shed *S. enterica* in feces than those large animal patients experiencing minimal stress (i.e., hospitalized for elective diagnostics or procedures). By identifying easy to measure factors or patient profiles that are associated with an increased probability of shedding – veterinarians can implement prevention strategies prior to agent detection, thereby limiting the potential for environmental contamination and nosocomial transmission.

In the second, we shifted our focus to factors associated with shedding of MDR-*Salmonella* among horses at a large referral practice in central Kentucky. While this study was much smaller – assessing 2 years of data – we were able conduct a follow-up study to address what it means to be shedding *Salmonella* for the shedding horse and its stablemates. We hypothesized that horses with longer durations of hospitalization and those receiving antimicrobial drugs (AMD) during hospitalization would be more likely to harbor MDR-*Salmonella* infections; that horses shedding MDR-strains would be less likely to survive 1 year past hospital discharge; and that previously hospitalized shedding patients would adversely affect health outcomes among stablemates (specifically hospitalization or occurrence of diarrhea). In the literature there is a single study looking at long-term health outcomes among previously hospitalized patients and their stablemates [1.4]. By asking this same question in a different population we hope to develop consistent evidence upon which to base management recommendations for patients shedding *Salmonella* after discharge from the hospital.

Lastly we focus on the environment – particularly the veterinary teaching hospital environment – and factors associated with its contamination. This was a longitudinal study that spans 10 years of routine surveillance of the hospital environment. We hypothesized that hospital environmental contamination with *S. enterica* would be more commonly associated

with the livestock hospital, multi-animal use areas, periods of high case load (specifically dairy cattle case load), and at times when there are *Salmonella* positive patients housed within the large animal facility (included the equine and livestock hospitals). Gaining a better understanding of factors associated with *S. enterica* contamination of the veterinary hospital environment will allow for more specific preventive measures to be implemented to not only protect the hospital, but to decrease the risk to patients and personnel.

The final chapter puts our results in context of current literature – summarizing our findings and reveals how each contributes to the progression of our understanding of the epidemiology of *Salmonella* in animal populations and their environments. *Salmonella* significantly impacts animal morbidity and mortality. Thus it deserves research efforts that will allow for veterinarians to put into practice evidence-based infection prevention strategies motivated by the epidemiology.

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2 CHAPTER 2: MANAGING *SALMONELLA* IN EQUINE POPULATIONS

2.1 INTRODUCTION

Congregating animals from multiple sources as occurs at veterinary hospitals, racetracks, equestrian events, and boarding and training facilities, increases the risk for transmission of infectious diseases such as *Salmonella* [2.1]. The purpose of this chapter is to provide equine practitioners with details relevant to effectively managing *Salmonella* in these populations. We will begin by focusing on the agent – *Salmonella enterica* – to develop an appreciation for its key features including the nuances of organism detection and test interpretation. We will then consider the fundamentals of veterinary infection control with the intent of developing a foundation that can be applied to both the hospital and field settings. Finally, we will demonstrate how infection control principles and understanding the epidemiology of *S. enterica* can facilitate managing transmission risks related to this organism in hospital populations and in the field setting. Detailed descriptions of bacteriology, pathophysiology and treatment are beyond the scope of this chapter.

Importance of *Salmonella* in equine populations

As one of the most common causes of epidemic disease in veterinary hospitals [2.2] and an agent frequently associated with on-farm contamination [2.3], significant efforts are made to control its transmission among animals, especially within equine hospitals. Unfortunately these efforts are predominantly based on first principles as many prevention methods in veterinary medicine have not been critically evaluated in clinical studies. Regardless, we know that outbreaks attributed to *Salmonella* can come at a great cost, both in case fatality rate and

financial expenditures, and presents a very clear risk to veterinary patients and personnel working with these animals [2.4, 2.5]. Veterinary practitioners have an ethical obligation to appropriately manage risks related to *Salmonella* in animal populations and their environment. There is a recognizable standard of practice with respect to infection control and due effort must be given to control and prevention of infectious disease transmission within animal populations and facilities [2.6].

When *Salmonella* spreads among patients, environmental contamination is predictably present – whether as cause or effect [2.4, 2.7-2.9]. Additionally, subclinical infections and shedding in the absence of disease is much more common than clinical infections [2.8, 2.10]. Unfortunately, testing strategies of relevant veterinary samples (i.e., fecal and environmental samples) for the presence of *Salmonella* is variable among laboratories and current testing methodology generally lacks in sensitivity; likely due to the intermittent nature and low level of organisms shed in animal feces. Therefore, testing strategies generally require testing of multiple samples, lengthy enrichment steps, and 3-5 days to realize results. In that time, significant environmental contamination and disease transmission can occur. Consequently, risk recognition and the ability to rapidly identify these patients are critical to effective management of populations and their environments.

2.2 *SALMONELLA* – THE BASICS

Salmonella enterica, a member of the family Enterobacteriaceae, is a Gram negative facultative anaerobic bacterium found colonizing the small intestine, cecum, and colon of both cold and warm blooded vertebrates. There are over 2400 serotypes which are distinguished by the presence of differing O-antigen (polysaccharide portion of lipopolysaccharide) and H-

antigen (filamentous portion of flagella or flagellin) on the bacterium surface. *S. enterica*, subspecies Enterica, the focus of this review, accounts for approximately 59% of all serotypes and is responsible for approximately 99% of salmonellosis in warm blooded animals [2.11, 2.12].

S. enterica is considered an opportunistic pathogen – more likely to cause clinical disease in situations of high exposure or patients that have an increased susceptibility such as neonates and patients with severe systemic illness. Transmission occurs by the fecal-oral route and can result in enterocolitis (i.e., diarrhea), bacteremia, or subclinical infection – with infection dependent upon the infective dose, host susceptibility, and the infecting serotype. As such, identifying subclinical fecal shedding, managing contacts among patients, and practicing effective personal and environmental hygiene are critical for protecting animals and people.

2.3 *SALMONELLA* TESTING AND INTERPRETATION

There are many methods available for the detection of *Salmonella enterica* in samples relevant to veterinary medicine – including enriched culture, polymerase chain reaction (PCR), and lateral flow immunoassays (LFIs) – all of which require varying levels of expertise, cost, and time to detection. Practitioners should be aware of the different testing methods available and know which method is being employed upon laboratory submission as this may affect test interpretation.

2.3.1 Culture of fecal samples

There are limitations to the detection of *Salmonella* when culturing fecal samples. Experimentally, the analytic sensitivity of equine fecal culture has been found to be as few as 4 cfu/gram of feces when enriched in tetrathionate broth [2.13] and 100 cfu/gram of feces when

enriched in selenite broth [2.14]. However, in practice, fecal culture is a relatively insensitive detection method. This is likely due to intermittent shedding of relatively few organisms per gram of feces [2.15, 2.16], as well as the heterogeneous distribution of organisms within fecal samples [2.17]. With this in mind, it can take up to 3 days to realize test results for a single fecal sample – due to lengthy culture processes (i.e., enriched cultures) – and typically 3-5 samples per animal to achieve reasonable sensitivity.

The reliability of bacterial culture for *S. enterica* detection can be affected by the type of sample (feces, swab, or rectal biopsy), heterogeneity of target organism in the sample, sample weight, intermittent shedding, bacterial culture method, and laboratory proficiency. In general, a fecal culture is a more sensitive detection method than a rectal swab – this is likely due to the sample weight/volume with a larger weight/volume resulting in higher test sensitivity [2.18]. Organisms such as *Salmonella* tend to cluster within a fecal sample rather than be homogeneously distributed – therefore testing of a small aliquot (e.g., swabs or <1 gram) may result in a false-negative test results as there is a higher probability that a single sample will not contain any *Salmonella* organisms even though the animal is actively shedding at low levels [2.17]. As stated, the relative sensitivity of culture increases with increasing sample weight and this can be improved upon by thoroughly mixing the sample (e.g., with a paddle blender) [2.17, 2.18]. Alternatively, culture of a rectal mucosal sample can be performed with a reported greater sensitivity than fecal culture – although given the invasive nature of the sample it may best be reserved for those "difficult-to-sample" cases which have scant or liquid feces with minimal solid material [2.19].

There are many different methods that can be used for aerobic culture of *Salmonella* which employ a wide variety of broth and solid culture media, as well as incubation times and temperatures [2.20]. These different methodological choices lead to differences in test accuracy and time until results are reported. This ought to be carefully considered by laboratories, and practitioners should have a general understanding of how methodological choices affect the ability to detect *Salmonella.* A detailed review of culture techniques for *Salmonella* is beyond the scope of this chapter. In general, using an enrichment step with *Salmonella* selective media (e.g., tetrathionate [TET], Rappaport-Vassiliadis [RV]) will improve overall test sensitivity – allowing *Salmonella* to grow while inhibiting the growth of competing bacteria, thus enabling detection on selective plating media (e.g., xylose-lysine-tergitol 4 [XLT4] or hektoen enteric agar). Pre-enrichment of samples with low bacterial burdens (e.g., environmental samples) with non-selective media (e.g., buffered peptone water [BPW]) can aid in recovery of bacteria that are damaged or stressed because of environmental conditions; but use with samples containing high bacterial burdens (i.e., fecal samples) may actually be counterproductive as this may allow overgrowth of competing bacteria resulting in a falsely negative test result. In general, samples should be kept refrigerated and processed as soon after collection as possible. However, the proportion of test-positive samples has been shown to not differ significantly when processed the same day, after 6 days of refrigeration (4°C), or after 14 days of freezing (-15°C) [2.21], suggesting that recovery is not greatly impaired, if at all, when samples are kept cool before cultures are initiated.

We have found that the proficiency of laboratories in their ability to detect *Salmonella* in enriched cultures can vary dramatically. Some of these differences are undoubtedly

attributable to use of less than optimal culture methods (e.g., very harsh enrichment media or lower culture temperatures). In addition, some laboratories can have substantially lower recovery rates than other laboratories when using the same culture methods, even among laboratories that routinely process fecal samples for *Salmonella* culture. This highlights the importance of asking laboratories to provide documentation of training and proficiency testing (e.g., "check" tests) when selecting a laboratory to perform *Salmonella* cultures.

2.3.2 Culture of environmental samples

Salmonella is an organism which is relatively hardy in damp environments that contain organic debris – exhibiting the ability to develop biofilms and environmental reservoirs which serve as potential sources for infection. When performing environmental surveillance the type of sample collection device and testing method should be carefully considered, as sensitivities of each will likely differ. For example, electrostatic wipes have been found to be an effective and more sensitive collection method as compared to sterile sponges for detection of Salmonella in the hospital environment [2.22, 2.23]. These differences can be attributed to both the collection method (the device used and the size of the surface area sampled) as well as the culture method. In general, sampling a larger surface area will not only provide a more representative sample but will likely be a more sensitive method for organism detection. Organisms in the environment may be "injured" due to harsh environmental conditions (e.g., drying and UV light) and due to exposure to disinfectants. As such, general practice is to perform a pre-enrichment step in a nutrient rich media (e.g., buffered peptone water [BPW]) prior to performing an enriched culture. Although this extends the lag time required to obtain

results, it allows for injured or damaged organisms to repair themselves before being exposed to the relatively harsh enrichment media – thereby improving overall testing results.

2.3.3 Polymerase chain reaction

Polymerase chain reaction (PCR) is generally considered to be a highly sensitive and specific method of *Salmonella* detection. Most PCR assays use probes that target highly conserved bacterial genes allowing detection of many different *Salmonella* serotypes without cross-reaction with other common bacteria (e.g., Enterobacteriaceae spp.) [2.24]. Polymerase chain reaction assays have been reported to have an analytic sensitivity of 100 cfu/gram of equine feces when testing overnight broth cultures or 1000 cfu/gram of feces when testing non-enriched samples. They are also generally more rapid than bacterial culture, providing results in 1-2 days compared to 2-5 days for many enriched culture methods [2.14, 2.24-2.26]. Consequently, PCR may be particularly useful with samples containing low numbers of organisms and for times when it is important to obtain results quickly (e.g., during epidemics). While PCR may be useful for earlier detection of shedding and environmental contamination as compared to culture, it does not necessarily detect viable organisms. Therefore, a positive PCR results may not necessarily be indicative of infection risk related to environmental contamination. It is also important to note that PCR does not replace the need for culture as it is very important to have more than dichotomous (positive/negative) results to facilitate epidemiological investigations and ongoing surveillance. It is impossible to determine the likelihood of healthcare-associated transmission unless additional information is available for strain differentiation, such as antimicrobial susceptibility, serogroup, serotype, or optimally the

pulse field gel electrophoresis (PFGE) profile (i.e., DNA "fingerprint"). As such, PCR testing should always be paired with culture of PCR-positive samples.

Polymerase chain reaction is often considered a more sensitive detection method than enriched culture for fecal and environmental samples. However, there is much debate as to the reason for this apparent higher positive detection rate, or even whether this is consistently true. In theory, PCR can detect non-viable organisms as well as degraded DNA, which may account for some of this difference. It is also clear that suboptimal laboratory methods and laboratory proficiency can likewise affect this observation. Additionally, low numbers of organisms contained within samples, or poor test specificity of PCR can also affect apparent test accuracy [2.26-2.28]. A caveat to using PCR as a method of detection for environmental samples is that disinfectants target different parts of bacterial organisms. For example quaternary ammonium and phenolic disinfectants target cytoplasmic membranes leaving DNA intact whereas bleach and formaldehyde degrade DNA which could theoretically lead to differences in PCR detection rates [2.29, 2.30].

2.3.4 Lateral flow immunoassays

Commercially-available lateral flow immunoassays (LIAs) have been developed for use in food safety microbiology, and have shown promise as practical alternatives to traditional culture and PCR methods for detection of *Salmonella* in animals and their environments [2.13, 2.31]. Lateral flow immunoassays have been shown to have an analytic sensitivity of ~4 cfu/gram from enriched cultures of experimentally-inoculated equine fecal samples, and can reliably detect *S. enterica* in 1-gram samples after only 18-hrs in selective broth culture [2.13]. The use of these tests does not require any specialized training or equipment – just the

purchase of an incubator and pre-made media are all that are necessary. Although there may be some differences in the ability to detect different strains (serotypes) of *Salmonella*, their low cost, ease of use, and reliability make them an appealing option for point-of-care testing in equine practice [2.32]. Just as with PCR, it is important to pair LFI testing with follow-up culture of samples that are LFI-positive. In addition to characterization of isolates, it also allows for epidemiological investigation and assessment of transmission risks in populations.

2.3.5 Testing strategy and test interpretation

Detecting *Salmonella* in equine practice can be challenging – as horses frequently shed low numbers of organisms and do so intermittently, except in extreme situations which may or may not be accompanied by clinical disease [2.15, 2.16]. Regardless of the analytical sensitivity of test methods, this causes the overall detection system (i.e., sample type combined with sample processing and detection method) to have poorer epidemiological sensitivity (i.e., lower probability of detecting truly infected/shedding horses). While testing larger sample volumes will, up to a limit, improve test sensitivity [2.18], it is also helpful to test multiple samples. Interpreting the results in parallel for multiple tests performed on the same patient has the benefit of greatly improving the overall sensitivity of the testing strategy.

Research suggests that a truly positive patient is more likely to culture-positive with increased number of samples tested [2.33]. The generally accepted application of this idea is that a minimum 3-5 negative cultures should be obtained in a short time frame (i.e., sampling at 12-24 hr intervals) to be reasonably sure that patients have a low risk of *Salmonella* shedding [2.15]. Assuming independence of test results, it has been previously reported that the sensitivity for a series of fecal cultures using selective enrichment were 44% for a single culture,

66% for two cultures, 82% for three cultures, and 97% for 5 cultures [2.34]. Thus, by obtaining a series of 3-5 negative cultures a practitioner can be reasonably confident that a horse is truly negative.

Regardless of the detection strategy employed – many different *Salmonella* serotypes can cause disease and the distribution of serotypes can change over time [2.35]. Thus it is important to ensure that the test being employed can detect many different serotypes, especially those commonly detected in a given geographic or practice location. In addition, when evaluating tests, how it performs on the bench top may differ from samples relevant to veterinary medicine (i.e., fecal and environmental samples), therefore methods should be appropriately validated and optimized for their intended use on veterinary relevant samples.

2.4 FUNDAMENTALS OF VETERINARY INFECTION CONTROL

Many of the practices used in veterinary infection control have not been scientifically evaluated. However we can learn from infection control strategies applied in human healthcare. In the Study on the Efficacy of Nosocomial Infection Control (SENIC), conducted in U.S. human healthcare facilities (1970-1976), the implementation of an infection control program reduced nosocomial infections by an estimated 32%. Importantly, the minimum components needed for programs to achieve this impressive reduction in infection risk were simply to identify a person to oversee infection control activities, conduct some type of surveillance activity, and maintain a system for reporting [2.36]. While similar data is lacking in veterinary medicine it is not unrealistic to presume similar measures may be effective. In a recent epidemic of multi-drug resistant *S*. Newport, an ineffective infection control program

was sighted as an important factor in the outbreak which resulted in patient fatalities, hospital closure, and an estimated financial cost of US$4.1 million [2.4].

Infection control is achieved through all efforts used to prevent the introduction and limit the spread of contagious pathogens within a facility or population – with the goal of eliminating sources of potentially pathogenic microorganisms and to disrupt infectious disease transmission. In veterinary hospital settings, this is a challenge as we are purposefully caring for patients with infectious diseases in the midst of animals whose resistance to disease may be compromised; and we are doing so in an environment where animals from many different farms congregate.

There are several types of preventive measures that can be used to decrease infectious disease transmission risk – including optimizing environmental and personal hygiene, and managing patient movement and contacts during hospitalization. While every equine facility is distinctive with its own physical and operational features, necessitating the molding of infection control efforts to each facility's specific needs, all programs are based on these shared infection control principles. Detailed descriptions for program development have been depicted elsewhere [2.37, 2.38]. In addition, there are many available online resources that can facilitate program development [2.39-2.41]. Although the choice of policies governing prevention efforts will be facility specific, it is important that they are designed with all animals in mind, not just those suspected of harboring an infectious disease. Consideration should be given to establishing distinct hospital areas for which to manage neonates, patients with severe disease (e.g., colic or systemic illness vs elective surgery), inpatients versus outpatients, and species

(e.g., horse vs cattle). There are many examples of this including intensive care units, isolation facilities, and having separate equine and livestock hospitals.

2.5 MANAGING *SALMONELLA* RISK IN HOSPITAL POPULATIONS

2.5.1 Patient Management

Managing *Salmonella* in populations can be particularly challenging, in part because of the wide diversity in clinical consequences of infection – ranging from asymptomatic, intermittent shedding to acute diarrhea and fever with neutropenia to septicemia and death. Additionally, horses recovering from naturally occurring acute salmonellosis can shed for extended periods of time – one-third shedding for up to 30 days [2.34], and can do so intermittently. Veterinarians have the challenge of caring for the patient standing before them but must consider the population of tomorrow in order to effectively control healthcare-associated infections (HCAIs).

2.5.1.1 Factors associated with epidemic disease

Nosocomial outbreaks of salmonellosis – representing a climactic meeting of patient and hospital factors – have been repeatedly shown to be a constant risk in all types of veterinary hospitals, resulting in significant morbidity and mortality among hospitalized patients and zoonotic infections in personnel [2.4, 2.7, 2.8, 2.30, 2.42]. Commonly, sub-standard environmental hygiene is identified as a contributing factor – including ineffective infection control policies, floor surfaces which allow contamination to accumulate, and use of porous, non-cleanable surfaces in other construction such as unsealed concrete and wood [2.4, 2.7, 2.30]. Additionally, common use equipment (e.g., buckets, nasogastric tubes, and rectal thermometers) and periods of high caseload with limited personnel have been found to impact

the occurrence of HCAIs [2.7, 2.9, 2.30]. In the course of epidemics, horses with severe disease, such as those with colic or undergoing abdominal surgery, are frequently identified as shedding *Salmonella* and likely contribute to ongoing environmental contamination and transmission among hospitalized patients [2.7, 2.9, 2.43, 2.44].

2.5.1.2 Factors associated with endemic disease

During outbreaks, there is typically widespread environmental contamination and it is not uncommon for patient and environmental isolates to be phenotypically similar (i.e., serotype and antimicrobial susceptibility) – this phenomenon has also been identified during times of endemic disease, suggesting animals to be a likely source for this contamination [2.4, 2.8, 2.23, 2.45].

Historically, *Salmonella* shedding among horses has been associated with a triad of clinical signs – diarrhea, fever and leukopenia – based on early studies and observation [2.46, 2.47]. Indeed, a recent case-control study lends support to this observation finding horses with acute colic with clinical signs of fever (rectal temperature >103°F), diarrhea and abnormal leukocyte count (≤4,500 cells/µl or leukocytosis ≥12,500 cells/µl) were more likely to shed *Salmonella* in feces and reflux in the first 5 days of hospitalization [2.48]. A meta-analysis of studies experimentally inoculating healthy animals (horses, cattle, sheep), found that on average, pyrexia occurred within 1.5 days of infection (95% CI 1.47, 1.55) and diarrhea occurred within 1.7 days of infection (95% CI 1.62, 1.83) [2.49]. The 43 studies included in this meta-analysis used inoculating doses from 10^4-10^{13} – in natural infection the infective dose is expected to be at least at the low end of this range and is likely to be lower. Thus, the average times to onset reported here are probably more rapid than what would be expected in natural

infections. Interestingly, this study also reported an average time to shedding of 1.3 days (95% CI 1.22, 1.39) after inoculation suggesting that by the time fever and/or diarrhea are apparent, animals are frequently shedding *Salmonella* in their feces. This emphasizes the utility of identifying specific factors or groups of factors associated with shedding that are easily recognizable, thus allowing prevention strategies to be implemented more rapidly – before it becomes an epidemic.

The duration from exposure to fecal shedding can be affected by serotype, inoculating dose, as well as the health status of the horse. Time to shedding varies by serotype, ranging from approximately 3 day to 5 days among naturally infected horses, depending on the infecting serotype [2.43]. This is in contrast to experimental inoculation which has shown shedding to occur within 1.3 days (95% CI 1.22, 1.39) [2.49] which suggests that infecting dose may also play a role as experimental inoculations are general at much higher doses than what would be expected to occur naturally. Not only can time to shedding vary by serotype, it may also be affected by health status. Days from admission to shedding among horses presenting for gastrointestinal disease can range from 1 day to 3.5 days for serotypes Saint Paul and Java, respectively [2.33], likely representing an increased susceptibility in this compromised subgroup of horses, but may be due to variation in virulence among serotypes and strains.

It has been suggested that horses with more severe disease are more likely to shed detectable quantities of *Salmonella* in their feces. In a recent study, horses admitted for acute colic (excluding those presenting with diarrhea) were more likely to shed *Salmonella* with surgical management versus medical management and with more severe disease (e.g., inflammatory and vascular compromising conditions) versus those with simple colic (e.g.,

simple obstruction and non-strangulating lesions) resolving with minimal medical management suggesting that horses with more severe disease are more likely to shed [2.48]. While this seems to make biological sense – it is important to note that over the years this has not been a consistent finding. Many studies have evaluated shedding risk among horses with gastrointestinal disease. Some studies reported an increased risk associated with abdominal surgery [2.33, 2.48] while others reporting no association with abdominal surgery [2.9, 2.50, 2.51]. In addition, while it is commonly believed that antimicrobial therapy is associated with an increased probability of shedding, this too has been inconsistently reported [2.9, 2.33, 2.50-2.52].

While horses with severe disease are probably more susceptible than healthy horses, they are also more likely to have fecal samples tested – thus extrapolating findings from studies observing limited patient populations should be done with caution. Studies to determine factors associated with endemic shedding among the general patient population have found that both patient and hospital factors may be important [2.52-2.55]. Patients with systemic illness – regardless of body system affected – and those having any one of the classic triad of clinical signs (fever, diarrhea, or leukopenia) have a higher likelihood of shedding, but these are not the only animals that shed *Salmonella*. In other words, these are specific indicators for identifying shedding, but they are not perfectly sensitive. Patient management factors may also play a role; with transportation distance (patients within 20 miles having a greater risk), antimicrobial therapy (specifically being treated with aminoglycosides), and duration of hospitalization affecting the probability of shedding [2.55]. Species or rearing circumstances may be an important hospital factor as well, with intensively managed cattle being much more

likely to shed than horses – an element that should not be overlooked when managing horses in a multi-species hospital [2.55]. Further, reports consistently show a seasonal occurrence to shedding – by and large it is highest in late summer and early fall and lowest in the spring [2.3, 2.15, 2.33, 2.35]. Thus studies evaluating a limited time-frame may underestimate risk factor contributions to overall patient shedding. There are also large regional differences in the shedding prevalence that has been found in similar species and rearing conditions – shedding appears to be much more likely to occur in the warmer and wetter regions of North America compared to cooler and dryer regions [2.3].

2.5.2 Sub-populations and *Salmonella* risk

Managing *Salmonella* in horse populations is challenging as horses can shed intermittently and often in the absence of clinical signs [2.10]. Patient shedding prevalence can vary markedly from as few as 0.5% up to 7% with horses tested on admission typically having a lower prevalence than horses tested throughout hospitalization [2.3, 2.15, 2.53, 2.54]. In contrast, horses admitted for elective procedures (i.e., musculoskeletal disease, cryptorchidism) and as hospital companions are less likely to shed [2.54] and healthy horses in the general equine population have an estimated shedding prevalence of 0.8% [2.3]. Thus differential patient management may be warranted for patient population subgroups identified as being at a high risk for *Salmonella* shedding on admission or throughout hospitalization.

2.5.2.1 Horses with gastrointestinal disease

Many facilities manage horses with gastrointestinal disease or colic separately from the general patient population as this subgroup has an increased likelihood of shedding with prevalence ranging from 4.3% up to 13% [2.33, 2.51, 2.54]. Factors associated with fecal

shedding among these patients includes transportation distance (travel time of greater than 1 hour), abnormal findings on nasogastric intubation, diarrhea, leukopenia (≤ 5000 WBC/µl), previous antimicrobial therapy, abdominal surgery, and duration of hospitalization [2.15, 2.33, 2.48, 2.51].

2.5.2.2 Horses with severe disease

Horses admitted to the critical care unit are also more likely to shed *Salmonella* during hospitalization [2.56]. This subgroup of horses, along with critical neonates, likely represents patients with greater disease severity – as well as susceptibility to infection – when compared to the general hospital population. Equine neonates present a particular challenge. Foals are typically unable to stand, require intensive management, and, for foals with gastrointestinal disease, are at higher risk for shedding *Salmonella* compared to adults [2.33]. For patients comprising the general inpatient population, approximately 70% of shedding risk can be attributed to systemic illness (i.e., the population attributable fraction) regardless of body system affected – with more severe disease having a higher probability of shedding [2.55].

2.5.3 *Salmonella* surveillance among patients in clinical practice

Routine surveillance by testing fecal samples may be an effective means to identify *Salmonella* shedding among the general inpatient population but careful consideration should be given to how this might be incorporated into an infection control program (including cost and ability to manage positive patients). Targeted surveillance of horses presenting for acute colic or diarrhea or developing diarrhea during hospitalization has been shown to be an effective method for identifying fecal shedding however research suggests that many horses can be shedding *Salmonella* in the absence of clinical signs [2.8, 2.29, 2.48]. A recent case-

control study found that most horses presenting for acute colic were identified as shedding *Salmonella* through routine untargeted surveillance of all inpatients (64.4%, excluding isolation patients) rather than on admission (6.8%) or targeted surveillance triggered by the infection control program (28.8%) [2.48]. Suggesting that, depending upon the types of patient seen at a practice, routine patient surveillance may be warranted.

Alternatively, a facility may find it more cost effective to focus on syndromic surveillance – a method that has been shown to be effective at detecting adverse events in hospitalized horses [2.57]. When applying this technique to horses with *Salmonella*, however, it may be a challenge as not all patients shedding *Salmonella* will develop signs. For example, historically *Salmonella* shedding has been associated with a triad of signs – diarrhea, fever, and leukopenia. However, in a recent study only an estimated 2.7% of shedding could be attributed to this constellation of signs (i.e., the population attributable fraction) – whereas approximately 70% of shedding could be attributed to either systemic illness or gastrointestinal disease [2.55]. Given this, practitioners may elect to differentially manage and conduct targeted surveillance of those patients with more severe disease or gastrointestinal disease.

Finally, environmental surveillance for *Salmonella* may also be a cost effective means for detecting patient shedding as environmental contamination is commonly detected near where positive patients are managed [2.23, 2.52]. This could be conducted as routine or periodic surveillance of high-traffic areas such as examination areas or alleyways. If contamination is detected, not only can infection control measures be heightened but more extensive patient testing could be undertaken to facilitate mitigation efforts.

2.5.4 Management of the hospital environment

Incorporating environmental surveillance into clinical practice is a common method for managing risks associated with *Salmonella* in populations. Active surveillance of patients and the environment can serve to detect endemic shedding among patients and to identify outbreaks early in their course – thereby limiting the overall consequences [2.8]. Research shows that isolates recovered at times of endemic and epidemic disease can be phenotypically linked (serotype and antimicrobial susceptibility) to animal isolates suggesting animals as a likely source for environmental contamination and ongoing transmission [2.23, 2.45]. In addition, recovery of genetically related *Salmonella* isolates during routine patient and environmental surveillance over an extended period of time suggests environmental persistence and nosocomial transmission – despite the implementation of a rigorous infection control program [2.58]. As such, environmental hygiene and surveillance are critical to eliminating reservoirs for infection within the hospital environment.

When incorporating environmental surveillance into practice, careful consideration should be given to locations being sampled and type of samples being collected (e.g., floor contact surface, hand-contact surface, or a composite sample of both those surfaces). For example, for those practicing in a mixed species practice, samples collected in areas used to manage livestock are more likely to be culture-positive, as are samples collected from floor-contact surfaces or composite samples (but hand-contact samples may be the more important with respect to transmission risk) [2.59]. In addition, sample collection and detection method, laboratory selection, and available resources (both financial and personnel) should be taken into consideration. Methods should be appropriately validated and optimized for their

intended use and practitioners should understand that different collection and testing methodologies can result in different test sensitivity [2.22]. In general, sampling a larger surface area will provide a more representative sample and will likely be a more sensitive method for detecting *Salmonella* in the environment.

Environments in veterinary hospitals can be frequently contaminated near where positive patients are managed (e.g., equine isolation, livestock hospital, calf isolation) with floor samples, floor drains, cracks and crevices being common sights for contamination [2.23, 2.29, 2.60, 2.61]. It is imperative to maintain non-porous, cleanable surfaces throughout the hospital environment as epidemics are commonly associated with in-stall matting and surfaces such as unsealed concrete and wood [2.4, 2.7, 2.30]. While environmental contamination cannot be completely eliminated, the goal is to reduce contamination of the environment with potential pathogens to a level that becomes biologically irrelevant. To gain meaningful information, environmental testing should be performed regularly to establish a baseline level of environmental contamination to which future findings can be compared. In this way potential environmental reservoirs of Salmonella can be detected and cleaning effectiveness can be continually monitored.

2.6 MANAGING *SALMONELLA* RISK IN THE FIELD SETTING

Salmonella is not just one of the most common causes of hospital outbreaks [2.2] – it is also frequently detected on equine operations and farms [2.3] and is a recognized cause of farm outbreaks and disease in personnel [2.62-2.64]. In general, horses in the general equine population are considered healthy – an estimated 0.8% shed *Salmonella* in their feces [2.3].

Though much of this chapter has focused on managing *Salmonella* risk in the hospital setting – the same infection control fundamentals equally apply. Namely, prevent disease introduction and transmission between facilities and among animals by breaking the cycle of transmission and practicing rigorous hygiene. On-farm infection control practices are largely owner dependent. In a survey of Colorado boarding facilities, only 50% of facility managers reported isolating new horses from resident horses and only 6.6% isolated resident horses returning to the farm after travel [2.65]. Among U.S. equine operations with at least 5 resident horses, approximately 78% had non-resident horses arriving on farm [2.66]. While the risk of exposure to non-resident horses increased with operation size, so too did the likelihood of implementing some biosecurity measures such as entry requirements for personnel.

Clearly, it is important for the practitioner to maintain a minimum standard of infection control – whether in the clinic or in the field. As the ambulatory practitioner is moving from farm to farm – it is critically important to maintain a high level of hygiene within the practice vehicle, with respect to multi-use equipment, and with outer attire worn on an individual farm. There are many online resources available to help facilitate on-farm infection control program development [2.39-2.41] as well as published resources on program development [2.37, 2.38] and outbreak investigation and control [2.67, 2.68].

2.7 CONCLUSION

Recently, an international panel of infection control experts identified critical needs for infection control in veterinary populations – specifically expanding the epidemiologic knowledge with respect to *Salmonella* in equine populations and its detection methodology [2.6]. Recognizing the challenges faced by practitioners in managing this agent is the first step

to improving its control. Despite the nature of this organism to be shed intermittently, at low levels, and often subclinically – due effort must be employed to mitigate associated risks, whether in the hospital or field setting. Veterinary practitioners have an ethical responsibility to appropriately manage risks related to *Salmonella enterica* in animal populations and their environment.

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3 CHAPTER 3: RAPID *SALMONELLA* DETECTION IN EXPERIMENTALLY-INOCULATED EQUINE FAECAL AND VETERIANRY HOSPITAL ENVIRONMENTAL SAMPLES USING COMMERCIALLY AVAILABLE LATERAL FLOW IMMUNOASSAYS

3.1 SUMMARY

Background: *Salmonella enterica* is the most commonly reported cause of outbreaks of nosocomial infections in large animal veterinary teaching hospitals and closure of equine hospitals. Rapid detection may facilitate effective control practices in equine populations. Shipping and laboratory testing typically requires ≥48-hours to obtain results. Lateral flow immunoassays (LFIs) developed for use in food safety microbiology provide an alternative that have not been evaluated for use with feces or environmental samples.

Hypothesis/Objectives: Identify enrichment methods that would allow commercially available rapid *Salmonella* detection systems (LFIs) to be used in clinical practice with equine fecal and environmental samples, providing test results in 18-24 hrs.

Study design: Experiment

Animals: n/a

Materials and Methods: Equine fecal and environmental samples were inoculated with known quantities of *S. enterica* serotype Typhimurium and cultured using two different enrichment techniques for feces and four enrichment techniques for environmental samples. Samples were tested blindly using two different LFIs and plated on agar media for confirmatory testing.

Results: In general, commercial LFIs had fewer false negative test results with enrichment of 1 g fecal samples in tetrathionate for 18 hrs, while all environmental sample enrichment

techniques had similar detection rates. The limit of detection from spiked samples, approximately 4 cfu/g, was similar for all methods evaluated.

Conclusions and clinical importance: This study demonstrated that commercially available LFIs may be useful tools to aid management of *S. enterica* in horses. The LFIs evaluated could reliably detect *S. enterica* within 18 hrs indicating they may be useful for rapid point-of-care testing in equine practice applications. Additional evaluation is needed using samples from naturally-infected patients and the environment to gain an accurate estimate of test sensitivity and specificity and further substantiate the true value of these tests in clinical practice.

3.2 INTRODUCTION

Salmonella enterica is the most commonly reported cause of outbreaks of nosocomial infections in large animal veterinary teaching hospitals and the most common cause of closure of equine hospitals at these facilities [3.1]. Congregating horses from multiple sources, as is common at breeding farms, racetracks, or equestrian events, is also associated with increased risks for spread of contagious diseases such as *Salmonella*. Additionally, horses returning home from veterinary hospitals or other facilities can serve as a source of infection for others. Control of *Salmonella* is further complicated by the fact that subclinical infection and shedding are much more common than clinical infections and horses can shed infectious doses of *Salmonella* in the absence of disease [3.2]. Further, significant environmental contamination is inevitably present when *Salmonella* spreads between horses, whether as a cause or effect, and it is well documented that environments in equine facilities that appear clean can still be contaminated with *Salmonella* [3.3-3.5].

Developing methods for point-of-care testing and performing objective comparisons of *Salmonella* detection methodologies were recently identified as critical needs for infection control in equine populations by an international panel of infection control experts [3.6]. Rapid and reliable testing methods for *S. enterica* in environmental and fecal samples are considered essential for facilitating effective infection control in horse populations. Enriched aerobic culture and polymerase chain reaction (PCR) are currently the most common detection methods employed by veterinarians to identify *Salmonella*. These typically require 48-96 hrs and 24-72 hrs from submission to reporting, respectively, in addition to time needed for transportation of samples to the laboratory. Unfortunately, there are several overarching problems for these tests when used to detect *Salmonella*, including poorly standardized and laborious testing methodologies, poor sensitivity, the lengthy time needed to obtain results, limited availability of tests that can be used in practice settings, and the costs for testing.

Recently, commercial tests have been developed for use in food safety microbiology which may provide a practical and useful alternative to traditional culture and PCR methods when practitioners are screening for *Salmonella*. Two of these commercial rapid diagnostic tests kits for *Salmonella enterica* detection are marketed for point-of-use testing of a variety of food items including, but not limited to, raw ground beef and chicken, beef and pork skin, chicken rinse water, and poultry feed [3.7, 3.8]. These rapid tests are immunoassays that use antibodies specific for surface antigens of *Salmonella* and colloidal gold-antibody conjugates incorporated into a lateral flow test strip. They are commercially available from the manufacturers for relatively low cost, simple to use and require minimal equipment. In theory, these lateral flow immunoassays (LFIs) could be economically employed in point-of-care testing

providing results with reasonable sensitivity in 24 hrs for fecal samples and within 24 to 48 hrs for environmental samples. This would allow more extensive yet less expensive use of screening to control *Salmonella* in veterinary settings. Cultures would still be needed to provide antimicrobial susceptibility information and for follow-up testing (e.g., genetic characterization) to allow identification of nosocomial transmission, but this could be limited to culture of samples which are test-positive with the rapid test strip.

Use of a rapid *Salmonella* detection system represents a savings not only in cost but, just as importantly, in time. Early detection of animal shedding or environmental contamination would allow facility managers to more effectively implement measures to decrease animal and zoonotic infections. The objectives of this study were to evaluate different enrichment methods for use with two commercially available lateral flow immunoassays for use with equine fecal and environmental samples. The protocols that we evaluated were designed so that they might be easily implemented at veterinary practices to obtain test results within 24-48 hrs of sample collection.

3.3 MATERIALS AND METHODS

Study Summary: Culture method optimization was undertaken for use with two commercially available lateral flow immunoassays in equine fecal and environmental samples to enable the detection of *S. enterica* within 24- and 48-hours, respectively (Figure 3.1). A *S. enterica* serotype Typhimurium isolate that had been previously recovered from a patient at the Colorado State University was used for inoculating 1-g and 10-g fecal samples; 10 samples inoculated with 10^0, 10^1, and 10^2 cfu/g (30 total), and five samples inoculated with 10^3 and 10^4cfu/g (10 total), and 10 uninoculated control samples were evaluated.

Fecal samples: A composite pool of feces was used in each round of this study to provide a uniform sample matrix and microbiome. This was created by collecting feces from each of 5 horses considered to have a very low risk for *Salmonella* infection as they were members of a university owned, isolated herd which is tested periodically and was repeatedly culture-negative prior to study initiation. A *S. enterica* serotype Typhimurium isolate that had been previously recovered from a patient at the Colorado State University was used for inoculating fecal samples. A stock culture was made by inoculating this isolate into 10 ml of tryptic soy broth[1] (TSB) and incubating overnight at 43°C. The bacterial concentration of the overnight culture was determined by plating 10-fold dilutions on blood agar[1] (TSA), incubating overnight at 43°C, and calculating an estimate for the original culture concentration (~$4x10^8$ cfu/ml).

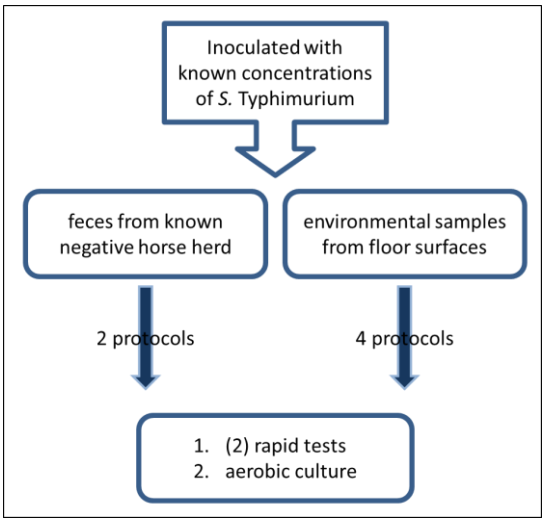

Figure 3.1 Overview of Culture Method Optimization

Ten-fold dilutions of overnight broth cultures were made and 1 ml/g were used to inoculate pooled 1-g and 10-g fecal samples to achieve concentrations of ~4×10^0 to 4×10^4 cfu per g of feces; uninoculated fecal samples served as negative controls. Ten uninoculated control samples, 10 samples inoculated with 10^0, 10^1, and 10^2 cfu/g (30 total), and five samples inoculated with 10^3 and 10^4cfu/g (10 total) were evaluated. All fecal samples were cultured using 2 different enrichment methods that were designed to detect *Salmonella* within 24 hrs when testing with LFIs. In the first method, 10-g fecal samples were enriched for 6 hrs in 100 ml of buffered peptone water[1] (BPW) at 43°C, then 0.1ml was passed into 10 ml of Rappaport-

Vassiliadis[1] (RV) broth and incubated for 18 hrs at 43°C. In the second, 1-g fecal samples were incubated in 10 ml of tetrathionate[1] (TET) for 18 hrs at 43°C. For comparison purposes, samples from broth enrichment cultures were also streaked for isolation on xylose-lysine-tergitol 4[2] (XLT4) agar and incubated for an additional 24 hrs at 43°C. A positive plate had at least 1 colony with characteristic morphology. In general, higher inoculum concentrations resulted in greater numbers of characteristic colonies. One isolate from each plate was selected for serogroup confirmation using commercially available grouping antisera. Study was approved by the Colorado State University Institutional Animal Care and Use Committee. Consent from the Colorado State University Veterinary Teaching Hospital (CSU-VTH) was obtained for animal use in this study.

Environmental samples: Environmental samples used in this experiment were collected from a high-traffic linoleum floor surface in an educational building adjacent to the CSU-VTH that was previously found to have a very low frequency of *S. enterica* contamination (6 ft x 22 ft area per each sample) using an electrostatic dust wipe[3] [3.9]. The same *S. enterica* serotype Typhimurium isolate was used for inoculating environmental samples. A stock culture was made by inoculating 10 ml of TSB and incubating overnight at 43°C. The bacterial concentration of the overnight culture was determined by plating 10-fold dilutions on blood agar, incubating overnight at 43°C, and calculating the original culture concentration (~$4x10^8$ cfu/ml). Ten-fold dilutions of overnight broth culture were used to inoculate environmental samples to achieve concentrations of ~4×10^0 to 4×10^3 cfu per ml BPW; uninoculated environmental samples served as controls. Five uninoculated control samples and 5 samples each inoculated with ~4×10^0, ~4×10^1, ~4×10^2, and ~4×10^3cfu/ml (20 total) were evaluated. Four different

enrichment methods were used prior to testing with LFIs. In the first, collection wipes were enriched for 6 hrs in 100 ml of BPW at 43°C. In the second, collection wipes were enriched for 6 hrs in 100 ml BPW at 43°C, then 0.1ml was passed into 10 ml of RV broth and incubated for 18 hrs at 43°C. In the third, collection wipes were enriched for 6 hrs in 100 ml BPW at 43°C, then 1 ml was passed into 9 ml TET for 18 hrs at 43°C. In the fourth, collection wipes were enriched for 6 hrs in 100 ml BPW at 43°C, then 1 ml was passed into 9 ml TET for 18 hrs at 43°C, then 0.1 ml was passed into 10 ml RV broth for 18 hrs at 43°C. For comparison purposes, samples from broth enrichment cultures were also streaked for isolation on XLT4 agar and incubated for an additional 24 hrs at 43°C.

Sample Testing: Two different LFIs, LFI-A[4] and LFI-B[5], were used to detect *S. enterica* in broth cultures of fecal samples and environmental samples as previously described. A positive test was defined as one where the test line had an equal or greater intensity as the control line on the test strip. Investigators were blinded (BAB and NRN) when determining the results of tests (i.e., they did not know the inoculum concentration of the initial samples, nor did they know the results of other tests for the same samples).

Non-specific cross-reactivity: Each commercial assay was evaluated for the potential of non-specific cross-reaction with non-target bacteria (i.e., non-*Salmonella*) by testing uninoculated (negative control) samples of feces and environmental samples, both before and after culturing with different broth media as previously described for inoculated samples.

Data Analysis: Data was entered into a computer spreadsheet and validated. Chi-square and Fisher's Exact tests were performed using a critical alpha of 0.05 for identification of statistical differences.[6]

3.4 RESULTS

Equine fecal samples: Overall, fewer false-negatives were found in results from the LFIs when using protocols for enriching 1-g fecal samples in TET for 18 hrs when compared to enriching 10-g samples in BPW for 6 hrs and then RV for 18 hrs (Table 3.1; $P<0.01$). *Salmonella* was detected in the majority of samples inoculated with 10^0 to 10^4 cfu/g when enriched overnight (18 hr) in TET, whereas there was imperfect detection for samples enriched using BPW-RV, especially when samples were inoculated with $<10^2$ cfu/g (Table 3.1). In comparing results when evaluating the same 1-g or 10-g samples, the proportions of false-negative results were similar for the LFIs and aerobic culture when samples were enriched as described. In 14 instances, at least one LFI was negative when samples were culture-positive, particularly when enriched using BPW-RV. In general, when this occurred, LFIs tended to be in agreement (i.e., both were negative); only in 4 instances were LFIs results discordant. However, using the overnight (18 hr) TET enrichment protocol, *Salmonella* was reliably detected in fecal samples inoculated with 10^2-10^4 cfu/g using both LFIs as well as culture.

Environmental samples: All enrichment methods used for environmental samples had similar proportions of false-negative results (Table 3.2); with no significant difference between BPW-RV, BPW-TET, and BPW-RV-TET ($P=0.33$). Samples inoculated with 10^2-10^3 cfu/ml were reliably detected with all four enrichment methods but detection was imperfect with inoculating doses $<10^2$ cfu/ml. Specifically, there were false-negatives results when samples were enriched with BPW (7 samples) or with BPW-TET (1 sample; Table 3.2). When comparing results for tests performed on the same environmental samples, all false-negative results occurred when using LFI-A and none occurred when using LFI-B. When samples were inoculated with $<10^2$ cfu/ml,

there were 8 false-negative tests, the majority of which occurred when enriched in non-selective media (i.e., BPW).

Test strip non-specific cross-reactivity: All of the negative control fecal and environmental samples were negative when using both commercial test strips as well as culture.

3.5 DISCUSSION

Results of this study suggest that commercially available LFIs may have potential to aid practitioners in management of *S. enterica* in horses through point-of-care testing of fecal and environmental samples. However, this initial experimental work needs to be expanded to demonstrate the utility in field settings.

Both rapid detection systems consistently detected an inoculating dose of approximately 4-40 cfu/g of feces when enriched in TET for 18 hrs at 43°C. Lateral flow immunoassays which are similar to LFI-A and LFI-B are routinely used in clinical practice to detect other target antigens. This type of product is even sold over-the-counter as diagnostics tests for human health conditions (e.g., pregnancy tests, HIV infection). The optimized methods identified in this study are relatively simple, and would not require extensive training or investment in specialized equipment other than a modest one-time purchase of a small incubator. Powder media used to make BPW is inexpensive and rehydrated BPW broth does not need to be autoclaved if it is made aseptically in a sterile container with sterile water and used within 4 hrs of mixing. High quality TET broth can be purchased in 10 ml volumes that would be ready to use with 1 g fecal samples or 1 ml BPW enriched environmental samples. All other materials (including disposable pipets and vials) are sold with the LFI kits which are relatively inexpensive; and the time needed to conduct testing with the LFIs is quite reasonable,

15-minutes for LFI-A and 10-minutes for LFI-B. These factors, combined with a relatively low cost (probably <$20 USD, including labor), may make LFIs a practical alternative to other methods of *Salmonella* testing in veterinary practice settings. Positive samples would still need to be cultured to allow for antimicrobial susceptibility testing for patient management, and serotype identification or genetic analysis when investigating relatedness of isolates obtained from different sources.

As expected, commercial test kits did not give false-positive test results when evaluating uninoculated control samples. However, there were differences in test performance when evaluating inoculated fecal and environmental samples after enrichment. False-negative reactions were common when testing environmental samples after non-selective enrichment (i.e., with 6-18 hr enrichment cultures in BPW), but false-negative reactions were not a problem when samples were tested after selective enrichment (i.e., with 6 hrs BPW pre-enrichment followed by either 18 hr enrichment in RV or TET). This difference may have been caused by non-*Salmonella* bacteria replicating faster than *Salmonella* (i.e., out-competing) in an unrestricted growth environment. False-positive reactions did not occur when using test strips to evaluate environmental or fecal samples. While traditional, reference-based estimates of epidemiological sensitivity and specificity could theoretically be calculated using these results, such estimates would lack external validity as this study used experimentally inoculated samples. Additionally, the small sample size would limit the accuracy of any estimates. Accordingly, traditional estimates of the epidemiological sensitivity and specificity were not determined. The limit of detection, however, was approximately 4 cfu per g of feces or 1 ml of sample. Given the well-known need for multiple cultures to achieve an acceptable negative

predictive value for *Salmonella* testing [3.10, 3.11], these results suggest that the limitation of the detection process are more to likely to result from the intermittent nature of fecal shedding rather than limitations in the analytical sensitivity of the detection methods. As a result more samples must be screened to achieve reliable detection of fecal shedding. This need for testing of multiple samples can represent a substantial cost to the owner or practitioner, which further adds to the importance of identifying reliable, low-cost tests that can be employed at the point-of-care, such as the LFIs evaluated in this study.

While traditional *Salmonella* culture methods typically employ longer incubation times (e.g., 24 hours for pre-enrichment) and lower incubation temperatures (37°C or 42°C), the purpose of this study was to develop a 24-hour test that could be implemented in clinical practice. In general, *Salmonella enterica* can withstand higher incubation temperatures than other competing bacteria, although some strains may be more susceptible to higher temperatures than others [3.12]. With this in mind, the choice of a higher incubation temperature allowed for shorter incubation times while still achieving limited numbers of false-negative and false-positive results when testing high-bacterial burden matrices such as feces.

These commercially available LFIs may be useful tools when managing *S. enterica* in equine populations. Using rapid, point-of-care testing could allow earlier detection of *S. enterica* shedding, thus decreasing risks for transmission and environmental contamination. In addition, these tests could be used to achieve a substantial direct cost savings in surveillance programs; rather than submitting all samples to a reference laboratory for testing, only those samples identified as positive using the rapid test would be submitted, thus decreasing the number of samples requiring costly culture and susceptibility testing. However, additional

evaluation is needed using samples from naturally-infected patients and contaminated environments to further substantiate the true value of these tests.

3.6 ENDNOTES

[1]BD Diagnostic Systems, Sparks, MD, USA
[2]XLT-4, Hardy Diagnostics, Santa Maria, CA, USA
[3]Swiffer®, Proctor & Gamble, Cincinnati, OH, USA
[4]Reveal® for *Salmonella* Test System, Neogen® Corporation, Lansing, MI, USA
[5]RapidChek® SELECT™ *Salmonella* Test System, SDIX, Newark, NE, USA
[6]SAS v9.3, SAS Inc., Carey, NC

Table 3.1: Results of testing feces experimentally inoculated with *Salmonella enterica* serotype Typhimurium using commercial immunoassays and aerobic culture

Enrichment Method	Inoculum Concentration (cfu/g)	Total Tests	Positive Tests		
			LFI-A[3]	LFI-B	Culture[5]
10 g in BPW-RV[1,6]	Control	10	0	0	0
	~4×10^{0}	10	0	0	2
	~4×10^{1}	10	0	0	6
	~4×10^{2}	10	2	3	5
	~4×10^{3}	5	4	3	5
	~4×10^{4}	5	4	4	5
1 g in TET[2,6]	Control	10	0	0	0
	~4×10^{0}	10	6	6	6
	~4×10^{1}	10	6	6	6
	~4×10^{2}	10	10	10	10
	~4×10^{3}	5	5	5	5
	~4×10^{4}	5	5	5	5

[1]10g fecal samples enriched for 6 hrs in 100ml of BPW at 43°C, then 0.1 ml was passed into 10 ml of RV broth and incubated for 18 hrs
[2]1 g fecal samples incubated in 9 ml of TET for 18 hrs
[3]Reveal® 2.0
[4] RapidChek® Select™
[5] Overnight aerobic culture on XLT4 agar at 43°C
[6]Significantly different ($P<0.01$)

TABLE 3.2: Results of testing environmental samples experimentally inoculated with Salmonella enterica serotype Typhimurium using commercial immunoassays and aerobic culture

Enrichment Method	Inoculum Concentration (cfu/g)	Total Tests	Positive Tests		
			LFI-A[5]	LFI-B[6]	Culture[7]
Environmental Sample in BPW[1]	Control	5	0	0	0
	~4×10 ^{0}	5	0	5	5
	~4×10 ^{1}	5	3	5	5
	~4×10 ^{2}	5	5	5	5
	~4×10 ^{3}	5	5	5	5
Environmental Sample in BPW-RV[2,8]	Control	5	0	0	0
	~4×10 ^{0}	5	5	5	5
	~4×10 ^{1}	5	5	5	5
	~4×10 ^{2}	5	5	5	5
	~4×10 ^{3}	5	5	5	5
Environmental Sample in BPW-TET[3,8]	Control	5	0	0	0
	~4×10 ^{0}	5	4	5	5
	~4×10 ^{1}	5	5	5	5
	~4×10 ^{2}	5	5	5	5
	~4×10 ^{3}	5	5	5	5
Environmental Sample in BPW-TET-RV[4,8]	Control	5	0	0	0
	~4×10 ^{0}	5	5	5	5
	~4×10 ^{1}	5	5	5	5
	~4×10 ^{2}	5	5	5	5
	~4×10 ^{3}	5	5	5	5

[1]Environmental sample in 100 mls BPW incubated for 6 hours at 43°C

[2]Environmental sample enriched for 6 hrs in 100ml of BPW at 43°C, then 0.1 ml was passed into 10 ml of RV broth and incubated for 18 hrs.

[3]Environmental sample enriched for 6 hrs in 100ml of BPW at 43°C, then 1 ml was passed into 10 ml of TET for 18 hrs.

[4]Environmental sample enriched for 6 hrs in 100ml of BPW at 43°C, then 1 ml was passed into 10 ml of TET for 18 hrs, then 0.1 ml was passed into 10 ml RV broth and incubated for 18 hrs.

[5]Reveal® 2.0
[6]RapidChek® Select™
[7]Overnight aerobic culture on XLT4 agar at 43°C
[8]Not significantly different (*P*=0.33)

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4 CHAPTER 4: DETECTION OF DIFFERENT SEROTYPES OF *SALMONELLA ENTERICA* IN EXPERIMENTALLY-INOCULATED EQUINE FECAL SAMPLES BY COMMERCIALLY AVAILABLE RAPID TESTS

4.1 SUMMARY

Background: *Salmonella enterica* can significantly impact management of animal facilities. Comprehensive screening is essential for effective control in high-risk populations. Availability of reliable point-of-care diagnostic tests would facilitate these efforts.

Hypothesis/Objectives: Compare the ability of commercially available rapid diagnostic assays (2 lateral-flow immunoassays [LFIs], DNA hybridization [DNAH], real-time PCR [qPCR]), and culture to detect common serotypes of *S. enterica* in feces.

Animals: n/a

Methods: In an experimental study, 112 *S. enterica* isolates were randomly selected from the 10 most common serotypes recovered at a veterinary hospital. Archived isolates were amplified in broth and standardized inocula (100 colony forming units [cfu]) were incubated with equine feces in tetrathionate broth (TET). Cultures were tested in a blinded fashion using LFIs, DNAH, qPCR, and culture.

Results: The LFIs detected 84% and 67% of isolates, respectively, but reactivity varied among serotypes. Both reacted poorly with serotype Cerro (Group K) isolates, and 1 LFI did not react with any serotype Mbandaka (Group C_1) or Montevideo (Group C_1) isolates. DNAH detected 94% of isolates, while culture and qPCR most reliably detected all serotypes. False-positive results were obtained for 4 negative-controls using DNAH and 1 negative-control using qPCR, but LFIs and culture had no false-positive results.

Conclusions and clinical importance: Culture, qPCR, and DNAH were effective in detecting most *Salmonella* isolates, but have limited application at point-of-care settings. LFIs are appealing as point-of-care tests because of low cost and ease-of-use, but limited detection of some serotypes needs to be evaluated with samples obtained from naturally infected animals.

4.2 INTRODUCTION

Salmonella enterica can have a significant impact on the management of animal facilities. In a survey of accredited Veterinary Teaching Hospitals, *Salmonella enterica* was reported to be the most common cause of outbreaks of nosocomial infections at equine hospitals with 71% of affected facilities restricting admissions and 39% closing completely to aid mitigation efforts [4.1]. Comprehensive screening and rapid detection of *S. enterica* in fecal and environmental samples are extremely important aids for effective control of this agent in high-risk animal populations (i.e., populations which congregate at veterinary hospitals, boarding facilities and equestrian events). Unfortunately, there are limitations with the most common detection methods (aerobic culture and real-time polymerase chain reaction [qPCR]) including cost, limited sensitivity, time needed to obtain results, and laborious testing methodologies. These factors limit availability of tests and limit the ability of veterinary practitioners to employ comprehensive testing and surveillance programs in at-risk animal populations. With these limitations in mind, we propose that an ideal test for the rapid detection of *S. enterica* in field applications may include the following: 1) availability as a point-of-care test, 2) minimal need for expensive equipment and specialized training, 3) providing results within 24 (hrs, 4) applicable in a variety of settings and regions, and 5) be applicable for use with specimens relevant to clinical settings (i.e., fecal and environmental samples in equine practice).

There are several commercially available assays that are marketed in North America for rapid detection of *S. enterica* in food safety and other applications, including lateral-flow immunoassays (LFIs) [4.2, 4.3], DNA hybridization (DNAH) assays [4.4], and real-time PCR (qPCR) assays [4.5]. However, sample matrices relevant to food safety generally have a low background bacterial burden. In contrast, equine feces has high numbers of background bacteria and other substances (e.g., PCR inhibitors) that can interfere with target organism detection, especially when hosts are shedding very low numbers of *Salmonella* bacteria. In prior investigations we identified an optimized culture technique for use with LFIs which allows for the detection of ~4cfu/gm in experimentally inoculated equine fecal samples within 24 hrs [4.6]. However, limited preliminary assessment of LFI assay reactivity with 1 strain (isolate) from each of 5 different *Salmonella enterica* serotypes (Typhimurium – serogroup B; Montevideo – serogroup C_1; Newport – serogroup C_2; Muenster – serogroup E_1; Cerro – serogroup K) suggested that the performance of LFIs might vary by strain and/or serotype [results not shown]. Variable ability to detect different *Salmonella* serotypes would be an important factor when considering use in veterinary practices.

Given the ease of use, low cost, and the potential utility LFIs demonstrated in prior investigations [4.6], we believe they may have greater applicability in practice than other commercially available rapid diagnostic tests such as DNAH and qPCR. However, thus far, it has not been shown that LFIs are more or less useful than DNAH, qPCR, or standard culture at detecting variable *S. enterica* serotypes. Therefore, the goal of this study was to evaluate variability in the ability to detect a variety of different clinical isolates of *Salmonella enterica*

using 4 different commercially available rapid tests (2 LFIs, a DNAH test, and a qPCR assay) and aerobic culture when inoculated into equine feces.

4.3 MATERIALS AND METHODS

Study Summary: The ability to detect different strains of *S. enterica* was evaluated using 4 commercially available rapid diagnostic testing systems (2 different LFIs, DNA hybridization, and real-time qPCR), and results were compared to aerobic culture (Figure 4.1). A total of 112 isolates were randomly selected from archived strains of the 10 most commonly isolated serotypes that had been recovered in a veterinary hospital. Standardized inocula were incubated with broth media and equine feces at a concentration which is consistent with low-level shedding in naturally infected horses and then tested using all tests. An additional 25 equine fecal samples that were not inoculated with *Salmonella* served as negative controls that underwent identical processing to aid in blinding. Random assignment of identification numbers to all samples and blinding were used to ensure that investigators were not aware of isolate identification when classifying test results. This study was approved by the Colorado State University Institutional Animal Care and Use Committee, and owner consent was obtained to collect samples that yielded *S. enterica* isolates and equine feces used in this study.

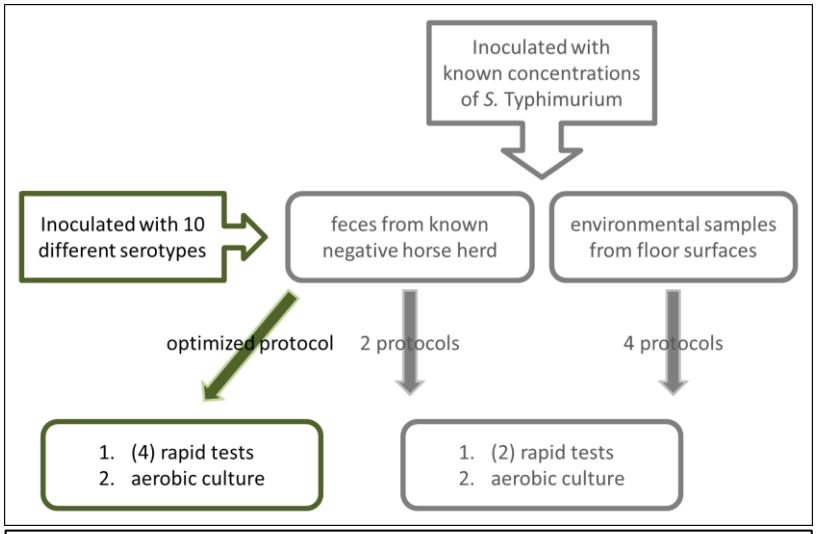

Figure 4.1 Overview of Serotype Reactivity and Method Optimization

Commercially Available Rapid Tests: Four commercially available rapid diagnostic testing systems were used in this study: 2 LFIs (LFI-A[7] and LFI-B[8]), a DNAH test[9], and a real-time PCR assay.[10] Both of these LFIs are sold in a small strip format, employing gold-labeled antibody specific to *Salmonella enterica* subsp. *enterica*. As the broth cultures flow through the test strip, an antibody-coated colloidal gold reagent, which provides a visible signal in the test, is rehydrated. If *Salmonella* antigens are present in the sample, they bind to the antibody-gold conjugate forming an antigen-antibody complex that is subsequently captured by a zone of anti-*Salmonella* antibody on the test strip forming a visible line (the sample test line). A second zone captures antibody-gold conjugate that is not bound in the first zone, thus forming a second visible line on the test strip (the control line). The DNA hybridization test is a multi-step test which lyses bacteria in the broth cultures, allowing the ribosomal RNA to bind to a horseradish peroxidase labeled indicator probe and a capture probe specific for *S. enterica*. The presence of *Salmonella* will result in a color change which is measured as a change in optical density (OD) by means of a 96-well plate reader. The qPCR test amplifies and detects a *S. enterica*-specific DNA sequence using specialized thermocycler equipment which contains sensors for measuring the fluorescence of the probe that is released during polymerization of DNA primers. The presence of *Salmonella* in the sample is determined based on a threshold level of detection which is reached during the exponential phase of DNA amplification.

Salmonella Isolates Used in Testing: *Salmonella* isolates used in this study were randomly selected from all isolates that had been recovered and archived as part of long-term surveillance conducted at the Colorado State University Veterinary Teaching Hospital (CSU-VTH). Briefly, in addition to testing clinical suspects, active surveillance has been used to

identify subclinical *Salmonella* shedding in large animal inpatients hospitalized at the CSU-VTH. Further, environmental samples are collected monthly from approximately 60 sites to identify environmental contamination that might be present in the Veterinary Teaching Hospital. Isolate serotyping was conducted at the USDA National Veterinary Services Laboratory (Ames, IA, USA) and susceptibility testing, by the Kirby-Bauer disc diffusion method, was performed by the Colorado State University Veterinary Diagnostic Laboratory (Fort Collins, CO, USA) (Table 4.1). Since 2002, more than 1,300 different *Salmonella* isolates have been collected and archived at -80°C. A formal random process was used to select 112 isolates from the 10 most common serotypes that have been saved in this archive since 2002 (these serotypes represent approximately 80% of all isolates in the archive). The sample size was selected arbitrarily. Probability sampling was used for selection of individual isolates with a maximum of 15 and a minimum of 5 isolates selected per serotype. Thus, serotypes with greater representation in the archive had greater probability of selection, but each isolate had an equal probability of selection (a simple random sample). A random number generator was used to assign a unique number to each isolate for selection and testing purposes. Only isolates from these 10 serotypes were eligible for selection, and isolate included in this study were limited to 1 per animal and only 1 unique isolate from the same environmental sampling date.

Salmonella Serotypes Tested: Isolates included in the study included Serogroup B: Typhimurium (n=10), Typhimurium var. 5- (formerly Typhimurium var Copenhagen; n=12); Serogroup C1: Mbandaka (n=9), Montevideo (n=15); Serogroup C2: Muenchen (n=5), Newport (n=10); Serogroup C3: Kentucky (n=9); Serogroup E: Meleagridis (n=15), Muenster (n=13); and Serogroup K: Cerro (n=14) [Table 4.1]. Seventy-four isolates included in these evaluations were

originally recovered from animal feces (including 63 that were isolated from cattle, 8 from horses, and 3 from New World camelids) and 38 isolates were originally recovered from environmental samples (including 19 recovered from the Livestock Hospital, 12 from the Equine Hospital, and 7 from core facilities or the Small Animal Hospital).

Salmonella Isolate Testing Using Lateral Flow Immunoassays and a DNA Hybridization Test: Archived *Salmonella* isolates stored in glycerol solution at -80°C were thawed and streaked for isolation on tryptic soy agar plates with 5% sheep blood (TSA).[11] For each isolate, a standardized inoculating dose of approximately 100 cfu per milliliter (ml) was developed by first creating a stock solution using sterile saline with approximately 10^8 cfu per ml based upon optical density (equivalent to a 0.5 McFarland standard), then performing 10-fold dilutions to achieve the desired concentration of approximately 100 cfu/ml of solution. The concentration of *Salmonella* in these samples was confirmed by enumerating colony counts after plating each dilution on TSA and incubating at 43°C for 18 hrs. A composite pool of feces was used in testing all samples in order to provide a uniform sample matrix and background microbiome. This was created by collection of approximately 100 gm of feces from each of 5 adult horses that were part of an isolated horse herd (housed in a dry paddock and having a history of being culture-negative for *Salmonella* with periodic testing), and mixed thoroughly to create 1 composite fecal pool. Uninoculated fecal samples were also tested to ensure that the original fecal pool was negative for *Salmonella*, or at least had a very low likelihood of containing detectable quantities of this target bacterium.

For each isolate, approximately 100 cfu in 1 ml culture broth and a 1 g sample of the composite fecal pool were added to 9 ml tetrathionate broth (TET)[11] and incubated at 43°C for

18 hrs [4.6]. After incubation, samples of TET broth were evaluated in parallel using the LFI-A and LFI-B. Both LFIs have a test line and control line; for the purposes of this study, results were classified as positive if the test line was at least as intense (i.e., darkly colored) as the control line on the test strip.

In addition, because the color of TET broth interferes with evaluation of optical density (OD), 1 ml of enriched sample was inoculated into 9 ml buffered peptone water (BPW) and incubated at 43°C for an additional 6 hrs before evaluation with the DNAH test. The OD of each sample was determined using an automated plate reader at 450 nm, with a positive test defined as an absorbance value ≥ 0.10, per manufacturer's recommendation.

Salmonella Isolate Testing Using Aerobic Culture: All TET and BPW broth cultures that were previously described were plated on xylose-lysine-tergitol 4 (XLT4)[12] agar plates and incubated at 43°C for 18-24 hrs. In addition, to help rule-out the possibility of contamination, 10% of isolates recovered from BPW culture broth were randomly selected for serogroup confirmation using commercially available grouping antisera. A 1 ml aliquot of all TET broth cultures was frozen at -80°C, then thawed and recultured on the day that qPCR testing was performed (within 2 weeks of being frozen).

Salmonella Isolate Testing Using Real-time PCR: For logistical reasons, samples were batch tested by qPCR after testing samples with the other rapid tests (within 2 weeks). As described, aliquots of all TET cultures were frozen at -80°C. After thawing, samples were processed using a commercially available DNA extraction kit[13] and qPCR kit[10] per manufacturer's directions [4.5]. This qPCR utilizes an internal positive control for inhibition detection. A positive qPCR result was defined as a test having a cycle threshold (Ct) number ≤ 35. If results suggested that PCR

inhibition had occurred, as evidenced by a negative sample Ct value in conjunction with a negative internal positive control Ct value, extracted DNA samples were diluted 1:10 in PCR-grade water and re-tested, based on manufacture's recommendation. Thawed TET broth cultures were also recultured on the day that qPCR testing was performed to help confirm that frozen samples were comparable to those previously tested with the other rapid tests.

Uninoculated Control Samples: Twenty-five 1 g aliquots of the pooled feces were not inoculated with *Salmonella* but were processed in a manner identical to that described for testing *S. enterica* isolates. Inclusion of these uninoculated control samples served several purposes, including aiding in the blinding process, allowing assessment of false-positive rates for the assays, and to confirm that the pooled feces was actually free of *Salmonella* prior to laboratory inoculation.

Data Analysis: After recording interpretations for all tests, randomly assigned study identification numbers were decoded, results were tabulated, and descriptive statistics calculated. While traditional, reference-based estimates of sensitivity and specificity might be calculated, these estimates would lack external validity due to the use of experimentally inoculated samples and therefore were not determined.

4.4 RESULTS

Overall, all experimental and control samples were correctly identified by at least 1 of the 4 commercially available tests evaluated in this study (Table 4.2). Of 112 *Salmonella* isolates tested, 39% (n=44) were negative on at least 1 rapid test (28 were negative on 1 test, 15 negative on 2 tests, and 1 isolate (serotype Cerro) was negative on 3 tests; Table 4.2). LFI-A detected 84% of isolates and LFI-B detected 67%, but reactivity varied among serotypes. A

majority of the misclassification was related to isolates from 2 serogroups. Both immunoassays reacted poorly with serotype Cerro (serogroup K) isolates, and LFI-B did not react with any serotype Mbandaka (serogroup C1) or Montevideo (serogroup C1) isolates (Table 4.2). DNAH detected 94% of isolates, and qPCR detected 99% of isolates tested. Culture most reliably detected *Salmonella* of all serotypes, as all samples of BPW were culture-positive, as were all TET broth samples that were cultured on 2 occasions (at the time of original processing and also after thawing frozen aliquots of the original enrichments). When isolates were not detected with 2 tests, 86% (12/14) of the time they were undetected with both LFIs, and isolates that were not detected with ≥2 tests were most commonly serotypes Cerro (9 isolates) or Montevideo (4 isolates). When comparing to aerobic culture results, 4 uninoculated control samples were test-positive (false-positive) when evaluated with DNAH and 1 control sample had false-positive results using qPCR, but there were no false-positive results with the LFIs.

One uninoculated control sample was test-positive on both LFIs, DNAH, qPCR and on culture. The isolate recovered from this culture was phenotypically evaluated (serotype and susceptibility profile) and found to match an environmental isolate included in the study. Evaluation of unique sample identification numbers and processing sequence allowed determination that the sample inoculated with this environmental isolate was handled immediately prior to processing of the uninoculated control sample. Thus, these results suggest that this individual sample was inadvertently contaminated during the study. The remaining 24 uninoculated control samples were culture-negative on 2 occasions, and tested negative with both LFIs and qPCR, suggesting that horses that provided feces used in this study were truly uninfected and that cross-contamination was not a significant issue in this study.

Evaluation of the serogroup for 10% (12/112) of isolates randomly selected from those recovered from BPW broth cultures found that all 12 isolates matched the phenotype of isolates that were used to inoculate fecal samples, suggesting that contamination was unlikely to have complicated interpretation of positive test results from inoculated samples.

4.5 DISCUSSION

The 4 rapid tests that were evaluated in this study were able to reliably detect the most common serotypes that have been recovered from large animal inpatients and their housing environment at the CSU-VTH using a 24 hr enrichment technique with experimentally inoculated equine feces. Lateral flow immunoassay-A appears to have the greatest promise for point-of-care testing in equine practice, when considering all factors (e.g., cost, ease of use, applicability in field settings, and reliability). Culture and qPCR were effective at detecting most *Salmonella* isolates, but these tests have limited potential for point-of-care use in private practice settings. These tests are also technically challenging to perform without specialized training. Additionally, the DNAH and qPCR tests require relatively expensive, specialized equipment, as compared to aerobic culture. While costs for materials and labor used to conduct aerobic culture are modest, they are substantial for DNAH and qPCR relative to the number of tests that need to be performed in a comprehensive surveillance system.

In contrast, immunoassays can be easily adapted for use as rapid point-of-care tests, and tests similar to LFI-A and LFI-B have been marketed for use in clinical practice and even as over-the-counter diagnostics sold for human health conditions. Subjectively, the LFIs were much easier to perform than any of the other rapid tests or culture. The method that was developed for use of these tests in veterinary practice is simple, and does not require extensive

training or specialized equipment other than a modest one-time purchase of a small incubator [4.6]. High quality culture media (TET broth) can be purchased in 10 ml volumes that would be ready to use with 1 gram fecal samples as demonstrated in this study. All other materials (including disposable pipets and vials) are sold with the test kits. The kits are relatively inexpensive, and personnel time needed to conduct testing with the LFIs is quite reasonable (15 minutes for LFI-A and 10 minutes for LFI-B). Combined, these factors make LFIs a practical alternative to other methods of *Salmonella* testing in veterinary practice settings as they are easy to use and relatively inexpensive.

Limited detection of some *Salmonella* serotypes is an important consideration regarding the practical utility of LFIs in clinical practice. While isolates of several serotypes were consistently detected with the LFIs used in this study, some strains were less reliably detected, especially with LFI-B. These findings confirm our preliminary observations that serotypes can have different reactivity in commercial immunoassays. As isolates tested were randomly selected from an isolate bank derived from long-term surveillance, we believe it likely that selected isolates represent different strains, although this was not confirmed through genetic evaluation. To our knowledge, variable ability of these tests to detect different serotypes has not been previously reported, even in literature regarding use of these products in food safety applications [4.2-4.4]. This may be due in part to differences in application and also in test interpretation. In food safety applications, manufacturers' of the LFIs used in this study recommend interpreting any visible color at the sample test line, no matter how intense, as indication of a positive test result. In previous work optimizing the culture method and interpretation of these LFIs for use in veterinary applications, we found that to minimize the

false-positive rate, the color of the sample test line should be as intense as the control test line [4.6]. This method of interpretation not only improved test specificity in samples relevant to veterinary medicine, but also provides an internal reference for classifying test results. This difference may also be related to differences in background microbiome, as sample matrices tested in food safety applications have relatively low background microbial contamination which is quite different from animal feces which can contain 10^{10}-10^{11} bacteria per gram [4.7].

This variability in detection of different serotypes is critically important to implementation of LFIs in clinical veterinary practice as their practical value will depend upon which serotypes are most likely to be detected in a particular population or region. Cumulatively, from 2009-2011, the USDA National Veterinary Services Laboratory reported that equine isolates most commonly submitted for serotyping included (in descending order of frequency): Javiana (group D1), Typhimurium (B), Newport (C2), Braenderup (C1), Anatum (E), Infantis (C1), 4,5,12:i:- (B), Typhimurium var 5- (B), Muenchen (C2), and Mbandaka (C1) [4.8-4.10]. These 10 serotypes represent about 65% (1367/2069) of all isolates submitted to USDA-NVSL during those 3 years. Five of these serotypes are included in the 10 serotypes that were selected for inclusion in this study based upon frequency of recovery at the CSU-VTH, which is a referral hospital for all species of animals. Because of differences in recovery in different regions as well as the frequency of *Salmonella* shedding in dairy cattle at the time they are admitted to this hospital, complete overlap in the 2 lists is not expected. It is relevant to note the particularly poor recognition of C1 strains when tested with LFI-B, and serotype Cerro was not detected well by either of the LFIs. Serotype Cerro has become a predominant strain recovered from cattle, but this trend has not been mirrored in other species, thus would not be

a serotype generally expected to be isolated from horses [4.11-4.13]. At this time, we are making no recommendations for the use of LFIs on cattle feces for the detection of *S. enterica*. Preliminary investigations found that both LFIs used in this study showed very poor specificity when used to detect *Salmonella* in feces collected from dairy and feedlot cattle that were being fed high concentrate rations (results not shown). However, if used in equine populations, LFI-A appears to have better reliability, though it will be important to test this assumption in practice using specimens from naturally infected horses. This becomes critically important as the distribution of serotypes has been shown to vary by geographic location and can change over time in the same geographic location [4.14, 4.15].

Overall, there was a low occurrence of false-positive test results. Both LFIs had correct results for all negative control samples and also correctly detected *Salmonella* in 1 negative control sample that was contaminated by an isolate with the same phenotype as an environmental isolate being used in this test assessment. However, both the DNAH and qPCR tests had lower specificity given the 4 false-positive test results and 1 false-positive test result, respectively. While aerobic culture results were repeatedly negative, increasing our confidence that these samples did not contain viable *Salmonella*, we cannot rule-out the possibility that these samples may have contained DNA fragments or non-viable organisms which resulted in a positive test.

While this study evaluated the occurrence of false-positive and false-negative test results under realistic conditions that mimicked an appropriate sample matrix and background microbiome, it is not possible to obtain a relevant estimation of test accuracy from this study. A more valid evaluation of the diagnostic (epidemiologic) sensitivity and specificity would

require application of tests as they would be employed in practice using specimens from a variety of naturally infected and uninfected animals, as opposed to inoculating a standard number of cfu from laboratory amplified strains into a common pool of feces. Test sensitivity and specificity will need to be evaluated for these rapid tests using studies that are relevant to practice settings before the true value to veterinarians can be fully understood.

Results of this study suggest that LFIs could be useful alternatives to traditional aerobic culture, DNAH, and qPCR methods for detection of *Salmonella*. As discussed, culture, qPCR and DNAH have significant limitations which restrict their ability to be used as point-of-care tests in veterinary practice. Additionally, current pricing suggests that LFIs could be utilized in practice to obtain test results within 24 hrs for approximately 3-5 times lower cost per test. Even if limitations in serotype reactivity for LFIs lead to some false-negative test results, lower costs per test would allow testing of more total samples which should improve the overall sensitivity of the surveillance system. However, it is important that aerobic culture be part of any surveillance system for *Salmonella* in order to provide phenotypic and genotypic information that inform us about the epidemiology of infections and antimicrobial susceptibility that can affect treatment decisions for patients. Thus, it is recommended that any LFI test-positive sample be submitted to a veterinary diagnostic laboratory for culture to allow phenotypic testing, and in special circumstances genotypic comparisons to aid in epidemiologic investigations.

4.6 ENDNOTES

[7]Reveal® for *Salmonella* Test System, Neogen® Corporation, Lansing, MI
[8]RapidChek® SELECT™ *Salmonella* Test System, SDIX, Newark, NE
[9]GeneQuence® for *Salmonella*, Neogen® Corporation, Lansing, MI
[10]MicroSEQ® *Salmonella* spp. Detection Kit, Applied Biosystems™, Carlsbad, CA

[11]BD Diagnostic Systems, Sparks, MD, USA
[12]Hardy Diagnostics, Santa Maria, CA, USA
[13]PrepSEQ™ Nucleic Acid Extraction Kit, Applied Biosystems™, Carlsbad, CA

Table 4.1: Phenotypes of *Salmonella* isolates used for inoculation of equine feces used in evaluating commercially available rapid tests and aerobic culture

Resistance Pattern	Serogroup B		Serogroup C1		Serogroup C2		Serogroup C3	Serogroup E1		Serogroup K
	Typhimurium	Typhimurium var. 5-	Mbandaka	Montevideo	Muenchen	Newport	Kentucky	Meleagridis	Muenster	Cerro
No Resistance	2		3	12	3	2	5	14	11	10
Amo, Amp, Ceft, Ceph, Chlor, Str, Sul, Tet	4	2				8	1			2
Amo, Amp, Chlor, Str, Sul, Tet	2	3								
Amp, Chlor, Str, Sul, Tet		2								
Amp, Chlor, Str, Sul, Tet, Tri									1	
Amp, Str, Sul, Tet	1	2								
Amp, Str, Sul	1	3								
Amik, Amp										1
Str, Sul, Tet			2							
Sul, Tet			1							
Sul			2	3	2		1	1		1
Tet			1				2		1	
Total	10	12	9	15	5	10	9	15	13	14

Amik = amikacin; Amo = amoxicillin; Amp = ampicillin; Ceft = ceftiofur; Ceph = cephalothin; Chlor = chloramphenicol; Str = streptomycin; Sul = sulfonamide; Tet = tetracycline; Tri = trimethoprim-sulfamethoxazole; Susceptibility determined by Kirby-bauer disc diffusion.

Table 4.2: Results of testing equine fecal samples experimentally-inoculated with different *Salmonella enterica* isolates using commercially available rapid tests and aerobic culture

Serogroup	Serotype	Total Isolates	Positive Test Results						
			LFI-A[a]	LFI-B[b]	DNAH[c]	qPCR[d]	TET Culture 1*	TET Culture 2*	BPW Culture**
B	Typhimurium	10	10	10	9	10	10	10	10
	Typhimurium var. 5-	12	12	11	12	12	12	12	12
C1	Mbandaka	9	8	0	9	8	9	9	9
	Montevideo	15	13	0	12	15	15	15	15
C2	Muenchen	5	5	5	5	5	5	5	5
	Newport	10	9	10	10	10	10	10	10
C3	Kentucky	9	7	9	7	9	9	9	9
E1	Meleagridis	15	14	14	15	15	15	15	15
	Muenster	13	12	13	13	13	13	13	13
K	Cerro	14	4	3	13	14	14	14	14
TOTAL		112	94	75	105	111	112	112	112

Inoculating dose and culture method: ~100 cfu in 1 gram of feces with 9 ml of TET broth, incubated for 18 hrs at 43°C

*Culture performed on different aliquots of same TET enrichment, plating on XLT-4 agar that was incubated for 24 hrs at 43°C

** Aliquots of TET enrichment were incubated in BPW for 6 hrs at 43°C, then plated on XLT-4 agar and incubated for 24 hrs at 43°C

BPW=buffered peptone water; DNAH=DNA hybridization; LFI=lateral flow immunoassay; qPCR=polymerase chain reaction; TET=tetrathionate

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5 CHAPTER 5: RISK FACTORS FOR LARGE ANIMAL INPATIENT SHEDDING OF *SALMONELLA ENTERICA* IN A VETERINARY TEACHING HOSPITAL

5.1 SUMMARY

Background: During nosocomial *Salmonella* outbreaks in veterinary hospitals there tends to be widespread environmental contamination. Previous work indicates patient isolates can have the same phenotype as environmental isolates, suggesting animals to be a likely source. Factors for animal shedding have been identified however many of these studies focus on a subset of inpatients with results being minimally generalizable to the general hospital population.

Hypothesis/Objective: 1) To determine factors associated with fecal shedding of *Salmonella* among large animal inpatients within the general hospital population; 2) do so in comparison to two different groups of patients – a group in which there is high confidence in negative status (having at least 3 negative cultures) and a group with potential for misclassification of shedding status (at least 1 negative culture); and 3) to demonstrate that the choice of comparison group can affect resultant associations.

Animals: All large animal inpatients admitted from March 2002 through December 2012 were eligible for this study.

Methods: Inpatients included in this case-control study had fecal samples collected and cultured, using standard techniques, as part of long-term infection control efforts. Factors related to patient stress and defense mechanisms were evaluated. Data on factors of interest were collected retrospectively from electronic medical records. Multivariable conditional

logistic regression was used to evaluate associations between animal factors and fecal shedding of *S. enterica*.

Results: During the study period, there were approximately 11,061 inpatients of which 5.9% (n=648) were fecal culture positive for *S. enterica*. The majority of culture-positive inpatients were bovine (72%) and equine (22%) with the remaining being New World camelid and small ruminant. Overall, 69.4% of patient shedding could be attributed to systemic illness (population attributable fraction) in this study.

Conclusions and clinical importance: The findings of this study will provide a better understanding of factors associated with fecal shedding in the general large animal inpatient population, allowing for the implementation of evidence based preventive measures. This information will be integral to risk management related to periods of epidemic as well as endemic disease.

5.2 INTRODUCTION

Salmonella enterica is one of the most commonly reported agents associated with healthcare-associated infections (HCAIs) in veterinary teaching hospitals, having the potential for a high case fatality rate and a substantial financial cost [5.1, 5.2]. With these outbreaks there tends to be widespread environmental contamination which likely contributes to ongoing transmission among patients [5.2, 5.3]. Previous work has shown that environmental isolates can have the same phenotype (sensitivity and antimicrobial susceptibility) as animal isolates suggesting animals to be a likely source for environmental contamination [5.4]. Researchers have suggested many factors that may be associated with animal shedding, but with inconsistent results [5.5-5.10]. In addition, previous reports generally focus on a limited

number of patients, often on a specific subgroup such as patients with colic or diarrhea, from a limited time period, and typically concentrate on horses. While results of these studies can be useful, it is prudent to exercise caution when extrapolating conclusions to the broader hospital population during times of endemic disease.

If the occurrence of epidemic disease in a hospital population is related to endemic rates of fecal shedding – then evaluating the general hospital population over an extended period of time, while conducting routine surveillance for fecal shedding, will allow for objective comparisons and potentially demonstrate factors that could be employed in routine prevention of HCAIs. At the Colorado State University Veterinary Teaching Hospital (CSU-VTH), routine fecal surveillance of large animal inpatients has been conducted since 2002, as part of long-term infection control efforts, which now presents the opportunity to gain a better understanding of factors associated with shedding among the general large animal inpatient population (i.e., endemic shedding).

Choosing the most relevant comparison group in a case-control study can be a difficult task. This is especially true when studies are conducted to determine factors associated with shedding at times of epidemic disease, when many patients may not have had fecal samples tested to determine shedding status and the focus is typically on a subpopulation of patients [5.11]. Routine surveillance of the general large animal inpatient population is relatively rare, thus case-control studies conducted to determine factors associated with endemic shedding are limited in their ability to select truly negative patients and may be restricted to a subpopulation of patients such as patients presenting for colic, diarrhea or having clinical signs indicative of salmonellosis [5.9]. The ability to identify truly negative patients is also hampered

by limitations in *Salmonella* detection methods which historically have poor sensitivity necessitating lengthy culture processes (i.e., enriched cultures) and testing of multiple samples per patient. Thus it can take up to 3 days to realize test results for a single sample – and typically 3-5 fecal cultures are performed per patient to achieve reasonable sensitivity. Based on routine surveillance at the CSU-VTH, culture-positive patients are typically identified on days 4-7 of hospitalization (having had at least 2 cultures), whereas culture-negative patients are more commonly hospitalized less than 3 days (will have had at least 1 negative culture) and greater than 8 days (will have had at least 3 negative cultures) [5.12]. Therein lays the difficulty – which group of culture negative patients is the better comparison and what affect will this choice have on overall conclusions?

The objectives of this study were, 1) to determine factors associated with fecal shedding of *Salmonella* among large animal inpatients within the general hospital population (i.e., endemic shedding); 2) to do so in comparison to two different groups of patients – a group in which there is high confidence in negative status (having at least 3 negative cultures) and a group with potential for misclassification of shedding status (at least 1 negative culture); and 3) to demonstrate that the choice of comparison group can affect resultant associations.

5.3 MATERIALS AND METHODS:

Study Overview: A case-control study was conducted to detect factors associated with *Salmonella enterica* fecal shedding among the general large animal inpatient population at the CSU-VTH from 2002-2012. During the study period there were over 11,000 inpatient admissions with a Salmonella prevalence of 5.9% (648/11,061). Cases included all Salmonella-positive inpatients (having at least 1 positive fecal culture). Controls were enrolled in two

groups – inpatients with at least 1 negative culture (1NEG patients) and inpatients with at least 3 negative cultures (3NEG patients). Data were derived from long-term surveillance conducted as part of the Infection Control Program and electronic medical records at the CSU-VTH. Conditional logistic regression was used to develop two multivariable models to determine factors that may be associated with fecal shedding of *Salmonella*.

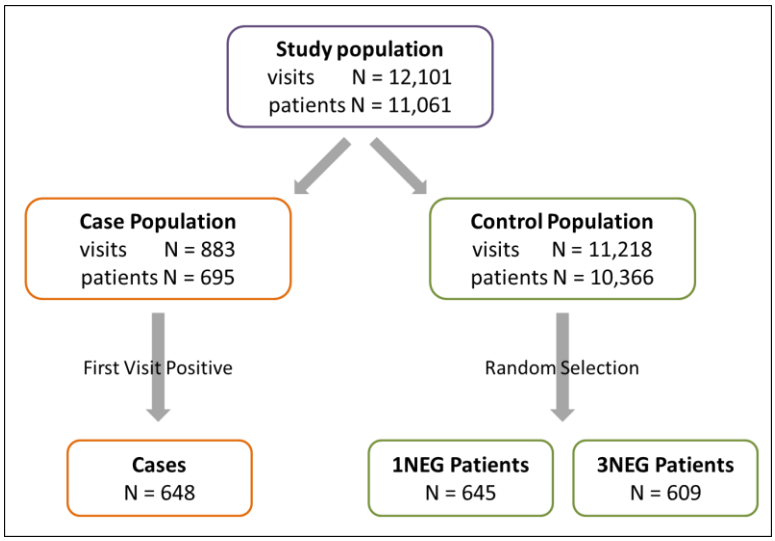

Figure 5.1: Derivation of study participants

Population and Study Sample: The study population included all large animal inpatients (i.e., had at least one night of hospitalization) from the species most commonly admitted (equine, bovine, New World camelid [NWC], and small ruminants [caprine and ovine]) to the CSU-VTH from April 2002 through December 2012 (n=11,061; Figure 5.1) The limited numbers of patients of other species were excluded. Cases were defined as large animal inpatients that were culture positive on at least 1 fecal culture obtained for routine surveillance during a single hospitalization (n=648). Control patients were randomly selected from all fecal culture-negative patients during the study period (n=10,413). Two control groups were included in this study; 1) large animal inpatients with at least 1 negative fecal culture obtained during routine surveillance (1NEG patients; n=645), and 2) large animal inpatients with at least 3 negative fecal cultures obtained during routine surveillance (3NEG patients; n=609). Cases and controls were matched on admission month and year. Each patient could only be enrolled once in the study

(i.e., once enrolled all other visits were disregarded) and each control could only belong to one comparison group.

Fecal Samples: Per hospital policy, fecal samples were collected from every large animal inpatient on admission and then 3 times weekly from April 2002 to June 2003 or twice weekly from July 2003 to Dec 2012 as part of ongoing surveillance at the CSU-VTH. All fecal samples were cultured using standard enriched techniques to detect the presence of *Salmonella enterica*. Briefly, 1 g fecal samples were enriched in 9 mls tetrathionate[14] (TET) broth for 18 hrs at 43°C, then streaked for isolation on xylose-lysine-tergitol 4[15] (XLT4) agar media and incubated for 18 hrs at 43°C. Suspect colonies were subcultured on trypticase soy agar containing 5% sheep blood[16] (TSA) and incubated 18 hrs at 43°C and tested for agglutination using commercial polyvalent and O group-specific antisera to confirm identification as S. enterica. Serotype determination was performed by the USDA National Veterinary Services Laboratory (NVSL, Ames, IA).

Potential Risk Factors: Variables considered to be potential risk factors were derived from invoice data (e.g., antimicrobial classes, maximum level of care during hospitalization) contained within the electronic medical records database, healthcare-associated infections (HCAIs) database, as well as data obtained at the time of fecal sample submission (e.g., body system(s) affected, leukopenia or fever in previous 48 hrs) and data contained within the infection control program database. At

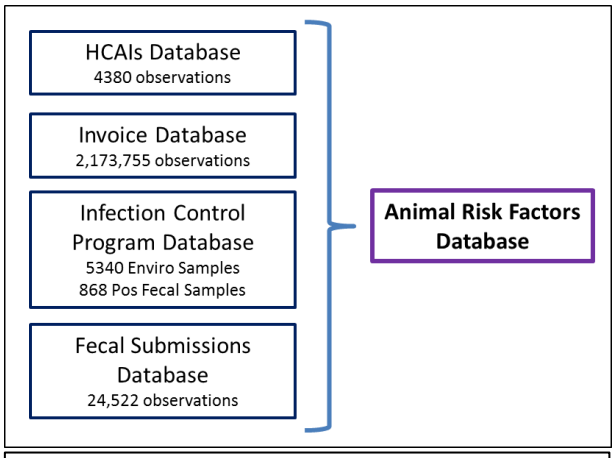

Figure 5.2 Derivation of animal risk factors database

fecal sample submission, data were recorded by caregivers via a web-based questionnaire (Appendix 1) regarding clinical status and treatment history for the 48 hrs prior to sample collection and was contained within the Infection Control Program database. Data was cross-validated from multiple sources to ensure accuracy (Figure 5.2).

Variables related to patient population characteristics: Independent variables representing patient characteristics included species (equine, bovine, New World camelid, small ruminant [caprine, ovine]), age (foal [< 1 year], yearling [1-2 years], adult [> 2 years]), sex (male, female), maximum degree of systemic illness during hospitalization (based on an ordinal rating of healthy/minimal, minor/moderate, or major systemic illness), body system(s) affected (normal [considered to be clinically normal/healthy], musculoskeletal, gastrointestinal, respiratory, renal, hepatic, reproductive, other – note: patients could have more than 1 affected system), transportation distance (≤ 20 miles, 20-50 miles, > 50 miles), leukopenia (equine WBC < 5000/µl; bovine, New World camelid, small ruminant < 5500/µl) within 48 hrs prior to fecal sample collection (yes, no, unknown [a complete blood count was not performed during hospitalization]), fever (equine > 101.5°F [38.6°C]; bovine, NWC, and small ruminant > 103°F [39.4°C]) within 48 hrs prior to fecal sample collection (yes/no), diarrhea (defined as loss of formed fecal consistency) within 48 hrs prior to fecal sample collection (yes/no), and reduced feed intake in pervious 48 hrs (yes/no).

Variables related to patient management and hospital factors: Independent variables representing patient management factors included route of antimicrobial used within 48 hrs prior to fecal sample collection (none, parenteral, oral, both), received any antimicrobials during hospitalization (yes/no), class of antimicrobial administered during hospitalization

(aminoglycoside, beta-lactam, any generation of cephalosporin, florfenicol, fluoroquinolone, macrolide, sulfas, tetracycline), class of gastroprotectant administered during hospitalization (antidiarrheal, H2-blocker, proton-pump inhibitor, mucosal protectant), general anesthesia in previous 48 hrs (yes/no), maximum level of care during hospitalization (routine care, level 1 – 4), and duration of hospitalization. Independent variables representing hospital factors included stabling location (equine main hospital, equine colic aisle, equine isolation, livestock hospital, calf isolation), service (livestock medicine and surgery, equine medicine, equine surgery, other ancillary services), and hospitalization during an outbreak defined as the identification of at least 2 patients (not from the same herd/flock) hospitalized within 10 days of each other with S. enterica isolates of the same phenotype (serotype and antibiogram).

Data Analysis: Data were entered in a spreadsheet, validated, and explored using descriptive statistics. Continuous variables were assessed for the assumption of normality in the logit scale; variables not meeting this assumption were categorized based on distributional quartiles or breakpoints with biological relevance. Conditional logistic regression was used to evaluate factors that might be associated with patients being culture-positive for *S. enterica* (the dependent variable for analyses), while controlling for matching on month and year of admission. All potential risk factors were screened for inclusion in the multivariable model building process using a critical $\alpha \leq 0.25$. The final multivariable model was identified using backwards selection with a critical $\alpha \leq 0.05$ for retention in the model. Confounding was identified by ≥20% change in parameter estimates when previously excluded variables were individually offered back to the multivariable model. When identified, confounding variables were forced into the multivariable models regardless of *P*-values. First-order interaction terms

for main effects variables included in final models were also evaluated. Odds ratios (OR) and profile likelihood 95% confidence intervals (95% CI) were calculated using the least squares mean estimates. In addition, analyses were stratified by species to assess species influence on parameter estimates. Crude population attributable fractions (PAFs) were estimated using parameter estimates (derived from univariable modelling of 1NEG patients) and population prevalence for variables of interest [5.13]. Pearson residuals and delta-betas were evaluated to assess model fit. All statistical analyses were conducted with a commercially available software program.[17]

5.4 RESULTS

Overall *Salmonella* shedding prevalence among large animal inpatients admitted during the study period was 5.9% (648/11,061) – with the highest prevalence occurring in bovine patients (73.5%; 476/648; Table 9.1); the majority of which were dairy breeds (98%; including Ayrshire, Brown Swiss, Holstein-Friesian, and Jersey breeds).

Among *Salmonella*-positive patients, the mean duration of hospitalization until detection of shedding was 2 days (median 1, IQR 1-3; range 1-35) with *Salmonella* detected most commonly in the first fecal sample (median 1; range 1-9). On average, the maximum number of fecal samples tested for fecal culture-positive patients was 1.7 (median 1, IQR 1-2; range 1-15); for 1NEG patients was 1.3 (median 1, IQR 1-2; range 1-3); and for 3NEG patients was 4.4 (median 3, IQR 3-5; range 3-25). There was a higher proportion of cattle among fecal culture-positive patients (73.5%; 476/648) compared to 1NEG patients (22.8%; 147/645) or 3NEG patients (4.1%; 25/609); whereas horses accounted for the majority of 1NEG patients (64.7%; 417/645) and 3NEG patients (83.4%; 508/609).

The duration of hospitalization was similar for cases and 1NEG patients, with a mean of 3.8 days (median 2, IQR 1-5) and 3.1 days (median 2, IQR 1-4), respectively; while it was notably longer for 3NEG patients with a mean of 11.7 (median 8, IQR 6-11).

Among patients with "unknown" leukopenia in the previous 48 hrs, the majority were bovine and equine patients (57.5% [543/945] and 35.7% [337/945], respectively) and occurred in patients hospitalized for ≤ 3 days (61.4%; 350/570), which was the most common duration of hospitalization among bovine patients (75.6%; 379/501).

During the study period there were 81 clusters of possible nosocomial transmission of *Salmonella* identified retrospectively among hospitalized patients (i.e., at least 2 patients from different farms with phenotypically similar isolates hospitalized within 10 days of each other). On average, this involved 3 patients (range 2 to 21) and continued for 13 days (range 1 to 64). Most commonly HCAIs involved bovine patients, accounting for approximately 52.7% (116/220) of all patients detected to be shedding phenotypically similar isolates.

Univariable and multivariable modeling with 1NEG patients (patients with at least 1 negative culture): Variables for hospitalization days, systemic illness, diarrhea in previous 48 hours, having a fever in previous 48 hours, leukopenia in previous 48 hours, route of antimicrobial drug administration, body system affected (specifically musculoskeletal, gastrointestinal, reproductive, other or normal), species, receiving a gastroprotectant in previous 48 hours, receiving any antimicrobial drugs in previous 48 hours, receiving aminoglycosides, beta-lactams, any generation of cephalosporin, or sulfas during hospitalization, age, sex, and transportation distance passed univariable screening and were included in multivariable model building (Table 5.1). The final multivariable model (Table 5.2)

included hospitalization days, species, systemic illness, and fever as main effects and an interaction between aminoglycoside and gastrointestinal system. Diarrhea and being hospitalized during an outbreak were forced into the model, regardless of *P*-value, because there was evidence of confounding in the final model.

Controlling for the effects of other variables in the model – cattle had a 15 times greater odds of shedding *Salmonella* as compared to horses (OR 15.8; 95% CI 10.3, 24.1) while the odds of shedding among New World camelid and small ruminants was no different than horses it was less than that for cattle. In general, there was a greater odds of shedding among patients hospitalized for 4 days or greater as compared to patients hospitalized 3 days or fewer (P<0.0001) with an almost 12 times greater odds among patients hospitalized for at least 15 days (OR 12.47; 95% CI 4.03, 38.60). The odds of shedding among patients with major or minor systemic illness were no different, but greater than that in healthy patients (P=0.04) and febrile patients had an almost 2 times greater odds of shedding as compared to afebrile patients (OR 1.9; 95% CI 1.2, 2.9). In addition, the odds of shedding among patients with gastrointestinal disease and receiving aminoglycosides was 2 times greater than for patients without gastrointestinal disease nor receiving aminoglycosides (OR 1.9; 95% CI 1.3, 2.6). In general, analyses stratified by species resulted in stronger measures of association for systemic illness and hospitalization days among horses.

Univariable and multivariable modeling with 3NEG patients (patients with at least 3 negative cultures): Variables for hospitalization days, systemic illness, diarrhea in previous 48 hrs, having a fever in previous 48 hrs, leukopenia in previous 48 hrs, having general anesthesia in previous 48 hrs, route of antimicrobial drug administration, body system affected (specifically

musculoskeletal, gastrointestinal, reproductive, respiratory, renal, other or normal), species, receiving a gastroprotectant in previous 48 hrs, receiving any antimicrobial drugs in previous 48 hrs, receiving aminoglycoside, beta-lactam, any generation of cephalosporin, tetracycline, fluoroquinolone, florfenicol, or sulfas during hospitalization, age, sex, transportation distance, and being hospitalized during an outbreak passed univariable screening and were included in multivariable model building (Table 5.1). The final multivariable model included hospitalization days, species, gastrointestinal disease, receiving aminoglycosides, having leukopenia, sex, and transportation distance. Variables for systemic illness, fever, age, musculoskeletal and reproductive systems, and being hospitalized during an outbreak were forced into the model, regardless of *P*-value, because there was evidence of confounding in the final model (Table 5.2). There were no significant first-order interactions among main effects in this model.

Controlling for the effects of other variables in the model – cattle had a 22 times greater odds of shedding compared to horses (22.3; 95% CI 10.2, 49.2) while the odds of shedding among New World camelid and small ruminant were no different from horses they were less than that for cattle. Patients hospitalized for at least 4 days were less likely to shed *Salmonella* as compared to patients hospitalized for 3 days or fewer ($P<0.0001$). In addition, there was an increased odds of shedding *Salmonella* for patients with gastrointestinal disease as compared to healthy patients ($P=0.01$), for patients that received aminoglycosides during hospitalization as compared to patients who did not (P=0.01), patients that were female as compared to male (P=0.01), and patients transported 20 or fewer miles as compared to patients transported 50 miles or greater ($P=0.01$). Interestingly, patients with "unknown" leukopenia were almost 3 times more likely to shed as compared to patients without leukopenia (OR 2.75; 95% CI 1.72,

4.41) while the odds of shedding among patients with leukopenia was no different than for patients without leukopenia. In general, analyses stratified by species resulted in stronger measures of association for hospitalization days among horses.

Population attributable fraction (PAF): Population attributable fraction (PAF) is the proportion of disease risk in a population that can be attributed to a particular risk factor or group of factors [5.13]– taking into account both the magnitude of effect and the prevalence of the risk factor in the population. Note that PAF is not dividing total risk among different factors rather it is giving a relative indication as to what proportion of disease may be prevented if that particular factor or constellation of factors were eliminated from the population (assuming the frequencies of all other factors in the population remain unchanged) and that factors can contribute to more than one attributable fraction. In this patient population, approximately 2/3 of the shedding risk can be attributed to each of systemic illness (PAF=69.4%; classified as healthy, minor, and major) or gastrointestinal disease (PAF=59.9%; e.g., colic, diarrhea). Only 2.7% of the shedding risk can be attributed to patients demonstrating the classic triad of signs typically associated with *Salmonella* shedding, diarrhea, fever, and leukopenia [5.14, 5.15]. When considering each clinical sign alone, the PAF for diarrhea, fever, and leukopenia were 73.8%, 30.9%, and 57.7%, respectively. Finally, approximately 70% of patient shedding in this population can be attributed to species – specifically cattle (PAF=68.7%).

5.5 DISCUSSION:

Factors associated with endemic shedding of *Salmonella* among the general large animal inpatient population are similar to those identified with targeted surveillance and during times of epidemic disease [5.6, 5.10, 5.16] – namely factors related to systemic disease; including fever and leukopenia, gastrointestinal disease, duration of hospitalization and species. Historically, *Salmonella* shedding has been assumed to be typified by patients exhibiting the classical triad of signs – diarrhea, fever, and leukopenia [5.14, 5.15]. While it is true that all 3 signs may be a specific indicator of clinical salmonellosis, the results reported here indicate that taken together this triad of signs is not a sensitive indicator of shedding risk (i.e., many other large animal inpatients shed *Salmonella* without showing all 3 signs). Because the frequency with which this complex of signs occurs together in a population can be quite low it results in a very poor detection rate upon which to base infection control policy. Alternatively, approximately 70% of patient shedding could be attributed to systemic illness – irrespective of the body system affected – suggesting that infection control efforts may best be directed at patients with severe disease.

In the study reported here two comparison groups were used to develop two different multivariable models – one control group consisting of patients with at least 1 negative culture (1NEG patients) and another consisting of patients with at least 3 negative cultures (3NEG patients) – based on common comparison groups reported in the literature [5.7, 5.8, 5.10, 5.16]. It is important to consider whether these two control groups represent different patient populations when they are being used in comparisons with the case population. For example, 1NEG patients generally had a similar duration of hospitalization as cases (mean 3.1 and 3.8

days, respectively) while 3NEG patients were hospitalized considerably longer (mean 11.7 days). As a result, the odds of shedding increased with duration of hospitalization when comparing to 1NEG patients, but decreased with duration of hospitalization when comparing to 3NEG patients. These are two very different answers – therefore the question becomes which provides the most relevant comparison upon which to base policy decisions?

We contend that the comparison group consisting of 1NEG patients (those with at least 1 negative culture) is the more appropriate comparison group (i.e., is more representative of the case population) although some patients may have been misclassified (i.e., classified as negative when they are truly shedding) due to the insensitive nature of the *Salmonella* culture methodology and the limited number of fecal cultures performed on some patients in this study. This misclassification is expected to be differential in this particular study and will likely attenuate the strength of associations (i.e., it is a nullifying bias). With this in mind, we can estimate the proportion of *Salmonella* shedding in this population that can be attributed to systemic illness – approximately 70% – and realize that severity of disease, as defined by systemic illness, is a likely candidate upon which to base infection control policy. If we manage patients with more severe systemic illness differently (i.e., cohort them in an area with heightened barrier precautions) then we will in effect have "detected" approximately two-thirds of shedding patients. This will presumably limit the potential for environmental contamination, which is typically seen when animals are shedding, as the lag time of 3-5 days (depending on testing methodology) to realize results has effectively been eliminated.

Although 3NEG patients may represent a slightly different population as compared to the case population – resultant associations do tend to support our assertion; that patients

with more severe disease are more likely to be shedding. While systemic illness was a confounder in this model, other variables that suggest disease severity, such as gastrointestinal disease, receiving aminoglycosides, and experiencing leukopenia, were associated with patient shedding. While experiencing leukopenia in the previous 48 hrs was associated with patient shedding – a stronger association was noted for patients with an unknown leukocyte count. We suspect this is related to the high proportion of shedding dairy cattle in our population that are hospitalized for less than 3 days and are medically managed without consulting complete blood counts (typically these patients present with a displaced abomasum). Additional factors associated with shedding in this model were sex (specifically being female) and transportation distance (specifically traveling ≤ 20 miles). Again we believe these associations to be related to our patient population – 25% being mostly lactating dairy cattle with a high shedding prevalence which typically reside within 20 miles of our facility. While this is specific to our practice area it highlights the importance of developing an infection control program that is specific to the facility and practice environment.

For facilities managing more than one species, consideration should be given to species segregation as a means of shedding risk mitigation, as again approximately 70% of patient shedding can be attributed to species in this study population (PAF=68.7%). It is important to interpret this in light of the patient population from which it was derived – specifically that the bovine case load at our facility is predominately dairy breeds and generally has a high shedding prevalence. While this is likely similar to other academic institutions (i.e., managing multiple species), it may not be so with respect to private practices. As the hospital population varies with regard to risk factors of interest, the results of this study become less generalizable. In

addition, consideration should be given to differences in facilities and infection control protocols as these will likely impact infectious disease transmission among inpatients.

While this was a retrospective study and relied on the quality of data recorded in the medical record it was based on routine fecal surveillance for an extended period of time. As such we believe that resultant associations do support our assertion that consideration of severity of disease should be a key component to policy development and will potentially allow for substantial risk mitigation with respect to endemic patient shedding of *Salmonella* and ultimately to the prevention of epidemic disease.

5.6 ENDNOTES

[14]Becton Dickinson and Co, Cockeysville, MD, USA
[15]Hardy Diagnostics, Santa Maria, CA, USA
[16]BD Diagnostic Systems, Sparks, MD, USA
[17]SAS v9.3, SAS Institute, Inc., Cary, NC

Table 5.1: Univariable conditional logistic regression analysis of factors associated with large anima inpatient fecal shedding of *Salmonella enterica*

		Case (N=648)	1 NEG (N=645)				2 NEG (N=609)			
Variable		**N**	**N**	**OR**	**95% CI**	***P*-value**	**N**	**OR**	**95% CI**	***P*-value**
Patient age	**foal**	77	77	0.96	0.68 – 1.35	0.04	58	1.23	0.85 – 1.77	0.01
	yearling	18	37	0.48	0.03 – 0.84		38	0.45	0.25 – 0.79	
	adult	553	531	ref			513	ref		
Sex	**male**	559	370	4.77	3.61 – 6.31	<0.001	274	7.81	5.88 – 10.39	<0.0001
	female	89	275	ref			335	ref		
Species	**bovine**	476	147	10.53	7.91 – 14.01	<0.001	25	87.82	52.63 – 146.55	<0.0001
	NWC	23	57	1.17	0.68 – 2.00		64	1.14	0.65 – 1.98	
	small ruminant	6	24	0.07	0.26 – 1.73		12	1.54	0.52 – 4.56	
	equine	143	417	ref			508	ref		
Systemic illness	**major**	128	83	4.85	3.32 – 7.08	<0.0001	158	1.67	1.16 – 2.40	<0.0001
	minor	432	303	4.59	3.41 – 6.18		287	3.07	2.24 – 4.19	
	healthy	88	259	ref			164	ref		
Hospitalization days	**≥ 15 days**	20	6	3.69	1.47 – 9.30	0.001	129	0.02	0.01 – 0.03	<0.0001
	8-14 days	52	29	2.10	1.27 – 3.33		229	0.09	0.06 – 0.13	
	4-7 days	157	139	1.28	0.98 – 1.67		202	0.02	0.01 – 0.04	
	≤ 3 days	419	471	ref			49	ref		
Maximum care level during hospitalization	**level 4**	17	10	1.55	0.69 – 3.50	<0.0001	33	0.25	0.13 – 0.46	<0.0001
	level 3	43	52	0.69	0.45 – 1.06		91	0.20	0.14 – 0.31	
	level 2	41	69	0.49	0.32 – 0.74		119	0.15	0.10 – 0.22	

	level 1	32	85	0.30	0.19 –	0.46		122	0.10	0.07 –	0.16		
	routine care	515	429	ref				244	ref				
Anesthesia in previous 48 hrs	**yes**	131	119	1.12	0.84 –	1.49	0.43	212	0.47	0.37 –	0.61	<0.0001	
	no	517	526	ref				397	ref				
Leukopenia in previous 48 hrs	**yes**	37	18	3.47	1.91 –	6.31	<0.0001	63	1.79	1.14 –	2.84	<0.0001	
	unknown	469	375	2.22	1.73 –	2.85		101	15.28	11.26 –	20.73		
	no	142	252	ref				445	ref				
Diarrhea in previous 48 hrs	**yes**	276	93	4.46	3.39 –	5.86	<0.0001	117	3.23	2.49 –	4.18	<0.0001	
	no	372	552	ref				492	ref				
Febrile in previous 48 hrs	**yes**	173	52	4.75	3.37 –	6.69	<0.0001	127	1.76	1.35 –	2.31	<0.0001	
	unknown	93	59	2.26	1.57 –	3.25		1	121.75	16.82 –	881.30		
	no	382	534	ref				481	ref				
Healthy	**yes**	41	86	0.42	0.28 –	0.63	<0.0001	109	0.28	0.19 –	0.41	<0.0001	
	no	607	559	ref				500	ref				
Gastrointestinal disease	**yes**	477	244	4.81	3.77 –	6.14	<0.0001	207	5.74	4.45 –	7.39	<0.0001	
	no	171	401	ref				402	ref				
Musculoskeletal disease	**yes**	97	213	0.35	0.27 –	0.47	<0.0001	345	0.14	0.11 –	0.19	<0.0001	
	no	551	432	ref				264	ref				
Renal disease	**yes**	9	8	1.11	0.43 –	2.87	0.84	23	0.36	0.17 –	0.80	0.01	
	no	639	637	ref				586	ref				

Respiratory disease	**yes**	56	33	1.75	1.12	–	2.73	0.01	yes	0.78	0.54	–	1.14	0.20
	no	592	612	ref					no	ref				
Reproductive disease	**yes**	65	51	1.30	0.89	–	1.91	0.14	33	1.99	1.28	–	3.10	0.002
	no	583	594	ref					576	ref				
Disease associated with other body systems	**yes**	68	87	0.75	0.53	–	1.05	0.09	134	0.42	0.30	–	0.58	<0.0001
	no	580	558	ref					475	ref				
GI protectant during hospitalization	**yes**	79	62	1.31	0.92	–	1.87	0.01	218	0.25	0.18	–	0.33	<0.0001
	no	569	583	ref					391	ref				
AMD during hospitalization	**yes**	354	321	1.22	0.98	–	1.52	0.08	461	0.39	0.30	–	0.50	<0.0001
	no	294	324	ref					148	ref				
AMD route of administration	**Parenteral**	306	260	1.30	1.04	–	1.64	0.004	243.00	0.67	0.51	–	0.87	<0.0001
	Both	37	31	1.30	0.79	–	2.16		173.00	0.11	0.07	–	0.16	
	Oral	11	30	0.41	0.20	–	0.84		45.00	0.12	0.06	–	0.24	
	None	294	324	ref					148.00	ref				
Aminoglycoside during hospitalization	**yes**	109	146	0.69	0.52	–	0.91	0.01	309	0.18	0.13	–	0.23	<0.0001
	no	539	499	ref					300	ref				
Beta-lactam during hospitalization	**yes**	320	259	1.46	1.17	–	1.82	0.001	395	0.54	0.43	–	0.67	<0.0001
	no	328	386	ref					214	ref				

Cephalosporin during hospitalization	**yes**	237	182	1.46	1.16	–	1.85	0.002	198	1.20	0.95	–	1.51	0.1343
	no	411	463	ref					411	ref				
Chloramphenicol during hospitalization	**yes**	8	9	0.86	0.33	–	2.26	0.76	20	0.39	0.17	–	0.89	0.03
	no	640	636	ref					589	ref				
Fluoroquinolone during hospitalization	**yes**	9	1	9.00	1.14	–	71.04	0.04	53	0.14	0.07	–	0.30	<0.0001
	no	639	644	ref					556	ref				
Sulfamethoxazole during hospitalizations	**yes**	30	48	0.61	0.38	–	0.97	0.04	126	0.18	0.12	–	0.27	<0.0001
	no	618	597	ref					483	ref				
Tetracycline during hospitalization	**yes**	37	44	0.82	0.52	–	1.30	0.39	112	0.24	0.16	–	0.36	<0.0001
	no	611	601	ref					497	ref				
Transportation distance	**≤ 20 miles**	471	287	4.70	3.52	–	6.29	<0.0001	124	17.06	12.20	–	23.85	<0.0001
	20-50 miles	95	118	2.31	1.60	–	3.34		144	2.79	1.93	–	4.04	
	≥ 50 miles	82	240	ref					341	ref				
Hospitalized during detected nosocomial transmission	**yes**	247	256	0.88	0.64	–	1.19	0.39	240	0.81	0.60	–	1.11	0.19
	no	401	389	ref					369	ref				

AMD = antimicrobial drug; CI = confidence interval; NWC = New World camelid; OR = odds ratio; ref = reference

Table 5.2: Final multivariable conditional logistic regression models of factors associated with large animal inpatient fecal shedding of *Salmonella enterica*

		Control Group 1 (≥ 1 negative culture)			Control Group 2 (≥ 3 negative cultures)		
Variable	**Category**	**OR**	**95% CI**	***P*-value**	**OR**	**95% CI**	***P*-value**
Hospitalization days	≥ 15 days	12.47	4.03 – 38.60	<0.0001	0.17	0.08 – 0.37	<0.0001
	8-14 days	2.51	1.37 – 4.61		0.13	0.07 – 0.24	
	4-7 days	1.87	1.28 – 2.72		0.30	0.18 – 0.53	
	≤ 3 days	ref			ref		
Systemic illness	major	1.89	1.10 – 3.26	0.04	CONFOUNDER		0.61
	minor	1.67	1.09 – 2.54				
	healthy	ref					
Species	bovine	15.80	10.34 – 24.14	<0.0001	22.32	10.15 – 49.11	<0.0001
	NWC	1.61	0.83 – 3.11		1.53	0.77 – 3.03	
	small ruminant	0.98	0.36 – 2.67		1.45	0.37 – 5.70	
	equine	ref			ref		
Febrile in previous 48 hours	yes	1.89	1.24 – 2.88	0.01	CONFOUNDER		0.13
	unknown	0.92	0.59 – 1.45				
	no	ref					
Gastrointestinal disease	yes	INTERACTION		0.07	2.10	1.21 – 3.66	0.01
	no				ref		
Aminoglycoside use during hospitalization	yes	INTERACTION		0.05	1.84	1.09 – 3.10	0.02
	no				ref		
Diarrhea in previous	yes	CONFOUNDER		0.14	NOT SIGNIFICANT		

48 hours	no					
Hospitalized during detected nosocomial transmission	yes	CONFOUNDER	0.95	CONFOUNDER		0.56
	no					
Leukopenic in previous 48 hours	unknown	NOT SIGNIFICANT		2.75	1.72 – 4.41	0.0001
	yes			1.17	0.62 – 2.20	
	no			ref		
Musculoskeletal disease	yes	NOT SIGNIFICANT		CONFOUNDER		0.10
	no					
Reproductive disease	yes	NOT SIGNIFICANT		CONFOUNDER		0.14
	no					
Patient age	Foal	NOT SIGNIFICANT		CONFOUNDER		0.52
	Yearling					
	Adult					
Transportation distance	≤ 20 miles	NOT SIGNIFICANT		1.58	0.96 – 2.60	0.05
	20-50 miles			0.83	0.49 – 1.42	
	≥ 50 miles			ref		
Sex	female	NOT SIGNIFICANT		1.68	1.12 – 2.54	0.01
	male					
GI*Aminoglycoside			0.01	NO FIRST ORDER INTERACTIONS		

GI Disease	Aminoglycoside				
yes	yes	1.94	1.31	–	2.59
yes	no	0.38	-0.03	–	0.78
no	yes	0.55	-0.01	–	1.11
no	no	ref			

CI = confidence interval; NWC = New World camelid; OR = odds ratio; ref = reference

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6 CHAPTER 6: FACTORS ASSOCIATED WITH EQUINE SHEDDING OF MULTI-DRUG RESISTANT *SALMONELLA ENTERICA* AND ITS IMPACT ON HEALTH OUTCOMES

6.1 SUMMARY

Background: *Salmonella enterica* can be an important factor in healthcare-associated epidemics and zoonotic disease in veterinary hospitals – with outbreaks of multi-drug resistant (MDR) *Salmonella* among equine patients resulting in high case fatality rates and substantial financial cost.

Hypothesis/Objectives: The objectives of this study were, 1) to determine factors associated with fecal shedding of MDR-*Salmonella*; and 2) to determine what effect *Salmonella* shedding may have on health outcomes of previously hospitalized horses and their stablemates.

Methods: Patients eligible for this case-control study included those having fecal cultures for *S. enterica* as part of a surveillance program from January 2011 through December 2012. Data regarding exposures of interest were collected retrospectively from medical records. Information on long-term outcomes was obtained by administering a phone survey to horse owners. Multivariable regression techniques were used to determine factors associated with shedding MDR-*Salmonella* and subsequent health outcomes.

Results: Equine patients enrolled in this study included 94 culture-positive (29 MDR and 65 susceptible) and 279 culture-negative (on at least 3 fecal samples) horses from 199 different farms. Horses experiencing diarrhea during hospitalization were more likely to shed *Salmonella* (OR 1.88; 95% CI 1.02, 3.45) compared to horses without diarrhea; and horses having decreased feed intake during hospitalization were more likely to shed MDR-strains (OR 5.95; 95% CI 1.21,

29.20) compared to horses with normal feed intake. In general, shedding *Salmonella* did not increase the long-term risk for non-survival, colic or abnormal feces after discharge of hospitalized horses nor did it increase the risk for hospitalization or abnormal feces in stablemates.

Conclusions and clinical relevance: In general, receiving antimicrobial therapy during hospitalization was not associated with shedding *Salmonella*, nor was it associated with shedding of MDR-strains. Additionally, shedding *Salmonella* did not decrease long-term survival or increase the occurrence of colic or abnormal feces in the hospitalized horse nor increase the risk for hospitalization or abnormal feces in stablemates. Despite these findings, in order to mitigate the exposure risk to other horses and personnel, it is still recommended to manage horses shedding *Salmonella* separately from other resident horses and to employ rigorous personal and environmental hygiene.

6.2 INTRODUCTION

Salmonella enterica can be an important factor in healthcare-associated epidemics and zoonotic disease in veterinary hospitals [6.1]. Outbreaks associated with multi-drug resistant (MDR) *Salmonella* among equine patients can result in high case fatality rates and substantial financial cost [6.2]. As such, routine surveillance to detect this organism among equine patients is commonly performed on targeted high-risk subgroups (e.g., gastrointestinal disease) and upon recognition of epidemic disease [6.2, 6.3], and less commonly performed continuously on all equine inpatients [6.4, 6.5]. While there are many reports suggesting patients are more likely to shed *Salmonella* in their feces at times of stress or systemic compromise [6.3, 6.5-6.8] there are no reports that indicate what factors may be important to horses shedding MDR-

strains; something that can result in infections that are much more difficult to treat in horses and humans alike.

While detecting shedding among hospitalized patients can be managed with increased infection control measures (i.e., segregation and/or barrier nursing precautions) within the hospital environment, it becomes a challenge to continue these precautions in the home environment. As such, gaining a better understanding of the risk shedding horses may pose to their stablemates will allow for improved recommendations for owners upon their animal returning to the resident farm from the hospital. A single study investigating health impacts after shedding *Salmonella* found that stablemates of horses shedding *Salmonella* during hospitalization were not at an increased risk for developing diarrhea [6.9]. While there are reports of *Salmonella* shedding during hospitalization not being associated with increased mortality during hospitalization [6.10], it may be associated with an increase in the long-term risk of death after discharge from the hospital [6.9].

With the relative paucity of evidence in the literature, it is very difficult to inform horse owners on what it means when their animal is shedding *Salmonella* – MDR or otherwise – with respect to that animals prognosis and risks to stablemates on resident farms. The objectives of this study were, 1) to determine factors associated with fecal shedding of MDR-*Salmonella*; and 2) to determine what effect *Salmonella* shedding may have on health outcomes of previously hospitalized horses and their stablemates.

6.3 MATERIALS AND METHODS

Study overview: A retrospective case-control study was undertaken to determine factors associated with equine shedding of MDR-*Salmonella enterica*. In total, 373 horses were

enrolled in the study, 94 cases (29 classified as MDR-strains) and 279 controls (Figure 6.1). Multinomial logistic regression was used to determine factors associated with patients shedding of MDR-*Salmonella*. In addition, a follow-up study was conducted to assess long-term outcomes associated with *Salmonella* shedding among previously +hospitalized horses and their stablemates. In total, 221 surveys were completed (59.2% response rate) and conditional logistic regression was used to determine factors associated with long-term health outcomes among previously hospitalized horses and their stablemates.

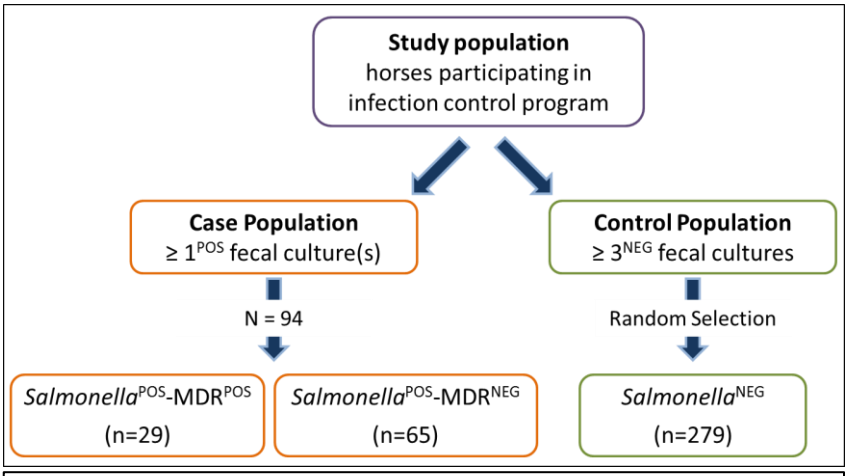

Figure 6.1 Derivation of study participants

Study population: Horses included in this study were selected from all patients hospitalized between January 2011 and December 2012 at a large equine referral practice in Kentucky. Study eligibility was restricted to those patients having participated in routine fecal surveillance for *S. enterica* (1 fecal sample daily for 3 days, then every Monday thereafter) as part of long-term infection control efforts. Cases included all horses during the study period which were culture-positive on at least 1 fecal sample during hospitalization (n=94). Cases were further classified as having MDR infections (*Salmonella*^{POS}-MDR^{POS}) if the patients' *Salmonella* isolates were resistant to at least 3 antimicrobial drugs (AMDs) based on the Kirby-Bauer disc diffusion method (n=29) or not MDR (*Salmonella*^{POS}-MDR^{NEG}) if resistant to fewer than 3 AMDs (n=65) when tested against 12 AMDs. Controls were selected randomly from all negative horses with

at least 3 negative fecal cultures (*Salmonella*^{NEG}); matching 3 controls to 1 case on year and month of admission (n=282). Of the 282 control horses selected for the study, 3 were sampled but had never been hospitalized and were therefore not included in the study – resulting in 279 culture-negative patients enrolled in the study.

Microbiological culture of fecal samples: All fecal samples were cultured using standard enriched techniques for the presence of *S. enterica*. Briefly, 1 gram fecal samples were enriched in 9 mls selenite[18] (SEL) broth for 18 hrs at 43°C, then streaked for isolation on Hektoen enteric[18] (HE) agar media and incubated for 18 hrs at 43°C. Suspect colonies were subcultured on trypticase soy agar containing 5% sheep blood[19] (TSA) and incubated 18 hrs at 43°C and tested with a commercial identification kit[19] per manufacturer's instructions. Antimicrobial susceptibility testing was performed on all isolates by the Kirby-Bauer disc diffusion method against a panel of AMDs (amikacin, ampicillin, cefazolin, cefotaxime, ceftiofur, chloramphenicol, enrofloxacin, gentamicin, imipenem, tetracycline, ticarcillin-clavulanate, and trimethoprim-sulfamethoxaz) [6.11].

Data Sources and Collection: Data were collected retrospectively from existing paper and electronic medical records and prospectively via owner questionnaire. Medical records were reviewed by 2 individuals to obtain information on exposure variables of interest occurring during hospitalization. Data were recording on pre-designed data collection forms to facilitate consistency in data collection (Appendix 2). These included both intrinsic patient factors and patient management factors. Patient factors included age (foal [≤ 1 year], adult [> 1year]), sex (male, female, unknown), breed (Thoroughbred, other), disease category (non-surgical colic, surgical colic, diarrhea, foaling complication, neonatal, other), diarrhea during hospitalization

(yes/no), fever (rectal temperature > 101°F [38.3°C]) during hospitalization (yes/no), leukopenia (< 5000 wbc/µL) during hospitalization (yes, no, unknown [complete blood count not performed during hospitalization), reduced feed intake during hospitalization (yes/no), and hospitalization days (≤ 3 days, ≥ 4 days). Management factors included date of admission and discharge, service managing the case (medicine, surgery, both), anesthesia or surgery during hospitalization (yes/no), maximum level of care during hospitalization (reported in the medical record as high [intensive care], surgical [moderate care], or low [low level care]), antimicrobial therapy during hospitalization (yes/no), class(es) of antimicrobial(s) received during hospitalization (aminoglycoside, beta lactam, cephalosporin, fluoroquinolone, macrolide, nitromidazoles, chloramphenicol, polymixin B, rifamycin, sulfa, tetracycline), gastrointestinal protectant received during hospitalization (yes/no), and gastrointestinal protectant class(es) (antidiarrheal, H2-blocker, proton pump inhibitor, mucosal protectant, probiotic).

Owner questionnaires were administered by two individuals to collect information regarding health outcomes of interest among previously hospitalized horses and their stablemates within a year of discharge from the hospital and to collect information on farm characteristics from July through December 2013. Attempts to contact horse owners were made via phone (maximum of 3 attempts before classifying as "unreachable") in order to obtain information using a pre-designed owner questionnaire (Appendix 3). Owners with multiple horses, at their request, were provided a written copy of the phone survey via fax or email to facilitate data collection on multiple horses. Surveys completed via fax or emails were checked for completion – if incomplete, study personnel performed a follow-up phone call to ascertain missing information. Outcome events that were investigated in the previously

hospitalized horses included horse survival (yes, no, unknown), occurrence of colic episodes after hospital discharge (yes, no, unknown; number of occurrences), and occurrence of abnormal feces (defined as cow-pie or watery consistency) after hospital discharge (yes, no, unknown; number of occurrences). Outcome events investigated regarding stablemates of the previously hospitalized horses included hospitalization of stablemates (yes, no, unknown) and occurrence of abnormal feces (as defined above) among stablemates (yes, no, unknown; number of adults, number of foals). Farm characteristics that were also recorded included number of horses housed on the property (on average), whether number of horses housed on the property changes (yes, no; minimum number, maximum number, average number in the month of June), management of resident horses returning from a hospital visit (separate housing, normal housing; number of days separated), management of resident horses returning from a show or event (separate housing, normal housing; number of days separated), and management of non-resident horses arriving to the property (separate housing, normal housing; number of days separated). Data collectors were blinded to the culture status of the horse and conducted the owner questionnaire prior to retrieving medical records data.

Data Analysis: Data were entered in a spreadsheet, validated, and explored using descriptive statistics. Continuous variables were categorized based on distributional quartiles or breakpoints with biological relevance. For categorical variables, simple comparisons were made with Chi-squared tests. Multinomial logistic regression (PROC SURVEYLOGISTIC[20]) was performed to evaluate whether factors might be associated with patient shedding MDR-*Salmonella*, controlling for clustering of patients by farm and matching by month and year. The assumption of independence of irrelevant alternatives (IIA) and parallel slopes, that the odds of

one level of the outcome is independent of the other available alternatives, as evaluated by Hausman's specification test, was met in this analysis. The dependent variable for this multivariable analysis was *Salmonella* culture status: *Salmonella*^{NEG}, *Salmonella*^{POS}-MDR^{NEG}, or *Salmonella*^{POS}-MDR^{POS}.

Secondarily, conditional logistic regression was performed using generalized estimating equations[20] to evaluate factors that might be associated with health outcomes of interest, controlling for clustering of horses by farm and matching by month and year. Health outcomes of interest included survival, occurrence of colic, or occurrence of abnormal feces among previously hospitalized horses during the follow-up period; and hospitalization or occurrence of abnormal feces in stablemates of previously hospitalized horses during the follow-up period. If the owner reported it was unknown whether the particular outcome had occurred during the follow-up period, the horse was excluded from analyses.

For each study outcome, univariable screening was performed on all variables of interest with a critical $\alpha \leq 0.25$ to be included in the multivariable model building process. The final multivariable model was identified using backwards selection with a critical $\alpha \leq 0.05$ for retention in the model. Confounding was identified by ≥20% change in parameter estimates when previously excluded variables were offered back to the multivariable models. When identified, confounding variables were forced into the multivariable models regardless of *P*-values. First-order interaction terms for main effects variables included in final models were also evaluated. Odds ratios (OR) and profile likelihood 95% confidence intervals (95% CI) were calculated from model parameter estimates.

6.4 RESULTS

The 373 horses included in the study resided on 199 different farms. The majority of farms (70.4%; n=140) provided information on 1 horse each, 24 farms (12.1%) provided information on 2 horses each, 7 farms (3.5%) provided information on 3 horses each, 9 farms (4.5%) provided information on 4 horses each, 10 farms (5.0%) provided information on 5 horses each, 2 farms (1.0%) provided information on 6 and 9 horses each, and 1 farm (0.05%) provided information on 7, 8, 10, 11, and 12 horses each.

The majority of study animals were adults (55.8%) greater than 1 year of age, females (69.9%), and Thoroughbreds (85.5%; Table 6.1). Most were hospitalized as companions to ill patients (26.8%; e.g., mares with ill foals) and for surgical colic (19.6%). In general, patients were hospitalized for at least 4 days or greater (60.9%), had a maximum care level of high (60.1%) requiring multiple treatments per day and experienced a fever (rectal temperature >101°F; 69.4%) at some point during hospitalization. However, the majority of horses did not experience diarrhea (55.5%) or leukopenia (< 5000 wbc/µL; 46.9%) during hospitalization.

Overall, owner response rate was 66.8% (133/199) representing 59.2% (221/373) of horses enrolled in this study. There was no statistically detectable difference in owner response rate for *Salmonella*^{POS} (57.4%; 54/94) or *Salmonella*^{NEG} (59.8%; 167/279) horses (P=0.68); nor any difference in owner response rate for *Salmonella*^{NEG} (59.8%; 167/279), *Salmonella*^{POS}-MDR^{NEG} (63.1%; 41/65), or *Salmonella*^{POS}-MDR^{POS} (44.8%; 13/29) horses (P=0.23). Questionnaires were unable to be completed for 20 horses, as owners were unable to be contacted within 3 attempts. Of questionnaires completed (n=353), the majority were administered by phone (58.4%; 206/353) with significantly fewer by email (8.5%; 30/353), and

fax (33.1%; 117/353; P<0.001). However there was no significant difference in owner response rate for *Salmonella*^{POS} or *Salmonella*^{NEG} horses by any administration method used (P=0.55); nor for *Salmonella*^{NEG}, *Salmonella*^{POS}-MDR^{NEG}, or *Salmonella*^{POS}-MDR^{POS} (P=0.23). There was no significant difference between participants and non-participants with respect to patient age (P=0.96), sex (P=0.78), or breed (P=0.24).

Factors associated with shedding MDR-*Salmonella*: Of the 373 horses enrolled in this study, 65 were classified as *Salmonella*^{POS}-MDR^{NEG}, 29 were classified as *Salmonella*^{POS}-MDR^{POS} and 279 were classified as *Salmonella*^{NEG}. Among all culture-positive horses, the majority were identified as shedding *Salmonella* on their first fecal sample (43.6%; 41/94) with comparatively fewer detected on sample 2 (18.1%; 17/94), sample 3 (19.1%; 18/94), sample 4 (8.5%; 8/94), sample 5 (9.6%; 9/94), and sample 6 (1.1%; 1/94).

Independent variables for diarrhea during hospitalization, reduced feed intake during hospitalization, and receiving an antidiarrheal (e.g., pepto bismuth, biosponge) during hospitalization passed univariable screening and were included in multivariable model building (Table 6.2). The final multivariable model included diarrhea and reduced feed intake as main effects. There was no confounding detected and there were no first-order interactions among main effects variables in this model. Controlling for the effects of other variables in the model, horses with diarrhea had an almost 2 times greater odds of shedding *Salmonella* (OR 1.88; 95% CI 1.02, 3.45) but were not more likely to shed MDR-*Salmonella* (OR 0.54; 95% CI 0.20, 1.47) as compared to horses without diarrhea during hospitalization. Horses with reduced feed intake during hospitalization had a decreased odds of shedding *Salmonella* (OR 0.66; 95% CI 0.28,

1.54) but an almost 6 times greater odds of shedding MDR-*Salmonella* (OR 5.95; 95% CI 1.21, 29.20) as compared to horses with normal feed intake during hospitalization.

Long-term risk for non-survival among formerly hospitalized horses: Of the 221 completed surveys, respondents did not own 24 horses at the time of the phone survey; all were excluded from this analysis. Of the 197 horses remaining (representing 122 farms; minimum 1 horse to maximum 7 horses), 12 were classified as *Salmonella*^{POS}-MDR^{POS}, 36 as *Salmonella*^{POS}-MDR^{NEG} and 149 as *Salmonella*^{NEG}. For the purposes of this analysis, all culture-positive horses were collapsed into a single category (*Salmonella*^{POS}) regardless of susceptibility results to improve model stability. Of the formerly hospitalized horses, 39 (19.7%) were reported to have died or were euthanized during the follow-up period; 11 (28.2%) having been *Salmonella*^{POS}.

Independent variables for disease category, leukopenia, reduced feed intake, receiving antimicrobial drugs during hospitalization, maximum care level during hospitalization, hospitalization days, breed, receiving a gastroprotectant during hospitalization, and having colic after being discharged from the hospital passed univariable screening and were included in multivariable model building (Table 6.3). The final multivariable model included maximum care level during hospitalization as the main effect. Fecal culture result was forced into the model, regardless of *P*-value, as it was the main exposure of interest. There was no confounding detected during the modeling process. Controlling for the effects of other variables in the model, horses receiving a high care level during hospitalization were 3.5 times more likely to die during the follow-up period (OR 3.52; 95% CI 1.14, 10.94) and horses receiving a moderate surgical level of care were 2 times more likely to die during the follow-up period (OR 2.05; 95%

CI 0.55, 8.88) as compared to horses receiving a low care level during hospitalization. Fecal culture status during hospitalization was not associated with non-survival (*P*=0.61).

Colic in formerly hospitalized horses: Of the 221 completed surveys, owners did not know if colic occurred after discharge for 17 previously hospitalized horses; all were excluded from this analysis. Of the 204 horses remaining (representing 127 farms; minimum 1 horse to maximum 9 horses), 13 were classified as *Salmonella*^{POS}-MDR^{POS}, 37 as *Salmonella*^{POS}-MDR^{NEG}, and 154 as *Salmonella*^{NEG}. Of formerly hospitalized horses, owners reported only 18 (8.8%) having the outcome of interest (4 *Salmonella*-positive and 14 *Salmonella*-negative) and thus we were unable to develop a final multivariable model. Univariable analysis revealed that formerly hospitalized horses that were culture-positive for *Salmonella* did not have an increased odds of experiencing colic after hospital discharge (OR 0.93, 95% CI 0.35, 2.55).

Abnormal feces in formerly hospitalized horses: Of the 221 completed surveys, owners did not know if abnormal feces occurred after discharge for 30 previously hospitalized horses; all were excluded from this analysis. Of the 191 horses remaining (representing 120 farms; minimum 1 horse to maximum 9 horses), 12 were classified as *Salmonella*^{POS}-MDR^{POS}, 37 as *Salmonella*^{POS}-MDR^{NEG}, and 142 as *Salmonella*^{NEG}. Of formerly hospitalized horses, owners reported only 9 (4.7%) having the outcome of interest (2 *Salmonella*-positive and 7 *Salmonella*-negative) and thus we were unable to develop a final multivariable model. Univariable analysis revealed that formerly hospitalized horses that were culture-positive for *Salmonella* did not have an increased odds of experiencing abnormal feces after hospital discharge (OR 0.87, 95% CI 0.24, 3.18).

Hospitalization of stablemates of formerly hospitalized horses: Of the 221 completed surveys, owners did not know whether stablemates of 14 formerly hospitalized horses were subsequently hospitalized; all were excluded from this analysis. Of the 207 horses remaining (representing 123 farms; minimum 1 horse to maximum 10 horses), 13 were classified as *Salmonella*^{POS}-MDR^{POS}, 40 as *Salmonella*^{POS}-MDR^{NEG}, and 154 as *Salmonella*^{NEG}. Owners reported that hospitalization of stablemates of formerly hospitalized horses occurred 155 times (74.9%) during the follow-up period; stablemates of 10 *Salmonella*^{POS}-MDR^{POS}, stablemates of 32 of *Salmonella*^{POS}-MDR^{NEG}, and stablemates of 113 of *Salmonella*^{NEG}.

For the purposes of risk-factor analysis, all *Salmonella*-positive horses were classified as *Salmonella*^{POS} regardless of susceptibility results to improve model stability. Variables for diarrhea, age, breed, sex, management of resident horses returning from a show or event, management of resident horses returning from the hospital, and management of non-resident horses passed univariable screening and were included in multivariable model building (Table 6.4). The final multivariable model included breed and management of resident horses returning from the hospital as the main effects. *Salmonella* culture status was forced into the model, regardless of *P*-value, as it was the main exposure of interest. There were no first-order interactions among main effects variables in this model. Controlling for the effects of other variables in the model, there was an almost 3 times greater odds of hospitalization of stablemates during the follow-up period if the previously hospitalized horse was a Thoroughbred as compared to all other breeds (OR 2.81; 95% CI 1.13, 7.00). There was also an almost 3 times greater odds of hospitalization of stablemates during the follow-up period if formerly hospitalized resident horses were housed separately upon returning to the farm as

compared to being housed normally (OR 3.30; 95% CI 1.18, 9.28). *Salmonella* culture status during hospitalization was not associated with hospitalization of stablemates during the follow-up period (*P*=0.30).

Abnormal feces in stablemates of formerly hospitalized horses: Of the 221 completed surveys, owners did not know if stablemates of formerly hospitalized horses experienced abnormal feces in the follow-up period for 16 horses; all were excluded from this analysis. Of the 205 horses remaining (representing 119 farms; minimum 1 horse to maximum 10 horses), 13 were classified as *Salmonella*^{POS}-MDR^{POS}, 37 were *Salmonella*^{POS}-MDR^{NEG}, and 155 were *Salmonella*^{NEG}. Owners reported that abnormal feces among stablemates of formerly hospitalized horses occurred 132 times (64.4%) during the follow-up period; stablemates of 13 *Salmonella*^{POS}-MDR^{POS}, stablemates of 25 *Salmonella*^{POS}-MDR^{NEG}, and stablemates of 100 *Salmonella*^{NEG}.

For the purposes risk-factor analysis, all *Salmonella*-positive horses were classified as *Salmonella*^{POS} regardless of susceptibility results to improve model stability. Variables for disease category, receiving aminoglycosides, beta lactams, sulfas, or rifamycins during hospitalization, age, breed, and the average numbers of horses on the farm passed univariable screening and were included in multivariable model building (Table 6.5). The final multivariable model included breed as the main effect. *Salmonella* culture status was forced into the model, regardless of *P*-value, as it was the main exposure of interest; and management of resident horses returning from the hospital was forced into the model, regardless of *P*-value, as a potential confounding variable. Controlling for the effects of other variables in the model, there was a 2.5 times greater odds of abnormal feces among stablemates during the follow-up

period if the previously hospitalized horse was a Thoroughbred as compared to all other breeds (OR 2.64; 95% CI 1.04, 6.68). *Salmonella* culture status during hospitalization was not associated with abnormal feces among stablemates during the follow-up period (*P*=0.89).

6.5 DISCUSSION

In general, horses experiencing diarrhea during hospitalization had an increased risk for shedding *Salmonella*, although they were less likely to shed MDR-strains. This finding is especially interestingly given that receiving antimicrobial therapy during hospitalization was not associated with shedding *Salmonella*, nor was it associated with shedding of MDR-strains. This was unexpected as some research suggests that antimicrobial use during hospitalization creates pressure on the gut microbiome, encouraging a shift to more resistant populations [6.12]. While this was not noted in the *Salmonella* isolates from the study reported here, this may have occurred in other gastrointestinal microflora.

Overall, horses shedding *Salmonella* during hospitalization were not less likely to survive or more likely to experience episodes of colic or abnormal feces during the year after hospital discharge. Rather the risk for long-term non-survival was increased for hospitalized horses receiving a moderate to high level of care – suggesting that it is the severity of disease that is likely the more important factor with respect to long-term survival. Our finding of no difference in risk for having colic or abnormal feces after hospital discharge among *Salmonella*-positive or negative horses is in agreement with a previous report [6.9]. This is important with respect to counseling owners on what it means when *Salmonella* shedding is detected in their animal.

Interestingly, stablemates were more likely to be hospitalized during the follow-up period if the previously hospitalized horse was a Thoroughbred or if the farm of residence managed resident horses separately (i.e., separate housing) upon return from the hospital. We suspect that this finding is related to the rigor of farm management among Thoroughbred farms in central Kentucky – farms with more rigorously managed populations will likely employ more rigorous infection control practices and be more likely to seek veterinary care for horses displaying clinical signs of disease. That being said, we also found that stablemates were more likely to experience abnormal feces during the follow-up period if the previously hospitalized horse was a Thoroughbred, but this was not associated with the fecal culture status of the previously hospitalized horse.

When extrapolating the results of this study it is important to keep in mind that data collection was reliant upon the quality of medical records and owner recall – which may result in information bias due to inaccurate accounts of outcome or exposure information. We tried to limit this by providing owners with the choice of "don't know" for many of the exposures and outcomes of interest and excluded animals from analyses for which this was the response. A unique attribute of this study was its focus on horses in central Kentucky with the majority of horses being Thoroughbred. While we do not believe there to be a biologically relevant difference among horse breeds with respect to risk for shedding *Salmonella*, this population of horses may differ from that in other regions.

The findings of this study provide more information for veterinarians when counseling owners on the repercussions of detecting *Salmonella* shedding in their horse. In general, shedding *Salmonella* did not decrease long-term survival or increase the occurrence of colic or

abnormal feces in the hospitalized horse nor increase the risk for hospitalization or abnormal feces in its stablemates. Despite this, it is still recommended to manage horses shedding *Salmonella* separately from other resident horses and employ rigorous personal and environmental hygiene to mitigate the exposure risk to other horses and personnel.

6.6 ENDNOTES

[18]Hardy Diagnostics, Santa Maria, CA, USA
[19] Labsco, Louisville, KY, USA
[20]SAS v9.3, SAS Institute, Inc., Cary, NC

Table 6.1: Descriptive statistics of all study horses (N=373)

		Fecal culture status					
		Salmonella^{POS}-MDR^{POS}		*Salmonella*^{POS}-MDR^{NEG}		*Salmonella*^{NEG}	
Variables	**Category**						
Age	foal (≤ 1 year)	14	48.3%	31	47.7%	120	43.0%
	adult (> 1 year)	15	51.7%	34	52.3%	159	57.0%
Sex	female	20	69.0%	43	66.2%	198	71.0%
	male	8	27.6%	21	32.3%	72	25.8%
	unknown	1	3.4%	1	1.5%	9	3.2%
Breed	Thoroughbred	26	89.7%	53	81.5%	240	86.0%
	other	3	10.3%	12	18.5%	39	14.0%
Maximum care level during hospitalization	high	18	62.1%	40	61.5%	166	59.5%
	surgical	1	3.4%	7	10.8%	39	14.0%
	low	10	34.5%	18	27.7%	74	26.5%
Disease category	non-surgical colic	4	13.8%	7	10.8%	35	12.5%
	surgical colic	7	24.1%	11	16.9%	55	19.7%
	diarrhea	5	17.2%	9	13.8%	23	8.2%
	foaling complications	1	3.4%	3	4.6%	16	5.7%
	neonatal	3	10.3%	6	9.2%	23	8.2%
	other	1	3.4%	11	16.9%	53	19.0%
	secondary patients	8	27.6%	18	27.7%	74	26.5%
Diarrhea during hospitalization	yes	12	41.4%	36	55.4%	118	42.3%
	no	17	58.6%	29	44.6%	161	57.7%
Fever (> 101°F) during hospitalization	yes	21	72.4%	47	72.3%	191	68.5%
	no	8	27.6%	18	27.7%	88	31.5%
Leukopenia (<5000 wbc/µL) during hospitalization	yes	11	37.9%	21	32.3%	77	27.6%
	no	11	37.9%	29	44.6%	135	48.4%
	unknown	7	24.1%	15	23.1%	67	24.0%
Hospitalization days	≥ 4 days	18	62.1%	42	64.6%	167	59.9%
	≤ 3 days	11	37.9%	23	35.4%	112	40.1%

NEG = culture-negative; POS = culture-positive; POS-MDR = culture-positive with multidrug-resistant strain

Table 6.2: Results of Multinomial logistic regression analysis of factors associated with hospitalized horses shedding MDR-*Salmonella* (n=373)

Multivariable Analysis							
Variable	**Category**	**Outcome**	**OR**	**95% CI**			***P*-value**
Diarrhea during hospitalization	yes	Pos	1.88	1.02	–	3.45	0.04
		Pos-MDR	0.54	0.20	–	1.47	
	no		ref				
Reduced feed intake during hospitalization	yes	Pos	0.66	0.28	–	1.54	0.05
		Pos-MDR	5.95	1.21	–	29.20	
	no		ref				

Univariable Analyses							
Variable	**Category**	**Outcome**	**OR**	**95% CI**			***P*-value**
Diarrhea during hospitalization	yes	Pos	1.69	1.03	–	2.78	0.10
		Pos-MDR	0.96	0.40	–	2.30	
	no		ref				
Reduced feed intake during hospitalization	yes	Pos	0.82	0.46	–	1.47	0.18
		Pos-MDR	2.27	0.87	–	5.91	
	no		ref				
Antidiarrheal during hospitalization	yes	Pos	1.33	0.72	–	2.46	0.21
		Pos-MDR	2.14	0.86	–	5.34	
	no		ref				

CI = confidence interval; OR = odds ratio; POS = culture-positive; POS-MDR = culture-positive with multidrug-resistant strain; ref = reference

Table 6.3: Results of logistic regression analysis of factors associated with long-term non-survival of horses shedding *Salmonella* (n=197)

Multivariable Analysis				
Variable	**Category**	**OR**	**95% CI**	***P*-value**
Care level maximum during hospitalization	high	3.52	1.14 – 10.94	0.03
	surgical	2.05	0.47 – 8.88	
	low	ref		
Fecal culture result of previously hospitalized horse	positive	1.25	0.55 – 2.87	0.61
	negative	ref		

Univariable Analyses				
Variable	**Category**	**OR**	**95% CI**	***P*-value**
Disease category	non-surgical colic	4.95	1.45 – 16.98	0.10
	surgical colic	2.37	0.71 – 7.91	
	diarrhea	0.46	0.05 – 4.33	
	foaling complications	5.74	1.09 – 30.33	
	neonatal	3.36	0.85 – 13.22	
	other	2.44	0.73 – 8.16	
	secondary patient	ref		
Leukopenia during hospitalization	yes	1.91	0.85 – 4.30	0.12
	unknown	0.63	0.21 – 1.88	
	no	ref		
Reduced feed intake during hospitalization	yes	2.35	1.19 – 4.64	0.01
	no	ref		
Received antimicrobial drugs during hospitalization	yes	1.72	0.72 – 4.10	0.20
	no	ref		
Care Level	high	3.53	1.13 – 11.01	0.03
	surgical	1.96	0.45 – 8.61	
	low	ref		
Received aminoglycosides during hospitalization	yes	1.80	0.87 – 3.71	0.12
	no	ref		
Received beta-lactams during hospitalization	yes	1.91	0.94 – 3.88	0.09
	no	ref		

Received tetracycline during hospitalization	yes	1.78	0.86	–	3.66	0.18
	no	ref				
Received chloramphenicol during hospitalization	yes	6.36	1.03	–	39.15	0.15
	no	ref				
Received sulfas during hospitalization	yes	0.46	0.10	–	2.08	0.25
	no	ref				
Received rifamycins during hospitalization	yes	6.31	1.03	–	38.66	0.16
	no	ref				
Received polymixin B during hospitalization	yes	8.27	0.75	–	91.04	0.22
	no	ref				
Hospitalization days	≥ 4 days	1.81	0.83	–	3.95	0.13
	≤ 3 days	ref				
Breed	Other	1.81	0.76	–	4.30	0.23
	Thoroughbred	ref				
Received gastroprotectants during hospitalization	yes	2.08	0.93	–	4.67	0.06
	no	ref				
Reported to have had at least 1 colic episode after hospital discharge	yes	3.01	1.17	–	7.71	0.17
	unknown	1.70	0.43	–	6.75	
	no	ref				

CI = confidence interval; OR = odds ratio; ref = reference

Table 6.4: Results of logistic regression analysis of factors associated with hospitalization of stablemates of previously hospitalized *Salmonella* shedding horses (n=207)

Multivariable Analysis				
Variable	**Category**	**OR**	**95% CI**	***P*-value**
Breed	Thoroughbred	2.81	1.13 – 7.00	0.049
	other	ref		
Management of resident horses returning from hospital	separate housing	3.30	1.18 – 9.28	0.04
	normal housing	ref		
Culture result of previously hospitalized horse	Positive	1.33	0.87 – 2.06	0.30
	Negative	ref		

Univariable Analyses				
Variable	**Category**	**OR**	**95% CI**	***P*-value**
Diarrhea during hospitalization	yes	0.78	0.55 – 1.11	0.20
	no	ref		
Age	foal	1.38	1.09 – 1.74	0.01
	adult	ref		
Breed	Thoroughbred	5.00	2.27 – 11.01	0.0001
	other	ref		
Sex	male	0.77	0.54 – 1.08	0.23
	unknown	0.52	0.20 – 1.38	
	female	ref		
Management of resident horses returning from show	separate housing	1.66	0.72 – 3.81	0.24
	normal housing	ref		
Management of resident horses returning from hospital	separate housing	5.77	2.42 – 13.78	0.0003
	normal housing	ref		
Management of non-resident horses arriving on the property	separate housing	0.31	0.11 – 0.87	0.05
	normal housing	ref		

CI = confidence interval; OR = odds ratio; ref = reference

Table 6.5: Results of logistic regression analysis of factors associated with abnormal feces among stablemates of previously hospitalized *Salmonella* shedding horses (n=205)

Multivariable analysis				
Variable	**Category**	**OR**	**95% CI**	***P*-value**
Breed	Thoroughbred	2.64	1.04 – 6.68	0.02
	other	ref		
Culture result of previously hospitalized horse	positive	1.01	0.86 – 1.19	0.89
	negative	ref		
Management of resident horses returning from hospital	separate housing	0.82	0.27 – 2.45	0.71
	normal housing	ref		

Univariable Analyses				
Parameter		**OR**	**95% CI**	***P*-value**
Disease category	non-surgical colic	0.88	0.52 – 1.50	0.22
	surgical colic	0.87	0.76 – 1.00	
	diarrhea	0.68	0.37 – 1.27	
	foaling complications	1.06	0.96 – 1.17	
	neonatal	1.19	0.99 – 1.44	
	other	1.18	0.87 – 1.61	
	secondary patient	ref		
Received aminoglycosides during hospitalization	yes	1.20	0.92 – 1.57	0.19
	no	ref		
Received beta-lactams during hospitalization	yes	1.25	0.95 – 1.64	0.13
	no	ref		
Received sulfas during hospitalization	yes	1.30	0.89 – 1.90	0.23
	no	ref		
Received rifamycins during hospitalization	yes	1.09	0.96 – 1.25	0.20
	no	ref		
Age	foal	1.05	0.98 – 1.12	0.18

	adult	ref				
Breed	Thoroughbred	2.44	1.16	–	5.00	0.004
	other	ref				
Average number of horses on resident farm	≥ 150 horses	2.96	1.06	–	8.26	0.02
	100-149 horses	3.19	1.24	–	8.20	
	50-99 horses	3.70	1.32	–	10.34	
	< 50 horses	ref				

CI = confidence interval; OR = odds ratio; ref = reference

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7 CHAPTER 7: RISK FACTORS FOR VETERINARY HOSPITAL ENVIRONMENTAL CONTAMINATION WITH *SALMONELLA ENTERICA*

7.1 SUMMARY

Background: Epidemics of healthcare-associated infections in veterinary hospitals are commonly attributed to Salmonella enterica and characteristically there is widespread environmental contamination identified during these times.

Hypothesis/Objectives: The objective of this study was to determine risk factors associated with environmental contamination of a veterinary hospital with S. enterica; and secondarily to determine a suitable analytic method to model such a complex ecology.

Methods: Environmental surveillance samples were collected from March 2003 through January 2013, using a commercially available electrostatic wipe, as part of a long-term infection control program. Sampling sites included both floor and hand-contact surfaces throughout the veterinary teaching hospital (VTH). Risk factors evaluated included hospital caseload, hospital use areas, severity of disease, presence of culture-positive inpatients and season. Data on risk factors of interest were collected retrospectively from the VTH electronic medical records database. Variable cluster analysis and principal components analysis were used to understand the underlying data structure and multicollinearity. Multivariable logistic regression was performed using generalized estimating equations to determine factors associated with environmental contamination with S. enterica while controlling for environmental sample clustering by sample date.

Results: During the study period a total of 5273 environmental samples were collected on 167 unique sampling dates (approximately 46 samples were collected monthly). Of the samples collected, a total of 8.2% (n=434) were culture-positive for S. enterica using standard culture techniques. In general, Salmonella was most likely to be detected in environmental samples collected in the livestock hospital and from floor samples. The probability of detecting Salmonella in the hospital environment was associated with livestock caseload, patient disease severity, the presence of patients shedding Salmonella, and is affected by the types and locations of environmental samples tested.

Conclusions and clinical importance: This study demonstrates the complex ecology of *Salmonella* in a veterinary hospital emphasizing the role latent (unmeasured) factors may play in driving endemic contamination to become hospital-wide and ultimately develop into an epidemic. Results of this study suggests that the probability of detecting *Salmonella* in the environment increases as the demand on personnel increases thereby emphasizing the need to remain vigilant in the practice of infection control measures that we know empirically will mitigate the risk for widespread environmental contamination and sustained transmission among patients (i.e., rigorous personal and environmental hygiene).

7.2 INTRODUCTION

Epidemics of healthcare-associated infections in veterinary hospitals are commonly attributed to *Salmonella enterica* and characteristically there is widespread environmental contamination identified during these times [7.1-7.4]. Further, environmental and patient isolates detected in the same timeframe will frequently be of the same phenotype (i.e., serotype and antimicrobial susceptibility) something that is appreciated during periods of

epidemic and endemic disease alike – suggesting a relationship among patients and the hospital environment [7.2, 7.5]. Despite incorporating rigorous personal and environmental hygiene practices into infection control programs, widespread outbreaks still occur with high case fatality rates and at considerable financial cost [7.3].

Salmonella is an important part of the ecology of larger veterinary teaching hospitals. In large hospitals, personnel tend to work in multiple areas (rather than individual areas) and the average person working is a veterinary student –considered to be a novice with respect to patient management and infection control practices. Additionally, all hospitals congregate compromised animals from many different farms thereby increasing the risk for patient shedding and infectious disease transmission [7.6, 7.7]. Our cursory understanding of this relationship is really based on snapshots of data obtained during epidemics or by use of targeted surveillance. While this gives us a very basic understanding that patients with severe disease (e.g., colic or diarrhea) and those that have experienced stressful situations are more likely to shed it does not really get to the heart of the matter [7.7-7.9]. By developing a deeper understanding of the forces at play, we can potentially determine a hospital profile that indicates a heightened risk for widespread contamination and sustained transmission among patients, thus allowing prevention strategies to be implemented before an outbreak is even detected.

The question becomes one of what drives endemic contamination to become hospital-wide and ultimately develop into an epidemic? Is it the immediate contamination pressure (i.e., what happens the day prior to the occurrence of environmental contamination); or is it cumulative contamination pressure (i.e., what happens 30 days prior to the occurrence of

contamination)? Further, how do we measure this contamination pressure on a hospital? Should we consider caseload (e.g., inpatients or outpatients) and types of patients (e.g., elective surgeries, systemically ill) – or should we consider factors related to the hospital (e.g., personnel, hospital type, season). When it comes to variables that we can measure, many of them are non-discriminatory in nature (i.e., "suitcase" variables). In other words these factors represent variance described by variables that are unmeasured or unidentified and are referred to as latent variables. While we can easily count caseload it becomes inordinately more difficult to tabulate the human effect – many human variables simply cannot be measured.

Applying traditional statistical modeling techniques to large complex data sets with non-discriminatory variables can result in unstable statistical models due to multicollinearity which occurs when individual variables or linear combinations of variables are highly correlated (i.e., not statistically independent) [7.10]. We see this as unstable parameter estimates (estimates that change drastically during the modeling process), large standard errors (incorrect variance estimates), and difficulties with the model convergence. Therefore, consideration should be given to using alternative techniques to aid in model development.

To reduce the impact that multicollinearity may have on statistical modeling, variable reduction techniques such as variable cluster analysis and principal component analysis can be used. Both of these approaches can be used to help understand which variables have important effects on the outcome of interest [7.11, 7.12]. Variable cluster analysis is a method that groups variables in a manner such that those in a cluster are more similar to each other than to variables in another cluster. In other words they are occupying similar space in the variance structure. As such, a variable can be selected that best represents the variance that is

embodied by that cluster thereby reducing the number of variables used in multivariable modeling while still accounting for the majority of the variance. Principal components analysis is a technique that creates new variables which are uncorrelated (i.e., orthogonal). Each principal component represents a proportion of variance from many different variables based on the variance space each variable occupies. The proportion of variance from each variable on a component suggests relationships among variables which may be indicative of latent variables. The new variables created (i.e., the principal components) represent the same amount of variance but do so with fewer variables thus reducing the number of variables used in the multivariable model building process while describing the majority of the variance.

The primary objective of this study was to determine factors associated with environmental contamination of a veterinary teaching hospital with *Salmonella enterica*; and secondarily to determine a suitable analytic method to model such a complex ecology.

7.3 MATERIALS AND METHODS

Environmental samples: Environmental samples were collected as part of long-term surveillance at the Colorado State University (CSU) -VTH from March 2003 through January 2013. As part of routine environmental surveillance, approximately 46 environmental samples were collected monthly at predetermined sites throughout the small animal, equine, and livestock hospitals using a commercially available electrostatic dust wipe [7.5]. Sites included common use areas such as hallways, treatment rooms, alleyways, teaching rounds rooms and technician offices. Sampling locations included hand-contact surfaces, floors, and composite samples of hand-contact surfaces and floors. All environmental samples were cultured using standard enriched techniques for the presence of *Salmonella enterica*, as previously described

[7.5]. Briefly, samples were pre-enriched in 90mls buffered peptone water[21] (BPW) for 24 hrs at 43°C, then 1 ml was passed into 9 mls tetrathionate enrichment broth[21] for 18 hrs at 43°C, then 0.10 ml was passed into 10 mls Rappaport-Vassiliadis R10 broth[21] for 18 hrs at 43°C, then streaked for isolation on XLT4 agar[22] media and incubated for 18 hrs at 43°C. Suspect colonies were subcultured on trypticase soy agar containing 5% sheep blood[21] and incubated 18 hrs at 43°C and tested for agglutination using commercial polyvalent and O group-specific antisera. Serotype determination was performed by the USDA National Veterinary Services Laboratory (NVSL, Ames, IA).

Fecal Samples: Fecal samples were collected from every large animal inpatient on admission then three times weekly from February 2003 to June 2003 or twice weekly from July 2003 to June 2011 as part of long-term surveillance at the CSU-VTH. All fecal samples were cultured using standard enriched techniques for the presence of *Salmonella enterica*. Briefly, 1 gram fecal samples were enriched in 9 mls tetrathionate broth for 18 hrs at 43°C, then streaked for isolation on XLT4 agar media and incubated for 18 hrs at 43°C. Suspect colonies were subcultured on trypticase soy agar containing 5% sheep blood and incubated 18 hrs at 43°C and tested for agglutination using commercial polyvalent and O group-specific antisera. Serotype determination was performed by the USDA National Veterinary Services Laboratory (Ames, IA).

Potential risk factors: Independent variables were derived from invoice data contained within the electronic medical records database as well as data contained within the Infection Control Program database, both maintained at the CSU-VTH, and the healthcare-associated infections (HCAIs) database (Figure 7.1). Data regarding environmental temperature were obtained from

the National Oceanic and Atmospheric Administration (NOAA; Figure 7.1). See Table 1 for independent variable definitions.

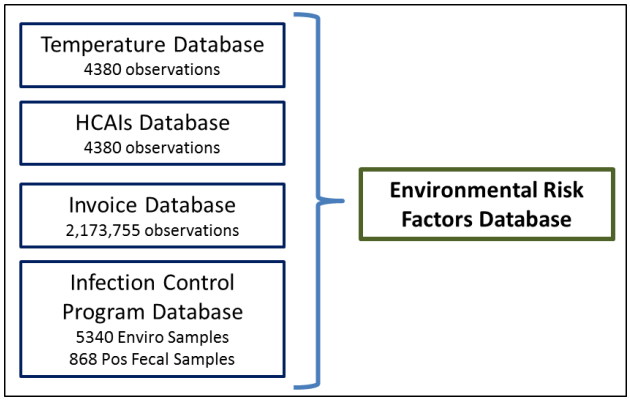

Figure 7.1 Derivation of environmental risk factors database

Factors related to hospital characteristics: Variables that were associated with hospital characteristics were evaluated, including hospital area (small animal hospital, equine hospital, livestock hospital), room use (single animal use, multiple animal use, personnel use), sample type (hand-contact surface sample, floor sample, composite of hand and floor), year (2002 through 2011), season (July-October, November-February, March-June), fecal sample frequency (3 times per week, 2 times per week), footwear hygiene (footbath, footmat, none) and occurrence of nosocomial infections during the study period.

Room use contained 3 categories. Single animal use was defined as those areas cleaned and disinfected between uses. Multi-animal use was defined as areas used by more than one patient without necessarily being cleaned and disinfected between uses (e.g., animal aisles). Personnel use was defined as areas inaccessible to patients (e.g., technician office, personnel rounds rooms).

Footwear hygiene contained 3 categories. Areas using footbaths was defined as those areas using dedicated footwear and disinfectant footbaths (livestock hospital, equine isolation facility). Areas using a foot mat were defined as areas using disinfectant foot mats with or without dedicated footwear (inpatient areas of the equine hospital). Finally, areas using

neither were defined as areas not requiring dedicated footwear or specific footwear hygiene practices (e.g., small animal hospital).

Nosocomial infections were defined as 2 or more patient isolates with the same phenotype (i.e., serotype and antimicrobial susceptibility) obtained from patients (not from the same herd/flock) hospitalized within 10 days of each other. The number of clusters identified within 1 week and 1 month of an environmental sampling date were calculated.

Factors related to patient population characteristics: Variables representing patient population characteristics that described the immediate contamination pressure (the day prior to environmental sample date) and the cumulative contamination pressure (the month prior to environmental sample date) on the veterinary hospital environment were evaluated. Variables describing patient population characteristics included the number of fecal culture-positive inpatients on the day prior to the environmental sample date, number of culture-positive inpatients the month prior to the environmental sample date, day prior and month prior inpatient case load, day prior and month prior outpatient case load, day prior and month prior total case load, total number of hospitalization days the month prior to environmental sample date and level of care the day prior and the month prior to environmental sample date. Patient population characteristic variables were determined by species including amphibian, avian, bovine, canine, equine, exotic large animals (i.e., Old World camelid), feline, New World camelid, other (caprine and ovine), reptiles, and small mammals. Additionally, patient population characteristics were determined for a species group including Small Animal (canine, feline), Equine, Livestock (bovine, caprine, new world camelid, ovine, porcine, exotic large animal), and Exotics (avian, amphibian, reptile, small mammal).

Environmental Temperature Data: Environmental temperature data was obtained for the study period from the National Climate Data Center from the National Oceanic and Atmospheric Administration (NOAA). Based on data from two different weather stations [Station GHCND:USC00053005 (Fort Collins, CO, US) and Station GHCND:USC00053006 (Fort Collins, 4 E, CO, US)], the average minimum, average maximum, and average difference in environmental temperature were calculated for the month prior to each environmental sampling date.

Data analysis: Data were entered in a spreadsheet, validated, and explored using descriptive statistics. Continuous variables were assessed for the assumption of normality on the logit scale; variables not meeting this assumption were categorized based on distributional quartiles or breakpoints with biological relevance. Logistic regression was performed using generalized estimating equations to evaluate factors that might be associated with the occurrence of a positive environmental culture, controlling for clustering of environmental samples by date. The dependent variable for this analysis was a positive environmental culture (yes/no).

Variables included in this study represent larger variable subsets for hospital factors and patient population factors including variables representing patient caseload and severity of disease (Table 7.1). Univariable screening was performed on all variables with a critical $\alpha \leq 0.25$ to be included in subset multivariable screening (Figure 7.2). Within each variable subset, multivariable screening using backwards selection was performed using a critical $\alpha \leq 0.20$ for retention in the subset multivariable model. Consideration was given to *P*-values and quasi-information criteria (QIC) in variable subset selection for inclusion in the final multivariable model building process.

All variables that passed univariable screening were also subjected to variable cluster analysis (PROC VARCLUS) to elucidate the underlying data structure. Variables with the lowest 1-R^2 ratio were selected as the best cluster representative when traditional modeling exhibited model instability [7.11]. In addition, all variables that passed univariable screening were also subjected to principal component analysis (PROC PRINCOMP) to assess multicollinearity. Variables loading on principal components with an Eigenvalue > 1.0 were also considered when traditional modeling exhibited model instability. Thus, final multivariable model development was based on *a priori* knowledge, biological sense, univariable associations, variable cluster analysis 1-R^2 ratio, variable loading on principal components, quasi-information criteria (QIC) and *P*-values.

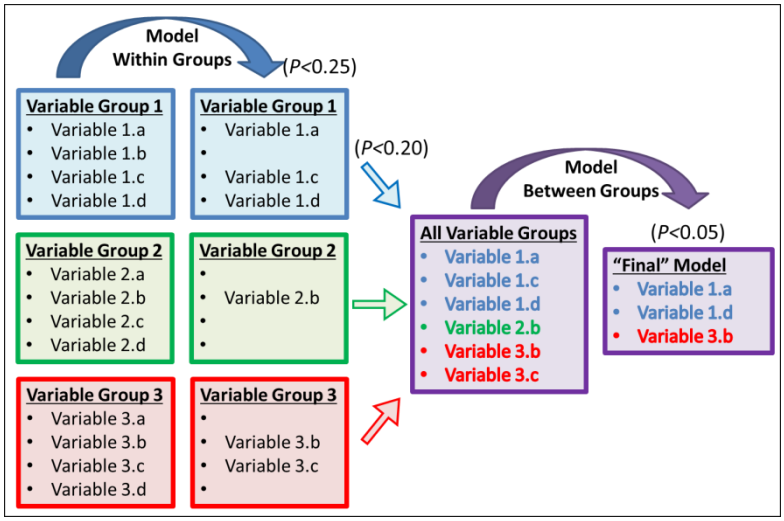

Figure 7.2 Subset multivariable screening/modeling process

The final multivariable model was identified using backwards selection with a critical $\alpha \leq 0.05$ for retention in the model. Confounding was identified by ≥20% change in parameter estimates when previously excluded variables were individually offered back to the multivariable model. When identified, confounding variables were forced into the multivariable models regardless of *P*-values. First-order interaction terms for main effects variables included in final models were also evaluated. Odds ratios (OR) and profile likelihood

95% confidence intervals (95% CI) were calculated using the least squares mean estimates. All statistical analyses were performed using commercially available software.[23]

7.4 RESULTS

During the study period, approximately 46 samples were collected monthly throughout the VTH (including the small animal, equine, and livestock hospitals) for a total of 5273 environmental samples, collected at 167 unique sampling dates. The majority of which were collected from the equine hospital (41.8%; n=2204/5273) and the remaining being divided between the small animal (30.7%; n=1619) and livestock (27.5%; n=1450) hospitals (Table 7.2). Of the samples collected, 8.3% (n=434) were culture-positive for *Salmonella enterica* using standard culture techniques. *Salmonella* was detected most frequently in samples collected from the livestock hospital (13%; n=188/5273) with fewer being detected in the small animal and equine hospitals, 9.8% (n=158/5273) and 4.0% (n=88/5273), respectively.

Environmental samples collected during this study were of three types: floor surfaces (n=3067), hand-contact surfaces (n=1321), and composite samples of floor and hand-contact surfaces (n=885). Of the different sample types, the majority were floor samples (58.2%) – representing the most common sample type collected in each of the small animal, equine, and livestock hospitals, 71.2%, 55.5%, and 47.7%, respectively.

Many variables characterizing the hospital (Table 7.2), patient population (Table 7.3), and severity of disease (Table 7.4) passed univariable screening and were subjected to both variable cluster analysis (Appendix 5) and principal component analysis (Appendix 6). Based on data exploration, descriptive statistics and univariable analyses, variables representing the immediate hospital pressure (i.e., day prior to environmental sample date) were eliminated

from inclusion in further model development due to the sparse nature of the data. In addition, data was too sparse for individual species contained within the small animal and exotics species groups. As such none of the individual small animal and exotics species passed into the multivariable model building process however individual species were considered for equine, bovine, and New World camelid.

The final multivariable model included bovine positive patient days, New World camelid inpatient caseload, equine outpatient caseload, equine care level 1 caseload, and New World camelid care level 2 caseload as main effects and an interaction between sample type and hospital. Season was forced into the model, regardless of *P*-value, as a potential confounding variable (Table 7.5). Controlling for effects of other variables in the model, the odds of detecting *Salmonella* in the environment was almost 2 times greater if the preceding month had at least 3 bovine positive patient days as compared to 2 or fewer (OR 2.10; 95% CI 1.05, 4.22); 2 times greater if the preceding month had at least 7 New World camelid inpatients as compared to 6 or fewer (OR 1.72; 95% CI 0.99, 2.98); and almost 2 times greater if the preceding month had at least 6 New World camelid patients at a care level 2 as compared to 5 or fewer (OR 1.87; 95% CI 1.04, 3.38). The odds of detecting *Salmonella* in the environment was 1.7 times greater if the preceding month had at least 86 equine outpatients as compared to 85 or fewer (OR 1.86; 95% CI 1.01, 3.43); and was approximately 2 times greater if the preceding month had least 74 equine patients at a care level 1 as compared to 73 or fewer (OR 2.24; 95% CI 1.25, 4.00).

In general, environmental samples collected in the livestock hospital and floor samples had a greater probability of being culture-positive. Within the livestock hospital, the odds of

detecting *Salmonella* was almost 2 times greater for floor samples (OR 1.81; 95% CI 1.11, 2.95) and for composite samples (OR 2.79, 95% CI 1.48, 5.27) as compared to hand-contact surface samples. Within the equine hospital, the odds of detecting *Salmonella* was also greater for floor samples (OR 1.50; 95% CI 1.07, 2.10), but was less likely for composite samples (OR 0.81; 95% CI 0.27, 2.43), as compared to hand-contact surface samples. Within the small animal hospital, the odds of detecting *Salmonella* in the environment was considerably greater for floor samples (OR 6.12; 95% CI 2.42, 15.48) and composite samples (OR 2.90, 95%CI 0.85, 9.96) as compared to hand-contact surface samples – likely related to central services incorporated into the small animal hospital that service both the equine and livestock hospitals.

7.5 DISCUSSION

This study demonstrates the complex ecology of *Salmonella* in a veterinary hospital emphasizing the role latent (unmeasured) factors may play in driving endemic contamination to become hospital-wide and ultimately develop into an epidemic. In general, the probability of detecting *Salmonella* in the hospital environment is associated with hospital and patient population characteristics; some of which are tangible such as hospital type (specifically the livestock hospital) or species (specifically large animal species) and many that can be easily measured such as the number of days a hospitalized patient was shedding *Salmonella* (i.e., positive patient days; specifically bovine) and caseload (specifically New World camelid inpatients and equine outpatients). The results of this study also suggest that the probability of detecting *Salmonella* in the environment increases as the demand on personnel increases (i.e., a busy hospital). We consider factors related to increased demand to be latent (unmeasured) variables which contribute to the complex hospital ecology but that simply cannot be measured

– the so-called "human effect." For example, cleaning frequency and number of patient contacts, which we know play a role in infectious agent transmission, and how personnel respond at times of increased demand (i.e., compliance with established protocols). While we have been limited in our understanding to data derived from epidemic disease and targeted surveillance, the study reported here provides some insight into the complicated nature of the environment in which we practice medicine on a daily basis.

Modeling complex relationships naturally leads to a complex data structure – something that must be accounted for in statistical model development. By using variable cluster analysis we can gain an appreciation for the data's structural complexity. In the present study, variables derived from the same species tended to cluster together. For example, equine outpatient caseload and inpatient caseload resided in the same cluster as did New World camelid outpatient caseload and inpatient caseload (Appendix 5). As a result, multivariable models containing the previous two or the latter two demonstrated characteristics of model instability. Thus, a single variable from each was selected as the best representative to move forward in the model building process. While the use of variable cluster analysis does reduce the number of variables considered in the analysis there can still be unidentified variables at play in the underlying data structure.

The use of principal components analysis really gets to the crux of this issue. Factors in this study that were easily measured tended to be non-discriminatory in nature (i.e., "suitcase" variables). While we were limited to these imperfect measures, use of alternative analytic methods (such as principal components analysis) allowed us to gain an understanding of which variables really represented unmeasured latent variables that may be contributing to the

overall ecology. In the present study, there were four principal components associated with the outcome – each explaining the variance as described by a unique constellation of variables (data not shown; Appendix 6). For example, the first principal component represented equine and New World camelid caseload and disease severity; the second represented positive patients and occurrence of HCAIs; the third represented the hospital factors of area use and type of hospital; and the final component also represented hospital factors, but it was the type of environmental sample collected and season that were associated with the probability of detecting environmental contamination.

The results of this study reflect the complexity of the question of what drives endemic contamination to become hospital-wide and ultimately develop into an epidemic. This study showed that the probability of detecting *Salmonella* in the hospital environment is associated with livestock caseload, patient disease severity, the presence of patients shedding *Salmonella*, and is affected by the types and locations of environmental samples tested. It also demonstrated the complexity of this relationship by highlighting the difficulties in using imperfect measures upon which to base interpretations and that there are unmeasurable latent factors that likely represent the human effect. The increased demand on personnel during times of high caseload and when caring for compromised patients likely affects compliance with infection control practices and creates more opportunity to transmit infectious agents between patients and among facilities. It is at these times that veterinarians and facility managers need to remain vigilant in the practice of infection control measures that we know empirically to work; namely rigorous personal and environmental hygiene and to segregate high-risk patients

as a means of mitigating widespread environmental contamination of the veterinary hospital environment.

7.6 ENDNOTES

[21]BD Diagnostic Systems, Sparks, MD, USA
[22]XLT-4, Hardy Diagnostics, Santa Maria, CA, USA
[23]SAS v9.3, SAS Inc., Carey, NC

Table 7.1: Variable descriptions

Variable		Definition
Hospital Factors	Hospital area	Small Animal Hospital, Equine Hospital, Livestock Hospital
	Room use	personnel use areas, single animal use areas, multi-animal use areas
	Sample type	type of environmental sample (hand-contact surfaces, floor surfaces, composite samples of hand and floor surfaces)
	Footwear hygiene	footmat, footbath, none
	Fecal culture frequency	3 times per week (Mar to Jun 2003) and 2 times per week (Jun 2003 to 2011)
	Season	July to October, November to February, March to June
	Year	2003 to 2011
	Average temperature	average outdoor air temperature the month prior to environmental sampling date
Population Factors by species[a,b] and species group[c] for the day prior to and the month prior to environmental sampling date		
Case Load	Inpatients	total number of inpatients
	Outpatients	total number of outpatients
	Total case load	total case load (inpatients and outpatients)
	Positive patients	total number fecal culture-positive patients
	Nosocomial infections	total number of identified nosocomial infections in patient population
Severity of disease	Level of care days	total days at each level of care (1-4)
	Hospital days	total number of hospitalization days

[a]Species = amphibian, avian, bovine, canine, caprine, exotic large animal, equine, feline, small mammals, new world camelids, other[b], reptile

[b]Other = caprine, ovine, porcine

[c]Species Groups = Small Animal, Exotics, Equine, Food Animal
Small Animal Species Group = canine, feline
Exotics Species Group = amphibian, avian, small mammals, reptile
Equine Species Group = equine
Food Animal Species Group = bovine, caprine, exotic large animal, new world camelid, ovine

Table 7.2: Univariable logistic regression results for hospital characteristics associated with hospital environmental contamination with *Salmonella enterica*

Variable	Category	n	N	% positive	OR	95% CI	*P*-value
Sample type	both	78	885	8.8%	2.23	1.51 – 3.31	<0.0001
	floor	296	3067	9.7%	2.21	1.63 – 2.99	
	hand	60	1321	4.5%	ref		
Hospital	livestock	188	1450	13.0%	3.25	1.92 – 5.49	0.001
	small animal	158	1619	9.8%	2.61	1.67 – 4.07	
	equine	88	2204	4.0%	ref		
Use	multi	246	2514	9.8%	2.02	1.40 – 2.91	0.001
	personnel	158	2038	7.8%	1.52	1.00 – 2.30	
	single	30	721	4.2%	ref		
Season	July-Oct	180	1592	11.3%	2.17	1.17 – 4.01	0.07
	Mar-Jun	153	1739	8.8%	1.43	0.79 – 2.57	
	Nov-Feb	101	1942	5.2%	ref		
HCAIs	≥ 3	33	232	14.2%	2.15	0.82 – 5.65	0.13
	1-2	154	1520	10.1%	1.74	1.01 – 3.01	
	0	247	3521	7.0%	ref		

CI = confidence interval; HCAI = healthcare-associated infection; OR = odds ratio; ref = reference

Table 7.3: Univariable logistic regression results for patient population characteristics associated with hospital environmental contamination with *Salmonella enterica*

Variable Subset	Variable	Category	N	OR	95% CI	*P*-value
Outpatients per month	bovine	> 19	2684	1.61	1.00 – 2.58	0.06
		≤ 19	2589	ref		
	equine	> 86	2579	1.84	1.14 – 2.96	0.02
		≤ 86	2694	ref		
	NWC	> 9	2761	1.95	1.20 – 3.17	0.01
		≤ 9	2512	ref		
Inpatients per month	equine	> 50	3935	1.60	0.86 – 2.96	0.11
		≤ 50	1338	ref		
	NWC	> 7	2609	1.67	1.03 – 2.71	0.05
		≤ 7	2664	ref		
Positive patients days per month	bovine	> 2	3800	2.01	1.14 – 3.56	0.01
		≤ 2	1473	ref		
	equine	> 1	2613	1.90	1.10 – 3.28	0.01
		≤ 1	2660	ref		
Positive patients per month	bovine	> 1	3739	1.70	1.01 – 2.88	0.04
		≤ 1	1534	ref		
	equine	> 1	2715	1.80	1.04 – 3.10	0.03
		≤ 1	2558	ref		
	NWC	≥ 1	787	1.81	0.79 – 4.12	0.26
		0	4486	ref		

CI = confidence interval; NWC = New World camelid; OR = odds ratio; ref = reference

Table 7.4: Univariable logistic regression results for patient severity of disease variables associated with hospital environmental contamination with *Salmonella enterica*

Variable Subset	Variable	Category	N	OR	95% CI	*P*-value
Level of care (patients per month)	equine level 1	> 73	2729	1.51	0.93 – 2.45	0.10
		≤ 73	2544	ref		
	equine level 2	> 20	4000	2.03	1.19 – 3.43	0.01
		≤ 20	1273	ref		
	equine level 3	> 16	2445	1.52	0.94 – 2.46	0.10
		≤ 16	2828	ref		
	NWC level 2	> 5	2548	1.62	0.99 – 2.64	0.07
		≤ 5	2725	ref		
	NWC level 3	≥ 1	2459	1.43	0.88 – 2.32	0.17
		0	2814	ref		
	other level 1	≥ 1	1163	1.84	1.00 – 3.39	0.11
		0	4110	ref		
	exotics level 1	> 1	3419	0.71	0.44 – 1.15	0.17
		≤ 1	1854	ref		
	small animal level 2	> 87	3953	1.43	0.85 – 2.39	0.16
		≤ 87	1320	ref		
	small animal level 3	> 21	2411	2.05	1.28 – 3.28	0.01
		≤ 21	2862	ref		
	small animal level 4	> 2	2281	0.69	0.43 – 1.11	0.13
		≤ 2	2992	ref		
Hospitalization days per month	equine	> 219	3910	2.21	1.15 – 4.26	0.01
		≤ 219	1363	ref		
	NWC	> 14	3860	1.99	1.14 – 3.48	0.01
		≤ 14	1413	ref		
	small animal	> 655	2583	1.47	0.91 – 2.37	0.12
		≤ 655	2690	ref		

CI = confidence interval; NWC = New World camelid; OR = odds ratio; ref = reference

Table 7.5: Final multivariable logistic regression model of factors associated with veterinary hospital environmental contamination with *Salmonella enterica*

Variable	Category	OR	95 % CI	*P*-value
Bovine positive patient days (mos prior)	≥ 3 days	1.84	0.99 – 3.42	0.04
	≤ 2 days	ref		
NWC inpatients (mos prior)	≥ 7 patients	1.99	1.18 – 3.36	0.01
	≤ 6 patients	ref		
Equine outpatients (mos prior)	≥ 86 patients	1.79	1.01 – 3.19	0.057
	≤ 85 patients	ref		
Equine care level 1 (mos prior)	≥ 74 patients	2.24	1.25 – 4.00	0.01
	≤ 73 patients	ref		
New World camelid care level 2 (mos prior)	≥ 6 patients	1.87	1.04 – 3.38	0.04
	≤ 5 patients	ref		
Sample Type	composite	INTERACTION		0.03
	floor			
	hand-contact	ref		
Hospital	livestock	INTERACTION		0.11
	small animal			
	equine	ref		
Season	July-October	CONFOUNDER		0.65
	March-June			
	November-February	ref		
Sample Type * Hospital				0.002
hand-contact sample	livestock	2.43	1.05 – 5.63	
	small animal	0.69	0.23 – 2.05	
	equine	ref		
composite sample	livestock	8.38	2.63 – 26.73	
	small animal	2.48	0.78 – 7.92	
	equine	ref		

floor sample	livestock	2.92	1.59 –	5.36
	small animal	2.81	1.74 –	4.56
	equine	ref		
equine	floor sample	1.50	1.07 –	2.10
	composite sample	0.81	0.27 –	2.43
	hand-contact sample	ref		
livestock	floor sample	1.81	1.11 –	2.95
	composite sample	2.79	1.48 –	5.27
	hand-contact sample	ref		
small animal	floor sample	6.12	2.42 –	15.48
	composite sample	2.90	0.85 –	9.96
	hand-contact sample	ref		

CI = confidence interval; NWC = New World camelid; OR = odds ratio; ref = reference

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8 CHAPTER 8: CONCLUSION

The relationship between *Salmonella enterica*, patients, and the hospital environment is a very complex ecology – creating considerable gaps in our understanding of its epidemiology. A deeper appreciation of its natural history in veterinary populations is critical to improving prevention efforts. The overarching goal of this dissertation was to build a foundation upon which to practice evidence-based prevention strategies with the purpose of reducing the transmission risk of *S. enterica* in the veterinary environment.

Managing *S. enterica* in populations really is dependent upon effective detection techniques – we cannot manage what we cannot measure. Development of point-of-care tests for *Salmonella* detection is crucial to the advancement of veterinary infection control [8.1]. With the goal of trying to fill this critical need, we undertook two different experimental studies. The objective of the first study was to optimize enrichment methods for use with commercially available lateral flow immunoassays (LFI) to detect *S. enterica* in equine fecal and environmental samples within 24 hours of sample collection. In conducting this study, we not only developed an effective culture protocol for use with these tests, but determined the analytic sensitivity of both LFIs and enriched culture to be approximately 4 cfu per gram of feces or ml of culture media, under experimental conditions [8.2]. This suggests that perhaps the relatively poor sensitivity for organism detection that is characteristically reported in the literature is more a consequence of intermittent shedding rather than shedding low numbers of organisms per gram of feces.

While LFIs for rapid *Salmonella* detection are very promising as point-of-care tests, we did note a limitation in the ability of these tests to detect different *Salmonella* serotypes. The objective of the second experimental study was to evaluate the ability to detect a variety of different clinical isolates of *S. enterica* (representing 10 different serotypes) using 4 different commercially available rapid tests (2 LFIs, a DNA hybridization test [DNAH], and a polymerase chain reaction [qPCR] assay) and aerobic culture when inoculated into equine feces. While culture, qPCR, and DNAH reliably detected all serotypes – these tests do not meet our ideal vision for a point-of-care test, requiring laborious techniques and specialized equipment. On the other hand, the two LFIs showed some serotype variability, detecting 84% and 67% of isolates tested, respectively, but could be easily implemented as point-of-care tests. Specifically, they were limited in detecting serotypes Cerro (serogroup K), and serotypes Mbandaka, and Montevideo (serogroup C_1). That said, these serotypes are not typically associated with horses and therefore are likely to not impact the usefulness of these tests in equine practice. However, serotype limited detection should be further evaluated in naturally infected samples. In fact, this is the focus of a study we have recently undertaken to estimate test sensitivity and specificity of LFIs in clinical practice.

Outbreaks of *Salmonella enterica* among hospitalized patients occur time and again. While considerable research has been done to improve our understanding of factors related to epidemic disease it is not clear from previous work whether these are equally applicable to periods of endemic disease. To put previous reports into perspective, we undertook a case-control study based on routine patient surveillance spanning 10 years. The objectives of this study were; 1) to determine factors associated with endemic fecal shedding of *S. enterica*

among large animal patients; 2) to do so in comparison to two different groups of patients – a group in which there is high confidence in negative status (having at least 3 negative cultures) and a group with potential for misclassification of shedding status (at least 1 negative culture); and 3) to demonstrate that the choice of comparison group can affect resultant associations.

Traditionally, prevention efforts have focused on patients with a triad of clinical signs – fever, diarrhea, and leukopenia. We found that only 2.7% of the shedding risk could be attributed to patients with these signs (i.e., the population attributable fraction). Though this triad of clinical signs may exhibit a strong association with shedding, they occur relatively infrequently in the population. Thus, by focusing prevention efforts on these signs alone, we are effectively not detecting the majority of shedding patients. We also found that approximately 70% of shedding risk can be attributed to each of systemic illness or gastrointestinal disease. Many hospitals segregate patients with gastrointestinal disease, but they likely do not do this with other systemically compromised patients such as those with pleuropneumonia or sepsis. Although it is a challenge to manage such patients with a heightened level of biosecurity (e.g., barrier nursing precautions or segregation), the results of this study suggest that it might be prudent to do so.

The bane of the case-control study is the selection of an appropriate control group. In the literature, there are two controls groups commonly used in studies on *S. enterica* – patients with one negative culture and patients with 3 negative cultures. This creates an interesting conundrum – by comparing to a patient with one negative culture there is likely some misclassification due to the insensitive nature of currently available detection methods. Alternatively, the delay in determining negative status for patients with three cultures may

actually create a control group that is not representative of the source population from which cases are drawn. We found that although some of the specific exposures associated with shedding may change, the general answer was the same. Patients with greater systemic compromise were more likely to shed *Salmonella* in their feces. Now there was a caveat to this – the control group with at least three negative cultures did differ from enrolled cases in a specific way – duration of hospitalization. By comparing to patients with three negative cultures, the likelihood of shedding actually decreased with hospitalization. Taken at face value, these results suggest that focusing infection control efforts during the first three days of hospitalization may be an effective strategy. However, we believe that the better comparison group in this study really is those patients with one negative culture. While there will likely be some misclassification, we expect this to be differential in nature and thus bias associations towards the null. In other words, the likelihood of shedding increases with duration of hospitalization and the reported strength of the association in this study likely underestimates the true effect. Interestingly, this brings us back to the general finding of this study – patients with more severe disease, and thus hospitalized for greater periods, are more likely to shed *Salmonella* in their feces. Therefore, due consideration should be given to these patients in infection control policy development.

Though we spend much time and effort trying to understand factors associated with shedding – and in fact do a variation of that in the subsequent study – we do not really know what fecal shedding of *Salmonella* truly means to the shedding patient or its stablemates. This next study was also a case-control study but had an additional follow-up component. Its objectives were; 1) to determine factors associated with fecal shedding of multi-drug resistant

(MDR)-*Salmonella*; and 2) to determine what effect *Salmonella* shedding may have on health outcomes of previously hospitalized horses and their stablemates. Again we found that horses experiencing gastrointestinal disease during hospitalization (specifically diarrhea) were more likely to shed *Salmonella* in their feces, although these were not more likely to be MDR-strains. While this is not novel, what was interesting is what we didn't find – that antimicrobial therapy during hospitalization was not associated with shedding of MDR-*Salmonella*. It is important to note that this was a relatively small study, focusing on one organism, in an equine population where the majority of horses (68%; 255/373) received antimicrobial therapy during hospitalization. This may have precluded our ability to detect an association between antimicrobial therapy and shedding MDR-*Salmonella*. The second interesting finding from this study was related to health outcomes. Long-term non-survival was associated with severity of disease, not whether or not the patient was shedding *Salmonella*, MDR or otherwise. Further, there was no association with *Salmonella* shedding and colic or abnormal feces post discharge; or an association with hospitalization or abnormal feces in stablemates. Not only is this consistent with a previous report [8.3], but it allows us to start to develop a foundation upon which to make recommendations to horse owners. The message is this – *Salmonella* shedding horses do not appear to increase disease risk among stablemates, *if reasonable precautions are taken*. In other words, we still recommend managing *Salmonella* shedding horses separately from resident horses and using rigorous personal and environmental hygiene to mitigate the exposure risk to personnel and other animals.

Environmental contamination is inevitably present during periods of epidemic disease and in fact can be phenotypically linked to patient isolates from the same time period [8.4].

This brings up an interesting question – Which comes first, environmental contamination or patient shedding? While we probably will never know the answer to this, we can try to gain a better understanding of this intricate relationship. The final study in this dissertation was a longitudinal study using data from 10 years of environmental surveillance. Its primary objective was to determine factors associated with environmental contamination of a veterinary teaching hospital with *S. enterica*; and secondarily to determine a suitable analytic method to model such a complex ecology. Simply stated, we found that what it really comes down to is the human effect. When veterinary hospitals experience high case load and are caring for patients with severe disease there is added demand on personnel likely resulting in decreased compliance with prevention measures. As a result, the probability of detecting *Salmonella* in the hospital environment increases. Additionally, we found that there is no perfect measure of this risk. In using variable cluster analysis and principal components analysis, it became obvious that what can be easily measured are really, in our opinion, "suitcase" variables. In other words, on the surface we know what we are measuring, but we have no idea what is contained within. While this study demonstrates the imperfect nature of available measures, it also suggests that a busy hospital is one with more opportunity for infectious disease transmission and it is at these times when we need to remain vigilant in the practice of infection control.

Control of *Salmonella enterica* in veterinary populations and their environments is a complex relationship between the agent, the host, and the environment. The research contained within this dissertation informs this very complex ecology. First, the available detection methods have an adequate analytic sensitivity, but they are likely hindered by intermittent shedding of the organism in animal feces. Second, patients with greater systemic

illness (i.e., disease severity) have a higher likelihood of shedding *S. enterica* in their feces. Third, patients shedding *Salmonella* are unlikely to increase disease risk among stablemates, provided appropriate precautions are taken. Fourth, despite having imperfect measures of risk, the probability of detecting *S. enterica* in the hospital environment increases as the demand on the hospital increases. There is still much to learn about the epidemiology and prevention of *S. enterica* – such as the role serotype may play and the continued development of accurate point-of-care tests – but there is now a broader foundation upon which to practice evidence based prevention strategies in veterinary medicine.

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APPENDIX 1: Routine fecal surveillance data collection form

CSU VTH Large Animal Salmonella Submission Form
a printed copy of this form is required for all salmonella submissions

Hospital Case: **827777** Owner: Animal:] Species: **EQU** Breed: **QTR** Sex: **F** VTH Clinician of record: **Dr. JOLYNN JOYCE**	Dlab Clinician of record: JoLynn Joyce Accession Number Sample #: 0 Technician:
Bill To: ◉ OWNER ○ HO8014 ○ Other:	
Sample Date: 05/20/2008 Sample Time:	
History: include major signs, sites of lesions, differential diagnosis, other laboratory tests (date and/or accession no.),antibiotic therapy	

Systemic Illness Summary in past 48 hours.	
○ **Healthy or Minimal Systemic Illness**	(Examples: Patients with minor lameness, arthroscopy, COPD, Mares admitted with foals, reproductive problems, etc.
○ **Minor or Moderate Systemic Illness**	(Examples: Patients with lacerations, recovering fractures, animals RTG recovering from a more severe illness like colic, LDA, FUO, mild respiratory infections, etc.)
○ **Major Systemic Illness**	(Examples: severe fractures, renal failure, liver failure, colic, severe strangles, pleuritis, pnemonia, colitis or enteritis, peritonitis, etc.)

Diarrhea of soft fecal consistency in past 48 hours.
○ Yes ○ No

Febrile in past 48 hours.(Equine > 101.5; Bovine > 103)
○ Yes ○ No ○ Unknown

Leukopenia in past 48 hours.(Equine WBC < 5000/ul; Bovine WBC < 5500/ul)
○ Yes ○ No ○ Unknown

Anesthesia or surgery in past 48 hours.
○ Yes ○ No

Antibiotics in past 48 hours.(check all that apply)
☐ None ☐ Oral ☐ Parenteral ☐ Topical or Ophthalmic

Significant reduction in dietary intake during past 48 hours.(inappetance or withholding of feed)
○ Yes ○ No

Body System affected.(check all that apply)
☐ Normal ☐ Musculoskeletal ☐ GI ☐ Respiratory ☐ Renal ☐ Hepatic ☐ Reproductive ☐ Other

Stabling Location.
○ Food Animal ○ Equine Main Barn ○ Colic Aisle ○ Main Isolation ○ Calf Isolation

Service.
○ Food Animal ○ Equine Medicine ○ Equine Surgery ○ Other (Opthalmology, Dermatology etc)

Other Comments: include information about patient's condition, other clinical signs or treatment

SUBMIT AND REVIEW | RESET FORM

APPENDIX 2: Medical records data collection form to obtain information on exposure variables of interest occurring during hospitalization

HEMI GI Disease Study - Medical Records Data

Study ID	376	Initials	kb	Collection Date	12/18/2013
Patient Nam				Farm Name	
Lab Sample Date	10/22/2012	Lab Sample ID	1210220023	Patient Zip Code	53714
Admission Date	10/12/2012	Discharge Date	10/22/2012	In-House Location	pcu

Service	Medicine	If other, please list		Sex	Mare
Breed				Age	Adult (>2 years)

Reduced feed intake during hospital visit?	no	Reduced intake due to	

Patient Care Level (max during visit)	Level 5
Diarrhea (soft feces) during visit?	yes

Febrile during visit (temp > 101 F)?	yes	Max temperature (F)	101.4

Presenting Complaint	fever/pheumonia
Disease Category	Respiratory

Anesthesia or surgery during hospital visit?	No
If Yes - Type?	

Leukopenic during hospital visit (WBC < 5000)?	no
Minimum Laboratory Value	

Antimicrobials during visit	Enrofloxacin (Baytril), Other, Potassium Penicillin (K-Pen)
Antimicrobials -- Other (list)	Metronidazole

Gastroprotectants during visit	Omeprazole, Other
Gastroprotectants -- Other (list)	yogurt and sacchromyces boulardii

Medical Records Notes	

APPENDIX 3: Owner questionnaire to collect information regarding health outcomes of interest among previously hospitalized horses and their stablemates

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Case Number: _____ Date: _____

HDM -- GI Disease Phone Interview

This is _____ calling from HDM. May I speak with [*horse owner*_____]?

Hello, my name is _____ calling from HDM. We are working on a project to learn more about colic and gastrointestinal disease in horses that come to our hospital. To get a representative sample, we are contacting owners who have had horses admitted to our hospital over the past year for both gastrointestinal and non-gastrointestinal diseases.

Is now a good time to talk? [*IF NOT*] May we schedule a time when it is better for you?
[*Record time and name for call back*] _____

I understand that [*name of horse*_____] was admitted to the HDM in [*month & year*_____].

- ☐ Yes [*next question*]
- ☐ No [*enter correction*] _____

This interview will take about _____ minutes to complete, it is voluntary, and your answers will be kept confidential. Is this OK?

- ☐ Yes [*continue*]
- ☐ No – When would be a better time to call back?
  [*Record name and time for callback*] _____

I am going to ask you a series of questions about the period of time following [*name of horse*_____] stay at our hospital in [*month & year*_____]. If you have questions at any time during this interview, or need anything clarified, please let me know. If you do not know the answer to any of the questions, it is perfectly acceptable to tell me that. In order to keep the questionnaire neutral, I did not review your horse's medical records prior to this phone call, so please excuse me if I ask questions that may be in the medical record.

QUESTION 1: Is [*name of horse*_____] still alive?

- ☐ Yes – Do you still own the horse?
  - ☐ Yes [*Go to QUESTION 2*]
  - ☐ No – When was the horse sold? _____
- ☐ No – I am sorry to hear about your loss. Is it OK for me to continue and ask a few more questions?
  - ☐ Yes – Thank you –

    When did the horse die? [*Enter date*] _____

    What was the cause of death? _____
    - ☐ Normal
    - ☐ Musculoskeletal (i.e., lameness, laminitis or founder, fracture)
    - ☐ GI (gastrointestinal, i.e., colic, diarrhea)
    - ☐ Respiratory (i.e., pneumonia, strangles)
    - ☐ Renal (i.e. kidney disease)
    - ☐ Hepatic (i.e. liver disease)
    - ☐ Reproductive
    - ☐ Other _____
  - ☐ No – Thank you for your time
- ☐ Don't know, horse was sold – When was the horse sold? _____

QUESTION 2: Did [*name of horse* _____] have any colic episodes since being discharged from our hospital?

- ☐ Yes – How many episodes? _____ How often [*daily/weekly/monthly*]? _____
  - ☐ Did your horse fully recover?
    - ☐ Yes
    - ☐ No – has continuing problems with colic
    - ☐ No – horse died/euthanized before it resolved
    - ☐ Don't know
- ☐ No [Go to Question 3]
- ☐ Don't know [Go to Question 3]

QUESTION 3: Did [*name of horse* _____] have abnormal feces following its hospitalization at our hospital?

- ☐ Yes – would you characterize the feces as being soft – a cow-pie consistency?
  - ☐ Yes – How many episodes? _____ How often [*daily/weekly/monthly*]? _____
    - ☐ Did your horse fully recover?
      - ☐ Yes
      - ☐ No – the horse still has soft feces
      - ☐ No – the horse died/euthanized before it resolved
      - ☐ Don't know
  - ☐ No

- ☐ Yes – would you characterize the feces as being watery – a diarrhea consistency?
  - ☐ Yes – How many episodes? _____ How often [*daily/weekly/monthly*]? _____
    - ☐ Did your horse fully recover?
      - ☐ Yes
      - ☐ No – the horse still has watery feces
      - ☐ No – the horse died/euthanized before it resolved
      - ☐ Don't know
  - ☐ No

- ☐ No [Go to Question 4]

- ☐ Don't know [Go to Question 4]

The next few questions will be about the other horses at your facility.

QUESTION 4: Does the number of horses housed on the same property as [*name of horse*_____] change substantially over the year?

☐ Yes – What is the minimum number of horses? [*Record number*] _____
What is the maximum number of horses? [*Record number*] _____
On any day in June, how many horses? [*Record number*] _____

☐ No – On average, how many horses? [*Record number*] _____
On any day in June, how many horses? [*Record number*] _____

QUESTION 5: To your knowledge, have any of the other horses on your property been hospitalized since [*name of horse* _____] was discharged from the hospital?

☐ Yes – approximately how many adults (≥ 6 mos of age)? [*Record number*] _____
approximately how many foals (< 6 mos of age)? [*Record number*] _____
☐ No
☐ Don't know

QUESTION 6: To your knowledge, have any of the other horses on your property have diarrhea or soft feces since [*name of horse*_____] was discharged from the hospital?

☐ Yes – approximately how many adults (≥ 6 mos of age)? [*Record number*] _____
approximately how many foals (< 6 mos of age)? [*Record number*] _____
☐ No
☐ Don't know

QUESTION 7: To your knowledge, how are horses housed when arriving at your facility…

Resident horse returning from a show/event

- ☐ Normal housing
- ☐ Separated from other horses – for how long [Number of *days*] _____
    - ☐ Separate housing?
    - ☐ Separate cleaning equipment?
- ☐ Other _____
- ☐ Don't know

Resident horse returning from a veterinary hospital

- ☐ Normal housing
- ☐ Separated from other horses – for how long [Number of *days*] _____
    - ☐ Separate housing?
    - ☐ Separate cleaning equipment?
- ☐ Other _____
- ☐ Don't know

Non-resident horse

- ☐ Normal housing
- ☐ Separated from other horses – for how long [Number of *days*] _____
    - ☐ Separate housing?
    - ☐ Separate cleaning equipment?
- ☐ Other _____
- ☐ Don't know

That is the end of our survey. Thank you very much for your time. We really appreciate your help in learning about gastrointestinal disease in horses that come to our hospital.

APPENDIX 4: Variable cluster analysis

Table: Variable cluster analysis for variables associated with veterinary hospital environmental contamination with *Salmonella enterica*

Cluster	Variable	Own Cluster	Next Closest	1-R^2 Ratio
Cluster 1	equine outpatients	0.5061	0.1715	0.5961
	equine inpatients	0.5535	0.0988	0.4954
	equine care level 1	0.3586	0.086	0.7017
	equine care level 2	0.4617	0.0731	0.5808
	equine care level 3	0.3941	0.0846	0.6619
	equine hospitalization days	0.5974	0.2651	0.5479
	small animal hospitalization days	0.3083	0.1479	0.8117
Cluster 2	HCAIs	0.4761	0.1323	0.6038
	bovine positive days	0.6666	0.0498	0.3509
	bovine positive patients	0.7281	0.0815	0.2960
	NWC positive patients	0.2276	0.0477	0.8111
Cluster 3	small animal care level 2	0.0361	0.0094	0.9730
	equine positive days	0.9699	0.0749	0.0325
	equine positive patients	0.9742	0.0929	0.0284
Cluster 4	sample type	0.1692	0.0543	0.8785
	NWC outpatients	0.519	0.2384	0.6316
	NWC inpatients	0.7163	0.1841	0.3477
	NWC hospitalization days	0.6381	0.1582	0.4298
Cluster 5	NWC care level 2	0.5961	0.1714	0.4874
	NWC care level 3	0.4889	0.0504	0.5382
	other care level 1	0.4982	0.0491	0.5277
	small animal care level 3	0.4782	0.2008	0.6529
Cluster 6	season	0.5769	0.0561	0.4483
	bovine outpatients	0.5558	0.0620	0.4736
	small animal care level 4	0.1792	0.0315	0.8475
Cluster 7	use	1	0.1405	0
Cluster 8	hospital	1	0.1405	0
Cluster 9	exotics care level 1	1	0.0139	0

NWC = New World camelid

APPENDIX 5: Principal components analysis

Table: Principal component analysis for variables associated with veterinary hospital environmental contamination with *Salmonella enterica*

VARIABLE	VARIABLE TYPE	Prin1	Prin2	Prin5	Prin14
bovine outpatients	case load	0.109	-0.047	-0.023	0.202
equine inpatients	case load	0.291	-0.087	-0.007	0.162
equine outpatients	case load	0.338	0.027	0.003	0.060
NWC inpatients	case load	0.223	0.075	0.038	0.016
NWC outpatients	case load	0.243	0.013	0.110	0.008
equine care level 3	disease severity	0.233	-0.113	0.118	0.219
equine care level 2	disease severity	0.260	-0.095	-0.030	0.215
small animal care level 2	disease severity	0.021	0.073	0.004	0.192
exotics care level 1	disease severity	0.003	-0.002	-0.228	0.174
other care level 1	disease severity	-0.032	0.224	-0.110	0.144
equine care level 1	disease severity	0.210	-0.153	0.118	0.115
small animal care level 3	disease severity	0.030	0.387	-0.053	0.091
equine hospitalization days	disease severity	0.314	-0.031	0.056	0.049
NWC hospitalization days	disease severity	0.188	0.126	-0.031	-0.011
NWC care level 2	disease severity	0.021	0.319	-0.056	-0.043
NWC care level 3	disease severity	-0.024	0.273	-0.074	-0.065
small animal hospitalization days	disease severity	0.225	-0.170	-0.031	-0.095
small animal care level 4	disease severity	-0.059	0.034	0.022	-0.132
smaple type	hospital	-0.100	0.084	0.282	0.679
season	hospital	-0.142	0.092	0.164	0.243
HCAIs	hospital	0.095	0.311	0.055	0.007
use	hospital	0.010	-0.008	0.597	-0.124
hospital	hospital	0.006	-0.013	0.594	-0.221
NWC positive patients	positive patients	0.010	0.231	0.117	0.097
equine positive patients	positive patients	0.160	0.196	-0.098	0.033
equine positive patient days	positive patients	0.176	0.193	-0.106	-0.008
bovine positive patients	positive patients	-0.063	0.387	0.101	-0.033
bovine positive patient days	positive patients	0.025	0.331	0.110	-0.149

NWC = New World camelid; prin = principal component

Colors indicate variable positive loading of >0.10 on the principal component.