# EPIDEMIOLOGY, SURVEILLANCE AND INFECTION CONTROL ASPECTS OF *SALMONELLA* INFECTIONS IN HOSPITALIZED HORSES

By

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| | |
|---|---|
| CFU | Colony forming unit |
| CT | Number of cycles required for the fluorescent signal to cross the threshold |
| °C | Degree Celsius |
| ∆Rn | Delta normalized reporter value of an experimental reaction minus the normalized reporter value of the baseline signal generated by the instrument |
| F | Fahrenheit |
| fL | Femtoliters |
| GI | Gastrointestinal |
| g/dL | Grams per deciliter |
| K/uL | Thousands per cubic milliliter |
| mEq/L | Milliequivalents per litre solvent |
| mg/dL | Milligrams per deciliter |
| mmol/L | Millimoles per liter |
| M/uL | Millions per cubic milliliter |
| mL | Milliter |
| pg | Picograms |
| PCR | Polymerase chain reaction |
| Spp | Species |
| U/L | Units per liter |

Abstract of Dissertation Presented to the Graduate School
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This research work addressed four objectives. The first part was a critical review of published studies that have contributed to the literature of epidemiology and infection control of nosocomial *Salmonella* infections in hospitalized horses. This review identified several knowledge gaps related to epidemiology, diagnostics, and surveillance and infection control of *Salmonella* infections in hospitalized horses. The review was followed by three investigations that addressed three of the identified knowledge gaps.

The second part was an investigation of the relationships between clinical parameters (clinical signs and procedures, hematological and plasma chemistry) and *Salmonella* shedding in horses with signs of GI disease and with or without diarrhea. This study was designed as a case-control study. Results revealed that high plasma triglycerides at admission, abdominal surgery, and season (summer) were associated with *Salmonella* shedding in horses without diarrhea. These parameters can be considered for evaluation in hospital biosecurity programs to identify equine GI inpatients at high risk of *Salmonella* shedding.

The third part was an investigation that assessed the diagnostic performance of a real-time polymerase chain reaction (PCR) assay for detection of *Salmonella* spp in

feces of hospitalized horses, compared to bacteriological culture. Fecal samples from horses classified as true-positives and true-negatives were used to assess the relative sensitivity and specificity of the PCR assay, respectively. Two primer sets were evaluated and results revealed that this PCR protocol was reliable, with a relatively high specificity (86-100%) and sensitivity (100%) when compared to bacteriological culture. This real-time PCR protocol can be used as a surveillance tool to detect *Salmonella* spp in fecal specimen of hospitalized horses.

The fourth part was a survey that assessed the awareness and relevance of a hospital surveillance and infection control program among referral veterinarians and clients who refer or send horses to a referral hospital for veterinary care. A total of 92 RDVMs and 594 clients participated in the survey. Study results revealed that most clients (61%) were not aware that the referral hospital operates an infection control program, and both RDVMs and clients considered it very important that a referral hospital operates a surveillance and infection control program.

# CHAPTER 1
# INTRODUCTION

Shedding of *Salmonella* spp in feces of hospitalized horses is an important problem for large animal hospitals because of the potential risk of nosocomial *Salmonella* infections. Consequences of outbreaks of nosocomial *Salmonella* infections can be significant and may include high mortality in equine inpatients and substantial financial losses.[1-6] Because of the potential risk of outbreaks of nosocomial *Salmonella* infections, several large animal hospitals have established surveillance and infection control programs to allow early detection of *Salmonella* shedding in hospitalized animals and timely implementation of appropriate preventative and control measures.

The frequency of horses shedding *Salmonella* during hospitalization can be higher among horses with signs of gastrointestinal (GI) tract disease without diarrhea compared to horses with diarrhea. *Salmonella*-positive horses without diarrhea present a threat to veterinary hospitals because bacteriological culture procedures for detection of *Salmonella* shedding are often completed after the patient has been discharged, causing a delayed response in implementation of infection control measures. Several epidemiologic studies have been conducted to identify exposure factors associated with *Salmonella* shedding in hospitalized horses. However in those studies, the analyses did not separate horses with diarrhea from those without diarrhea. As a result, the reported risk factors apply to a combined population of horses with and without diarrhea. Exposure factors associated with *Salmonella* shedding in horses with or without diarrhea have not been independently investigated. This information is important as it can further improve current hospital surveillance programs for early detection of *Salmonella* shedding and allow rapid implementation of infection control measures.

Several PCR protocols have been developed and evaluated for potential use in hospital surveillance programs for early detection of *Salmonella* spp in feces of hospitalized horses. The advantage of using PCR protocols to monitor *Salmonella* shedding in feces of hospitalized horses is that test results can be obtained more quickly, compared to bacteriological culture (≤ 24 hours versus 3-5 days), thereby allowing an earlier implementation of preventative measures such as isolation and barrier nursing that minimize the risk of a potential outbreak of nosocomial *Salmonella* infection.[7,8] However, despite the availability of rapid and cost-effective PCR tests for detection of *Salmonella*, its acceptance as a surveillance tool for monitoring community-acquired and nosocomial *Salmonella* infections in horses has been limited. The main reasons for not using PCR testing as a surveillance tool are that in previous studies [9-13] sampling methods used to establish the gold standard for true-positives and true-negatives were not objective. This led to potential misclassification bias and inconclusive results. In particular, there was a high frequency of *Salmonella*-PCR false-positive results in horses without clinical signs of salmonellosis that tested negative to *Salmonella* spp by bacteriological culture on multiple fecal samples. These issues were perhaps due to the use of primers that were non-specific or cross-reactive with other bacteria. For PCR to be considered as a surveillance tool, these limitations need to be addressed through use of correct sampling approaches, consistent standard definitions for true-positives and true-negatives, and use of a real-time PCR protocol with primers and probes that have demonstrated no evidence of cross-reactivity with other enteric and non-enteric organisms.

At a referral hospital, referring veterinarians and clients play a vital role in a hospital surveillance and infection control program. Veterinarians participate in the program by referring clients to a tertiary care hospital because of its specialized expertise and need for specialized care. In turn, clients bring high risk patients such as those with signs of gastrointestinal (GI) disease that may be targeted in a hospital surveillance and infection control program. At a referral hospital, information about a hospital surveillance and infection control program and its importance to RDVMs and clients is promoted in various ways. At the UF LAH, educational efforts about the program include provision of an infection control brochure to all new clients to educate them about the infection control measures that are instituted at the hospital to optimize patient care. One element of an animal disease surveillance system is evaluation and feedback. To have an efficient program, it is important that performance of the program is evaluated and feedback is collected from all individuals involved in the program including clinicians, hospital personnel, RDVMs and clients. Considering the role played by RDVMs and clients, it is important to know the level of awareness and perceptions about the relevance of a hospital surveillance and infection program among this group. This information can be used to guide decision-making on hospital policy issues related to surveillance and infection control, and streamline costs and efficiency of services provided to clients.

## Research Objectives and Approach

### Objective 1: Review of Epidemiology and Infection Control Aspects of Nosocomial *Salmonella* Infections in Hospitalized Horses

The purpose of this objective was to review published studies that have contributed to the literature of epidemiology and infection control aspects of nosocomial *Salmonella* infections in hospitalized horses. The review is presented as part of Chapter 2 and was structured in 3 parts: 1) surveillance and infection control; 2) epidemiological research; and 3) discussion. The first part consists of six elements case definition, environmental contamination, sampling, laboratory diagnosis, risk management, and guidelines for surveillance and infection control. The second part was limited to a review of published studies that examined exposure factors associated with nosocomial *Salmonella* infections in hospitalized horses. Finally, the third part was a discussion section relevant to infection control issues and areas of research that can further improve existing hospital surveillance and infection control programs.

### Objective 2: Exposure Factors Associated with *Salmonella* Shedding among Hospitalized Horses with or without Diarrhea

The objective of this study was to investigate the relationships between clinical signs, hematological and plasma chemistry parameters, and clinical procedures (before admission, at admission, and during hospitalization) and *Salmonella* shedding in hospitalized horses with signs of GI disease, with or without diarrhea. A case-control study approach was used to identify investigated exposure factors associated with *Salmonella* shedding. The study population included adult horses with signs of GI disease and the sample of study animals included case horses with diarrhea, case horses without diarrhea, and control horses.

## Objective 3: Diagnostic Performance of Real-Time PCR for Diagnosis of *Salmonella* Shedding in Hospitalized Horses

The objective of this study was to assess the diagnostic performance of a real-time PCR assay for detection of *Salmonella* spp in feces of hospitalized horses, compared to bacteriological culture. The study approach involved several steps. First, to ensure that primers and probes used in the real-time PCR assay were specific and did not cross-react with other enteric or non-enteric organisms, two sets of primers and probes were selected from published PCR studies after conducting a bioinformatics analysis. Thereafter, the real-time PCR assay was validated using each set of selected primers and probes. Validation steps included optimization of the primer/probe concentrations, assessment of analytical sensitivity and specificity, and determination of the assay detection limit using Phosphate buffered saline (PBS) and fecal samples spiked with *Salmonella* organisms. Analytical sensitivity of three commercially available DNA extraction kits was evaluated to determine the most optimal kit which was later used to extract DNA from the study samples.

To assess the diagnostic sensitivity (detection of true-positives) of the real-time PCR assay, fecal samples from horses with signs of GI disease (including colic, diarrhea, or fever and leukopenia) that were classified as *Salmonella*-culture positive on at least one fecal samples were used in the analysis. To assess the diagnostic specificity (detection of true-negatives), fecal samples from two different groups of horses were used in the analysis. The first group included fecal samples from horses without signs of GI disease that were classified as *Salmonella*-culture negative on five fecal samples. The second group included fecal samples from horses with signs of GI disease that were classified as *Salmonella*-culture negative on at least three fecal

samples. A real-time PCR assay with a sensitivity ≥ 90% and specificity ≥ 95% for detection of *Salmonella* spp in fecal specimens of study horses was considered an appropriate diagnostic tool.

### Objective 4: Awareness and Relevance of Hospital Surveillance and Infection Control Services among Referral Veterinarians and Clients

The objective of this study was to assess the awareness and relevance of a hospital surveillance and infection control program among referral veterinarians and clients who refer or send horses to a referral hospital for veterinary care. An electronic and mail survey was conducted and a convenience sampling approach was used to select eligible RDVMs and clients. Two separate 2-page questionnaires were designed for RDVMs and clients. Initially an electronic (email) survey was sent to all enrolled RDVMs (n=242) and clients (n=483) that had email addresses and a remainder email was sent 10-days later to urge the respondents to complete the survey. Data for the respondents were entered into an excel database for analysis. A mail survey was later mailed out to all enrolled RDVMs (n=225) and clients (n=2612) that did not have an email address but had a physical mailing address (and were never contacted during the time when the electronic survey was administered). One month was allowed for responses to be returned by mail. Data for the respondents were entered into an excel database for analysis.

# CHAPTER 2
# LITERATURE REVIEW

## Etiology

Salmonellosis is caused by a variety of strains of *Salmonella* species, all of which are Gram-negative, facultative anaerobic, motile, non-lactose fermenting, rod-shaped bacteria that belong to the family Enterobacteriaceae.[14] All *Salmonella* species are classified as *S. enterica* or *S. bongori.* Salmonellae are subdivided into serogroups and then further into serotypes or serovars based on testing with specific antisera.[15] The most important serogroups in veterinary medicine are A, B, C, D, and E.[16] Within the species of *Salmonella enterica*, over 2,000 different serotypes of veterinary and medical importance affect the gastrointestinal tract and result in diarrhea alone, or diarrhea in conjunction with fever, anorexia, depression, and shock.[14] The most frequently isolated serotypes in horses include *S. enterica* serovar Typhimurium*, S. enterica* ser. Anatum*, S. enterica* ser. Newport*, S. enterica* ser. Krefeld and *S. enterica* ser. Agona.[17] The only host-adapted serovars reported for horses is *Salmonella* sp. ser. Abortusequi, causing abortion between 5 and 10 months of gestation.[18]

## Pathogenesis

Once salmonellae infect a host, the first line of defense encountered is the acid barrier of the stomach.[19] Organisms that survive the acid barrier travel to the small intestine, where they are exposed to secretory products of the intestine (such as IgA, defensins, bile salts, and intestinal mucus) and to intestinal microflora that prevent bacteria from penetrating enterocytes.[20] Environmental factors in the intestinal lumen (such as oxygen concentration, osmolarity, and pH) affect the expression of *Salmonella*

invasion genes (which determine the release of bacterial products required for invasion of host cells).[21]

For successful progression of a systemic infection and colonization of the host after oral infection, penetration of the gut epithelium is an important initial step. Microfold cell (M cells) located in the follicle-associated epithelium in the peyer's patches are the primary intestinal epithelial cell type targeted for invasion by *Salmonella* in the mouse[22], and in cattle, *S* Typhimurium invades both M cells and enterocytes with no predilection for a particular cell type.[23] In adult horses, *Salmonella* primarily infects the cecum and proximal colon, causing typhlocolitis, and the ability to disseminate beyond the intestine and cause enteric fever is limited.[24] In foals, however, salmonellosis is often associated with bacteremia. The ability of *Salmonella* to cause enterocolitis depends on the ability of the bacteria to invade the gastrointestinal mucosa.[25,26] After crossing the intestinal mucus layer, salmonellae interact with both enterocytes and M cells.[21] Once attached to the enterocytes or M cells, the organisms are rapidly internalized. Salmonellae have the ability to induce endocytocis (ruffling) in otherwise nonphagocytic cells and enterocytes. This process involves the formation of large membranes around the bacteria by the host cell (enterocytes and M cells), as well as cytoskeletal rearrangements within the host cell itself. Once inside the cells, salmonellae migrate toward the submucosa of the small intestine where they interact with macrophages and lymphocytes.[27] Salmonellae have the ability to survive within macrophages and use them to spread beyond the intestine.[19] They are transported via the lymphatics to draining lymphoid nodes and then to the thoracic duct, where they enter the systemic circulation.

During the asymptomatic phase of the infection, the organisms are localized to the

intestine and replicate within macrophages and epithelial cells. After becoming enveloped by macrophages, vacuoles called phagolysosomes are formed from the fusion of the ends of membrane ruffles and lysosomes contained within the macrophage. When a critical number of organisms have replicated, clinical signs result from the secretion of cytokines by the infected cells.[28] A characteristic feature of *Salmonella* infection is the induction of an early inflammatory response in the intestinal epithelium, resulting in the infiltration of polymorphonuclear leukocytes. The induction of such a response is likely due to the production of cytokines or other proinflammatory molecules by natural killer cells and macrophages.[28] The inflammatory response contributes to the pathophysiology of the infection, characterized by inflammatory diarrhea.[27]

**Diarrhea associated with salmonellosis.** Diarrhea associated with salmonellosis has multiple causes.[24] *Salmonella* cytotoxins inhibit protein synthesis in mucosal cells, causing morphologic damage and altered permeability.[29] Virulent *Salmonella* also produce an enterotoxin similar to the heat-labile toxin (LT) produced by *Escherichia coli.*[30,31] The enterotoxin contributes to but is not required in the pathogenesis of diarrhea.[32,33] *Salmonella* enterotoxin increases secretion of chloride and water by colonic mucosal cells in many species, including horses, by increasing intracellular cyclic adenosine monophosphate concentrations.

The ability of virulent salmonellae to cause diarrhea appears to be associated most closely with the ability to invade enterocytes and to trigger an inflammatory reaction in intestinal tissue.[26,34] Effector proteins (SopB) injected into enterocyte cytosol by the type III secretory system of invading salmonellae stimulate chloride and fluid

secretion.[35] *Salmonella* invasion of enterocytes is also a potent activator of inflammatory chemokine and cytokine production, resulting in the recruitment of leukocytes, particularly neutrophils, and activation of resident macrophages and mast cells. Products of these activated leukocytes, including prostaglandins, leukotrienes, reactive oxygen metabolites, and histamine, are potent stimulators of chloride secretion in the colon of many species.[25,36-38] The enteric nervous system integrates the diverse processes of pathogen recognition, triggering of the inflammatory response, and induction of enterocyte fluid secretion.[38] Many of the inflammatory mediators stimulate colonic secretion by prostaglandin-dependent mechanisms, resulting in increased intracellular cyclic adenosine monophosphate or calcium concentrations or both in mucosal cells.[36] In addition, these mediators and the enteric nervous system may stimulate secretion by prostaglandin-independent mechanisms, inhibit sodium and water absorption, cause motility disturbances, and potentiate tissue injury, all of which enhance the pathogenicity and dissemination of *Salmonella* and contribute to the pathogenesis of diarrhea.[36,38] Neutrophils recruited to the mucosa by signals generated by the infected enterocytes physically contribute to mucosal injury by producing a variety of products that are lethal to pathogens but are also toxic to host cells.[39,40] Moreover, neutrophils attracted to infected epithelial cells accumulate beneath the monolayer, lifting it off the basement membrane in sheets. Neutrophils also migrate across the epithelial monolayer in potentially massive numbers. Transepithelial migration of neutrophils increases the permeability to macromolecules, bacterial products, and even bacteria.[40] Potentially massive losses of electrolytes, water, and protein can occur depending on bacterial and host factors. Perhaps most devastatingly,

mucosal injury and altered permeability allow systemic absorption of bacterial products and dissemination of bacteria, resulting in systemic inflammatory responses such as occur with endotoxemia and bacteremia.[24]

## *Salmonella* Virulence Mechanisms

During a long-standing association with its hosts, *Salmonella enterica* has evolved a sophisticated virulence mechanism for interaction with the host and modulation of host cell functions. This virulence mechanism includes a large number of genes that are necessary for successful pathogenesis of *Salmonella* infections.[41] Many of these virulence genes protect *Salmonella* against damage generated by defense mechanisms of the innate and adaptive immune system of the infected host, or allow *Salmonella* to cope with nutritional deprivation imposed by specific host environments.[42]

Several of the *Salmonella* virulence genes are clustered in certain areas of the chromosome known as "*Salmonella* pathogenicity islands" (SPI). To date, five SPI have been described and two of them, SPI-1 and SPI-2, contain a large number of genes encoding type III secretion systems for virulence proteins. The type III secretion system is a pathway by which virulence proteins are transported from the bacterial cytoplasm to the extracellular space. Type III secretion systems are structurally and functionally related to the flagella assembly systems, and typically contain more than 20 different protein subunits that are located in the inner and outer membrane as well as in the periplasm and cytoplasm of the bacterial cell.[43]

The ability to invade non-phagocytic cells and the intracellular survival and replication of *Salmonella* in phagocytes of the host are two hallmarks of *Salmonella* pathogenesis.[42] *Salmonella* are the only species that contain two type III secretion systems, which are encoded by two distinct gene clusters (SPI-1 and SPI-2). These two

type III secretion systems play different roles during pathogenesis; with SPI-1 being required for initial penetration of the intestinal mucosa and SPI-2 necessary for subsequent systemic stages of infection.[43] The SPI-1 encoded type III secretion system translocates effector proteins into the cytosol of host cells. This system is required for invasion of non-phagocytic host cells (including epithelial cells) and enteropathogenesis,[44,45] while the SPI-2 encoded type III secretion system is required for intracellular survival in macrophages.[22]

The molecular function of type III secretion systems is secretion of substrate proteins which fall into different functional classes.[42] One subset of substrate proteins is required to allow the translocation of a second subset of substrate proteins into the eukaryotic target cell. This second subset of substrate proteins is referred to as effector proteins. SPI-1 encoded effector proteins include SipA, SipB, SipC, SipD, SptP, SopA, and SopD, while SPI-2 encoded effector proteins include SseB and SpiC. Other effector proteins encoded outside the SPI locus include SopB, SopE, and SopE2. There is evidence for a third subset of substrate proteins that are not translocated but appear to have regulatory functions.[42]

Expression of *Salmonella* virulence genes is regulated by factors imposed on the pathogen by host microenvironments. Environmental conditions that affect the ability of *Salmonella* to enter host cells include oxygen levels, osmolarity, bacterial growth state, and pH.[46] The actual mechanisms, how the environmental signals are sensed, and how they influence gene expression, are not fully elucidated.[42] The effects of these environmental factors are mediated by regulation of expression of transcriptional

regulator HilA.[47] HilA expression is regulated by genes encoded on SPI-1, SPI-4, and SPI-5.[48]

### *Salmonella* Virulence Genes Required for Invasion of Intestinal Epithelial Cells

During the intestinal phase of *Salmonella* infection, the SPI-1 encoded type III secreted proteins play a crucial role, affecting invasion of intestinal cells, induction of inflammatory cell recruitment, and fluid secretion.[45] Studies have demonstrated that disruption of the SPI-1 encoded type III protein secretion system decreases or abolishes the ability of *S* Typhimuirum to invade the intestinal epithelium, which correlates with its ability to elicit an inflammatory response and thereby induce diarrhea.[48,49-51] Upon contact with intestinal epithelial cells, *S* Typhimurium translocates bacterial effector proteins into the host cell cytosol via the SPI-1 encoded type III secretion system. Some of these proteins have kinase, phosphatase, or actin-binding activity, and once in the epithelial cell cytosol, they alter host cell signaling pathways that promote changes in the cytoskeleton. Pseudopods or membrane ruffles are formed on the host epithelial cell membrane, with consequent bacterial internalization and changes in host cell gene expression.[52]

The SPI-1 encoded effector protein, SipC, acts as translocase and translocates itself into the host cytosol via the SPI-1 encoded type III secretion system. This protein bundles actin filaments and nucleates actin polymerization which results in cytoskeletal rearrangements within the infected epithelial cell.[52] SipA, another SPI-1 encoded type III secretion protein, is not required for invasion but inhibits depolymerization of actin filaments by directly binding to and thereby decreasing the critical concentration of actin.[53] Therefore, SipC is essential for actin nucleation and bundling of actin filaments whereas SipA acts by enhancing the efficiency of this process.[54]

Other effector proteins encoded by genes outside the SPI-1 locus, including SopE and its homologue SopE2, play a role in recruiting the actin-nucleating complex to the membrane ruffles, thereby contributing to the invasion phenotype.[55] SopE protein targets host cell signal transduction pathways by acting as a nucleotide exchange factor for the GTPases CDC42 and Rac1.[56] The activation of CDC42 and Rac1 results in the reorganisation of the actin cytoskeleton, leading to the uptake of *Salmonella*.

SptP, is an SPI-1 encoded type III secretion protein that acts by reversing the cytoskeletal changes induced by *Salmonella* during invasion, restoring the normal structure.[57] Though SptP disrupts the actin cytoskeleton of epithelial cells, it is not required for cell invasion.[58] However, SptP is required to switch off actin reorganization during host cell invasion thereby contributing to termination of the invasion process.[57] The effect of SptP antagonizing the action of other bacterial effector proteins clearly indicates that *Salmonella* is able to finely regulate cellular pathways in favor of its own purposes.

Bacterial uptake by epithelial cells is quickly followed by changes in host cell gene expression. Transcription activators for the production of pro-inflammatory cytokines including IL-8 are induced that provoke inflammatory responses.[59] IL-8 is a chemo-attractant for neutrophils. Infiltration of neutrophils into the lamina propria occurs shortly after infection with *S* Typhimurium, and is followed by massive migration of neutrophils through the epithelium into the intestinal lumen.[23,60] Some studies have indicated that IL-8 recruits neutrophils to the subepithelial space rather than through transepithelial migration into the intestinal lumen.[61,62] *Salmonella*-induced IL-8 secretion by epithelial cells is dependent on the mitogen-activated protein kinase pathway and activation of the

transcription factor NF-kB, and requires a functional SPI-1 encoded type III secretion system. The *Salmonella* proteins, SipA and SopA, are involved in induction of neutrophil transepithelial migration. These two SPI-1 encoded effector proteins are translocated into the cytosol of the host cell via the SPI-1 type III secretion system.[63]

Depending on the species infected, *Salmonella* infection may or may not result in enteritis and diarrhea. In mouse, no diarrhea occurs and the response to infection is mostly with mononuclear infiltration in the intestine.[64] In calves, *S* Typhimurium infection results in enteritis in which neutrophils are the primary inflammatory cells involved.[64] A characteristic feature of *Salmonella*-induced enteritis is an intense intestinal secretory and inflammatory response including the induction of polymorphonuclear cell transmigration through the intestinal epithelium in humans.[65] The SPI-5 encoded gene, SopB, has been linked to the pathogenesis of diarrhea[66,67] and its expression is dependent on the regulator sirA, which is also an activator of the SPI-1 regulator hilA.[68] SopB has inositol phosphate phosphatase activity[69] and is not required for invasion, but contributes to diarrheal symptoms by triggering loss of electrolytes and fluid secretion of epithelial cells of the intestine.[67] SopB is thought to mediate fluid secretion by increasing chloride secretion.[70] The *Salmonella* protein SopD, which is also secreted in an SPI-1 dependent manner, has an additive effect to SopB in the induction of enteritis.[71] Another protein, SopA, influences the intestinal inflammatory response by a mechanism distinct from SopB and SopD. SopA is involved in induction of neutrophils, a phenomenon that is not influenced by SopB or SopD.[72] The effector proteins SipA, SopA, SopB, SopD, and SopE2 have been demonstrated to act in concert to induce diarrhea.[73]

**Survival of *Salmonella* Following Intestinal Invasion**

*Salmonella* is a facultative intracellular pathogen that can survive and replicate inside host phagocytic cells, most prominently in macrophages,[74] and can utilize phagocytes as vehicles for dissemination to other tissues such as the spleen and liver.[75] The ability to grow within macrophages and polymorphonuclear phagocytes is a prerequisite for *Salmonella* virulence.[74,76-79] Once *Salmonella* have crossed the epithelial layer that separates the intestinal lumen from host tissue, the bacteria first encounter the cells residing in the Peyer's patches and the mesenteric lymph nodes.[80-82] The cells encountered include resident phagocytes and dendritic cells. Macrophages in particular are crucial to the initial defense against *Salmonella*; serving to ingest bacteria and generate inflammatory signals in response to infection.[83-85] After uptake by a phagocytic cell, *Salmonella* does not enter the intracellular cytoplasm but resides inside the phagosomal vacuole. In this environment, *Salmonella* has to be able to cope with environmental changes such as rapid decrease in pH and nutritional deprivation that are restrictive for the growth of phagocytosed bacteria. In addition, phagosomes containing ingested particles usually enter the degradative pathway of the cell. After fusion with lysosomes to form a phagolysosome, phagocytosed bacteria will encounter toxic substances such as hydrolytic enzymes (proteases, lysozyme), small cationic proteins (i.e., defensins), and enzymes that produce reactive forms of oxygen to cause the so-called oxidative burst (superoxide radical, hydroxyl radical), as well as reactive nitrogen intermediates.[42]

The strategy by which *Salmonella* modifies the phagosome to survive and replicate is not yet fully understood. Suggested mechanisms include inhibition of the fusion of *Salmonella*-containing vacuoles with lysosomes,[86] delay in acidification of the

phagosome,[87] or the generation of *Salmonella*-containing vacuoles with a composition different from that of normal phagocytes,[88] and avoidance of the NADPH oxidase-dependent killing by macrophages.[89] An SPI-2 encoded substrate protein, SpiC, has been shown to interfere with normal cellular vesicle trafficking.[90] Therefore, SPI-2 may be involved in the altered characteristics of the *Salmonella*-containing vesicles. The molecular functions of SPI-2 have not been characterized in as much detail as those of SPI-1.[42]

*Salmonella* can induce death of host phagocytic cells. *Salmonella*-induced macrophage cell death is largely due to expression of genes associated with invasion. SipB protein has been identified as the bacterial protein responsible for induction of apoptosis.[91] SipB is translocated into the host cell cytosol via the SPI-1 encoded type III secretion system, where it binds to and activates caspase-1, an intracellular cysteine protease also known as IL-1β converting enzyme. Once activated, caspase-1 cleaves and activates IL-1β.[91] Therefore, *Salmonella*-induced macrophage apoptosis results in release of active IL-1β, which is thought to play a significant role in *Salmonella*-elicited inflammation.

**Multi-Drug Resistant *Salmonella* Strains in Horses**

Multi-drug resistant (MDR) *Salmonella* infections in horses have been documented. In two previous outbreak investigations of nosocomial salmonellosis in horses, cattle, and alpacas, multi-drug-resistant strains of *Salmonella* Newport were identified as the causative strain.[5,92,] In one study, 17 of 54 horses infected with the outbreak strain died during hospitalization (but it was not clear how many horses were affected with diarrhea).[5] In another study, 1 of 2 horses infected with the outbreak strain developed enterocolitis, diarrhea, septicemia and subsequently died.[92] Several

Studies in humans have demonstrated that infections with drug-resistant non-typhoid *Salmonella* serotypes are associated with excess morbidity and mortality.[93-100] Multi-drug resistance (MDR) typically occurs as a result of accumulation of multiple mutations and/or the horizontal transfer of resistance genes on mobile genetic elements such as plasmids and transposons along with novel genetic elements such as integrons and resistance islands.[101-104]

Most of the antimicrobial drug resistance determinants of *Salmonella* are encoded on plasmids and on the multidrug resistance region of SPI-1.[105] Plasmids are small, circular, self-replicating DNA elements that are capable of transfer via conjugation.[103,106] Resistance genes encoded in plasmids are often located within genetic elements called transposons. These elements possess the transposase function that enables the transposon to recombine into the bacterial chromosome or plasmids.[103] Horizontal transfer of plasmids to other cells often provides a selective advantage to the organism if the plasmid carries genes encoding antimicrobial resistance, persistence, environmental adaptability, heavy metal resistance, and/or virulence.[103,107-109] Plasmids are classified by incompatibility (Inc) groups, which are named as such because two members of the same Inc group cannot be stably maintained in a bacterium during cell division.[110] In *Salmonella* isolated in the United States, IncA/C plasmids have been associated with an expansion of MDR in several serovars of *Salmonella*. These plasmids are often large (150–200 kb) and some can carry genes encoding for resistance to 10 or more antimicrobials.[111]

Multi-drug efflux pumps of the resistance nodulation-division (RND) family encoded on SPI-1 play a major role in providing both intrinsic and elevated levels of

resistance to a wide range of noxious compounds in Gram-negative bacteria.[112,113] In *Salmonella*, increased expression of the multi-drug AcrAB-TolC efflux pump has been shown to be a significant determinant of microbial resistance.[114] This system confers innate multiple antibiotic resistance.[115] Disruption of the multi-drug AcrAB-TolC efflux pump has been shown to result in decreased pathogenicity associated with reduced expression of genes located on SPI-1 and other attributes required for anaerobic growth, motility, and host cell invasion.[116] The mobility of SPI-1 coupled with the ability of various antibiotic resistance genes to be integrated and lost from the chromosomal resistance locus allows for the transfer of stable antibiotic resistance to most of the commonly used antibiotics and adaptation to new antibiotic challenges.[114]

A class of mobile genetic elements, the integrons, are considered efficient means by which transfer of resistance markers among unrelated bacterial populations can be facilitated as part of a transposon of the Tn*21* family or independently on broad host range plasmids.[114,117] Integrons contain a recently described system for site-specific recombination, similar to that in temperate bacteriophages.[118] These structures are naturally occurring gene expression systems that can potentially capture and integrate one or more gene cassettes and convert them into functionally expressed genes. It is these gene cassettes that encode open reading frames (ORFs) corresponding to the resistance determinants of several antimicrobial agents. MDR-encoding integrons are widespread in *S*. Typhimurium DT104, other phage types of *S*. Typhimurium as well as other serovars of *Salmonella* and many other Gram-negative bacteria.[106,119-121]

## Host Immunity against *Salmonella* Infections

Host factors that restrict gastrointestinal colonization and invasion by pathogens include gastric pH, commensal gastrointestinal flora, gastrointestinal motility, the

mucosal barrier and mucosal immunity.[24,25,122] Gastric acidity is an important defense mechanism preventing live organisms from reaching the intestine. Altering the gastric pH with histamine receptor antagonists, for example, may increase susceptibility to infection. Gastrointestinal flora inhibits the proliferation and colonization of *Salmonella* by secreting bacteriocins, short-chain fatty acids, and other substances that are toxic to *Salmonella*. In addition, elements of the normal flora compete for nutrients and space, especially on the mucosa.[25] Being predominantly anaerobic, the normal flora maintain a low oxidation-reduction potential in the environment of the large intestine, which inhibits the growth of many bacterial pathogens.[123] The importance of normal host gastrointestinal ecology is illustrated by the fact that disturbances of the colonic flora with antibiotics, changes in feed, ileus, or other underlying gastrointestinal disease greatly increase the susceptibility of the host to infection by *Salmonella*, often resulting in serious disease. After surviving these host factors, *Salmonella* encounters other factors at the intestinal mucosal surface including mucus which forms a physical barrier, lysozyme, lactoferrin, and lactoperoxidase.[124]

The immune status of the host may be one of the most important factors determining not only the susceptibility to *Salmonella* infections but also the degree of invasion and subsequent outcome of the infection.[24] During the initial stages of *Salmonella* infection, the host's innate immune response is conducted by natural killer cells, natural killer T cells, neutrophils and macrophages.[125] This first line of defense involves production of high levels of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) produced primarily by natural killer cells and macrophages. Macrophage derived cytokines act by enhancing the bactericidal capacity of phagocytes, facilitating

antigen presentation, and influencing the T helper cell polarization of the immune response.[125] Opsonizing antibodies and activation of the complement cascade are important in fighting systemic invasion by *Salmonella* by increasing the efficiency of phagocytosis and by direct bactericidal activity.[24]

The adaptive immune response with both humoral and cell-mediated immune responses, is involved in the acquired resistance to *Salmonella* infection.[17] The humoral response involves production of IgA by plasma cells. IgA is the principal antibody isotype involved in mucosal immunity, and acts by binding to surface antigens and preventing attachment and penetration of salmonellae.[20] Cell-mediated immunity is activated by the different antigens expressed in *Salmonella*, which induce a specific T helper cell response. On the basis of differential cytokine expression, both T helper 1 (Th1) and T helper 2 (Th2) type responses can be identified in most cases of infection by facultative intracellular pathogens in mice.[28] Th1 cells produce cytokines such as interferon γ (IFN-γ) and interleukin 12 (IL-12), which regulate cell-mediated protective immune responses against intracellular organisms. In contrast, Th2 cells produce cytokines such IL-4, IL-5, IL-10 and IL-13 which regulate humoral immune responses which may not be protective.[28,126]

*Salmonella* are intracellular bacteria which can survive in macrophages and dendritic cells.[27] Consistent with the notion that Th1/Th2 ratio predicts the competence of the response to intracellular pathogens, there is some early evidence that invasive *Salmonella* generally elicit a Th1 immune response; however, there are also reports describing both Th1 and Th2 responses to *Salmonella* in mice, with the predominant response reflecting the particular pathway of antigen processing (MHC-II versus MHC-I

dependent) in macrophages and dendritic cells.[28] Another adaptive immune response in the early stages of infection is cell-mediated immunity by αβ T lymphocytes, which function as effector cells, and γδ T lymphocytes that function as regulatory cells in the late stages of disease in mice.[127]

## Epidemiology and Infection Control Aspects of Nosocomial *Salmonella* Infections in Hospitalized Horses

Over the past thirty years, outbreaks of nosocomial *Salmonella* infections in hospitalized horses have been reported, with varying consequences. In 1981-1982, the University of California-Davis, Veterinary Medicine Teaching Hospital experienced an outbreak of nosocomial salmonellosis due to *Salmonella enterica* serotype Saintpaul.[1] The outbreak extended for 10 months, resulting in severe disruption of hospital routine and temporary closure of the hospital for 3 months to facilitate remodeling and disinfection. In 1995, an outbreak of nosocomial salmonellosis due to *S.* Infantis at Colorado State University resulted in temporary closure of the hospital for 3 months and an estimated $500,000 in lost revenues and facility renovation.[2,128] In 1996, an outbreak of equine salmonellosis due to *S.* Typhimurium occurred at the Michigan State University.[3] Unique features of this outbreak included a high case fatality, zoonotic infection, and closure of the hospital for 1 month for complete disinfection. In 2000, an outbreak due to a multi-drug resistant strain of *S.* Typhimurium occurred at Purdue University,[4] the hospital was closed for 3 months. In this outbreak, estimated losses included equine mortality, $250,000 to $300,000 for cleaning and disinfection, reduced caseload, and impaired student education. In 2004, an outbreak of equine salmonellosis due to *S.* Newport occurred at the University of Pennsylvania veterinary teaching hospital.[129,130] The hospital was closed for 3 months, and students had to go out-of-state

for large animal clinical training. Estimated losses during this outbreak included equine mortality and over $4 million for hospital renovations, cleaning and disinfection procedures, as well as a reduced caseload for 10 months following re-opening. The most recent reported outbreak of salmonellosis was associated with *S.* Newport at Colorado State University veterinary teaching hospital in 2006.[92] In this outbreak, a total of 8 animals (4 alpacas, 2 horses, 1 goat, 1 cow) were found to be shedding the outbreak strain, all but 1 shed *Salmonella* in the absence of clinical signs or before the onset of disease. Unlike previous outbreaks reported above, in this outbreak the hospital was not closed. The threat of closure was avoided primarily because of aggressive surveillance and mitigation strategies.[92]

**Surveillance and Infection Control**

Control and prevention of outbreaks of nosocomial *Salmonella* infections in hospitalized horses requires careful formulation, implementation, and evaluation of a hospital surveillance and infection control program. For an early detection system, the monitoring of nosocomial *Salmonella* infections in hospitalized horses is fundamental. From a hospital risk management point of view, a working case definition of nosocomial infection must be formulated, even when *Salmonella* serotyping data are not readily available.

**Case definition for nosocomial *Salmonella* infections**

In general, salmonellosis is suspected to be of nosocomial origin when an infection is identified after animals have been hospitalized for 72 hours or longer, and when the serotype and antimicrobial susceptibility pattern of *Salmonella* isolates from the primary and nosocomial cases are the same. However, in published epidemiologic studies, the criteria used to define nosocomial *Salmonella* infections in horses have varied. For

example, in one study, an outbreak was considered nosocomial in origin because a relatively large number of cases shedding the same serotype were detected within a short period of time, and the serotype involved had seldom previously been isolated from hospitalized animals at that hospital.[1] In another study, infection with *S.* Krefeld or *S.* Typhimurium was considered nosocomial when the mean time from admission to shedding was ≥ 4 days.[131]

In a recent study,[132] horses were classified as nosocomial *Salmonella* cases using the following criteria: Nosocomial cases were horses that tested negative for *Salmonella* in samples obtained at the time of admission and tested positive in samples collected ≥ 48 hours after admission, and the primary case or source of nosocomial infection was an index case that had positive results for *Salmonella* in a sample collected at the time of admission, and shared the same serotype and antibiogram as the isolate from the nosocomial case, and there was an overlap between admission and discharge dates of the primary and nosocomial cases. Another source of nosocomial infection was environmental contamination. Horses that had negative results for *Salmonella* in samples obtained at the time of admission but had positive results thereafter and shared the same serotype and antibiogram as a *Salmonella*-positive environmental sample collected during the period of hospitalization were also classified as nosocomial cases. When environmental contamination was attributed as the source of infection, the nosocomial case was never exposed to the primary case associated with the environmental contamination (i.e. there was no overlap between admission and discharge dates of the primary and nosocomial cases because the primary case had

already been discharged from the hospital or euthanized before the nosocomial case was admitted).

In addition to phenotyping methods such as serotyping and antimicrobial susceptibility testing, genotyping methods such as pulsed field gel electrophoresis (PFGE), plasmid profile analysis, and phage typing have been used to determine if *Salmonella* isolates are genetically related, providing further evidence of nosocomial infection.[3,4,133,134] In a study that followed an outbreak of equine salmonellosis, presence of plasmids, examination of antibiotic profiles, and production of bacteriocins and hemagglutination of erythrocytes were used to indicate that equine and environmental isolates had a common origin.[133] In another study, PFGE was used in addition to serotyping and antimicrobial susceptibility testing, to characterize the outbreak strain and isolates were considered nosocomial if the PFGE pattern was similar to that of the outbreak strain.[3] Another outbreak investigation used PFGE and phage typing to identify the outbreak strain, in addition to serotyping and antimicrobial susceptibility testing.[4] Finally, in a study that examined the epidemiological relationship between *Salmonella* cases by comparing isolates from a veterinary hospital over a period of six years, PFGE, presence of the insertion element IS*200*, plasmid profiles, and antimicrobial resistance patterns were used to show that isolates from the veterinary hospital originated from a common source.[134]

From a hospital risk management point of view, a working case definition must be formulated even when *Salmonella* serotyping data are not readily available. At the University of Florida: Large Animal Hospital (UF LAH), the serotyping of *Salmonella* isolates is performed at the US Department of Agriculture: National Veterinary Services

Laboratory in Ames, Iowa, and it may take 4 weeks or longer to obtain the serotyping results. Thus, it becomes imperative to have a working case definition that excludes serotyping data but permits early detection of nosocomial *Salmonella* infections, so that enhanced infection control measures can be implemented to reduce the risk of disease transmission. At the UF LAH, the following two working case definitions are used based on two potential sources of nosocomial infection: another in-patient (index case) or environmental contamination (Figure 2-1). First, if the source of nosocomial infection is another patient, then a horse will be considered a suspect for nosocomial *Salmonella* infection or colonization when: (i) it tests positive on a fecal sample that is collected ≥ 48 hours after admission; (ii) the serogroup of the isolate from the suspect nosocomial case is the same as that of the isolate from the suspect primary case; and (iii) the antimicrobial susceptibility pattern of the isolate from the suspect nosocomial case is similar to that of the isolate from the suspect primary case. In addition, the following two parameters are evaluated: (iv) if there is a temporal overlap of admission and discharge dates of the suspected nosocomial and primary cases; and (v) there is a spatial overlap of housing location for the suspected nosocomial and primary cases (e.g., if both the suspected nosocomial and primary cases are housed in the same barn). The first four conditions above (i, ii, iii, iv) are required, but the last one (v) may or may not affect the decision to classify a horse as a nosocomial case. Second, if environmental contamination is considered the source of infection, then a horse will be considered a suspect for nosocomial *Salmonella* infection when: (i) it tests positive on a fecal sample that is collected ≥ 48 hours after admission; (ii) the serogroup of the isolate from the suspect nosocomial case is the same as that of the environmental isolate; (iii) the

antimicrobial susceptibility pattern of the isolate from the suspect nosocomial case is the same as that of the environmental isolate; and (iv) there is no time overlap between admission and discharge dates of the suspect nosocomial and primary cases; however, the *Salmonella* isolate recovered from the suspect primary case shares the same serogroup and antimicrobial susceptibility pattern as a *Salmonella*-positive environmental sample collected during the period of hospitalization. These two working definitions of nosocomial *Salmonella* infection have served well at the UF LAH over the last 10 years to identify nosocomial *Salmonella* infections, and to justify the need to implement enhanced infection control measures to reduce the risk of disease transmission in hospitalized horses. A retrospective analysis using *Salmonella* serotyping results obtained ≥ 4 weeks or longer after each suspect nosocomial case was identified, revealed that the frequency of misclassification of nosocomial cases was ~ 1%.[135]

**Environmental contamination as a source of nosocomial *Salmonella* infections in hospitalized horses**

The environment can be an important source and reservoir for exposure and transmission of *Salmonella* in veterinary hospitals. In several reports of nosocomial outbreaks of salmonellosis, environmental contamination was implicated in the spread of *Salmonella* among patients and in the persistence of salmonellae in the hospital environment.[2,4,133,134] In one study, persistence of *S.* Typhimurium in the environment was identified as the source of nosocomial infection for several horses.[3] In that study, *S.* Typhimurium was isolated from hospital personnel, shared equipment, and stalls. A hospitalized foal was identified as the point-source of infection. To confirm if the environment was a source of nosocomial infection, serotype information, antimicrobial

susceptibility, and PFGE patterns were used to compare the environmental isolates and the isolate recovered from the point-source foal. In another study, environmental contamination was suggested to be the source of infection for other horses during an outbreak of salmonellosis due to *S.* Typhimurium in a university hospital.[4] The point source of environmental contamination was a horse that presented with colic. *Salmonella* Typhimurium was isolated from stall drains, surgery pads, forklift tires, and the ambulatory garage floor. Similarities of isolates based on serotyping, antibiogram, phagetyping and PFGE patterns were used to indicate that a common source strain of *S.* Typhimurium was responsible for environmental contamination. In a different study, environmental contamination contributed to the wide spread nature of infection during an outbreak of *S.* Infantis in horses and food animals.[2] *Salmonella* Infantis was isolated from hospital workers' hands, rectal thermometers, mice trapped in the hospital facility, and mats in stalls and recovery rooms; the original source of *S.* Infantis was not determined.

Hospital environment can serve as a reservoir, leading to persistence of salmonellae in hospital environments over protracted periods. In a study that followed an outbreak of salmonellosis due to *S.* Infantis at a veterinary hospital, isolates obtained from patients or the hospital environment over a period of 9 years were compared using antimicrobial drug susceptibility patterns, PFGE, and presence of integrons to determine whether isolates were epidemiologically related.[128] Results of the genetic analysis in combination with clear epidemiological links indicated that environmental contamination arising from the outbreak persisted across years despite rigorous hygiene and biosecurity precautions and may have led to subsequent nosocomial infections. In

another study at a veterinary hospital, 28 isolates of multi-drug resistant (MDR) *S*. Heidelberg obtained from horses over a period of six years were compared using PFGE, IS*200* element profiles, antimicrobial resistance patterns, plasmid profiles, and phage typing.[134] The PFGE patterns indicated that the MDR isolates from the veterinary hospital originated from a common source. The isolates also had indistinguishable IS200 profiles, the same antimicrobial resistance pattern and had at least one plasmid in common. In that study, no environmental isolates were tested. The isolation of a single strain of MDR *S.* Heidelberg from horses admitted to that hospital during the six year period is likely explained by persistence of this strain in the hospital environment.

**Sampling**

In most veterinary hospitals with surveillance programs in place, horses known to be at a high risk of shedding *Salmonella* at admission and during hospitalization are targeted for routine sampling. This sub-population includes horses that present with signs of gastrointestinal tract disease such as colic, horses with diarrhea, and horses with fever and leucopenia. At Colorado State University, fecal samples are obtained from all bovine inpatients for *Salmonella* culture, as well as from all equine colic patients at arrival and every other day after that.[136] At Michigan State University, fecal samples are collected on the day of admission and at various times thereafter from all horses with evidence of gastrointestinal tract abnormalities for diagnosis of *Salmonella* using bacteriological culture.[137] Additionally, at the attending clinician's discretion, fecal samples are collected from horses without clinical signs of gastrointestinal tract disease that are considered to be at risk for shedding salmonellae such as neonatal foals with systemic disease, mare and foal pairs when only one of the pair has diarrhea, and horses treated with antimicrobials for long periods. At Purdue University, fecal samples

are collected from horses that present with diarrhea, or that develop diarrhea with leucopenia or fever after admission to test for *Salmonella* using bacteriological culture.[4]

Several university veterinary hospitals have environmental sampling as part of their surveillance and infection control programs. At Colorado State University, stalls in which *Salmonella*-positive animals are housed are cultured before being made available for use with other patients.[128] At Michigan State University, stalls used for horses with diarrhea or that shed salmonellae in their feces are sampled and tested using bacteriological culture.[137] Environmental samples are also collected from other hospital areas that are considered at risk for *Salmonella* contamination such as surgery rooms, anesthesia induction and recovery rooms.

**Laboratory diagnosis**

**Detection of *Salmonella* spp using bacteriological culture.** Most veterinary hospitals with a surveillance and infection control program use bacteriological culture for detection of *Salmonella* spp. colonization or infection in horses, as well as environmental contamination of hospital facilities. While bacteriologic culture is the most common diagnostic procedure used for identification of horses infected with *Salmonella*, culture procedures are not standardized among veterinary microbiology laboratories.[138] For example, in a recent study,[132] 1 to 2 g of fresh feces was placed in 10 mL of selenite cystine broth (ratio, 1:10 to 1:5), and the broth was incubated at 37$^{o}$C overnight for selective enrichment of *Salmonella*. This laboratory procedure differs from that reported in another study[131] in which investigators placed 5 g of fresh feces in 100 mL of selenite broth (ratio, 1:20) and incubated the broth at 37$^{o}$C overnight. These differences can affect the epidemiologic sensitivity (i.e., false negatives) for diagnosis of *Salmonella*. For example, in one study, weight of the fecal sample obtained from pigs had an effect on

detection of *Salmonella*; sensitivity increased from 32% to 63% when weight of the fecal sample increased from 1 to 10 g (diluted 1:9 [wt:wt] with buffer peptone water solution).[139] It is difficult to know how those results in swine can be extrapolated to horses. It is particularly difficult to assess how much misclassification of infected or non-infected horses may have occurred in previous epidemiologic studies. The study in swine used only 1 sample per pig. In the equine study that used 1 to 2 g of fresh feces,[132] the median number of samples collected was 3 for control horses and 4 for nosocomial case horses. Assuming the concentration of *Salmonella* in the first fecal sample collected in that study population was similar to that in swine,[139] it is possible the surveillance system may have missed *Salmonella* shedders at admission because of low sensitivity.

Although bacteriological culture is considered the gold standard for detection of *Salmonella* spp in fecal specimens in horses,[6,8,10,92] it has some limitations. One limitation of using bacteriological culture to monitor community-acquired (infection contracted outside a hospital or infection present on admission) and nosocomial *Salmonella* infections in horses is the time required (3 to 5 days) to obtain positive laboratory results. In the absence of overt clinical signs (diarrhea or fever and leucopenia), this prolonged detection time creates a delay in implementation of appropriate infection control measures such as isolation which are necessary to minimize the risk of nosocomial infections and environmental contamination. In addition, serotyping of *Salmonella* isolates is often conducted at a reference laboratory (USDA National Veterinary Services Laboratory in Ames, IA), and it takes 2 weeks or longer to obtain the results.

A second limitation of bacteriological culture is suboptimal sensitivity.[8] In a previous study that compared the sensitivity of bacteriological culture and PCR, all PCR-positive horses were detected after a total of 3 samples/horse were submitted, whereas as many as 5 samples/horse were required to identify all culture-positive horses.[11] In that study, 71 (65%) of 110 hospitalized horses were positive by PCR, compared with 11 (10%) horses that were positive by culture. Fifty six of 71 (79%) horses that were test positive by PCR were identified as test positive after the first sample from each study horse was submitted, 63/71 (89%) were test positive by PCR after testing of the second sample, and all (71/71;100%) were identified as test positive after testing of the third sample. Seven of 11 (64%) culture-positive horses were identified after testing of the first sample for each study horse, 8/11 (73%) were identified as culture-positive after testing of the second sample, and 9/11 (82%) were identified as culture-positive after testing of the third sample. Bacteriological culture of multiple fecal samples from horses has been shown to yield a greater proportion of *Salmonella*-positive results compared to single fecal samples.[140] A requirement of multiple samples to accurately assess an animal's shedding status is not best suited for hospital infection control programs because of the lag in time between identification of *Salmonella*-positive horses and isolation from the general hospital population, and the cost of performing multiple cultures.[8] The prolonged time required for definitive identification of *Salmonella* spp and the suboptimal sensitivity of bacteriological culture underscore the need for a more rapid and sensitive method for detection of *Salmonella* spp in hospital surveillance programs.

**Detection of *Salmonella* spp using PCR.** Several PCR protocols have been developed and evaluated for potential use in hospital surveillance programs for early detection of *Salmonella* in hospitalized horses. The advantage of using PCR protocols to monitor *Salmonella* shedding in feces of hospitalized horses is that test results can be obtained more quickly, compared to bacterial culture (≤ 24 hours versus 3-5 days), thus allowing an earlier implementation of preventative measures such as isolation and barrier nursing and, therefore, minimizing the risk of a potential outbreak of nosocomial infection.[7,8] Several studies (Table 2-1) have reported different estimates of sensitivity and specificity of real-time PCR protocols for detection of *Salmonella* spp in fecal samples of horses. These studies[9,10,12,13] concluded that PCR is a rapid, sensitive, and specific assay for detection of *Salmonella* spp that can be an alternative to conventional culture methods for surveillance, and can reduce the risk of nosocomial infections through the provision of highly accurate and rapid pathogen detection.

Despite the availability of rapid and highly sensitive real-time PCR tests for detection of *Salmonella*, its acceptance as a surveillance tool has been very limited. One of the limitations to the acceptance of PCR testing is that sampling methods used to establish the gold standard for true-positives and true-negatives have been non-objective[10,13] and this can lead to misclassification bias and inconclusive results. These sampling limitations are showed in two previous real-time PCR studies that targeted the invA gene. In the first study, a specificity estimate of 339/345 or 98% was reported for PCR when compared to culture.[13] However, in that study, fecal samples used to estimate specificity were collected from horses and the environment of pens of a horse feedlot and the clinical status of study horses was not reported. Therefore it is not clear

if horses in that study had colic with or without clinical signs of salmonellosis. In addition, only one fecal sample was collected from each study horse. In a second study that also compared the diagnostic performance of PCR to culture, specificity estimates reported were 904/905 or 99% when fecal samples without enrichment were used and 889/905 or 98% when fecal samples with enrichment were used.[10] In that study, horses used to assess specificity included horses with or without colic, and only one fecal sample was tested from each horses. It is possible that some of these horses with colic were infected with *Salmonella* and could not be detected based on one culture result only. In these two previous studies,[10,13] the lack of a gold standard definition for true-negatives, and the use of horses that tested culture-negative on one fecal sample only may have led to misclassification bias which produces inconclusive results.

Additional previous studies that compared the diagnostic performance of conventional PCR with culture identified more limitations on the proposed use of PCR as a surveillance tool for *Salmonella* in hospital infection control programs. An early study compared the diagnostic performance of PCR and culture for detection of *Salmonella* spp in fecal samples collected from horses with and without clinical signs of salmonellosis.[11] The PCR protocol used primers for the highly conserved segment of the histidine transport operon gene of *S.* Typhimurium. Overall, more fecal samples were classified as positive using PCR, compared to culture. Among equine outpatients without clinical signs of salmonellosis, 26/152 (17%) tested positive by PCR and 0/152 (0%) tested positive by culture. In addition, among hospitalized horses, 71/110 (65%) tested positive by PCR and 11/110 (10%) tested positive by culture. This study revealed that PCR can yield many false-positive results (especially among equine outpatients

without clinical signs of salmonellosis). In a hospital setting, the consequences of false-positive results are significant because equine in-patients with a positive result must be placed in isolation stalls, increasing the cost of hospital fees. Another study[8] compared the diagnostic performance of PCR and culture for detection of *Salmonella* spp in equine feces. The PCR protocol used the same primers as those by Cohen et al. (1996).[11] Results from this study were very informational because the analysis was focused on 116 horses without clinical signs of GI disease from which ≥ 5 fecal samples were collected and cultured for *Salmonella* spp; the median number of samples collected per horse was 8. A total of 87 (75%) horses had 1 or more samples classified as positive by PCR versus 11 (9.5%) by culture. In this study, if culture were used as the gold standard, the specificity of the PCR protocol used would be very low. Among the 105 horses that were culture-negative on ≥ 5 samples, only 29 (28%) were PCR negative for all samples tested. The authors concluded that the reasons why some fecal samples (from which *Salmonella* spp cannot be cultured) are PCR positive need to be determined before PCR can be incorporated into *Salmonella* surveillance programs for hospitalized horses.

Another potential limitation of using PCR as a surveillance tool is that *Salmonella* can lose the target genomic sequence in a PCR assay, resulting in false-negative results.[12,137] Environmental isolates of *S*. Senftenberg and *S*. Litchfield which carry a deletion encompassing a vast segment of the centisome 63 region of the *Salmonella* chromosome have been reported.[141] This deletion includes the entire inv, spa, and hil loci, which are required for entry of *Salmonella* spp into mammalian cells. Deletion of

genes that are targeted in PCR assays creates ambiguity in the interpretation of PCR false-negative results.

An important consideration that should be taken into account before selecting PCR as a surveillance tool is that antibiogram and serotype data can only be obtained if PCR is used in combination with bacteriological culture. If an enrichment step is used prior to DNA extraction and PCR amplification, part of the enrichment broth can be stored and later tested using bacteriological culture followed by antimicrobial susceptibility testing and serotyping of samples that test positive by PCR. Antibiogram and serotyping data are needed to establish if there is an epidemiological relationship between *Salmonella* cases or isolates (from potential primary and nosocomial cases), thus providing evidence that *Salmonella* infection or colonization is nosocomial in origin. In addition, this information can facilitate therapeutic decisions by clinicians in cases where antimicrobial therapy is deemed necessary.

Use of real-time PCR protocols has the potential to improve the diagnostic performance of conventional PCR protocols used for diagnosis of *Salmonella* spp in equine feces. The use of a sequence-specific fluorogenic probe in addition to conventional PCR primers allows for greater specificity for detecting a target sequence.[142] In addition, the fluorogenic nature of the probe allows for rapid detection, because a fluorescent signal can be detected as the reaction progresses, negating the need for post-PCR assay sample processing. Another advantage of real-time PCR over conventional PCR is that it can minimize post amplification cross-contamination or reduce the risk of ‘carry over’ contamination because closed tube systems are used.[10,13]

Enrichment of fecal specimen prior to PCR amplification can improve sensitivity of a PCR assay for diagnosis of *Salmonella* spp in equine feces. In one study in which enrichment was used prior to PCR amplification, PCR detected $10^0$ colony forming units (CFU) of *S.* Enteritidis/g of feces,[143] and in another study, performed by the same author, where enrichment was not used, PCR detected $10^3$- $10^4$ CFU of *Salmonella* spp/g of feces.[7] The PCR method that used enrichment was more sensitive than the PCR technique that did not involve use of enrichment.[143] In another study where real-time PCR was used to detect *Salmonella* spp in 911 fecal and enriched broth samples, three of the 911 (0.3%) fecal samples tested positive while 22 (2.4%) enriched broth samples tested positive.[10]

The type of DNA extraction method used can affect sensitivity, specificity, level of cross-contamination, cost, labor, and time required for a PCR assay. In a previous PCR study that compared the relative sensitivity and specificity of three commercially available DNA extraction kits, three different sensitivities and specificities were reported: 100 and 98.1%; 80 and 100%; and 93.3 and 100%.[12] The kit with a sensitivity and specificity of 100 and 98.1% was considered optimal and was selected for use in that PCR assay. In another study, a DNA extraction method was reported to reduce chances of cross-contamination during extraction and to be less expensive and required less labor and time compared to DNA extraction methods used in other PCR assays.[7,143,144] In addition, this method of DNA extraction did not require the use of potentially noxious organic compounds (e.g., phenol, chloroform, and isoamyl alcohol).[143]

**Risk management**

Several studies have demonstrated that isolation, cleaning and disinfection, and traffic control can be effective in the control of *Salmonella* outbreaks in

hospitals.[2,3,145,146] The most commonly used protocol has been isolation of horses that pose a risk to other horses; these include horses with clinical signs of salmonellosis (diarrhea, fever and leucopenia), and horses that are suspected or confirmed to be *Salmonella*-positive. Most veterinary hospitals maintain isolation units for this purpose, and horses are considered infectious and contagious until proven otherwise. A number of methods are employed to prevent and control microbial contamination during isolation, including the use of barrier nursing precautions such as examination gloves, protective coveralls or gowns, disposable boots when handling infected horses, and foot baths or foot mats. Foot baths and foot mats have been shown to be effective in reducing bacterial contamination in veterinary hospital environments when used properly.[147,148]

In addition to barrier nursing precautions, promoting personal hygiene habits among hospital personnel can raise awareness of the importance of such behaviors in controlling nosocomial disease.[146] These behaviors include washing hands or using alcohol-based hand wipes or lotions between patients, cleaning boots, avoiding walking on animal's hay, wearing clean clothing, wearing gloves when handling contaminated wounds or soiled bandages, cleaning up manure promptly, and not bringing personal pets into barn areas. One of the most important aspects of personal hygiene is washing one's hands before and after coming into contact with any patient. Automatic or treadle-operated sinks and soap dispensers or alcohol based hand disinfectant solutions should be available at key locations in the hospital (such as isolation facilities).

Effective cleaning and disinfection of contaminated environments is one of the most important measures in preventing and controlling the spread of *Salmonella* in

veterinary hospitals. Thorough cleaning of areas with fecal contamination such as stalls, water buckets or automatic watering apparatuses and drains is recommended.[146,149] The use of bleach in the environment after initial cleaning procedures is effective for additional elimination of environmental bacteria, and it has been shown to be the most effective product in eliminating detectable *Salmonella* organisms from hospital surfaces.[137] Several guidelines for the use of different disinfectants and disinfection techniques for materials, stalls and horse facilities have been published.[137,146,150,151]

Traffic control measures have been used to prevent and control the spread of *Salmonella* organisms. Elements of traffic control include the designation of individuals to deal with infected/isolated animals only, restricting the number of hospital personnel or attendants entering isolation stalls, the cleaning of healthy horse stalls before cleaning stalls of sick animals, and control of excessive and unnecessary movement between and through barns.[2] Traffic should always flow from cleaner to less clean areas. Other management practices recommended for controlling microbial contamination include: use of separate equipment (thermometers, nasogastric tubes, twitches) and cleaning tools (grooming tools, manure carts, forks, brooms, and shovels) for suspicious or confirmed *Salmonella*-positive animals and their stalls.[152] Animals should not be moved from one stall to another without due consideration for infection control protocols.[146]

**Guidelines for surveillance and infection control**

Guidelines for surveillance and infection control programs that are tailored to the needs and limitations of veterinary hospitals have been published.[92,132,136,146,153-155] The following is a summary of surveillance and infection control procedures at the UF LAH.

The objective is to reduce the risk of an outbreak of nosocomial *Salmonella* infections in horses and food animals.

The UF LAH has an infection control committee and an infection control officer (ICO). The committee consists of 10 members including clinicians, a microbiologist, an epidemiologist, hospital personnel (veterinary technicians), and the ICO. The committee meets quarterly and its responsibilities include formulation, implementation, and evaluation of hospital surveillance and infection control protocols and policy. The responsibilities of the ICO include: overseeing sample collection from hospitalized horses and the hospital environment; microbiologic procedures; collection, analysis and reporting of epidemiological data; and the implementation of all infection control protocols. In addition, the ICO periodically conducts training and orientations on surveillance and infection control practices to new hospital personnel, faculty, residents, interns, and DVM students. The ICO is supervised by a senior hospital epidemiologist, who also serves as chair of the infection control committee.

All equine patients that present with or later develop signs of gastrointestinal (GI) disease are targeted for early detection of shedding of *Salmonella* spp in feces at the time of admission and during hospitalization. A fecal sample (or swab) is collected from the rectum of each horse within 12 hours after admission and submitted for bacteriological culture; thereafter, additional samples are collected from the stall floor (or rectum if possible) each morning prior to stall cleaning, every Monday and Thursday during hospitalization. In the past, horses were sampled and tested at admission and every 48 hours (i.e., Monday, Wednesday, and Friday) during hospitalization but because of funding limitations, a maximum of two fecal samples per animal per week

are now collected. Fecal samples are submitted to the microbiology laboratory (located within the hospital complex) for bacteriological culture of *Salmonella* spp. On average a negative result is reported within 2-3 days after the sample is submitted. In contrast, a positive result is reported within 4-5 days due to additional required testing procedures (e.g., subculturing, serogrouping, and antimicrobial susceptibility testing).

In addition to sampling hospitalized horses, routine environmental sampling is carried out monthly or bi-monthly to evaluate cleaning and disinfection procedures, or more frequently during periods of high *Salmonella* activity in the hospital. During each event of routine environmental sampling, 25 of 100 hospital sites are targeted for sampling, and bacteriological culture is used to recover *Salmonella* from the environmental samples. The selection of sites is not random; instead, sites considered to be at high risk of contamination are sampled. This sample size is considered sufficient to detect (with 95% confidence) one or more sites with evidence of *Salmonella* contamination if the prevalence of contamination among the 100 sites is 10% or higher, which can be expected during an outbreak situation.[92] Furthermore, stalls used by horses that were *Salmonella*-positive are cultured, and the stall must test negative before other patients are placed in such stalls (unless there is no other stall available, then that stall is used after proper cleaning and disinfection procedures have been completed). Also, a new round of environmental samples is collected (eg, n = 25) whenever there is evidence that a nosocomial *Salmonella* infection has occurred in the hospital, or when a *Salmonella*-positive environmental sample is reported following routine environmental sampling; the objective is to assess cleaning and disinfection

procedures and the magnitude of potential residual environmental contamination of hospital facilities.

Horses known to be shedding *Salmonella* spp or that exhibit signs of diarrhea or fever and leucopenia are housed in an isolation unit. Horses that present with signs of GI disease such as colic and do not exhibit signs of diarrhea, or fever and leucopenia, and are not known to have tested positive for *Salmonella* spp are housed in one barn assigned to patients with gastrointestinal disease or the intensive care unit. Horses that present for other complaints such as orthopedic, reproductive, or ophthalmologic problems are housed in another barn. Horses that exhibit signs of diarrhea or fever and leucopenia are sometimes housed in the GI barn under ward or stall isolation (i.e., isolated in the individual stall) if the isolation unit is full. To notify hospital personnel that a stall in the colic barn is under ward isolation, a rope barricade and isolation sign is placed around the stall entrance. Isolation procedures include the use of gloves, plastic boots, gowns, footbaths by all hospital personnel when attending to patients housed under isolation. Gloves, plastic boots, gowns, and footbaths are placed outside the patient stalls and can be readily accessed. Foot mats containing a quaternary ammonium compound are placed at all points of entry and exit in all barns, the isolation unit, and different hallways within the LAH. Hand hygiene is strongly encouraged including washing of hands and using alcohol-based hand disinfectants. Sinks and soap dispensers, and alcohol-based hand disinfectant solutions are available at key locations within the hospital.

Every time there is evidence of nosocomial *Salmonella* infection or potential nosocomial *Salmonella* infection, enhanced infection control measures are instituted

immediately for all hospitalized patients. The enhanced infection control measures include mandatory use of gloves, plastic boots, and footbaths by all hospital personnel when attending to every patient in the hospital. In addition, there is restricted movement of patients, for example, patients are not allowed to get out of stalls to graze or exercise. Rigorous cleaning and disinfection of the hospital (stalls, equipment) is conducted and thereafter, environmental sampling is conducted immediately to assess the magnitude of potential contamination of hospital facilities and equipment. Enhanced infection control measures are suspended after laboratory and epidemiologic data reveal no further evidence of nosocomial *Salmonella* infection or hospital environment contamination.

Communication is an integral part of a hospital surveillance and infection control program. At the UF LAH, information on the hospital infection control status is relayed quickly and clearly to all stakeholders including clinicians, clients or animal owners, referral veterinarians (RDVM), hospital personnel, DVM students, the infection control committee, hospital board, and media. Figure 2-2 shows communication channels in place at the UF LAH, where the ICO plays a pivotal role in communication. First, every time an in-patient tests positive for *Salmonella* spp, the laboratory immediately notifies both the attending clinician and the ICO. The attending clinician then informs the client or animal owner and the referral veterinarian about the infection status of the in-patient. Clients are educated on the potential risk to humans and other animals on the farm, and how to manage that patient after hospital discharge. At the time of discharge, clients are given a *Salmonella* fact sheet by the attending clinician. The fact sheet includes a brief description of the surveillance program at the hospital, symptoms of clinical

salmonellosis, and measures that should be implemented to reduce the risk of infection of humans and other animals at the farm. If *Salmonella*-positive laboratory results are reported after the patient has been discharged, then the clinician relays the information to clients through a telephone call. In addition, all new hospital clients are given a brochure that describes the hospital surveillance and infection control program. The aim is to inform clients about the surveillance and infection control procedures that are implemented at the hospital to optimize patient care.

Second, the ICO makes an assessment to determine if the source of infection is an in-patient with a community-acquired infection or a potential nosocomial infection. The ICO establishes communication with the hospital epidemiologist to confirm the source of infection and to define infection control measures that are considered most appropriate. If the patient is classified as a primary case, then the ICO communicates and coordinates with hospital personnel to ensure that appropriate infection control measures are implemented for that patient such as isolation and barrier nursing precautions. However, if the patient is classified as a potential nosocomial case, then enhanced hospital infection control measures are instituted as previously described. Before implementing enhanced measures, the ICO has to seek approval from at least two members of the infection control committee. Next, the ICO immediately notifies (by email) all clinicians, veterinary technicians, DVM students, and the barn crew about the new infection control status in the hospital and the enhanced measures that must be observed.

Third, the ICO is responsible for scheduling hospital infection control committee meetings every three months (or more frequently). The main objectives of these

meetings are to provide committee members with a summary report of hospital surveillance and infection control activities for the past three months and to review and update existing standard operating procedures, as well as the performance of key surveillance parameters (ie., frequency of nosocomial cases, compliance in sample collection from horses at admission, rapid reporting of laboratory results, efficacy of cleaning and disinfection). Draft meeting minutes are prepared and submitted to all committee members by the infection control committee chair within three days for review and approval. Ten days after the meeting, the minutes are submitted to the hospital board by the committee chair for approval. Finally, when necessary, communication with the media is performed with the assistance of the UF College of Veterinary Medicine: Office of Public Relations.

Referring veterinarians (RDVMs) and clients/animal owners play an important role in a hospital surveillance and infection control program. Veterinarians participate in the program by referring clients to a tertiary care hospital because of its specialized expertise and need for specialized care. In turn, clients bring high risk patients such as those with signs of gastrointestinal disease that may be targeted in a hospital surveillance and infection control program. Information about a hospital surveillance and infection control program and its importance to RDVMs and clients can be delivered through various channels. At the UF LAH, educational efforts include provision of an infection control brochure to all new clients to educate them about the infection control measures that are instituted at the hospital to optimize patient care. In addition, every time a patient tests positive for *Salmonella,* the attending clinician immediately updates the affected clients and RDVMs about the patient *Salmonella* shedding status and

infection control measures that were implemented in the hospital for that patient. At the time of discharge, clients with *Salmonella*-positive horses are given a *Salmonella* fact sheet by the attending clinician. The purpose of the fact sheet is to educate the client about the potential risks of having a *Salmonella*-positive horse on a farm and measures that should be implemented at the farm-level to reduce the risk of exposure to humans and other farm animals. An important element of an animal disease surveillance system is evaluation and feedback. Evaluation of the performance of a hospital surveillance program may involve assessing the frequency of nosocomial infections within a given period of time, frequency of environmental contamination with selected pathogens, compliance level by hospital clinicians and personnel, and level of awareness about the program among RDVMs and clients who refer or bring patients to the hospital. To operate an efficient program, it is important to solicit feedback from all individuals involved in the program including clinicians, hospital personnel, RDVMs and clients. Considering the role played by RDVMs and clients, it is important to know the level of awareness and perceptions about the relevance of a hospital surveillance and infection program among this group. This information can be used to guide decision-making on hospital policy issues related to surveillance and infection control, and streamline costs and efficiency of services provided to clients.

**Epidemiologic Research**

Identification of risk factors associated with nosocomial *Salmonella* infection in hospitalized horses is important, so that effective preventative and control measures can be instituted to reduce the risk of disease transmission and potential outbreaks. Initial studies conducted in the 1980s and 1990s in California provided an initial epidemiologic framework for investigation of risk factors associated with nosocomial

*Salmonella* infection in hospitalized horses. A first study investigated an outbreak of nosocomial salmonellosis due to *S.* Saintpaul in a veterinary hospital.[1] In this study, a presenting complaint of colic, nasogastric intubation, and treatment with antibiotics were associated with isolation of *S.* Saintpaul. In a second study conducted at the same hospital, a presenting complaint of colic, nasogastric intubation, and treatment with antibiotics were again associated with isolation of *Salmonella.*[156] Finally, in a third study, diagnosis of large colon impaction, withholding feed, number of days fed bran mash, treatment with potassium penicillin G, and increase in mean daily ambient temperature were identified as risk factors for nosocomial *Salmonella* infection.[131]

A recent epidemiologic study identified abdominal surgery as a risk factor for nosocomial *Salmonella* infection in horses.[132] There are several explanations for the observed association between abdominal surgery and nosocomial *Salmonella* infection in horses. In horses that undergo abdominal surgery, the large intestine may be evacuated and lavaged, feed may be withheld or changed, antimicrobial drugs are usually administered, and various degrees of ileus may develop.[131,157-161] These events can cause stress in surgical patients and alter the gastrointestinal physiology and microflora. In humans and mice, abdominal surgery has been associated with severe alterations of host-defense mechanisms.[162-166] These alterations suppress the innate immune system during the perioperative period and cause substantial impairment of cell-mediated immunity. In view of the effects of abdominal surgery on the immune system in humans and mice, it is possible that surgical stress similarly suppresses the innate and adaptive immune systems of equine surgical patients. As a result, abdominal surgery may increase their susceptibility to nosocomial *Salmonella* infections. Studies

are necessary to confirm if abdominal surgery is a predisposing factor for nosocomial *Salmonella* infection in hospitalized horses.

While a high caseload has been suspected as a predisposing factor for nosocomial *Salmonella* infection in hospitalized horses, previous epidemiologic studies[1,131,132,156] failed to identify high caseload as a risk factor. A study in Florida[132] explained that the lack of an association between a high caseload and nosocomial *Salmonella* infections could be attributed to the fact that the number of horses shedding *Salmonella* at the time of admission during periods of high or low caseload was not different. Another explanation was that the hospital surveillance and infection control program was well-established and the degree of personnel compliance was acceptable. It is possible that high caseload may have an effect on risk of nosocomial infection in hospitalized horses when the number of shedders in the hospital is high and infection control standards are suboptimal.

In veterinary hospitals, horses that present with diarrhea at admission or that develop diarrhea during hospitalization are placed in isolation to reduce the risk of *Salmonella* transmission to other inpatients. Three published studies have showed, however, that the frequency of hospitalized horses that were classified as positive to *Salmonella* shedding and did not have diarrhea at admission or during hospitalization can be high. For example, in one study, among 33 horses from which *Salmonella* Saintpaul was isolated, 27 (88%) did not have diarrhea.[1] In another study, among 131 horses from which *Salmonella enterica* was isolated, 125 (95%) did not have diarrhea.[3] Finally, in a third study, among 233 horses from which *Salmonella enterica* was isolated, 197 (85%) did not develop diarrhea.[167] Surveillance data collected at the

University of Florida Large Animal Hospital (UF LAH) over the last four years (2007-2010) revealed that 52 of 67 adult equine inpatients with signs of GI disease that tested positive for *Salmonella* did not have diarrhea at admission or during hospitalization. Horses that test positive for *Salmonella* and do not have diarrhea at admission or during hospitalization present a threat because most horses are detected after discharge and there is no opportunity to implement infection control measures during hospitalization. This subpopulation of horses can also present a threat to other horses and humans in the community when the equine patient returns home. Exposure factors associated with *Salmonella* shedding in horses with or without diarrhea have not been investigated. Information on exposure factors associated with *Salmonella* shedding in horses without diarrhea is important because it can help improve current hospital surveillance programs for early detection of *Salmonella* shedding in GI horses without diarrhea and allow timely implementation of infection control measures.

**Discussion**

In previous outbreaks where hospitals had to close, the lack of a case definition for nosocomial *Salmonella* infections, day-to-day monitoring of nosocomial cases, routine environmental sampling, infection control protocols, and guidelines for hospital closure due to an increased number of nosocomial *Salmonella* infections, all contributed to the onset of outbreaks of *Salmonella* infections in hospitalized horses. A question that is dreaded and yet warrants discussion by directors of hospital infection control programs and hospital administrators is when to temporarily close a hospital due to an increased number of nosocomial infections in horses. Currently, there are no published reports with standardized criteria or guidelines to decide when a hospital should be temporarily closed to stop a nosocomial outbreak of *Salmonella* infections in hospitalized horses. A

review of previous outbreak reports revealed several parameters that can be taken into consideration (Table 2-2). The consequences of previous nosocomial outbreaks of *Salmonella* infections in horses clearly justify the need to formulate standards for hospital surveillance and infection control measures, including specific guidelines for when to close a hospital operation to prevent unnecessary morbidity and mortality in hospitalized horses. Three key parameters that can be used by hospital administrators when making a decision to temporarily close a veterinary hospital include: (i) weekly number of nosocomial *Salmonella* cases with or without clinical disease; (ii) case fatalities; and (iii) zoonotic disease.

Exposure factors associated with *Salmonella* shedding in horses with or without diarrhea have not been investigated. Previous epidemiological studies identified several factors associated with *Salmonella* shedding in horses including colic,[1,131,156,168] transportation,[160,169] clinical procedures such as change in diet,[167] antimicrobial therapy,[152,160,170] abdominal surgery,[145,152] surgically treated small colon impactions,[171] clinical findings such as diarrhea at admission or within ≤ 6 hour after hospitalization,[169,167] fever during hospitalization,[167] leucopenia within ≤ 6 hour after hospitalization and abnormal results of nasogastric intubation.[169] In addition, four studies identified exposure factors associated with nosocomial *Salmonella* infection in hospitalized horses including a presenting complaint of colic,[1,156] nasogastric intubation,[1,156] treatment with antibiotics,[1,156] diagnosis of large colon impaction,[131] withholding feed,[131] number of days fed bran mash,[131] treatment with potassium penicillin G,[131] increase in mean daily ambient temperature,[131] and abdominal surgery.[132] However, in all those previous studies, the analyses did not separate horses

with and without diarrhea. Therefore the factors identified in those studies apply to a combined population of horses with and without diarrhea. Information on exposure factors associated with *Salmonella* shedding in horses without diarrhea is important because it can help improve current hospital surveillance programs for early detection of *Salmonella* shedding and allow timely implementation of infection control measures.

Although time to obtain *Salmonella* test results by bacteriologic culture is a limitation, bacteriologic culture is a good diagnostic tool to use in hospitals where compliance with surveillance and infection control protocols is good. Clinicians often argue that bacteriologic test results have limited value for patient care because laboratory results are often reported days after the inpatient has been discharged and other patients may have been exposed by the time laboratory results are reported. While this argument is valid, hospital surveillance and infection control protocols are designed to manage the infection control status of a hospital rather than that of individual inpatients.

The proposed use of PCR protocols as a surveillance tool for early detection of *Salmonella* fecal shedding in horses is a good idea, provided the frequency of false positives is low, acceptable, and does not significantly affect the cost of hospital fees. The study by Ward et al.[8] justifies the need to further investigate the reasons why horses without clinical signs of salmonellosis that are classified as negative (i.e., after five consecutive fecal samples using bacteriological culture) test positive by PCR. In addition, for PCR to be accepted as a surveillance tool, the issue of non-objective sampling methods used in previous PCR studies[10,13] needs to be addressed in future studies. Different sampling methods can affect the diagnostic performance assessment

of diagnostics tests. While assessing specificity, it's important that a gold standard definition for true-negatives that considers horses without clinical signs of salmonellosis that are sampled multiple times is used. Bacteriological culture of multiple fecal samples from horses has been shown to yield a greater proportion of *Salmonella*-positive results compared to single fecal samples.[140]

In an effort to standardize hospital surveillance and infection control programs and to institute a measure of accountability, the reporting of nosocomial *Salmonella* infections in hospitalized horses (and other species) should be considered as one parameter of excellence in hospital veterinary care. Reporting of nosocomial *Salmonella* infections may encourage improvement in the quality of patient care, and the overall efficiency of a hospital surveillance and infection control program.

In light of the recent epidemiologic finding[132] that abdominal surgery was identified as a risk factor for nosocomial *Salmonella* infection in hospitalized horses, there is need to further investigate this finding to confirm if surgical stress suppresses the innate and adaptive immune systems of equine surgical patients, thereby increasing their susceptibility to nosocomial *Salmonella* infections. Results from additional studies will help determine if abdominal surgery is a predisposing factor for nosocomial *Salmonella* infections in hospitalized horses, and justify the need to enhance infection control measures (eg, isolation and use of gloves, gowns, plastic boots, and footbaths) to protect equine inpatients that undergo abdominal surgery.

Referring veterinarians and clients play an important role in a hospital surveillance and infection control program. Considering the role played by RDVMs and clients, it's important to identify and characterize their level of awareness and perceptions about

the relevance of a hospital surveillance and infection control program. No studies have been conducted to assess the level of awareness and relevance of a hospital surveillance and infection control program among referring veterinarians and clients. This information can be used to justify the need for enhanced education efforts among referring veterinarians and clients, and guide decision-making on policy issues related to hospital surveillance and infection control.

The following knowledge gaps were identified after a critical review of the literature on epidemiology and infection control of nosocomial *Salmonella* infections in hospitalized horses:

1. There is no standard case definition for nosocomial *Salmonella* infections that can be used as a guideline when implementing surveillance and infection control procedures.
2. There are no published reports with standardized criteria or guidelines to decide when a hospital should be temporarily closed to stop a nosocomial outbreak of *Salmonella* infections in hospitalized horses.
3. Exposure factors associated with *Salmonella* shedding in horses with or without diarrhea have not been investigated.
4. For PCR to be accepted as a surveillance tool, It is important to assess the diagnostic performance of a PCR assay using objective sampling methods; with horses defined as true-negatives used to estimate specificity that are sampled multiple times. In addition, the PCR assay should have a low frequency of PCR false-positive results that is considered acceptable.
5. Further studies to confirm if surgical stress suppresses the innate and adaptive immune systems of equine surgical patients need to be conducted.
6. There is need to assess the level of awareness and relevance of a hospital surveillance and infection control program among referring veterinarians and clients.

Three of the knowledge gaps identified in this literature review were addressed in this research work. First, an investigation of the exposure factors associated with *Salmonella* shedding in hospitalized horses with or without diarrhea was conducted.

Second, an investigation was performed to assess the diagnostic performance of a real-time PCR assay for detection of *Salmonella* spp in fecal specimens of hospitalized horses, compared to bacteriological culture. Finally, a survey was performed to assess the level of awareness and relevance of a hospital surveillance and infection control program among referring veterinarians and clients.

Table 2-1. Reported sensitivity and specificity estimates of real-time PCR protocols for detection of *Salmonella* spp in equine fecal samples

| Primer probe | Sensitivity % (# samples tested) | Specificity % (# samples tested) | Reference |
|---|---|---|---|
| *spaQ gene* | 100.0 (80) | 97.3 (150) | Kurowski et al. 2002[12] |
| *invE-A gene* | 80.0 (45) | 98.6 (729) | Gentry-Weeks et al. 2002[9] |
| *hisJ gene* | 93.0 (15) | 85.5 (291) | Gentry-Weeks et al. 2002[9] |
| *invA gene* | 100.0 (28) | 98.3 (345) | Bohaychuck et al. 2007[13] |
| *invA gene* | 100.0 (6) | 98.2 (895) | Pusterla et al. 2009[10] |

Table 2-2. Large animal hospitals that have closed because of an outbreak of nosocomial *Salmonella* infections and surveillance parameters of interest:

| Hospital | Duration of hospital closure | Parameters that might have influenced the decision to close the hospital | Reference |
|---|---|---|---|
| 1 | 3 months | • Increased number of nosocomial cases in horses: 33 cases diagnosed with *S.* Saintpaul.<br>• 20 of 33 cases showed clinical signs of salmonellosis.<br>• 2 of the 33 cases died. | Hird et al. 1984[1] |
| 2 | 3 months | • 34 of 137 food animal and equine inpatients tested positive for the outbreak strain: *S.* Infantis. | Tillotson et al. 1997[2] |
| 3 | 1 month | • 18 of 138 inpatients tested positive for the outbreak strain: *S.* Typhimurium.<br>• 8 of 18 affected horses were euthanized or died.<br>• 1 zoonotic infection reported. | Schott et al. 2001[3] |
| 4 | 3 months | • 28 horses tested positive for the outbreak strain *S.* Typhimurium.<br>• 14 horses infected with *S.* Typhimurium died or were euthanized. | Ward et al. 2005[4] |

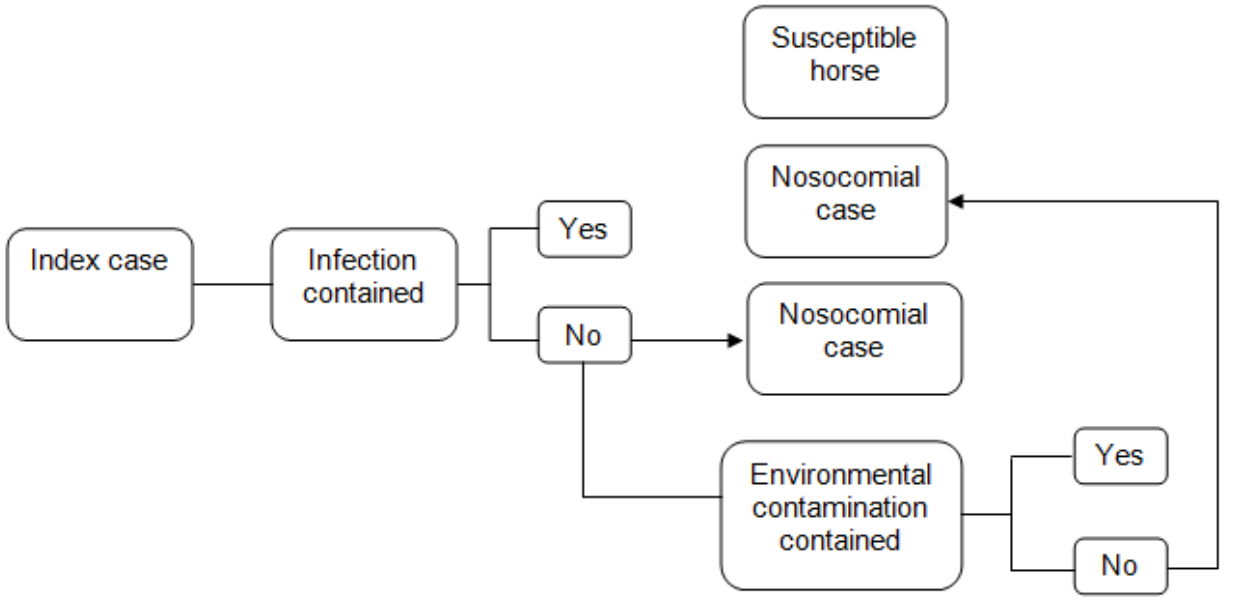


Figure 2-1. A diagram showing two potential sources of nosocomial infection: an inpatient index case or environmental contamination.

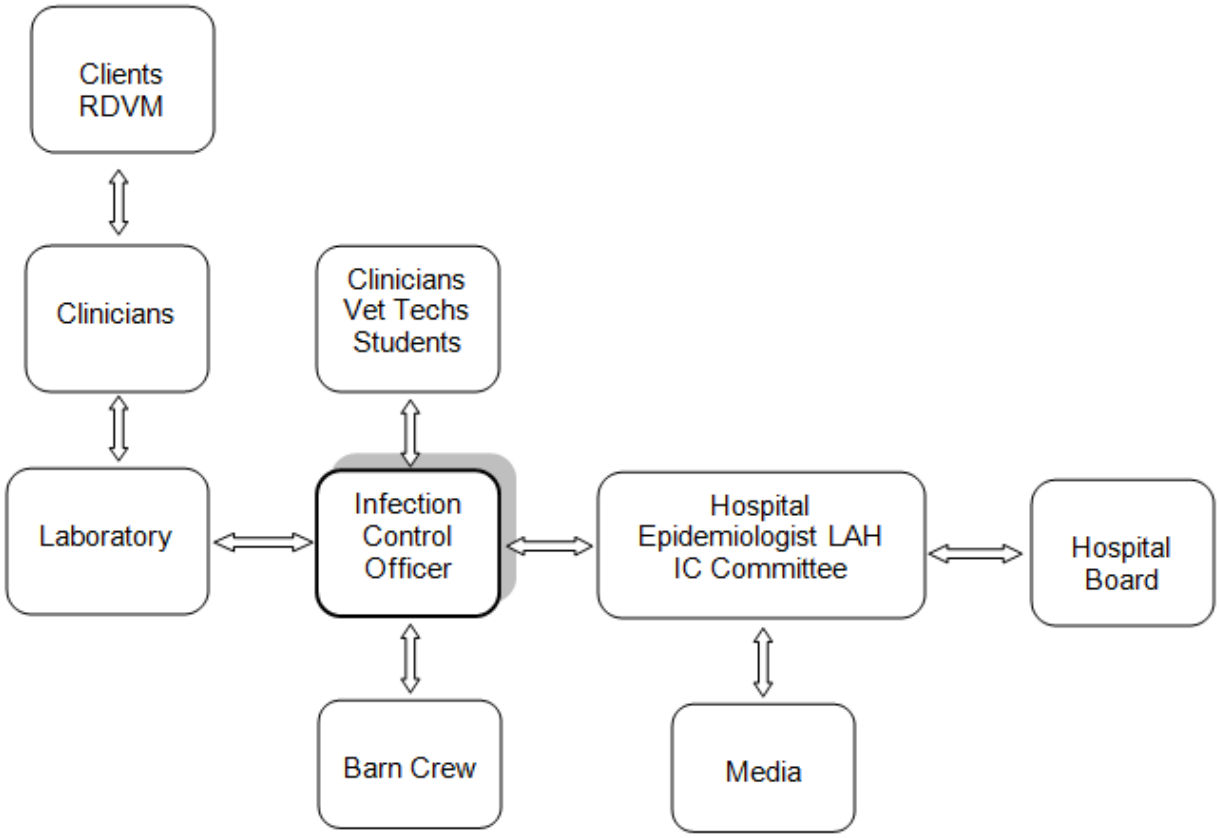


Figure 2-2. Surveillance and infection control communication channels at the University of Florida, Large Animal Hospital.

# CHAPTER 3
# EXPOSURE FACTORS ASSOCIATED WITH SALMONELLA SHEDDING AMONG HOSPITALIZED HORSES WITH OR WITHOUT DIARRHEA

Despite implementation of biosecurity standard operating procedures, veterinary hospitals have experienced outbreaks of salmonellosis causing high mortality in equine inpatients and substantial financial losses.[1-6] As a result, epidemiologic studies have been conducted to identify exposure factors associated with *Salmonella* shedding in hospitalized horses and to help formulate and implement improved biosecurity standard operating procedures. Several studies have identified hospitalized horses with colic to be at high risk of shedding *Salmonella*.[1,131,156,168] Other factors associated with *Salmonella* shedding include transportation,[160,169] clinical procedures such as change in diet,[167] antimicrobial therapy,[152,160,170] abdominal surgery,[145,152] surgically treated small colon impactions,[171] clinical findings such as diarrhea at admission or within ≤ 6 hour after hospitalization,[167,169] fever during hospitalization,[167] leucopenia within ≤ 6 hour after hospitalization and abnormal results of nasogastric intubation.[169] In addition, four studies identified presenting complaint of colic,[1,156] nasogastric intubation,[1,156] treatment with antibiotics,[1,156] diagnosis of large colon impaction,[131] withholding feed,[131] number of days fed bran mash,[131] treatment with potassium penicillin G,[131] increase in mean daily ambient temperature,[131] and abdominal surgery as exposure factors associated with nosocomial *Salmonella* infection in hospitalized horses.[132]

In veterinary hospitals, horses that present with diarrhea at admission or that develop diarrhea during hospitalization are placed in isolation to reduce the risk of *Salmonella* transmission to other inpatients. Three published studies have showed, however, that the frequency of hospitalized horses that were classified as positive to *Salmonella* shedding and did not have diarrhea at admission or during hospitalization

can be high. For example, in one study, among 33 horses from which *Salmonella* Saintpaul was isolated, 27 (88%) did not have diarrhea.[1] In another study, among 131 horses from which *Salmonella enterica* were isolated, 125 (95%) did not have diarrhea.[3] Finally, in a third study, among 233 horses from which *Salmonella enterica* was isolated, 197 (85%) did not develop diarrhea.[167]

Surveillance data collected at the University of Florida Large Animal Hospital (UF LAH) over the last four years (2007-2010) revealed that 52 of 67 adult equine inpatients with signs of gastrointestinal (GI) disease (colic or diarrhea) that tested positive for *Salmonella* did not have diarrhea at admission or during hospitalization. On average, duration of hospitalization for equine inpatients with signs of GI disease at the UF LAH is 5 days and bacteriologic culture procedures for detection of *Salmonella* require 4-5 days before a positive result is reported. Using existing hospital surveillance protocols, horses that test positive for *Salmonella* and do not have diarrhea at admission or during hospitalization present a threat because most horses are detected after discharge and there is no opportunity to implement infection control measures during hospitalization. This subpopulation of horses can also present a threat to other horses and humans in the community when the equine patient returns home.

To our knowledge, exposure factors associated with *Salmonella* shedding in horses with or without diarrhea have not been investigated. This information is important as it can further improve current hospital surveillance programs for early detection of *Salmonella* shedding and permit rapid implementation of infection control measures. The objective of this study was to examine the relationships between clinical signs, hematological and plasma chemistry parameters, as well as clinical procedures (before

admission, at admission, and during hospitalization) and *Salmonella* shedding in adult horses with signs of GI disease that tested positive for *Salmonella* and had or did not have diarrhea, compared to adult horses with signs of GI disease that tested negative for *Salmonella* and did not have diarrhea.

## Materials and Methods

### Study Population

All equine patients admitted to the UF LAH between January 1, 2007, and December 31, 2010, with signs of GI disease (colic; diarrhea; diarrhea and fever; diarrhea and anorexia; anorexia and fever; anorexia) that were tested for *Salmonella enterica* shedding in fecal specimens were initially considered for inclusion in the study. Foals (< 1 year old) were excluded. The rationale for excluding foals was that exposure factors for diarrhea in foals can differ from those for adult horses. Furthermore, UF LAH surveillance data for the study period revealed that nearly all foals admitted with signs of GI disease were foals affected with diarrhea upon admission, limiting our ability to study an adequate number of foals without diarrhea. Horses classified as nosocomial infections (n=6; serotypes isolated were Mbandaka, Miami, Litchfield, Meleagridis, Newport, and a non-identified serotype) were excluded because our interest was to extrapolate study results to equine in-patients with evidence of community-acquired *Salmonella* infections only. The criteria used to define nosocomial *Salmonella* infections in this study have been reported in detail elsewhere.

### Hospital Surveillance and Infection Control Procedures

During the study period, all equine patients admitted to the UF LAH with signs of GI disease (colic or diarrhea or anorexia or fever) were targeted for early detection of fecal shedding of *Salmonella enterica* at the time of admission and during

hospitalization. Details of the surveillance and infection control procedures at the UF LAH are described elsewehere.[6,132] Briefly, a fecal sample or swab (if fecal sample is not available) is collected per rectum from each horse within 12 hours after admission and submitted for bacterial culture. Thereafter, additional fecal samples are collected from the stall floor each morning prior to cleaning at 48 hour intervals until the patient is discharged from the hospital. Fecal samples collected outside regular business hours are refrigerated at 4°C prior to laboratory submission. For some horses, additional fecal samples are collected at the discretion of the attending clinician (eg, every 12 to 24 hours). Any equine patient that tests positive for *Salmonella enterica* or develops diarrhea, or fever and leucopenia is placed in an isolation barn. Isolation procedures include use of barrier nursing precautions (such as gloves, plastic boots, gowns, and footbaths) for personnel attending to patients. In addition to sampling hospitalized horses, routine environmental sampling is carried out monthly or bi-monthly to evaluate cleaning and disinfection procedures, or more frequently during periods of high *Salmonella enterica* activity in the hospital. During each event of routine environmental sampling, 25 hospital sites are targeted for sampling, and bacteriological culture is used to recover *Salmonella enterica* from the environmental samples. A hospital infection control officer is responsible for overseeing collection of fecal samples, microbiologic procedures, and collection and analysis of epidemiologic data.

**Study Design**

A case-control study approach was used to examine the relationships between clinical signs, hematological and plasma chemistry parameters, and clinical procedures before admission, at admission (day 1), and during hospitalization (days 2 and 3) and *Salmonella* shedding in GI horses with or without diarrhea. Exposure factors during

hospitalization were measured on hospital days 2 and 3 only because duration of hospitalization was different among study horses and testing procedures (eg, hematological and plasma chemistry parameters) were not required by the attending clinician in most horses after day 3.

**Selection of cases**

Two sets of case horses were used in this study: case horses that presented with signs of GI disease with or without diarrhea and tested positive for *Salmonella enterica*. Cases with diarrhea (n = 19) were further divided into 2 groups: (i) horses that tested positive for *Salmonella enterica* on fecal samples collected ≤ 2 days after admission (n = 14), and (ii) horses that tested negative on the first two fecal samples but positive on the third and later fecal samples collected ≥ 4 days after admission (n = 5). This group of horses tested negative on at least 2 consecutive fecal samples collected ≤ 3 days after admission. Diarrhea was defined as 3 consecutive bowel movements in which feces do not sit on top of the bedding.

Cases without diarrhea (n = 52) were also divided into 2 groups: (i) horses that tested positive for *Salmonella enterica* on fecal samples collected ≤ 2 days after admission (n = 25) and (ii) horses that tested negative on the first two fecal samples but positive on the third and later fecal samples collected ≥ 4 days after admission (n=21). Six case horses without diarrhea did not meet these inclusion criteria and were excluded because they tested negative on only one fecal sample (within ≤ 2 days after admission) but positive on fecal samples collected ≥ 4 days after admission.

The reason for creating the two subgroups of case horses with or without diarrhea that tested positive for *Salmonella enterica* on fecal samples collected ≤ 2 days after admission or ≥ 4 days after admission was to examine the relationship between

investigated exposure factors and *Salmonella* shedding at two defined exposure periods of interest: a) before admission and at admission (day 1) and b) on days 2 and 3 after admission.

**Selection of controls**

Control horses were defined as horses that tested negative for *Salmonella enterica* on 3 or more consecutive fecal samples and did not have diarrhea before or during the entire period of hospitalization. Control horses were randomly selected based on year of admission. Selection of number of control horses was based on the total number of cases horses without diarrhea (n = 52) diagnosed during 2007-2010 (ie, 10 cases in 2007, 11 in 2008, 18 in 2009, and 13 in 2010). Up to 2 control horses per case were selected in 2007 and 2008 and one control horse per case horse in 2009 and 2010. A lower caseload in 2009 and 2010 limited our ability to select two controls for each case horse in those two years. Thus, the ratio of controls-to-cases was 1:1. The final enrollment included 73 controls: 20 from year 2007, 22 from 2008, 18 from 2009, and 13 from 2010. The program research randomizer was used to generate random numbers and select control horses.[a]

**Microbiologic Procedures for Detection of *Salmonella* Spp**

Bacterial culture of fecal samples for detection of *Salmonella enterica* was performed at the UF Veterinary Clinical Microbiology Laboratory as previously described.[6,132] Briefly, for selective enrichment, 1 to 2 g of fresh feces or a rectal swab was placed in 10 mL of selenite cystine broth, and incubated at 37°C for 24 hours. The following day, a sample of the selenite cystine broth was subcultured on Hektoen

[a] Research Randomizer, Social Psychology Network, Middletown, Conn. Available at: www.randomizer.org; accessed March 15th 2011.

enteric agar plates. Plates were incubated at 37°C for 24 hours. Non-lactose fermenting, hydrogen sulfide-producing colonies were selected and isolated. These colonies were then inoculated on urea agar and lysine-iron agar slants and incubated at 37°C for 24 hours. Identification of urease-negative and hydrogen sulfide-producing organisms was established through use of a commercially available identification system. Serogroup of *Salmonella* isolates was determined by means of agglutination; polyvalent (A through I and Vi) and group-specific (A through E) *Salmonella* O antisera were used. *Salmonella* isolates were tested for antimicrobial susceptibility using the minimal inhibitory concentration method with commercially prepared plates and the Kirby Bauer disk diffusion method. Serotyping of *Salmonella* isolates was performed at the USDA National Veterinary Services Laboratories in Ames, Iowa.

**Data Collection**

A questionnaire was developed to collect epidemiologic data for each study horse during three exposure periods of interest: before admission, at admission (day 1), and during hospitalization (days 2 and 3). Briefly, data collected before admission included host factors (patient age, sex, breed), clinical findings (diarrhea (yes/no), fever (yes/no), leucopenia (yes/no), and clinical procedures (history of antimicrobial use (yes/no; types used), anti-inflammatory use (yes/no; types used), sedative use (yes/no; types used), antispasmodic use (yes/no; types used), nasogastric intubation (yes/no), laxative use (e.g., mineral oil) (yes/no; types used), stomach protectant use (e.g., omeprazole) (yes/no; types used), intestinal protectant use (e.g., di-tri-octahedral smectite) (yes/no; types used), IV fluid use (yes/no; types used), and rectal exam findings). Data collected at admission included admission date, season of year at admission

(winter/spring/summer/fall), presenting complaint, clinical findings (diarrhea, fever, leucopenia, heart rate (beats/min), color of mucous membranes (pink/dark pink/injected/icteric), capillary refill time, plasma chemistry and complete blood count results (normal/low/high), and clinical procedures (nasogastric intubation, rectal exam findings, abdominal ultrasound findings). Data collected during hospitalization included clinical findings (diarrhea, fever, leucopenia, plasma chemistry and complete blood count results), clinical procedures (nasogastric intubation, rectal exam findings, abdominal ultrasound findings, type of feed given, withholding of feed (yes/no; duration), laxative use, stomach protectant use, intestinal protectant use, IV fluid use, general anesthesia (yes/no), abdominal surgery (yes/no), anatomic location of surgical lesion, type of surgical lesion, type of surgical procedure, antimicrobial use, anti-inflammatory use, sedative use, antispasmodic use, final diagnosis), discharge date, duration of hospitalization, and number of fecal samples collected. For case horses, information on *Salmonella* isolates was collected including serogroup, serotype, fecal sample number that first tested positive, date when first positive fecal sample was reported, and number of hospital days when first test-positive sample was collected.

**Data Analysis**

The Kruskal-Wallis test[b] was used to compare continuous variables (number of fecal samples collected and duration of hospitalization) among case horses with diarrhea, case horses without diarrhea, and control horses.

[b] PROC NPAR1WAY, SAS 9.2, SAS Institute Inc, Cary, North Carolina.

Unconditional logistic regression was used to model the odds of *Salmonella* shedding (yes, no) using the LOGISTIC procedure in SAS.[c] In the univariable analysis, variables with a *P* value < 0.10 were considered eligible for multivariable analysis. To determine the best fitting model, the variable with the smallest *P* value in the univariable analysis was entered into the model first. Thereafter, each of the remaining variables was added to the model containing the first variable to test whether its addition significantly improved the fit of the model. The variable with the highest likelihood ratio statistic ($X^2$ test with one degree of freedom) was selected for addition to the model and the process was then repeated; variables had to have a *P* value ≤ 0.10 to be retained in the model. To determine whether the main effects model was continuous in the logit for the variable of number of samples tested, we examined the coefficients using indicator variables based on quartiles of the distribution. Because of the small sample size used in each model, interaction terms between exposure factors were not tested. In Models 2 and 3, exposure variables retained in the model were examined for confounding by adding each of the variables to the model and assessing the changes in the odds ratios (ie, > 20%) of the remaining variables in the model. The logistic regression model was assessed for goodness of fit using the Hosmer-Lemeshow test, while the ability to discriminate between horses that tested positive to *Salmonella* shedding versus those that did not was determined by using the receiver operating characteristic (ROC) curve. In the final model, adjusted OR and 95% confidence intervals were reported.

To accomplish the study objective, three models were developed. Model 1 included 14 case horses that tested positive for *Salmonella* on fecal samples collected ≤

[c] PROC LOGISTIC, SAS 9.2, SAS Institute Inc, Cary, North Carolina.

2 days after admission and had diarrhea. Model 2 included 25 case horses that tested positive for *Salmonella* on fecal samples collected ≤ 2 days after admission and did not have diarrhea. Model 3 included 21 case horses that tested positive for *Salmonella* on fecal samples collected ≥ 4 days after admission and did not have diarrhea. In this third model, the variable for number of samples tested was forced in the model because it can increase the likelihood of detecting horses that test positive to *Salmonella* shedding. In each of the three models examined, the same set of control horses was used.

A fourth model of case horses that tested positive for *Salmonella* on fecal samples collected at ≥ 4 days after admission and had diarrhea was not examined because the sample size was too small (n=5).

In this study, the number of case horses used was small; therefore, we limited the number of variables offered to each of the final models (based on the number of cases). Use of number of events per variable less than 10 can lead to biased parameter estimates in a logistic regression model.[172]

## Results

The median age for case horses with diarrhea (n=14) was 12 years (1$^{st}$ quartile = 4.5 & 3$^{rd}$ quartile = 17.25); for case horses without diarrhea (n=46) was 9.5 years (6 and 13); and for control horses (n=73) was 8 (6 and 12).

Among case horses with diarrhea (n=14), 9 were female, 1 was male, and 4 were geldings. Among case horses without diarrhea (n=46), 19 were female, 2 were male, and 25 were geldings. Among control horses (n=73), 33 were female, 8 were male, and 32 were geldings.

Among case horses with diarrhea (n=14), the most common breeds were Thoroughbred (3), American Paint horse (3), Arabian (3), Quarter Horse (2), and others

(3). Among case horses without diarrhea (n=46), the most common breeds were Thoroughbred (11), Quarter Horse (11), Warmblood (7), American Paint horse (3), Arabian (3), and others (11). Among control horses (n=73), the most common breeds were Quarter Horse (19), Thoroughbred (16), Warmblood (9), American Paint horse (6), Arabian (3), American Miniature (3), Pasofino (3), and others (14).

Among case horses with diarrhea (n=14), 7 presented for colic, 3 for diarrhea, 2 for diarrhea and fever, 1 for diarrhea and anorexia, and 1 for anorexia and fever. Among case horses without diarrhea (n=46), 45 presented for colic and 1 for anorexia. Among control horses (n=73), 71 presented for colic and 1 for anorexia and fever, and 1 for anorexia.

Among case horses with diarrhea (n=14), 11 were medically treated and 3 were surgically treated. Among case horses without diarrhea (n=46), 25 were medically treated and 21 were surgically treated. Among control horses (n=73), 35 were medically treated and 38 were surgically treated.

Among 14 case horses with diarrhea, the most commonly isolated *Salmonella enterica* serotypes were Anatum (n = 2) and Muenchen (n=2) (Table 3-1). Among 46 cases without diarrhea, the most commonly isolated serotype was Newport (n=13).

In 2007, 12 isolates were reported and the most common serotype was Anatum (n=2). In 2008, 17 isolates were reported and the most common serotypes were Anatum (n=3), Newport (n=3), Miami (n=2), and Saintpaul (n=2). In 2009, 19 isolates were reported and the most common serotypes were Newport (n=6), Muenchen (n=3) and Anatum (n=2). In 2010, 12 isolates were reported and the most common serotypes were Newport (n=3) and Muenchen (n=2).

The median number of fecal samples collected was not different between cases with diarrhea (3 samples; 1st quartile = 3 & 3rd quartile = 5), cases without diarrhea (3.5 samples; 2.75, 5), and controls (3 samples; 3, 4) ($P$ = 0.66). The median duration of hospitalization was not different between cases with diarrhea (5 days; 1st quartile = 4 & 3rd quartile = 7.5; range = 2, 10), cases without diarrhea (7 days; 4 and 9; 2, 39), and controls (6 days; 5 and 7; 1, 10) ($P$ = 0.48).

The median number of days of hospitalization when a horse was first identified as positive was 2.5 days (1st quartile = 1 & 3rd quartile = 4; range = 1, 6) for cases with diarrhea and 3 days (2 and 6; 1, 12) for cases without diarrhea. The median number of the fecal sample that first tested positive was significantly different between cases with diarrhea (median = 1.5; 1st quartile = 1 & 3rd quartile = 3) and cases without diarrhea (3; 2, 3) ($P$ = 0.01).

No horses with or without diarrhea had both fever and leucopenia at admission.

Positive *Salmonella* culture results for 32 of the 46 case horses without diarrhea were completed and reported after discharge. Among case horses with or without diarrhea that tested positive to *Salmonella* shedding, the median number of days between time of fecal sample collection and reporting of results for case horses was 5 days (1st quartile = 5, 3rd quartile = 6; range 4, 14) for positive samples and 2 days for negative samples (2 and 6; range 2, 19).

### Model 1: Case Horses that Tested Positive for *Salmonella* at ≤ 2 Days after Admission and had Diarrhea

In the univariable analysis, the following variables were associated with *Salmonella* shedding ($P \leq 0.05$) (Table 3-2): Before admission: antimicrobial and anti-inflammatory use. At admission: high triglycerides (> 45 mg/dL), low plasma sodium (<

134 mEq/L), low plasma protein (< 5.9 g/dL), and high mean corpuscular hemoglobin concentration (> 38.8 g/dL). Multivariable analysis was not performed for model 1 because of the small number of cases.

**Model 2: Case Horses that Tested Positive for *Salmonella* ≤ 2 Days after Admission and did not have Diarrhea**

In the univariable analysis, the following variables had values of $P \leq 0.10$ (Table 3-3). Before admission: sedative use. At admission: high blood urea nitrogen (> 22 mg/dL), high triglycerides, high plasma protein (> 7.9 g/dL), high red blood cell count (> 10 M/uL), and neutropenia (< 2.2 K/uL).

In the multivariable analysis, high plasma protein concentration at admission (OR = 2.85; 95% CI = 0.91, 8.91; $P = 0.07$; Table 3-5) and high triglycerides at admission (OR = 3.37; 95% CI = 1.15, 9.87; $P = 0.02$; Table 3-5) were associated with *Salmonella* shedding ≤ 2 days after admission. No confounding effect was observed when the variable year of admission was added to the final model. The model fit the data well as indicated by the Hosmer-Lemeshow goodness of fit test (0.73; $df = 4$; $P = 0.94$). However, the area under the curve for the ROC was 0.67 indicating that the model has low predictive ability. In this model, the variable number of samples tested was not forced into the final models because the median number of samples among cases and controls was the same (median = 3).

**Model 3: Case Horses that Tested Positive for *Salmonella* at ≥ 4 Days after Admission and did not have Diarrhea, and Tested Negative on at least 2 Fecal Samples Collected ≤ 3 Days after Admission**

In the univariable analysis, the following variables had values of $P \leq 0.10$ (Table 3-4). At admission: high alkaline phosphatase (> 228 U/L), high creatine kinase (> 729 U/L), low chloride (< 96 mEq/L), high mean corpuscular hemoglobin (> 18.7 pg), high

corpuscular hemoglobin (> 17.9 pg), elevated heart rate (41- 60 beats per min), and season. During hospitalization: antimicrobial use, leucopenia, fever and leucopenia, withholding of feed, stomach protectant use, general anesthesia, abdominal surgery, low plasma protein on hospital day 2, and highly elevated heart rate on day 3 (> 60 beats per min). The variables for mean corpuscular hemoglobin and corpuscular hemoglobin were correlated; therefore only mean corpuscular hemoglobin was selected for inclusion in the multivariable model.

In the multivariable analysis, two final models were examined. In the first model (Model A; Table 3-6), after adjusting for number of samples, the variable for abdominal surgery was retained in the final model for *Salmonella* shedding ≥ 4 days after admission in horses without diarrhea (OR = 2.79; 95% CI = 0.86, 9.01; *P* = 0.08). The model fit the data well, although the Hosmer-Lemeshow statistic almost reached statistical significance (8.78; *df* = 4; *P* = 0.06). The area under the curve for the ROC was 0.73 indicating that the model has moderate predictive ability. No confounding effect was observed when the variable for year of admission was added to the final model.

In the second model (Model B; Table 3-6), after adjusting for number of samples, season (summer) (OR = 4.92; 95% CI = 1.46, 16.57; *P* = 0.01) was associated with *Salmonella* shedding ≥ 4 days after admission. The model fit the data well as indicated by the Hosmer-Lemeshow goodness of fit test (4.97; *df* = 4; *P* = 0.28). The area under the curve for the ROC was 0.77 indicating that the model has moderate predictive ability. No confounding effect was observed when the variable for year of admission was added to the final model.

## Discussion

The present study examined the relationships between clinical signs, hematological and plasma chemistry parameters, and clinical procedures, and *Salmonella* shedding in GI horses with or without diarrhea. Following a univariable analysis, among horses that tested positive for *Salmonella* ≤ 2 days after admission and had diarrhea, use of antimicrobials and anti-inflammatories before admission, high plasma triglycerides, low plasma sodium, low plasma protein, and high mean corpuscular hemoglobin concentration at admission were associated with *Salmonella* shedding. Following a multivariable analysis, among horses that tested positive for *Salmonella* ≤ 2 days after admission and without diarrhea, high plasma protein concentration and high plasma triglycerides at admission were associated with *Salmonella* shedding. Among horses without diarrhea that tested positive for *Salmonella* ≥ 4 days after admission, abdominal surgery and season were associated with *Salmonella* shedding.

Our study had several limitations. First, the sample size used in this study was small and it limited the number of exposure factors that could be examined in the epidemiologic analyses, and it also affected the precision of the estimated associations between exposure factors measured and *Salmonella* shedding. Second, bacteriologic culture procedures used in the present study differed from those used in other studies.[1-6] Methods used to culture *Salmonella* are highly variable among laboratories,[132,138]with differences such as weight of fecal sample which affect the sensitivity of bacteriologic culture.[139] Third, bacteriological culture of multiple fecal samples from horses has been shown to yield a greater proportion of *Salmonella*-positive results compared to single fecal samples.[140] This is an inherent limitation in observational studies that are based on

hospital surveillance data. For example, at the UF LAH,[132,152] equine inpatients on average remain hospitalized for 5 days and a total of 5 or more fecal samples are rarely collected for detection of *Salmonella* shedding. Fourth, assessment of exposure factors during hospitalization was limited to the first 3 days because duration of hospitalization was different among study horses and frequency of testing procedures (eg, hematological and plasma chemistry parameters) was not consistent in study horses after day 3. Fifth, in this study, pre-admission data were collected from history provided by clients, most of these data were missing and the accuracy of this information was not assessed. Thus, it is difficult to determine the magnitude of pre-admission exposure misclassification potentially present in this study. Sixth, the study population was limited to adult equine patients with GI disease; therefore our study results cannot be generalized to the entire equine population at the UF LAH. Finally, there are differences between the UF LAH and other veterinary hospitals (ie, climate and housing conditions, surveillance and infection control measures, pathogen infection control pressure) which make the extrapolation of our study findings to other hospitals difficult.

In the present study, among horses without diarrhea, *Salmonella enterica* serotype Newport was the most commonly isolated serotype. The Newport isolates were susceptible to all antimicrobials tested. *Salmonella* Newport has been previously reported in equine inpatients affected with colic but without diarrhea at the UF LAH.[132,152] In contrast, in two previous outbreak investigations of nosocomial salmonellosis in horses, cattle, and alpacas, multi-drug-resistant strains of *Salmonella* Newport were identified as the causative strain at other hospitals.[5,92] In one study, 17 of 54 horses infected with the outbreak strain died during hospitalization (but it is was not

clear how many horses were affected with diarrhea).[5] In another study, 1 of 2 horses infected with the outbreak strain developed enterocolitis, diarrhea, septicemia and subsequently died.[92] A comparison of this and previous studies[5,92] shows that infections with drug resistant S Newport strains are associated with diarrhea and mortality in hospitalized horses, compared to susceptible *S* Newport strains. Several studies in humans have demonstrated that infections with drug-resistant non-typhoid *Salmonella* serotypes are associated with excess morbidity and mortality.[97,99]

In the present study, among 46 case horses without diarrhea, no horses had fever and leucopenia at admission. This finding is relevant at the UF LAH because (following hospital infection control procedures) horses that exhibit a combination of fever and leucopenia are housed in an isolation unit.[132,152] This hospital policy is based on the fact that fever and leucopenia are clinical signs associated with *Salmonella* infection in horses.[173-176] While this policy is logical, this study shows that its relevance among equine inpatients without diarrhea is limited. In this study, 32 of 46 case horses without diarrhea were classified as *Salmonella* shedders after discharge from the hospital. At the UF LAH, clients and referring veterinarians with horses that are reported as *Salmonella*-culture positive after discharge are informed about the *Salmonella*-shedding status by the attending clinician by telephone. During the same time, they are educated about the risks of having a *Salmonella*-positive horse on the farm to humans and other animals, and are advised on how to manage the patient at the farm (including isolation measures). The fact that many *Salmonella* shedders without diarrhea were detected after discharge and were not placed in isolation during hospitalization justifies the need

to re-evaluate the risk-based surveillance approach used at the UF LAH for detection of *Salmonella* shedders in equine inpatients.

Model 1: Case horses that tested positive for *Salmonella* at ≤ 2 days after admission and had diarrhea - In the univariable analysis, among horses that tested positive for *Salmonella* ≤ 2 days after admission and had diarrhea, use of antimicrobials or anti-inflammatories, as well as high plasma triglycerides, low plasma sodium, low plasma protein, or high mean corpuscular hemoglobin concentration at admission were associated with *Salmonella* shedding. The small number of cases in this analysis limited our ability to adequately examine the association between these exposure factors and *Salmonella* shedding using multivariable logistic regression. Another limitation in this analysis is that exposure factors associated with *Salmonella* shedding in horses with diarrhea (ie, low plasma protein or low plasma sodium) may also apply to horses affected with diarrhea but with no evidence of *Salmonella* infection. However, we offer several explanations for the associations observed in the univariable analysis. The association between antimicrobial use and *Salmonella* shedding has been reported in previous studies.[1,131,156] Administration of antibacterial agents, therapeutically or prophylactically, causes disturbances in the ecologic balance between the host and its normal microflora.[177] Antimicrobial drugs eliminate intestinal flora that are antagonistic to *Salmonella* organisms,[178] giving a competitive advantage to *Salmonella* and this may explain the observed association between exposure to antimicrobial drugs and *Salmonella* shedding. The association between anti-inflammatory use and *Salmonella* shedding may be explained by confounding factors. Misclassification due to recall bias

by clients or RDVMs is not likely. According to clinicians, most horses with signs of GI disease receive anti-inflammatories before admission.

The clinical parameters low plasma protein and low plasma sodium concentration have been documented in diarrheic horses experimentally infected with *Salmonella.*[175,176,179,180] The association between low plasma protein and *Salmonella* shedding in horses with diarrhea is likely explained by enteric protein loss through an impaired gastrointestinal mucosa caused by *Salmonella* infection. The intestinal damage caused by *Salmonella* infection results in mucosal disruption, permeability changes, and extensive cell loss especially in the large bowel which facilitates the release of plasma constituents.[176] *Salmonella* enterotoxin interferes with the function of mediators of active colonic secretion including cyclic AMP (cAMP) and prostaglandins E1 and E2,[31,181,182] and disrupts the sodium-potassium-chloride cotransport system on the basolateral membrane of colonic epithelial cells.[37,181,182] These derangements result in a decrease in sodium absorption, increase in chloride secretion from mucosal cells into the lumen, and secretion of water into the intestinal lumen.[37] The finding of high mean corpuscular hemoglobin concentration in horses with diarrhea might be explained by hemoconcentration due to the effect of low sodium or hyposmolality that may occur in horses with diarrhea and/or anorexia. The high plasma triglycerides may be indicative of anorexia caused by *Salmonella* infection. *Salmonella* infection in horses can lead to depression, anorexia, and hypoglycemia.[173-176]

Model 2: Case horses that tested positive for *Salmonella* ≤ 2 days after admission and did not have diarrhea - In the multivariable analysis, among case horses without diarrhea that tested positive for *Salmonella* shedding ≤ 2 days after admission, high

plasma protein concentration and high triglyceride concentration at admission were associated with shedding. Hyperproteinemia has been reported in *Salmonella*-positive horses with diarrhea,[175] but not in *Salmonella*-positive horses without diarrhea. *Salmonella* infection in horses causes depression, reduction in appetite and/or anorexia,[173-176] which may result in inadequate water intake. Deprivation of water can contribute to dehydration manifested as hypovolemia,[183] which is associated with several clinical findings including hyperproteinemia.[184-186] Hyperproteinemia associated with dehydration is characterized by a normal albumin/globulin ratio, because water loss concentrates all plasma proteins proportionally.[184] In the present study, the finding of hyperproteinemia was likely attributed to dehydration as all horses with hyperproteinemia (8 cases and 13 controls) had a normal albumin/globulin ratio. The finding of high plasma triglycerides in horses that tested positive ≤ 2 days after admission and did not have diarrhea can be explained by anorexia in horses infected with *Salmonella enterica*[173-176] or a presenting complaint of colic.

Model 3: Case horses that tested positive for *Salmonella* at ≥ 4 days after admission and did not have diarrhea - In the multivariable analysis, the variables for abdominal surgery and season (summer) were retained in the final model. Abdominal surgery can be a risk factor for *Salmonella* shedding in community-acquired infections as shown in this study, and in horses with hospital-acquired infections.[132] In this investigation, 16 of 21 case horses tested positive for *Salmonella* at ≥ 4 days after admission and had abdominal surgery. The most common *Salmonella* serotype was Newport (n=4) (one in each year), followed by Saintpaul (n=3), Anatum (n=3), Rubislaw (n=2), and others (n= 9). All 16 case horses were ruled out as nosocomial cases based

on the temporal and spatial distribution of *Salmonella* cases (which includes serotype, antimicrobial resistance pattern data) identified by the UF LAH surveillance program, as well as the *Salmonella* environmental contamination status of the hospital. Horses that undergo abdominal surgery suffer substantial amounts of stress,[122] which may be attributed to insult by the surgical procedure itself and accompanying events such as evacuation and lavage of the large colon or cecum, change or withholding of feed,[131] and administration of antimicrobial drugs.[160] In humans and mice, surgical stress has been shown to impair the immune system.[163-165] It is not known if surgical stress (surgical procedure) impairs the immune response system in hospitalized horses that undergo abdominal surgery. The accompanying events can cause disturbances in the ecologic balance between the host and its normal microflora[177]resulting in alteration of function of normal gastrointestinal flora antagonistic to *Salmonella*.[178]

In this study, season (summer months) was also associated with *Salmonella* shedding in horses that tested positive at ≥ 4 days after admission and did not have diarrhea. The association between season and *Salmonella* shedding is documented, with higher frequency of cases reported in summer and fall.[3,157,187] An association between an increase in ambient temperature and risk of nosocomial *Salmonella* infection has also been described.[131] Increase in ambient temperature may increase the risk of infection by favoring survival and multiplication of *Salmonella* organisms in the environment or by compromising immune responses of the host.[131,188]

In summary, this study makes an attempt to characterize *Salmonella* shedding in equine GI in-patients with or without diarrhea. Among horses with diarrhea, results on investigated exposure factors associated with *Salmonella* shedding are inconclusive

because confounding of exposure factors on *Salmonella* shedding could not be examined. Among horses without diarrhea, high plasma concentrations of triglycerides at admission, abdominal surgery, and season can be predisposing factors for *Salmonella* shedding.

Table 3-1. *Salmonella* serotypes isolated from cases with or without diarrhea

| Serotype | Cases with diarrhea (n=14) | Cases without diarrhea (n=46) |
|---|---|---|
| Newport | 1 | 13 |
| Saintpaul | 0 | 3 |
| Mbandaka | 1 | 1 |
| Rubislaw | 0 | 3 |
| Anatum | 2 | 6 |
| Braenderup | 1 | 2 |
| Thompson | 1 | 1 |
| Miami | 1 | 1 |
| Cerro | 1 | 0 |
| Uganda | 1 | 0 |
| Gaminara | 1 | 0 |
| Muenster | 1 | 0 |
| Muenchen | 2 | 3 |
| Meleagridis | 0 | 2 |
| Talahassee | 0 | 2 |
| Agona | 0 | 0 |
| Lomalinda | 0 | 1 |
| Others* | 1 | 8 |

* Rough O:mt:-, 4,5,12:1:-, Not known

Table 3-2. Univariable unconditional logistic regression analysis of investigated exposure factors at admission associated with *Salmonella* shedding ≤ 2 days after admission in horses with diarrhea

| Variable | Cases with diarrhea n = 14 | Controls n = 73 | OR* (95% CI)** | *P* value |
|---|---|---|---|---|
| Season | | | | |
| Winter and Spring | 7 | 45 | Reference | NA |
| Summer and Fall | 7 | 28 | 1.60 (0.50, 5.07) | 0.41 |
| | | | | |
| Prior to admission | | | | |
| Antimicrobial use | | | | |
| No | 8 | 68 | Reference | NA |
| Yes | 6 | 4 | 12.75 (2.95, 55.00) | < 0.001 |
| Anti-inflammatory use | | | | |
| No | 4 | 5 | Reference | NA |
| Yes | 10 | 67 | 0.18 (0.04, 0.81) | 0.02 |
| Sedative use | | | | |
| No | 7 | 39 | Reference | NA |
| Yes | 7 | 33 | 1.18 (0.37, 3.71) | 0.77 |
| Fever | | | | |
| No (< 101.5F) | 7 | 43 | Reference | NA |
| Yes (> 101.5F) | 4 | 6 | 4.09 (0.91, 18.28) | 0.06 |
| Leucopenia | | | | |
| No (≥ 5.5 K/uL) | 2 | 1 | Reference | NA |
| Yes (< 5.5 K/uL) | 2 | 2 | ND | ND |
| Nasogastric intubation | | | | |
| No | 7 | 30 | Reference | NA |
| Yes | 7 | 40 | 0.75 (0.23, 2.36) | 0.62 |
| Laxative use | | | | |
| No | 10 | 41 | Reference | NA |
| Yes | 4 | 31 | 0.52 (0.15, 1.84) | 0.31 |
| Stomach protectant use | | | | |
| No | 13 | 68 | Reference | NA |
| Yes | 1 | 4 | 1.30 (0.13, 12.66) | 0.81 |
| Intestinal protectant use | | | | |
| No | 14 | 72 | ND | ND |
| Yes | 0 | 0 | ND | ND |
| | | | | |
| At admission | | | | |
| Fever | | | | |
| No | 13 | 67 | ND | ND |
| Yes | 1 | 5 | ND | ND |
| Leucopenia | | | | |
| No | 7 | 59 | Reference | NA |
| Yes | 4 | 12 | 3.86 (0.92, 16.19) | 0.06 |

Table 3-2. Continued

| Variable | Cases with diarrhea n = 14 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| At admission | | | | |
| Fever and leucopenia | | | | |
| No | 14 | 73 | ND | ND |
| Yes | 0 | 0 | ND | ND |
| Nasogastric intubation | | | | |
| No | 2 | 8 | Reference | NA |
| Yes | 12 | 62 | 0.77 (0.14, 4.10) | 0.76 |
| Albumin | | | | |
| Normal (2.8-4.2 g/dL) | 8 | 60 | Reference | NA |
| Low (< 2.8) | 2 | 3 | 5.00 (0.72, 34.63) | 0.10 |
| High (> 4.2) | 0 | 4 | ND | ND |
| Creatinine | | | | |
| Normal (1.1-2.0 mg/dL) | 7 | 45 | Reference | NA |
| Low (< 1.1) | 0 | 3 | ND | ND |
| High (> 2.0) | 4 | 19 | 1.70 (0.42, 6.75) | 0.44 |
| Blood urea nitrogen | | | | |
| Normal (9-22 mg/dL) | 6 | 49 | Reference | NA |
| Low (< 9 ) | 0 | 1 | ND | ND |
| High (> 22) | 4 | 17 | 1.92 (0.48, 7.63) | 0.35 |
| Total carbondioxide | | | | |
| Normal (21-34 mEq/L) | 7 | 56 | Reference | NA |
| Low (< 21) | 3 | 8 | 3.00 (0.64, 14.02) | 0.16 |
| High (> 34) | 0 | 2 | ND | ND |
| Anion gap | | | | |
| Normal (10-20 mEq/L) | 9 | 53 | Reference | NA |
| Low (< 10) | 1 | 4 | 2.33 (0.21, 25.24) | 0.48 |
| High (> 20) | 1 | 9 | 0.87 (0.09, 7.95) | 0.90 |
| Alkaline phosphatase | | | | |
| Normal (69-228 U/L) | 5 | 46 | Reference | ND |
| Low (< 69) | 0 | 0 | ND | ND |
| High (> 228) | 5 | 21 | 2.19 (0.57, 8.38) | 0.25 |
| Aspartate aminotransferase | | | | |
| Normal (148-322 U/L) | 4 | 33 | Reference | NA |
| Low (< 148) | 1 | 1 | 5.00 (0.28, 88.53) | 0.27 |
| High (> 322) | 5 | 32 | 0.78 (0.23, 2.62) | 0.68 |
| Total bilirubin | | | | |
| Normal (0.3-1.9 mg/dL) | 2 | 16 | Reference | NA |
| Low (< 0.3) | 0 | 0 | ND | ND |
| High (> 1.9) | 8 | 51 | 1.25 (0.24, 6.52) | 0.78 |
| Globulin | | | | |
| Normal (2.4-4.9 g/dL) | 8 | 55 | Reference | NA |
| Low (< 2.4) | 0 | 3 | ND | ND |
| High (> 4.9) | 1 | 9 | 0.76 (0.08, 6.86) | 0.81 |

Table 3-2. Continued

| Variable | Cases with diarrhea n = 14 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| At admission | | | | |
| Albumin/Globulin ratio | | | | |
| Normal (0.6-1.4) | 9 | 63 | ND | ND |
| Low (< 0.6) | 0 | 2 | ND | ND |
| High (>1.4) | 0 | 2 | ND | ND |
| Glucose | | | | |
| Normal (62-128 mg/dL) | 4 | 34 | Reference | NA |
| Low (< 62) | 0 | 1 | ND | ND |
| High (> 128) | 6 | 31 | 1.64 (0.42, 6.38) | 0.47 |
| Triglycerides | | | | |
| Normal (5-45 mg/dL) | 4 | 52 | Reference | NA |
| Low (< 5) | 0 | 0 | ND | ND |
| High (> 45) | 7 | 14 | 5.67 (1.40, 22.92) | 0.01 |
| Magnesium | | | | |
| Normal (1.3-1.9 mg/dL) | 7 | 51 | Reference | NA |
| Low (< 1.3) | 3 | 12 | 1.82 (0.40, 8.09) | 0.43 |
| High (> 1.9) | 0 | 4 | ND | ND |
| Gamma-glutamyl transferase | | | | |
| Normal (17-50 U/L) | 8 | 44 | Reference | NA |
| Low (< 17) | 0 | 4 | ND | ND |
| High (> 50) | 2 | 19 | 0.57 (0.11, 2.98) | 0.51 |
| Creatine kinase | | | | |
| Normal (134-729 U/L) | 8 | 38 | Reference | NA |
| Low (< 134) | 0 | 1 | ND | ND |
| High (> 729) | 2 | 28 | 0.33 (0.06, 1.72) | 0.19 |
| Sodium | | | | |
| Normal (134-143 mEq/L) | 2 | 50 | Reference | NA |
| Low (< 134) | 8 | 15 | 13.6 (2.60, 71.02) | 0.002 |
| High (> 143) | 1 | 1 | ND | ND |
| Potassium | | | | |
| Normal (2.2-5.3 mEq/L) | 6 | 67 | ND | ND |
| Low (< 2.2) | 4 | 0 | ND | ND |
| High (> 5.3) | 0 | 0 | ND | ND |
| Chloride | | | | |
| Normal (96-105 mEq/L) | 4 | 44 | Reference | NA |
| Low (< 96) | 6 | 23 | 2.86 (0.73, 11.20) | 0.12 |
| High (> 105) | 0 | 0 | ND | ND |
| Calcium | | | | |
| Normal (10.9-13.4 mg/dL) | 7 | 49 | Reference | NA |
| Low (< 10.9) | 3 | 15 | 1.40 (0.32, 6.09) | 0.65 |
| High (> 13.4) | 0 | 2 | ND | ND |
| Color of mucus membranes | | | | |
| Pink | 8 | 49 | Reference | NA |
| Icteric/yellow | 1 | 1 | 5.44 (0.31, 95.21) | 0.24 |
| Red/injected/toxic line | 4 | 23 | 0.94 (0.26, 3.39) | 0.93 |

Table 3-2. Continued

| Variable | Cases with diarrhea n = 14 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| At admission | | | | |
| Capillary refill time | | | | |
| Normal (≤ 2) | 8 | 50 | Reference | NA |
| Abnormal (> 2) | 5 | 23 | 1.35 (0.40, 4.60) | 0.62 |
| Heart rate | | | | |
| Normal (28-40 beats/min) | 2 | 12 | Reference | NA |
| Elevated (41-60) | 8 | 47 | 1.02 (0.19, 5.44) | 0.98 |
| High (> 60) | 4 | 14 | 1.71 (0.26, 11.05) | 0.57 |
| Lactate (blood gas) | | | | |
| Normal (≤ 1.5 mmol/L) | 5 | 26 | Reference | NA |
| Abnormal (> 1.5) | 5 | 33 | 0.78 (0.20, 3.01) | 0.72 |
| Plasma protein | | | | |
| Normal (5.9-7.9 g/dL) | 5 | 44 | Reference | NA |
| Low (< 5.9) | 7 | 10 | 6.18 (1.71, 22.30) | 0.005 |
| High (> 7.9) | 1 | 10 | 0.88 (0.09, 8.15) | 0.91 |
| Packed cell volume | | | | |
| Normal (30-48%) | 7 | 43 | Reference | NA |
| Low (< 30) | 1 | 9 | 0.70 (0.07, 6.36) | 0.75 |
| High (> 48) | 5 | 13 | 2.45 (0.68, 8.75) | 0.16 |
| White blood cell count | | | | |
| Normal (5.5-11.0 K/uL) | 5 | 45 | Reference | NA |
| Low (< 5.5) | 4 | 12 | 2.26 (0.58, 8.82) | 0.23 |
| High (> 11.0) | 2 | 14 | 0.47 (0.05, 4.09) | 0.49 |
| Fibrinogen | | | | |
| Normal (100-400 mg/dL) | 7 | 53 | Reference | NA |
| Low (< 100) | 0 | 0 | ND | ND |
| High (> 400) | 3 | 13 | 1.74 (0.39, 7.69) | 0.46 |
| Percent Neutrophils | | | | |
| Normal (28-83%) | 7 | 51 | Reference | NA |
| Low (< 28) | 1 | 2 | 2.54 (0.21, 30.57) | 0.46 |
| High (> 83) | 2 | 15 | 0.67 (0.13, 3.39) | 0.63 |
| Percent Monocytes | | | | |
| Normal (1-11%) | 7 | 65 | Reference | NA |
| Low (< 1) | 0 | 0 | ND | ND |
| High (> 11) | 3 | 2 | 13.92 (1.97, 98.06) | 0.008 |
| Toxicity | | | | |
| No | 4 | 43 | Reference | NA |
| Yes | 6 | 21 | 3.07 (0.78, 12.06) | 0.10 |
| Percent lymphocytes | | | | |
| Normal (20-59%) | 3 | 23 | Reference | NA |
| Low (< 20) | 7 | 44 | 1.86 (0.43, 7.92) | 0.39 |
| High (> 59) | 1 | 0 | ND | ND |

Table 3-2. Continued

| Variable | Cases with diarrhea n = 14 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| At admission | | | | |
| Neutrophils | | | | |
| Normal (2.2-8.1 K/uL) | 7 | 42 | Reference | NA |
| Low (< 2.2) | 3 | 8 | 2.39 (0.50, 11.37) | 0.27 |
| High (>8.1) | 1 | 21 | 0.26 (0.03, 2.30) | 0.22 |
| Neutropenia | | | | |
| No (≥ 2.2 K/uL) | 8 | 63 | Reference | NA |
| Yes (< 2.2) | 3 | 8 | 3.17 (0.68, 14.64) | 0.13 |
| RBC | | | | |
| Normal (6.7-10 M/uL) | 5 | 52 | Reference | ND |
| Low (< 6.7) | 1 | 9 | 0.63 (0.07, 5.56) | 0.68 |
| High (> 10) | 3 | 7 | 2.44 (0.53, 11.06) | 0.24 |
| Hemoglobin | | | | |
| Normal (11.2-16.2 g/dL) | 5 | 51 | Reference | NA |
| Low (< 11.2) | 1 | 6 | 1.03 (0.11, 9.65) | 0.97 |
| High (> 16.2) | 4 | 11 | 2.26 (0.59, 8.67) | 0.23 |
| Hematocrit (calculated) | | | | |
| Normal (30-43%) | 6 | 45 | Reference | NA |
| Low (< 30) | 1 | 10 | 0.51 (0.05, 4.44) | 0.54 |
| High (> 43) | 3 | 12 | 1.27 (0.30, 5.35) | 0.74 |
| Mean corpuscular volume | | | | |
| Normal (37.5-50.0 fL) | 9 | 61 | Reference | NA |
| Low (< 37.5) | 1 | 2 | 3.38 (0.27, 41.30) | 0.33 |
| High (> 50.0) | 0 | 3 | ND | ND |
| Mean corpuscular hemoglobin | | | | |
| Normal (14-18.7 pg) | 8 | 63 | Reference | NA |
| Low (< 14) | 1 | 2 | 2.83 (0.23, 33.75) | 0.41 |
| High (> 18.7) | 1 | 3 | 1.88 (0.18, 19.70) | 0.59 |
| Mean corpuscular hemoglobin concentration | | | | |
| Normal (36.4-38.8 g/dL) | 6 | 52 | Reference | NA |
| Low (< 36.4) | 0 | 7 | ND | ND |
| High (> 38.8) | 5 | 12 | 4.54 (1.11, 18.45) | 0.03 |
| Corpuscular hemoglobin concentration mean | | | | |
| Normal (34.9-37.6 g/dL) | 3 | 46 | Reference | NA |
| Low (< 34.9) | 0 | 1 | ND | ND |
| High (> 37.6) | 7 | 21 | 5.11 (1.20, 21.73) | 0.02 |
| Corpuscular hemoglobin | | | | |
| Normal (13.7-17.9 pg) | 5 | 58 | Reference | NA |
| Low (< 13.7) | 1 | 1 | 7.00 (0.40, 122.04) | 0.18 |
| High (>17.9) | 4 | 9 | 3.11 (0.79, 12.23) | 0.10 |

Table 3-2. Continued

| Variable | Cases with diarrhea n = 14 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| At admission | | | | |
| Red cell distribution width | | | | |
| Normal (16.3-19%) | 6 | 57 | Reference | NA |
| Low (< 16.3) | 1 | 0 | ND | ND |
| High (> 19) | 3 | 11 | 2.59 (0.56, 11.95) | 0.22 |
| Hemoglobin distribution width | | | | |
| Normal (1.5-2.1 g/dL) | 4 | 37 | Reference | NA |
| Low (< 1.5) | 0 | 0 | ND | ND |
| High (> 2.1) | 6 | 30 | 1.85 (0.47, 7.16) | 0.37 |
| Platelet count | | | | |
| Normal (100-250 K/uL) | 8 | 60 | Reference | NA |
| Low (< 100) | 2 | 3 | 5.00 (0.72, 34.63) | 0.10 |
| High (> 250) | 0 | 3 | ND | ND |
| Mean platelet volume | | | | |
| Normal (5.6-10.4 fL) | 9 | 64 | Reference | NA |
| Low (< 5.6) | 0 | 0 | ND | ND |
| High (> 10.4) | 1 | 3 | 2.37 (0.22, 25.31) | 0.47 |
| Icterus | | | | |
| Normal (5-25 Units) | 5 | 50 | Reference | NA |
| Low (< 5) | 0 | 1 | ND | ND |
| High (> 25) | 5 | 14 | 3.57 (0.90, 14.11) | 0.06 |
| Year of admission | | | | |
| 2007 | 3 | 20 | Reference | NA |
| 2008 | 6 | 22 | 1.81 (0.40, 8.25) | 0.43 |
| 2009 | 2 | 18 | 0.74 (0.11, 4.94) | 0.75 |
| 2010 | 3 | 13 | 1.53 (0.26, 8.81) | 0.62 |

* OR = crude odds ratios; ** 95% CI = 95% confidence interval; NA = Not applicable; ND = Not determined

Table 3-3. Univariable unconditional logistic regression analysis of investigated exposure factors at admission associated with *Salmonella* shedding ≤ 2 days after admission in horses without diarrhea

| Variable | Cases with diarrhea n = 25 | Controls n = 73 | OR* (95% CI)** | *P* value |
|---|---|---|---|---|
| Season | | | | |
| Winter and Spring | 14 | 45 | Reference | NA |
| Summer and Fall | 11 | 28 | 1.26 (0.50, 3.16) | 0.61 |
| | | | | |
| Prior to admission | | | | |
| Antimicrobial use | | | | |
| No | 23 | 68 | Reference | NA |
| Yes | 1 | 4 | 0.73 (0.07, 6.95) | 0.79 |
| Anti-inflammatory use | | | | |
| No | 4 | 5 | Reference | NA |
| Yes | 20 | 67 | 0.37 (0.09, 1.52) | 0.16 |
| Sedative use | | | | |
| No | 18 | 39 | Reference | NA |
| Yes | 6 | 33 | 0.39 (0.14, 1.10) | 0.07 |
| Fever | | | | |
| No (< 101.5F) | 11 | 43 | Reference | NA |
| Yes (> 101.5F) | 3 | 6 | 1.95 (0.42, 9.08) | 0.39 |
| Leucopenia | | | | |
| No (≥ 5.5 K/uL) | 2 | 1 | Reference | NA |
| Yes (< 5.5 K/uL) | 3 | 2 | 0.75 (0.03, 14.97) | 0.85 |
| Nasogastric intubation | | | | |
| No | 9 | 30 | Reference | NA |
| Yes | `13 | 40 | 1.08 (0.40, 2.86) | 0.87 |
| Laxative use | | | | |
| No | 14 | 41 | Reference | NA |
| Yes | 9 | 31 | 0.85 (0.32, 2.21) | 0.74 |
| Stomach protectant use | | | | |
| No | 21 | 68 | Reference | NA |
| Yes | 1 | 4 | 0.80 (0.08, 7.64) | 0.85 |
| Intestinal protectant use | | | | |
| No | 22 | 72 | ND | ND |
| Yes | 0 | 0 | ND | ND |
| | | | | |
| At admission | | | | |
| Fever | | | | |
| No | 20 | 67 | Reference | NA |
| Yes | 4 | 5 | 1.54 (0.26, 9.02) | 0.62 |
| Leucopenia | | | | |
| No | 15 | 59 | Reference | NA |
| Yes | 7 | 12 | 2.29 (0.77, 6.83) | 0.13 |

Table 3-3. Continued

| Variable | Cases with diarrhea n = 25 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| At admission | | | | |
| Fever and leucopenia | | | | |
| No | 22 | 70 | ND | ND |
| Yes | 0 | 0 | ND | ND |
| Nasogastric intubation | | | | |
| No | 3 | 8 | Reference | NA |
| Yes | 22 | 62 | 0.94 (0.23, 3.88) | 0.93 |
| Albumin | | | | |
| Normal (2.8-4.2 g/dL) | 18 | 60 | Reference | NA |
| Low (< 2.8) | 2 | 3 | 2.00 (0.31, 12.75) | 0.46 |
| High (> 4.2) | 1 | 4 | 0.75 (0.07, 7.07) | 0.80 |
| Creatinine | | | | |
| Normal (1.1-2.0 mg/dL) | 12 | 45 | Reference | NA |
| Low (< 1.1) | 0 | 3 | ND | ND |
| High (> 2.0) | 10 | 19 | 1.97 (0.72, 5.34) | 0.18 |
| Blood urea nitrogen | | | | |
| Normal (9-22 mg/dL) | 11 | 49 | Reference | NA |
| Low (< 9 ) | 0 | 1 | ND | ND |
| High (> 22) | 10 | 17 | 2.62 (0.94, 7.25) | 0.06 |
| Total carbondioxide | | | | |
| Normal (21-34 mEq/L) | 15 | 56 | Reference | NA |
| Low (< 21) | 4 | 8 | 1.57 (0.42, 5.78) | 0.49 |
| High (> 34) | 1 | 2 | 1.57 (0.13, 18.30) | 0.71 |
| Anion gap | | | | |
| Normal (10-20 mEq/L) | 18 | 44 | Reference | NA |
| Low (< 10) | 2 | 3 | 1.62 (0.25, 10.58) | 0.60 |
| High (> 20) | 2 | 8 | 0.61 (0.11, 3.16) | 0.55 |
| Alkaline phosphatase | | | | |
| Normal (69-228 U/L) | 14 | 46 | Reference | NA |
| Low (< 69) | 0 | 0 | ND | ND |
| High (> 228) | 7 | 21 | 1.09 (0.38, 3.11) | 0.86 |
| Aspartate aminotransferase | | | | |
| Normal (148-322 U/L) | 9 | 33 | Reference | NA |
| Low (< 148) | 0 | 1 | ND | ND |
| High (> 322) | 12 | 32 | 1.37 (0.51, 3.70) | 0.52 |
| Total bilirubin | | | | |
| Normal (0.3-1.9 mg/dL) | 7 | 16 | Reference | NA |
| Low (< 0.3) | 0 | 0 | ND | ND |
| High (> 1.9) | 14 | 51 | 0.62 (0.21, 1.82) | 0.39 |
| Globulin | | | | |
| Normal (2.4-4.9 g/dL) | 18 | 55 | Reference | NA |
| Low (< 2.4) | 1 | 3 | 0.88 (0.08, 8.93) | 0.91 |
| High (> 4.9) | 1 | 9 | 0.29 (0.03, 2.45) | 0.25 |

Table 3-3. Continued

| Variable | Cases with diarrhea n = 25 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| At admission | | | | |
| Albumin/Globulin ratio | | | | |
| Normal (0.6-1.4) | 20 | 63 | Reference | NA |
| Low (< 0.6) | 0 | 2 | ND | ND |
| High (>1.4) | 1 | 2 | 1.57 (0.13, 18.30) | 0.71 |
| Glucose | | | | |
| Normal (62-128 mg/dL) | 9 | 34 | Reference | NA |
| Low (< 62) | 0 | 1 | ND | ND |
| High (> 128) | 12 | 31 | 1.46 (0.54, 3.94) | 0.45 |
| Triglycerides | | | | |
| Normal (5-45 mg/dL) | 12 | 52 | Reference | NA |
| Low (< 5) | 0 | 0 | ND | ND |
| High (> 45) | 10 | 14 | 3.09 (1.10, 8.63) | 0.03 |
| Magnesium | | | | |
| Normal (1.3-1.9 mg/dL) | 18 | 51 | Reference | NA |
| Low (< 1.3) | 1 | 12 | 0.21 (0.02, 1.76) | 0.15 |
| High (> 1.9) | 2 | 4 | 1.29 (0.22, 7.58) | 0.77 |
| Gamma-glutamyl transferase | | | | |
| Normal (17-50 U/L) | 14 | 44 | Reference | NA |
| Low (< 17) | 4 | 4 | 2.77 (0.62, 12.28) | 0.17 |
| High (> 50) | 3 | 19 | 0.43 (0.11, 1.66) | 0.22 |
| Creatine kinase | | | | |
| Normal (134-729 U/L) | 11 | 38 | Reference | NA |
| Low (< 134) | 0 | 1 | ND | ND |
| High (> 729) | 10 | 28 | 1.23 (0.46, 3.30) | 0.67 |
| Sodium | | | | |
| Normal (134-143 mEq/L) | 13 | 50 | Reference | NA |
| Low (< 134) | 9 | 15 | 2.30 (0.82, 6.44) | 0.11 |
| High (> 143) | 0 | 1 | ND | ND |
| Potassium | | | | |
| Normal (2.2-5.3 mEq/L) | 21 | 67 | ND | ND |
| Low (< 2.2) | 0 | 0 | ND | ND |
| High (> 5.3) | 0 | 0 | ND | ND |
| Chloride | | | | |
| Normal (96-105 mEq/L) | 15 | 44 | Reference | NA |
| Low (< 96) | 6 | 23 | 0.76 (0.26, 2.23) | 0.62 |
| High (> 105) | 0 | 0 | ND | ND |
| Calcium | | | | |
| Normal (10.9-13.4 mg/dL) | 15 | 49 | Reference | NA |
| Low (< 10.9) | 6 | 15 | 1.30 (0.43, 3.96) | 0.63 |
| High (> 13.4) | 0 | 2 | ND | ND |

Table 3-3. Continued

| Variable | Cases with diarrhea n = 25 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| At admission | | | | |
| Color of mucus membranes | | | | |
| Pink | 14 | 49 | Reference | NA |
| Icteric/yellow | 0 | 1 | ND | ND |
| Red/injected/toxic line | 11 | 23 | 1.67 (0.65, 4.25) | 0.27 |
| Capillary refill time | | | | |
| Normal (≤ 2) | 16 | 50 | Reference | NA |
| Abnormal (> 2) | 9 | 23 | 1.22 (0.47, 3.17) | 0.67 |
| Heart rate | | | | |
| Normal (28-40 beats/min) | 3 | 12 | Reference | NA |
| Elevated (41-60) | 16 | 47 | 1.36 (0.34, 5.44) | 0.66 |
| High (> 60) | 6 | 14 | 1.71 (0.35, 8.37) | 0.50 |
| Lactate (blood gas) | | | | |
| Normal (≤ 1.5 mmol/L) | 11 | 26 | Reference | NA |
| Abnormal (> 1.5) | 11 | 33 | 0.78 (0.29, 2.10) | 0.63 |
| White blood cell count | | | | |
| Normal (5.5-11.0 K/uL) | 15 | 35 | Reference | NA |
| Low (< 5.5) | 5 | 11 | 1.06 (0.31, 3.58) | 0.92 |
| High (> 11.0) | 3 | 13 | 0.53 (0.13, 2.16) | 0.38 |
| Packed cell volume | | | | |
| Normal (30-43%) | 12 | 48 | Reference | NA |
| Low (< 30) | 0 | 6 | ND | ND |
| High (> 43) | 8 | 13 | 2.46 (0.83, 7.28) | 0.10 |
| Plasma protein | | | | |
| Normal (5.9-7.9 g/dL) | 12 | 52 | Reference | NA |
| Low (< 5.9) | 2 | 6 | 1.44 (0.25, 8.05) | 0.67 |
| High (> 7.9) | 8 | 13 | 2.66 (0.90, 7.86) | 0.07 |
| Fibrinogen | | | | |
| Normal (100-400 mg/dL) | 15 | 53 | Reference | NA |
| Low (< 100) | 1 | 0 | ND | ND |
| High (> 400) | 4 | 13 | 1.08 (0.30, 3.82) | 0.89 |
| Percent Neutrophils | | | | |
| Normal (28-83%) | 17 | 51 | Reference | NA |
| Low (< 28) | 1 | 2 | 1.50 (0.12, 17.60) | 0.74 |
| High (> 83) | 3 | 15 | 0.60 (0.15, 2.32) | 0.46 |
| Percent Monocytes | | | | |
| Normal (1-11%) | 17 | 65 | Reference | NA |
| Low (< 1) | 0 | 0 | ND | ND |
| High (> 11) | 4 | 2 | 7.64 (1.29, 45.31) | 0.02 |
| Toxicity | | | | |
| No | 11 | 43 | Reference | NA |
| Yes | 9 | 21 | 1.67 (0.60, 4.66) | 0.32 |

Table 3-3. Continued

| Variable | Cases with diarrhea n = 25 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| At admission | | | | |
| Percent lymphocytes | | | | |
| Normal (20-59%) | 9 | 23 | Reference | NA |
| Low (< 20) | 12 | 44 | 0.69 (0.25, 1.89) | 0.47 |
| High (> 59) | 0 | 0 | ND | ND |
| Neutrophils | | | | |
| Normal (2.2-8.1 K/uL) | 15 | 34 | Reference | NA |
| Low (< 2.2) | 4 | 7 | 1.29 (0.32, 5.09) | 0.71 |
| High (>8.1) | 4 | 18 | 0.50 (0.14, 1.74) | 0.27 |
| Neutropenia | | | | |
| No (≥ 2.2 K/uL) | 16 | 63 | Reference | NA |
| Yes (< 2.2) | 6 | 8 | 2.95 (0.89, 9.72) | 0.07 |
| RBC | | | | |
| Normal (6.7-10 M/uL) | 14 | 52 | Reference | NA |
| Low (< 6.7) | 1 | 9 | 0.35 (0.04, 2.96) | 0.33 |
| High (> 10) | 6 | 7 | 2.71 (0.80, 9.12) | 0.10 |
| Hemoglobin | | | | |
| Normal (11.2-16.2 g/dL) | 15 | 51 | Reference | NA |
| Low (< 11.2) | 0 | 6 | ND | ND |
| High (> 16.2) | 6 | 11 | 1.85 (0.58, 5.85) | 0.29 |
| Hematocrit (calculated) | | | | |
| Normal (30-43%) | 15 | 45 | Reference | NA |
| Low (< 30) | 0 | 10 | ND | ND |
| High (> 43) | 6 | 12 | 1.50 (0.47, 4.69) | 0.48 |
| Mean corpuscular volume | | | | |
| Normal (37.5-50.0 fL) | 20 | 61 | Reference | NA |
| Low (< 37.5) | 1 | 2 | 1.52 (0.13, 17.72) | 0.73 |
| High (> 50.0) | 0 | 3 | ND | ND |
| Mean corpuscular hemoglobin | | | | |
| Normal (14-18.7 pg) | 19 | 63 | Reference | NA |
| Low (< 14) | 1 | 2 | 1.47 (0.12, 17.07) | 0.75 |
| High (> 18.7) | 1 | 3 | 0.98 (0.09, 9.94) | 0.99 |
| Mean corpuscular hemoglobin concentration | | | | |
| Normal (36.4-38.8 g/dL) | 16 | 50 | Reference | NA |
| Low (< 36.4) | 4 | 7 | 1.57 (0.41, 5.94) | 0.50 |
| High (> 38.8) | 1 | 11 | 0.25 (0.03, 2.06) | 0.19 |
| Corpuscular hemoglobin concentration mean | | | | |
| Normal (34.9-37.6 g/dL) | 16 | 47 | Reference | NA |
| Low (< 34.9) | 0 | 1 | ND | ND |
| High (> 37.6) | 5 | 20 | 1.12 (0.32, 3.96) | 0.85 |

Table 3-3. Continued

| Variable | Cases with diarrhea n = 25 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| At admission | | | | |
| Corpuscular hemoglobin | | | | |
| Normal (13.7-17.9 pg) | 16 | 58 | Reference | NA |
| Low (< 13.7) | 1 | 1 | 3.15 (0.18, 52.69) | 0.42 |
| High (>17.9) | 4 | 9 | 1.40 (0.38, 5.03) | 0.60 |
| Red cell distribution width | | | | |
| Normal (16.3-19%) | 16 | 57 | Reference | NA |
| Low (< 16.3) | 0 | 0 | ND | ND |
| High (> 19) | 5 | 11 | 1.61 (0.49, 5.34) | 0.42 |
| Hemoglobin distribution width | | | | |
| Normal (1.5-2.1 g/dL) | 13 | 37 | Reference | NA |
| Low (< 1.5) | 0 | 0 | ND | ND |
| High (> 2.1) | 8 | 30 | 0.75 (0.27, 2.07) | 0.59 |
| Platelet count | | | | |
| Normal (100-250 K/uL) | 19 | 60 | Reference | NA |
| Low (< 100) | 1 | 3 | 1.05 (0.10, 10.72) | 0.96 |
| High (> 250) | 0 | 3 | ND | ND |
| Mean platelet volume | | | | |
| Normal (5.6-10.4 fL) | 21 | 64 | ND | ND |
| Low (< 5.6) | 0 | 0 | ND | ND |
| High (> 10.4) | 0 | 3 | ND | ND |
| Icterus | | | | |
| Normal (5-25 Units) | 14 | 50 | Reference | NA |
| Low (< 5) | 1 | 1 | 3.05 (0.18, 51.20) | 0.43 |
| High (> 25) | 5 | 14 | 1.09 (0.34, 3.42) | 0.88 |
| Year of admission | | | | |
| 2007 | 5 | 20 | Reference | NA |
| 2008 | 3 | 22 | 0.54 (0.11, 2.58) | 0.44 |
| 2009 | 11 | 18 | 2.44 (0.71, 8.39) | 0.15 |
| 2010 | 6 | 13 | 1.84 (0.46, 7.31) | 0.38 |

* OR = crude odds ratios; ** 95% CI = 95% confidence interval; NA = Not applicable; ND = Not determined

Table 3-4. Univariable unconditional logistic regression analysis of investigated exposure factors (during hospitalization on days 2 and 3) associated with *Salmonella* shedding ≥ 4 days after admission in horses without diarrhea

| Variable | Cases with diarrhea n = 21 | Controls n = 73 | OR* (95% CI)** | *P* value |
|---|---|---|---|---|
| Season | | | | |
| Winter and Spring | 7 | 45 | Reference | NA |
| Summer and Fall | 14 | 28 | 3.21 (1.15, 8.93) | 0.02 |
| | | | | |
| Prior to admission | | | | |
| Antimicrobial use | | | | |
| No | 21 | 68 | ND | ND |
| Yes | 0 | 4 | ND | ND |
| Anti-inflammatory use | | | | |
| No | 1 | 5 | Reference | NA |
| Yes | 20 | 67 | 1.49 (0.16, 13.52) | 0.72 |
| Sedative use | | | | |
| No | 10 | 39 | Reference | NA |
| Yes | 11 | 33 | 1.30 (0.49, 3.44) | 0.59 |
| Fever | | | | |
| No (< 101.5F) | 15 | 43 | Reference | NA |
| Yes (> 101.5F) | 1 | 6 | 0.47 (0.05, 4.29) | 0.51 |
| Leucopenia | | | | |
| No (≥ 5.5 K/uL) | 0 | 1 | ND | ND |
| Yes (< 5.5 K/uL) | 0 | 2 | ND | ND |
| Nasogastric intubation | | | | |
| No | 10 | 30 | Reference | NA |
| Yes | 11 | 40 | 0.82 (0.31, 2.19) | 0.70 |
| Laxative use | | | | |
| No | 9 | 41 | Reference | NA |
| Yes | 12 | 31 | 1.76 (0.66, 4.70) | 0.25 |
| Stomach protectant use | | | | |
| No | 18 | 68 | Reference | NA |
| Yes | 3 | 4 | 2.83 (0.58, 13.81) | 0.19 |
| Intestinal protectant use | | | | |
| No | 21 | 72 | ND | ND |
| Yes | 0 | 0 | ND | ND |
| | | | | |
| At admission | | | | |
| Fever | | | | |
| No | 20 | 67 | ND | ND |
| Yes | 1 | 5 | ND | ND |
| Leucopenia | | | | |
| No | 19 | 59 | ND | ND |
| Yes | 0 | 12 | ND | ND |

Table 3-4. Continued

| Variable | Cases with diarrhea n = 21 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| At admission | | | | |
| Fever and leucopenia | | | | |
| No | 19 | 70 | ND | ND |
| Yes | 0 | 0 | ND | ND |
| Nasogastric intubation | | | | |
| No | 1 | 8 | Reference | NA |
| Yes | 18 | 62 | 2.32 (0.27, 19.82) | 0.44 |
| Albumin | | | | |
| Normal (2.8-4.2 g/dL) | 18 | 60 | Reference | NA |
| Low (< 2.8) | 1 | 3 | 1.11 (0.10, 11.34) | 0.92 |
| High (> 4.2) | 0 | 4 | ND | ND |
| Creatinine | | | | |
| Normal (1.1-2.0 mg/dL) | 14 | 45 | Reference | NA |
| Low (< 1.1) | 0 | 3 | ND | ND |
| High (> 2.0) | 5 | 19 | 0.91 (0.28, 2.90) | 0.87 |
| Blood urea nitrogen | | | | |
| Normal (9-22 mg/dL) | 15 | 49 | Reference | NA |
| Low (< 9 ) | 0 | 1 | ND | ND |
| High (> 22) | 4 | 17 | 0.76 (0.22, 2.63) | 0.67 |
| Total carbondioxide | | | | |
| Normal (21-34 mEq/L) | 16 | 56 | Reference | NA |
| Low (< 21) | 3 | 8 | 1.31 (0.31, 5.53) | 0.71 |
| High (> 34) | 0 | 2 | ND | ND |
| Anion gap | | | | |
| Normal (10-20 mEq/L) | 16 | 53 | Reference | NA |
| Low (< 10) | 1 | 4 | 1.14 (0.11, 11.72) | 0.90 |
| High (> 20) | 2 | 9 | 0.86 (0.16, 4.42) | 0.85 |
| Alkaline phosphatase | | | | |
| Normal (69-228 U/L) | 17 | 46 | Reference | NA |
| Low (< 69) | 0 | 0 | ND | ND |
| High (> 228) | 2 | 21 | 0.25 (0.05, 1.21) | 0.08 |
| Aspartate aminotransferase | | | | |
| Normal (148-322 U/L) | 13 | 33 | Reference | NA |
| Low (< 148) | 0 | 1 | ND | ND |
| High (> 322) | 6 | 32 | 0.47 (0.16, 1.40) | 0.17 |
| Total bilirubin | | | | |
| Normal (0.3-1.9 mg/dL) | 7 | 16 | Reference | NA |
| Low (< 0.3) | 1 | 0 | ND | ND |
| High (> 1.9) | 11 | 51 | 0.49 (0.16, 1.48) | 0.20 |
| Globulin | | | | |
| Normal (2.4-4.9 g/dL) | 17 | 55 | Reference | NA |
| Low (< 2.4) | 2 | 3 | 2.15 (0.33, 13.99) | 0.42 |
| High (> 4.9) | 0 | 9 | ND | ND |

Table 3-4. Continued

| Variable | Cases with diarrhea n = 21 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| At admission | | | | |
| Albumin/Globulin ratio | | | | |
| Normal (0.6-1.4) | 17 | 63 | Reference | NA |
| Low (< 0.6) | 0 | 2 | ND | ND |
| High (>1.4) | 2 | 2 | 3.70 (0.48, 28.27) | 0.20 |
| Glucose | | | | |
| Normal (62-128 mg/dL) | 11 | 34 | Reference | NA |
| Low (< 62) | 0 | 1 | ND | ND |
| High (> 128) | 8 | 31 | 0.79 (0.28, 2.24) | 0.66 |
| Triglycerides | | | | |
| Normal (5-45 mg/dL) | 17 | 52 | Reference | NA |
| Low (< 5) | 1 | 0 | ND | ND |
| High (> 45) | 1 | 14 | 0.22 (0.02, 1.82) | 0.16 |
| Magnesium | | | | |
| Normal (1.3-1.9 mg/dL) | 11 | 51 | Reference | NA |
| Low (< 1.3) | 6 | 12 | 2.19 (0.69, 6.92) | 0.18 |
| High (> 1.9) | 2 | 4 | 2.19 (0.36, 13.27) | 0.39 |
| Gamma-glutamyl transferase | | | | |
| Normal (17-50 U/L) | 14 | 44 | Reference | NA |
| Low (< 17) | 2 | 4 | 1.56 (0.26, 9.34) | 0.62 |
| High (> 50) | 3 | 19 | 0.49 (0.12, 1.88) | 0.30 |
| Creatine kinase | | | | |
| Normal (134-729 U/L) | 15 | 38 | Reference | NA |
| Low (< 134) | 0 | 1 | ND | ND |
| High (> 729) | 4 | 28 | 0.36 (0.10, 1.20) | 0.09 |
| Sodium | | | | |
| Normal (134-143 mEq/L) | 14 | 50 | Reference | NA |
| Low (< 134) | 5 | 15 | 1.21 (0.37, 3.92) | 0.74 |
| High (> 143) | 0 | 1 | ND | ND |
| Potassium | | | | |
| Normal (2.2-5.3 mEq/L) | 19 | 67 | ND | ND |
| Low (< 2.2) | 0 | 0 | ND | ND |
| High (> 5.3) | 0 | 0 | ND | ND |
| Chloride | | | | |
| Normal (96-105 mEq/L) | 8 | 44 | Reference | NA |
| Low (< 96) | 11 | 23 | 2.63 (0.92, 7.44) | 0.06 |
| High (> 105) | 0 | 0 | ND | ND |
| Calcium | | | | |
| Normal (10.9-13.4 mg/dL) | 11 | 49 | Reference | NA |
| Low (< 10.9) | 8 | 15 | 2.37 (0.80, 6.98) | 0.11 |
| High (> 13.4) | 0 | 2 | ND | ND |
| Color of mucus membranes | | | | |
| Pink | 15 | 49 | Reference | NA |
| Icteric/yellow | 0 | 1 | ND | ND |
| Red/injected/toxic line | 6 | 23 | 0.85 (0.29, 2.48) | 0.76 |

Table 3-4. Continued

| Variable | Cases with diarrhea n = 21 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| At admission | | | | |
| Capillary refill time | | | | |
| Normal (≤ 2) | 13 | 50 | Reference | NA |
| Abnormal (> 2) | 8 | 23 | 1.33 (0.48, 3.67) | 0.57 |
| Heart rate | | | | |
| Normal (28-40 beats/min) | 7 | 12 | Reference | NA |
| Elevated (41-60) | 10 | 47 | 0.36 (0.11, 1.15) | 0.08 |
| High (> 60) | 4 | 14 | 0.48 (0.11, 2.08) | 0.33 |
| Lactate (blood gas) | | | | |
| Normal (≤ 1.5 mmol/L) | 7 | 26 | Reference | NA |
| Abnormal (> 1.5) | 10 | 33 | 1.12 (0.37, 3.36) | 0.83 |
| Plasma protein | | | | |
| Normal (5.9-7.9 g/dL) | 17 | 44 | Reference | NA |
| Low (< 5.9) | 3 | 10 | 0.77 (0.19, 3.16) | 0.72 |
| High (> 7.9) | 0 | 10 | ND | ND |
| Packed cell volume | | | | |
| Normal (30-48%) | 17 | 43 | Reference | NA |
| Low (< 30) | 1 | 9 | 0.31 (0.03, 2.66) | 0.28 |
| High (> 48) | 2 | 13 | 0.43 (0.08, 2.12) | 0.30 |
| White blood cell count | | | | |
| Normal (5.5-11.0 K/uL) | 15 | 45 | Reference | NA |
| Low (< 5.5) | 0 | 12 | ND | ND |
| High (> 11.0) | 4 | 14 | 1.02 (0.28, 3.65) | 0.97 |
| Fibrinogen | | | | |
| Normal (100-400 mg/dL) | 18 | 53 | ND | ND |
| Low (< 100) | 1 | 0 | ND | ND |
| High (> 400) | 0 | 13 | ND | ND |
| Percent Neutrophils | | | | |
| Normal (28-83%) | 12 | 51 | Reference | NA |
| Low (< 28) | 0 | 2 | ND | ND |
| High (> 83) | 7 | 15 | 1.98 (0.66, 5.93) | 0.22 |
| Percent Monocytes | | | | |
| Normal (1-11%) | 18 | 65 | Reference | NA |
| Low (< 1) | 0 | 0 | ND | ND |
| High (> 11) | 1 | 2 | 1.80 (0.15, 21.06) | 0.63 |
| Toxicity | | | | |
| No | 9 | 43 | Reference | NA |
| Yes | 7 | 21 | 1.59 (0.52, 4.86) | 0.41 |
| Percent lymphocytes | | | | |
| Normal (20-59%) | 5 | 23 | Reference | NA |
| Low (< 20) | 14 | 44 | 1.46 (0.46, 4.57) | 0.51 |
| High (> 59) | 0 | 0 | ND | ND |

Table 3-4. Continued

| Variable | Cases with diarrhea n = 21 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| At admission | | | | |
| Neutrophils | | | | |
| Normal (2.2-8.1 K/uL) | 14 | 42 | Reference | NA |
| Low (< 2.2) | 0 | 8 | ND | ND |
| High (>8.1) | 5 | 21 | 0.71 (0.22, 2.29) | 0.57 |
| Neutropenia | | | | |
| No (≥ 2.2 K/uL) | 19 | 63 | ND | ND |
| Yes (< 2.2) | 0 | 8 | ND | ND |
| RBC | | | | |
| Normal (6.7-10 M/uL) | 14 | 52 | Reference | NA |
| Low (< 6.7) | 4 | 9 | 1.58 (0.43, 5.82) | 0.48 |
| High (> 10) | 1 | 7 | 0.50 (0.05, 4.44) | 0.54 |
| Hemoglobin | | | | |
| Normal (11.2-16.2 g/dL) | 14 | 51 | Reference | NA |
| Low (< 11.2) | 2 | 6 | 1.16 (0.21, 6.34) | 0.85 |
| High (> 16.2) | 3 | 11 | 0.95 (0.23, 3.84) | 0.94 |
| Hematocrit (calculated) | | | | |
| Normal (30-43%) | 14 | 45 | Reference | NA |
| Low (< 30) | 3 | 10 | 0.95 (0.23, 3.90) | 0.95 |
| High (> 43) | 2 | 12 | 0.53 (0.10, 2.62) | 0.43 |
| Mean corpuscular volume | | | | |
| Normal (37.5-50.0 fL) | 16 | 61 | Reference | NA |
| Low (< 37.5) | 1 | 2 | 1.90 (0.16, 22.37) | 0.60 |
| High (> 50.0) | 2 | 3 | 2.54 (0.39, 16.52) | 0.32 |
| Mean corpuscular hemoglobin | | | | |
| Normal (14-18.7 pg) | 13 | 63 | Reference | NA |
| Low (< 14) | 1 | 2 | 2.26 (0.19, 26.65) | 0.51 |
| High (> 18.7) | 5 | 3 | 7.55 (1.62, 35.12) | 0.009 |
| Mean corpuscular hemoglobin concentration | | | | |
| Normal (36.4-38.8 g/dL) | 13 | 50 | Reference | NA |
| Low (< 36.4) | 0 | 7 | ND | ND |
| High (> 38.8) | 6 | 11 | 2.09 (0.65, 6.73) | 0.21 |
| Corpuscular Hemoglobin concentration mean | | | | |
| Normal (34.9-37.6 g/dL) | 9 | 47 | Reference | NA |
| Low (< 34.9) | 0 | 1 | ND | ND |
| High (> 37.6) | 10 | 20 | 2.61 (0.92, 7.39) | 0.07 |
| Corpuscular hemoglobin | | | | |
| Normal (13.7-17.9 pg) | 9 | 58 | Reference | NA |
| Low (< 13.7) | 2 | 1 | 11.45 (0.95, 137.39) | 0.05 |
| High (>17.9) | 8 | 9 | 5.09 (1.61, 16.04) | 0.005 |

Table 3-4. Continued

| Variable | Cases with diarrhea n = 21 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| At admission | | | | |
| Red cell distribution width | | | | |
| Normal (16.3-19%) | 15 | 57 | Reference | NA |
| Low (< 16.3) | 0 | 0 | ND | ND |
| High (> 19) | 4 | 11 | 1.38 (0.38, 4.95) | 0.61 |
| Hemoglobin distribution width | | | | |
| Normal (1.5-2.1 g/dL) | 12 | 37 | Reference | NA |
| Low (< 1.5) | 0 | 0 | ND | ND |
| High (> 2.1) | 7 | 30 | 0.71 (0.25, 2.05) | 0.53 |
| Platelet count | | | | |
| Normal (100-250 K/uL) | 16 | 60 | Reference | NA |
| Low (< 100) | 2 | 3 | 2.50 (0.38, 16.25) | 0.33 |
| High (> 250) | 1 | 3 | 1.25 (0.12, 12.84) | 0.85 |
| Mean platelet volume | | | | |
| Normal (5.6-10.4 fL) | 18 | 64 | Reference | NA |
| Low (< 5.6) | 0 | 0 | ND | ND |
| High (> 10.4) | 1 | 3 | 1.18 (0.11, 12.09) | 0.88 |
| Icterus | | | | |
| Normal (5-25 Units) | 14 | 50 | Reference | NA |
| Low (< 5) | 1 | 1 | 3.62 (0.21, 61.21) | 0.37 |
| High (> 25) | 4 | 14 | 1.03 (0.29, 3.58) | 0.95 |
| | | | | |
| During hospitalization | | | | |
| Antimicrobial use | | | | |
| No | 3 | 29 | Reference | NA |
| Yes | 18 | 44 | 3.95 (1.06, 14.64) | 0.03 |
| Anti-inflammatory use | | | | |
| No | 1 | 9 | Reference | NA |
| Yes | 20 | 64 | 2.81 (0.33, 23.57) | 0.34 |
| Sedative use | | | | |
| No | 0 | 1 | ND | ND |
| Yes | 21 | 72 | ND | ND |
| Fever (day 2 or 3) | | | | |
| No | 16 | 45 | Reference | NA |
| Yes | 5 | 28 | 0.50 (0.16, 1.52) | 0.22 |
| Leucopenia | | | | |
| No | 1 | 12 | Reference | NA |
| Yes | 5 | 6 | 10.00 (0.94, 105.92) | 0.05 |
| Fever and leucopenia | | | | |
| No | 3 | 16 | Reference | NA |
| Yes | 3 | 2 | 8.00 (0.91, 70.27) | 0.06 |
| Nasogastric intubation | | | | |
| No | 10 | 34 | Reference | NA |
| Yes | 11 | 37 | 1.01 (0.38, 2.67) | 0.98 |

Table 3-4. Continued

| Variable | Cases with diarrhea n = 21 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| During hospitalization | | | | |
| Feed withholding | | | | |
| No | 5 | 7 | Reference | NA |
| Yes | 16 | 66 | 0.33 (0.09, 1.20) | 0.09 |
| Laxative use | | | | |
| No | 13 | 50 | Reference | NA |
| Yes | 7 | 23 | 1.17 (0.41, 3.32) | 0.76 |
| Stomach protectant use | | | | |
| No | 13 | 63 | Reference | NA |
| Yes | 8 | 10 | 3.87 (1.28, 11.70) | 0.01 |
| Intestinal protectant use | | | | |
| No | 20 | 70 | Reference | NA |
| Yes | 1 | 3 | 1.16 (0.11, 11.83) | 0.89 |
| General anesthesia | | | | |
| No | 5 | 35 | Reference | NA |
| Yes | 16 | 38 | 2.94 (0.97, 8.89) | 0.05 |
| Abdominal surgery | | | | |
| No | 5 | 35 | Reference | NA |
| Yes | 16 | 38 | 2.94 (0.97, 8.89) | 0.05 |
| Location of surgical lesion | | | | |
| Large intestine | 4 | 5 | Reference | NA |
| Small intestine | 11 | 28 | 0.49 (0.11, 2.17) | 0.34 |
| Small & large intestine | 1 | 3 | 0.41 (0.03, 5.70) | 0.51 |
| Type of surgical lesion | | | | |
| Strangulating | 2 | 5 | Reference | NA |
| Non-strangulating | 14 | 31 | 1.12 (0.19, 6.54) | 0.89 |
| Antispasmodic use | | | | |
| No | 8 | 33 | Reference | NA |
| Yes | 13 | 40 | 1.34 (0.49, 3.62) | 0.56 |
| Plasma protein (day 2) | | | | |
| Normal (5.9-7.9 g/dL) | 6 | 36 | Reference | NA |
| Low (< 5.9) | 13 | 25 | 3.12 (1.04, 9.31) | 0.04 |
| High (> 7.9) | 0 | 2 | ND | ND |
| Plasma protein (day 3) | | | | |
| Normal (5.9-7.9 g/dL) | 9 | 30 | Reference | NA |
| Low (< 5.9) | 7 | 16 | 1.45 (0.45, 4.64) | 0.52 |
| High (> 7.9) | 0 | 0 | ND | ND |
| Packed cell volume (day 2) | | | | |
| Normal (30-48) | 14 | 48 | Reference | NA |
| Low (< 30) | 4 | 9 | 1.52 (0.40, 5.70) | 0.53 |
| High (> 48) | 1 | 6 | 0.57 (0.06, 5.15) | 0.61 |

Table 3-4. Continued

| Variable | Cases with diarrhea n = 21 | Controls n = 73 | OR (95% CI) | *P* value |
|---|---|---|---|---|
| During hospitalization | | | | |
| Packed cell volume (day 3) | | | | |
| Normal (30-48) | 13 | 37 | Reference | NA |
| Low (< 30) | 2 | 8 | 0.71 (0.13, 3.79) | 0.69 |
| High (> 48) | 0 | 1 | ND | ND |
| Color of mucus membranes (day 2) | | | | |
| Pink | 16 | 55 | Reference | NA |
| Icteric/yellow | 0 | 5 | ND | ND |
| Red/injected/toxic line | 5 | 13 | 1.32 (0.40, 4.26) | 0.64 |
| Color of mucus membranes (day 3) | | | | |
| Pink | 18 | 64 | Reference | NA |
| Icteric/yellow | 0 | 3 | ND | ND |
| Red/injected/toxic line | 3 | 5 | 2.13 (0.46, 9.79) | 0.32 |
| Capillary refill time (day 2) | | | | |
| Normal (≤ 2) | 16 | 50 | Reference | NA |
| Abnormal (> 2) | 5 | 23 | 0.67 (0.22, 2.08) | 0.49 |
| Capillary refill time (day 3) | | | | |
| Normal (≤ 2s) | 16 | 57 | Reference | NA |
| Abnormal (> 2s) | 5 | 13 | 1.37 (0.42, 4.41) | 0.59 |
| Heart rate (day 2) | | | | |
| Normal (28-40 beats per min) | 3 | 14 | Reference | NA |
| Elevated (41-60) | 14 | 45 | 1.45 (0.36, 5.79) | 0.59 |
| High (> 60) | 4 | 14 | 1.33 (0.25, 7.08) | 0.73 |
| Heart rate (day 3) | | | | |
| Normal (28-40 beats per min) | 6 | 24 | Reference | NA |
| Elevated (41-60) | 11 | 42 | 1.04 (0.34, 3.19) | 0.93 |
| High (> 60) | 4 | 3 | 5.33 (0.93, 30.50) | 0.05 |
| Year of admission | | | | |
| 2007 | 4 | 20 | Reference | NA |
| 2008 | 8 | 22 | 1.81 (0.47, 6.97) | 0.38 |
| 2009 | 6 | 18 | 1.66 (0.40, 6.87) | 0.47 |
| 2010 | 3 | 13 | 1.15 (0.22, 6.01) | 0.86 |
| Number of fecal samples tested | | | 1.67 (1.11, 2.51) | 0.01 |

* OR = crude odds ratios; ** 95% CI = 95% confidence interval; NA = Not applicable; ND = Not determined

Table 3-5. Multivariable analysis of investigated exposure factors at admission associated with *Salmonella* shedding ≤ 2 days after admission in horses without diarrhea

| Variable | β | SE | OR | 95% CI | *P* |
|---|---|---|---|---|---|
| Plasma protein | | | | | |
| Low versus normal | -0.02 | 0.91 | 0.97 | 0.16, 5.88 | 0.97 |
| High versus normal | 1.04 | 0.58 | 2.85 | 0.91, 8.91 | 0.07 |
| Triglycerides | | | | | |
| High versus normal | 1.21 | 0.54 | 3.37 | 1.15, 9.87 | 0.02 |

Table 3-6. Multivariable analysis of investigated exposure factors (during hospitalization) associated with *Salmonella* shedding ≥ 4 days after admission in horses without diarrhea

| Variable | β | SE | OR | 95% CI | *P* |
|---|---|---|---|---|---|
| Model A | | | | | |
| Abdominal surgery | | | | | |
| Yes vs. No | 1.02 | 0.59 | 2.79 | 0.86, 9.01 | 0.08 |
| Number of fecal samples tested | 0.53 | 0.21 | 1.70 | 1.11, 2.60 | 0.01 |
| Model B | | | | | |
| Season | | | | | |
| Summer and Fall vs. Winter & Spring | 1.59 | 0.61 | 4.92 | 1.46, 16.57 | 0.01 |
| Number of fecal samples tested | 0.65 | 0.23 | 1.92 | 1.20, 3.06 | 0.005 |

# CHAPTER 4
# DIAGNOSTIC PERFORMANCE OF REAL-TIME PCR FOR DIAGNOSIS OF SALMONELLA SHEDDING IN HOSPITALIZED HORSES

Shedding of *Salmonella* spp in feces of hospitalized horses is an important problem for large animal hospitals because of the potential risk of outbreaks of nosocomial *Salmonella* infections. Outbreaks of nosocomial *Salmonella* infections in hospitalized horses can result in temporary hospital closure, loss of revenue, mortality in horses, zoonotic infections, and reduced caseload.[1-4,6,92,128-130,132]To reduce the risk of outbreaks of nosocomial *Salmonella* infections among hospitalized horses, several veterinary teaching hospitals have established surveillance and infection control programs.

In most veterinary hospitals with a surveillance and infection control program, bacteriological culture is considered the gold standard,[6,8,10,92] and is the most frequently used diagnostic technique for detection of *Salmonella* spp in fecal specimens of horses.[6] A major limitation of using bacteriological culture to monitor community-acquired and nosocomial *Salmonella* infections in horses is the time required (3 to 5 days) to obtain laboratory results. In the absence of overt clinical signs (diarrhea or fever and leucopenia), this prolonged detection time creates a delay in implementation of appropriate infection control measures (such as isolation and barrier nursing) which are necessary to minimize the risk of nosocomial infections and environmental contamination.

Several studies have described PCR-based tests for detecting *Salmonella* spp in fecal specimens in horses. One advantage of using PCR tests is that results can be obtained in 24 hours, thus allowing an earlier implementation of appropriate infection control measures. However, despite the availability of rapid and cost-effective PCR

tests for detection of *Salmonella*, its acceptance as a surveillance tool for monitoring community-acquired and nosocomial *Salmonella* infections in horses has been limited.

Two previous studies compared the diagnostic performance of conventional PCR and culture and revealed a number of limitations of PCR as a surveillance tool.[8,11] In one study, the study population included horses with and without clinical signs of salmonellosis and this conventional PCR assay targeted the transport operon gene of *Salmonella*.[11] In that study, among equine outpatients without clinical signs of salmonellosis, 26/152 (17%) tested positive by PCR and 0/152 (0%) tested positive by culture, but only one fecal sample was collected from this study population to test for *Salmonella*. The possibility exists that the 26 horses that tested positive by PCR were colonized with *Salmonella* and had these been tested two or more times by culture, the true status of these samples may have been *Salmonella*-culture positive. A gold standard definition for true-negatives that considers horses without clinical signs of salmonellosis that are sampled and tested by culture on only one fecal sample likely produces inconclusive results. Bacteriological culture of multiple fecal samples from horses has been shown to yield a greater proportion of *Salmonella*-positive results compared to single fecal samples.[140] In a second conventional PCR study that targeted the same gene, the study population included hospitalized horses without signs of GI disease from which multiple fecal samples were collected at 24-hour intervals.[8] Results revealed a high frequency of *Salmonella*-PCR positive results. In that study, the analysis for specificity included 105 horses without clinical signs of salmonellosis that tested *Salmonella*-culture negative on ≥ 5 samples during hospitalization. If culture were used as the gold standard, the epidemiologic specificity of the PCR assay used would

be very low, as only 29 horses (27.6%) were classified as PCR negative. In that study many false-positive results were yielded by that particular protocol.[8] Given the limited bacterial sequencing information available at that time, it is possible that the primers used in that study were not as specific as presumed and may have cross-reacted with other bacteria.

Two additional previous studies used real-time PCR technology to compare the diagnostic performance of PCR and culture and revealed similar limitations. In one study that targeted the invA gene, a specificity estimate of 339/345 or 98% was reported for the PCR assay.[13] However, in that study, the fecal samples used to estimate specificity were collected from horses and the environment of pens of a horse feedlot. The clinical status of study horses was not reported and it was not clear if horses in that study had colic with or without clinical signs of salmonellosis. In addition, only one fecal sample was collected from each study horse. In a second study that targeted the same gene, a relative specificity of 889/905 (98%) was reported for the PCR assay.[10] The analysis for specificity included 905 horses with and without clinical signs of salmonellosis that tested *Salmonella*-culture negative on one fecal sample under enrichment. In these two previous real-time PCR studies,[10,13] the lack of a gold standard definition for true-negatives, and the use of horses that tested culture-negative on only one fecal sample may have led to misclassification bias producing inconclusive results.

Different sampling methods can affect the diagnostic performance assessment of diagnostic tests. When assessing the specificity of a PCR assay, it is important that a gold standard definition for true-negatives be consistently considered. In particular one that defines negative horses as those horses without clinical signs of salmonellosis that

are sampled multiple times and test negative by culture. In addition, it is important that a PCR assay defines what frequency of PCR false-positives is acceptable. In a hospital setting, the consequences of false-positive results can be considerable because equine in-patients with a positive result must be placed in isolation stalls, increasing the cost of hospital fees. For PCR to be accepted as a surveillance tool, the aforementioned limitations in previous conventional and real-time PCR studies need to be addressed.

The objective of this study was to assess the diagnostic performance of real-time PCR assays for detection of *Salmonella* spp in fecal specimens of hospitalized horses, compared to bacteriological culture. The limitations observed in previous PCR studies were addressed in this study in an attempt to 1) define in silico the specificity and sensitivity of diagnostic PCR protocols on current bacterial bioinformatical databases, 2) develop a case-definition for each population of true-positive and true-negative *Salmonella* horses, and 3) develop an algorithm for PCR testing that is reliable, with a high relative specificity, sensitivity, and accuracy.

## Materials and Methods

### Study Population

The study was conducted at the University of Florida Large Animal Hospital (UF LAH). The hospital has an on-going surveillance program for detection of community-acquired and nosocomial *Salmonella* infections in hospitalized horses. All equine patients with or without clinical signs of gastrointestinal (GI) disease admitted to the UF LAH during a one-year period from January to December 2011 were eligible for inclusion in the study. Horses hospitalized for less than 48 hours or with less than three fecal samples collected for bacteriological culture were excluded. The study protocol

was approved by the Institutional Animal Care and Use Committee at the University of Florida (Protocol # 201104908).

**Study Design**

Figure 4-1 is a chart that presents the overall sampling approach that was used to estimate the relative sensitivity and specificity of the real-time PCR test for diagnosis of *Salmonella* in equine feces.

To assess the diagnostic sensitivity (detection of true-positives) of the real-time PCR assay, fecal or swab samples from 20 horses with signs of GI disease (colic, diarrhea, or fever and leucopenia) that were classified as *Salmonella*-culture positive were used in the analysis. To assess the diagnostic specificity (detection of true-negatives), fecal samples from two different groups of horses were used in the analysis. The first group included fecal samples from 30 horses without signs of GI disease that were classified as *Salmonella*-culture negative on 5 fecal samples. The second group included fecal or swab samples from 43 horses with signs of GI disease that were classified as *Salmonella*-culture negative on at least three fecal samples.

Bacteriological culture was used as the gold standard test in this study because it is the most frequently used diagnostic method for detection of *Salmonella* spp in fecal specimens of horses.[6,8,10] True-positive horses were defined as GI equine inpatients that tested *Salmonella*-positive by culture on at least one fecal or swab sample. True-negative horses were defined as non-GI or GI equine inpatients that had five fecal samples or at least three fecal or swab samples collected respectively, and tested *Salmonella* culture-negative on all samples.

The rationale for sampling and testing each study horse at least three times is that the probability of detecting *Salmonella* spp in fecal samples increases with the number

of samples tested. Temporal sensitivity is the probability of detecting a pathogen in a given time period and refers to the ability of a diagnostic test to detect the pathogen early, as opposed to late.[189] Assuming the epidemiologic sensitivity of the PCR assay is 90% (relative to culture), the probability of detecting a horse infected with *Salmonella* spp upon admission (sample 1) is 0.90, and the probability that the horse would fail to test positive (false negative), would be 1 – 0.90 = 0.10. If the horse failed to test positive upon admission and is tested again 24 hours later (sample 2), the probability that the horse would then test positive would be 0.10 x 0.90, or 0.09. The cumulative probability that the horse would be detected after two samples would be the probability of detection at admission (sample 1) plus 24 h later (sample 2), or 0.90 + 0.09 = 0.99. Finally, if the horse failed to test positive upon admission and 24 h later (samples 1 and 2), the probability that the horse would then test positive 48 h after admission (sample 3) would be 0.10 x 0.10 x 0.90, or 0.009. Thus, the cumulative probability that the horse would be detected after three samples would be the probability of detection at admission (sample 1) plus 24 h after admission (sample 2) plus 48 h after admission, or 0.90 + 0.09 + 0.009 = 0.999.

A sample size of 20 horses classified as *Salmonella* true-positive and 30 horses classified as *Salmonella* true-negative would provide 95% confidence and 80% power to estimate a desired real-time PCR assay sensitivity of 90% (95% confidence interval = 76.8, 100.0) and a specificity of 96% (90.2, 100).

**Fecal Sample Collection**

For GI horses, fecal sample collection was performed as part of the UF LAH surveillance program for early detection of fecal shedding of *Salmonella* spp at the time of admission and during hospitalization. A fecal sample or rectal swab (if fecal sample

was not available) was collected from each study horse within 12 h of admission (sample 1) and submitted for bacteriological culture. Thereafter, additional fecal samples (samples 2, 3, and more) were collected from the stall floor each morning prior to cleaning at 72-96 hour intervals until the patient was discharged from the hospital. Fecal samples collected outside regular business hours were refrigerated at 4°C prior to laboratory submission (for a maximum of 24 h). For some horses, additional fecal samples were collected at the discretion of the attending clinician (eg, every 12 to 24 hours).

For non-GI horses, only fecal samples were collected (ie, no fecal swabs collected) and submitted for bacteriological culture. The first fecal sample (sample 1) was collected within 12 h of admission from the stall floor of each stall housing the study horse by one of the study investigators. Thereafter, additional samples (samples 2-5) were collected at 12 h intervals during hospitalization.

As part of bacteriological culture procedures, all fecal or swab samples collected from study horses were enriched in 10 mL of Hajna Tetrathionate broth (with Iodine-Iodide) (TTB) for 24 h at 37°C. The following day, the broth was sub-cultured on Hektoen Enteric Agar (HEA) plates. The remainder of the broth (approximately 9.5 mL) was aliquoted and stored at -80°C to be used later for *Salmonella* DNA extraction and real-time PCR testing.

**Microbiological Procedures**

Bacteriologic culture of fecal samples for detection of *Salmonella* spp was performed at the UF Veterinary Clinical Microbiology Laboratory as previously described.[132] Briefly, for selective enrichment, 1 to 2 g of fresh feces was placed in 10 mL of TTB, and incubated at 37°C for 24 hours. The following day, a sample of the TTB

was sub-cultured on HEA plates. Plates were incubated at 37°C for 24 hours. Non-lactose fermenting, hydrogen sulfide-producing colonies were selected and isolated. These colonies were then inoculated on urea agar and lysine-iron agar slants and incubated at 37°C for 24 hours. Identification of urease-negative and hydrogen sulfide-producing organisms was established through use of a commercially available identification system. Serogroup of *Salmonella* isolates was determined by means of agglutination; polyvalent (A through I and Vi) and group-specific (A through E) *Salmonella* O antisera were used. *Salmonella* isolates were tested for antimicrobial susceptibility using the minimal inhibitory concentration (MIC) method with commercially prepared plates and the Kirby Bauer disk diffusion method. Serotyping of *Salmonella* isolates was performed at the USDA National Veterinary Services Laboratories in Ames, Iowa.

**Real-time PCR Assay Procedures**

**Bioinformatics analysis**

The goal for the bioinformatic analysis was to determine, in silico, the specificity of the primers and probes previously investigated for detection of *Salmonella* spp in equine fecal samples. Several targets were included consisting of the histidine transport operon, invE, sip, spaQ, and invA gene.[10-13] Primer and probe sequences from published studies were blasted using the National Centre for Biotechnology Information (NCBI) database to determine the degree of similarity and homology of *Salmonella* against bacterial nucleotide sequences. Individual primers and probes were blasted and compared using the following information: number of hits, organisms hit, description (chromosome/plasmid), sequence similarity scores, query coverage, expected values, and percent maximal identity score. In addition, conventional PCR primers and real-time

PCR primers and probes were blasted as a combination (ie., forward/reverse and forward/probe/reverse). In the end, two sets of primers and probes were selected from published real-time PCR studies that targeted *Salmonella* DNA in equine fecal samples.

After selection of the two sets of primers and probes, the target gene was cut into five overlapping fragments and blasted to verify the existence of sequence alignments with non-*Salmonella* organisms. In addition, the existence of sequence alignments with non-*Salmonella* organisms when using the histidine transport operon gene (Genbank Accession Number V01373.1; 3700 bp) was investigated. This investigation was important because this gene had been used in previous studies[8,11] that produced a high proportion of PCR false-positive results (26/152 or 17%[11] and 76/105 or 72%[8]), when bacteriologic culture results were used as the gold standard.

**Optimization of primer and probe concentration**

The two selected sets of primers and probes were optimized using the Applied Biosystems® Fast 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA) and SYBR green and TaqMan probe PCR chemistries. First, each set of primers was evaluated using SYBR green. The rationale for using SYBR green chemistry was to determine if non-specific binding occurred (based on dissociation curves). For each set of primers, a primer optimization matrix (Table 4-1) was used to determine the minimal primer concentration that yielded the minimal $C_T$ and maximal $\Delta$Rn. *Salmonella* Typhimurium DNA was used as a template and deionized water as a negative control. Samples were amplified using the following thermal cycling protocol with SYBR green: 20 s at 95°C; 3 s at 95°C for 40 cycles, 30 s at 60°C; 15 s at 95°C, 1 min at 60°C, 15 s at 95°C, and 15 s at 60°C. Optimal primer concentrations were determined and

dissociation curve analysis was performed to examine for non-specific binding (absence of erroneous peaks/presence of different melting temperature from the specific product).

Each set of primers was evaluated using TaqMan probe chemistry to determine the optimal primer/probe concentrations. A primer optimization matrix (Table 4-1) was used to determine the minimal primer concentration that yielded the minimal $C_T$ and maximal ΔRn. Thereafter, using the selected primer concentration, the probe concentration was varied from 50 to 300 nmols (50/150/200/250/300 nmols) to determine the optimal probe concentration that yielded the minimal $C_T$ and maximal ΔRn. *Salmonella* Typhimurium DNA was used as a template and deionized water as a negative control. Samples were amplified using the following thermal cycling protocol: 20 s at 95°C, 3 s at 95°C for 40 cycles and 30 s at 60°C.

A third set of primers and probe that universally targets the 16S rRNA gene of bacteria was used as a control of the efficiency of DNA purification and amplification and as an indicator of fecal inhibition.[190] This third set of primers and probe were optimized following the same approach as previously described.

The efficiency of the PCR assay was determined for each set of primers/probes by using seven 10-fold dilutions of *Salmonella* DNA (10 ng/µL) to generate standard curves. For both sets of primers/probes, the slope of the log-linear phase and $R^2$ values were examined to verify if the amplification of the PCR assay was efficient.

**Analytical specificity and sensitivity of the PCR assay**

Analytical specificity of the assay (using the two selected sets of primers/probes) and cross-reactivity with non-*Salmonella* enteric and non-enteric organisms was determined as described previously[10] with modifications. Enteric and non-enteric organisms tested included *E. coli, Klebsiella pneumoniae, Bordetella bronchiseptica,*

*Streptococcus equi* subsp*. zooepidemicus, Staphylococcus aureus, Clostridium difficile*, *Enterobacter cloacae, Enterobacter aerogenes, Pasteurella multocida, Pseudomonas aeruginosa, Enterococcus faecalis, Enterococcus casseliflavus, Paeni bacillus, Bacteroides fragilis, Proteus mirabilis, and Streptococcus equi* subsp*. equi.* All the non-*Salmonella* strains were obtained from the University of Florida Shands Diagnostic Microbiology laboratory and collections at College of Veterinary Medicine.

The analytical sensitivity of the assay (using the two selected sets of primers/probes) was determined using 13 *Salmonella* isolates of equine origin from 5 serogroups: serogroup B (*S.* Saintpaul and *S.* Typhimurium); serogroup C1 (*S.* Mbandaka, *S.* Braenderup); serogroup C2 (*S.* Newport*, S.* Litchfield, and *S.* Muenchen); serogroup D (*S.* Enteriditis, *S*. Miami, *S*. Javiana) and serogroup E (*S.* Anatum, *S*. Meleagridis, *S*. Muenster). All *Salmonella* strains were obtained from the UF Veterinary Hospitals Microbiology laboratory.

All *Salmonella* and non- *Salmonella* strains were grown on CBA plates for 24 h at 37°C. A colony from the agar plates was inoculated in TSB or Thioglycollate with H & K broth (*Bacteroides fragilis*) for 24 h at 37°C. Broth was aliquoted, stored at -20°C and later used for DNA extraction using DNeasy™ tissue kit (Qiagen, Valencia, CA). Real-time PCR was performed in duplicates for each isolate. *Salmonella* Typhimurium DNA was used as a positive control and deionized water as a negative control.

**Comparison of the analytical sensitivity of DNA extraction kits**

Three commercially available DNA extraction kits (DNeasy™ tissue kit, Qiagen, Valencia, California; UltraClean® fecal kit, MOBIO, Carlsbad, California; and QIAamp® DNA Stool Mini Kit, Qiagen, Valencia, California) were selected and compared to determine the technique with the optimal analytical sensitivity (lowest $C_T$ or highest

number of *Salmonella* gene copies/uL DNA). The DNeasy™ tissue kit was selected for evaluation because it was reported in a previous study to have optimal sensitivity (compared to other kits) during extraction of *Salmonella* DNA from equine feces.[12] The UltraClean® fecal kit and the QIAamp® DNA Stool Mini Kit were selected for evaluation because these kits are manufactured specifically for DNA extraction from fecal samples. The DNA extraction kit with the highest analytical sensitivity (lowest $C_T$ or highest number of *Salmonella* gene copies/uL DNA) was used to extract DNA from all study samples.

A *Salmonella* Typhimurium isolate obtained from the UF Veterinary Hospitals Microbiology laboratory was streaked on CBA plates and incubated for 24 h at 37°C. The following day, one colony from the CBA plate was inoculated in 5 mL of TSB and incubated for 24 h at 37°C. The following day, ten-fold dilutions of the 5 ml TSB were performed in phosphate buffered saline (PBS). An aliquot of 50 uL of each dilution was spread on HEA plates using the standard spread plate technique and the agar plates were incubated at 37°C for 24 h. The following day, *Salmonella* colony forming units (CFU) on each plate were enumerated and the dilution with approximately $10^9$ CFU/mL was determined. Using a predetermined number of CFU ($10^9$ CFU/mL), 1 ml of the selected dilution was used to spike 1 g of horse feces. The spiked horse feces were collected from a clinically normal horse. A horse was classified as clinically normal based on physical examination findings and absence of signs of GI disease, diarrhea, fever, or leucopenia, and after testing *Salmonella*-negative by bacteriological culture and real-time PCR on five consecutive fecal samples collected at 24 h intervals. Each spiked fecal sample was diluted in 10 ml of TTB and incubated at 37°C for 24h.

Thereafter, the 10 mL of TTB was aliquoted into nine samples (1 mL each) and stored for DNA extraction using the three different kits (three samples per kit). Three trials of the above experiment were performed. Real-time PCR was performed on the DNA samples and $C_T$ (number of cycles required for the fluorescent signal to cross the threshold) for the 3 different extraction kits were compared for each trial and with all trials combined to determine the kit with the lowest $C_T$. The kit with the lowest $C_T$ values was selected and used to extract DNA from all the study samples.

**Evaluation of PCR assay detection limit**

The detection limit of the PCR assay was evaluated using *Salmonella* organisms in liquid media (PBS) and fecal samples. The PCR assay detection limit using *Salmonella* organisms suspended in PBS was determined as described previously[10,143,144] with modifications. A *Salmonella* Typhimurium isolate (UF Veterinary Hospitals Microbiology laboratory, Gainesville, FL) was streaked on CBA plates and incubated for 24 h at 37°C. The following day, one colony from the CBA plate was inoculated in 5 mL of TSB and incubated for 24 h at 37°C. This was then diluted 10-fold in PBS. An aliquot of 50 uL of each dilution was spread on HEA plates using the standard spread plate technique and the agar plates were incubated at 37°C for 24 h. The following day, *Salmonella* colony forming units on each HEA plate were enumerated twice and the dilution with approximately $10^9$ CFU/mL was determined. Using the selected dilution with $10^9$ CFU/mL, a ten-fold dilution was performed with PBS. The dilutions were aliquoted, stored at -20°C, and later DNA was extracted using the selected optimal DNA extraction kit. Three trials of this experiment were performed. Real-time PCR was performed in duplicates for each DNA sample using both sets of

selected primers and probes. *Salmonella* Typhimurium DNA was used as a positive control and deionized water as a negative control.

The PCR assay detection limit using spiked fecal samples was determined as described previously with modifications.[143,144] Enumeration of *Salmonella* colony forming units ($10^9$ CFU/mL), was performed as described under evaluation of PCR assay detection limit using PBS (above). Using the selected dilution (with $10^9$ CFU/mL), a ten-fold dilution was performed with PBS. One mL of each dilution was used to spike 1 g of horse feces. The spiked horse feces were collected from a clinically normal horse. A horse was classified as clinically normal based on physical examination findings and absence of signs of GI disease, diarrhea, fever, or leucopenia, and after testing *Salmonella*-negative by bacteriological culture and real-time PCR on five consecutive fecal samples collected at 24 h intervals. Each spiked fecal sample was diluted in 10 mL of TTB and incubated at 37°C for 24h. The following day, the TTB was aliquoted, stored at -20°C, and later DNA was extracted using the selected optimal DNA extraction kit. Three trials of this experiment were performed. Real-time PCR was performed in duplicates for each DNA sample using both sets of selected primers and probes. *Salmonella* Typhimurium DNA was used as a positive control and deionized water as a negative control.

**Detection of *Salmonella* DNA in study samples**

To minimize observation bias, each broth sample was independently masked before DNA extraction and real-time PCR testing. After PCR testing, results where unmasked before data analysis.

Each study sample was tested in duplicates using the two selected primer sets. Results were reported qualitatively (positive or negative). A threshold cycle value of 37

was considered a positive result for each of the two selected primer sets based on the standard curve for each primer set where the lowest concentration of template was detected at a threshold cycle value of 37 (1x10e-5 ng/ul; *Salmonella* DNA).

**Data Collection**

For each study horse, the following data were collected: medical record number, admission date, discharge date, duration of hospitalization, age, gender, breed, presenting complaint, clinical findings at admission and during hospitalization (colic: yes, no; diarrhea: yes, no; fever: yes, no; leucopenia: yes, no), clinical procedures during hospitalization (treatment with antimicrobials: yes, no; surgery: yes, no), diagnosis at time of discharge, type of sample collected (fecal sample or rectal swab), fecal sample identification number (i.e., 1,2,3), date fecal sample was collected, number of fecal samples collected, culture results (*Salmonella* positive or negative), and PCR results (*Salmonella* positive or negative). For horses that tested positive by bacteriological culture, information on *Salmonella* isolates was collected including serogroup, serotype, fecal sample number that first tested positive, hospital day when first test-positive sample was collected, and number of hospital days when first test-positive sample was collected.

**Data Analysis**

The Wilcoxon Signed Rank test was used to compare mean $C_T$ values for the UltraClean® fecal kit and QIAamp® DNA Stool Mini Kit to determine the kit with the lowest $C_T$. These data were analyzed as paired data at each of the 3 trials performed (each trial on a separate plate) because efficiency of a PCR reaction and therefore $C_T$ values can vary from plate to plate.

Among GI inpatients, the epidemiologic sensitivity of the PCR assays were calculated by dividing the number of horses that tested positive to PCR on 1 or more samples by the total number of horses classified as true-positives. Among, GI and non-GI inpatients, the epidemiologic specificity was calculated by dividing the number of horses that tested negative to PCR on all samples by the total number of horses classified as true-negatives.

The positive predictive value of the PCR assays were calculated by dividing the number of horses that tested positive to both PCR and culture on 1 or more samples by the total number of horses that tested positive to PCR. The negative predictive value of the PCR assays were calculated by dividing the number of horses that tested negative to both PCR and culture on 1 or more samples by the total number of horses that tested negative to PCR.

## Results

### Bioinformatics Analysis

A summary of the blast results for individual primers and probes are shown in Table 4-2. Two sets of primers and probes were selected from published PCR studies performed in horses. The criteria for selection involved blasting the published primers and probes to determine the degree of similarity and homology of *Salmonella* against bacterial nucleotide sequences.

In addition, published conventional PCR primers and real-time PCR primers and probes were blasted as a combination (ie., forward/reverse and forward/probe/reverse). When primers for the histidine transport gene (Cohen et al. 1996)[11] were blasted as forward/reverse, the number of *Salmonella* hits/total number of organism hits was 33/264 or12%. When each set of real time primers and probes were blasted as

forward/probe/reverse, the number of *Salmonella* hits/total number of organism hits was highest for invA (78/100 or 78%; Pusterla et al. 2009[10]) followed by sip (24/41 or 59%; Kurowski et al. 2002[12]), invA (67/152 or 44%; Bohaychuk et al. 2007[13]), spaQ (47/145 or 32%; Kurowski et al. 2002[12]), and invE (2/12 or 16%; Kurowski et al. 2002[12]).

Overall, the two sets of primers and probes that had a combination of maximal identity with *Salmonella* spp and least identity with non-*Salmonella* organisms were those published by Pusterla et al. 2009[10] and Bohaychuk et al. 2007.[13] Nucleotide sequences of the two selected sets of primers and probes, and the universal bacteria primer used as a control are shown in Table 4-3. The selected probes target the invasion A (invA) gene of *Salmonella.* The invA gene is located on pathogenecity island 1 of the *Salmonella* chromosome and is essential for epithelial invasion, and is present in all invasive strains of *Salmonella* and absent from closely related organisms such as *E. coli.*[191-193] To verify the existence of sequence alignments with non-*Salmonella* organisms, the invA gene (Genbank accession number U43271; 1950 bp) was cut into five overlapping fragments and blasted. Results are presented in Table 4-4. The invA gene had sequence alignment with one non-*Salmonella* organism (*Photobacterium profundum*; a Gram-negative bacteria found mainly in marine environments).

The existence of sequence alignments with non-*Salmonella* organisms for the histidine transport operon gene (Genbank accession number V01373.1; 3700 bp) was also investigated. This investigation was performed because this gene had been used in previous studies that produced a high proportion of PCR false-positive results (26/152 or 17% and 76/105 or 72%, respectively), when bacteriologic culture results were used as the gold standard.[8,11] A summary of the blast results are shown in Table 4-4. Results

from this investigation revealed that the histidine transport operon gene had sequence alignments with several non-*Salmonella* organisms (including *Escherichia coli, Enterobacter cloacae, Shigella sonnei*, *Shigella flexneri, Shigella boydii, Escherichia fergusonii, Citrobacter koseri, Citrobacter rodentium, Cronobacter turicensis*, Serratia sp., *Burkholderia cenocepacia*, *Enterobacter asburiae, Dickeya dadantii, Pantoea ananatis, Pectobacterium wasabiae, Pseudomonas syringae, Pseudomonas brassicacearum, Pantoea vagans, and Erwinia pyrifoliae*).

**Optimization of Primer and Probe Concentration**

First, each set of selected primers was evaluated using SYBR green. For each set of primers, a primer optimization matrix was used to determine the minimal primer concentration that yielded the minimal $C_T$ and maximal $\Delta$Rn. Primer (forward and reverse) concentrations of 300, 100, and 30 nmols for primers 1, 2 and 3 respectively were optimal. For each of the three primer sets, non-specific binding was not observed at the different primer concentrations. Figures 4-2, 4-3 and 4-4 show dissociation curves for primer sets 1-3.

Each set of primers and probes was evaluated using TaqMan probe chemistry. A primer optimization matrix was used to determine the minimum primer concentration that yielded the minimum $C_T$ and maximum $\Delta$Rn. Thereafter, using the selected primer concentration, varying probe concentrations were used to determine the optimal probe concentration (that yielded the minimum $C_T$ and maximum $\Delta$Rn). Optimal primer (forward and reverse) concentrations for primers 1, 2, and 3 were 300, 100, and 30 nmols respectively. Optimal probe concentrations for probe 1, 2, and 3 were 250, 100, and 250 nmols respectively.

The efficiency of the PCR assay was determined for each set of primers/probes by using seven logs of 10 fold dilutions of *Salmonella* DNA (10 ng/µL) to generate standard curves. For each set of primers/probes, the slope of the log-linear phase and $R^2$ values showed that amplification of the PCR assay was efficient, as shown in figure 4-5 (Primer set 1: Slope = -3.55, Intercept = 18.12, $R^2$ = 0.99) and figure 4-6 (primer set 2: Slope = -3.56, Intercept = 19.38, $R^2$ = 0.99) .

**Analytical Specificity and Sensitivity**

The two selected sets of primers/probes were specific for *Salmonella*; there was no cross-reactivity with any of the enteric and non-enteric non-*Salmonella* organisms that were tested. All 13 *Salmonella* isolates tested positive using the two selected sets of primers/probes.

**Comparison of the Analytical Sensitivity of DNA Extraction Kits**

For primer sets 1 and 2, mean $C_T$ values for the three DNA extraction kits (DNeasy™ tissue kit, UltraClean® fecal kit, and QIAamp® DNA Stool Mini Kit) were compared for each of the 3 trials that were performed in triplicates to determine the kit with the lowest $C_T$ (Tables 4-5 and 4-6). The UltraClean® fecal kit had significantly lower mean $C_T$ values compared to the QIAamp® DNA Stool Mini Kit using primer set 1 ($P$ < 0.009) or primer set 2 ($P$ < 0.009). No $C_T$ results were reported for the DNeasy™ tissue kit, suggesting that this kit could not extract *Salmonella* DNA from horse fecal samples that were spiked and enriched. When this experiment was re-run using samples in which PBS was spiked with *S*. Typhimurium and horse fecal samples that were spiked with *S*. Typhimurium and enriched, $C_T$ values were reported for spiked PBS and still no $C_T$ values were reported for spiked fecal samples. Results of the third primer set (universal bacteria primer) used as a control of the efficiency of DNA purification

showed that no bacterial DNA was extracted from spiked horse fecal samples that were enriched but bacterial DNA was extracted from spiked PBS. On the basis of the results of the three DNA kits, the UltraClean® fecal kit had the highest sensitivity (lowest mean $C_T$ values) and was used to extract DNA from all the study samples. DNeasy™ tissue kit was not used further for extraction of *Salmonella* DNA from horse fecal samples that were spiked and enriched.

**PCR Assay Detection Limit**

The assay detected as low as < 1 colony forming unit when *Salmonella* DNA was purified from PBS using the UltraClean® fecal DNA extraction kit.

The assay detected as low as < 1 colony forming unit when *Salmonella* DNA was purified from spiked fecal samples using the UltraClean® fecal DNA extraction kit.

**Detection of *Salmonella* DNA in Study Samples**

Study fecal samples were collected from 93 horses over a 12 month period and the 93 horses included 20 horses that presented with GI signs and tested positive for *Salmonella* on at least 1 fecal or swab sample, 43 horses that presented with GI signs and tested negative for *Salmonella* on at least 3 fecal or swab samples, and 30 horses that presented without GI signs and tested negative for *Salmonella* on 5 fecal samples.

Horse breeds sampled included the following: Quarter Horses (19); Thoroughbred (16); Cross (9); Warm-blood (8); Percheron (5); American Paint Horse (4); Arabian (3); Hanoverian (3); Pony (3); Appaloosa (3), Tennessee Walking Horse (3); and Others (17). Median age of study horses was 9 yrs ($1^{st}$ quartile = 3.5, $3^{rd}$ quartile = 13; range = ≤ 1 yr, 33). Thirty-five of the 93 sampled horses were females, 46 were castrated males, and 12 were non-castrated males.

The median number of samples collected from horses that presented with GI signs and tested positive was 3 (1st quartile = 2, 3rd quartile = 4.5), for horses that presented with GI signs and tested negative was 4 (1st quartile = 3, 3rd quartile = 5), and for horses that presented without GI signs and tested negative was 5 (1st quartile = 5, 3rd quartile = 5).

Horses that were classified as true-positives (with GI signs and tested positive for *Salmonella,* n=20) presented for colic (18/20), diarrhea (1), and fever (1). Horses that were classified as true-negatives (without GI signs and tested negative, n=30) presented with lameness problems (8), ocular problems (16; melting corneal ulcer (7), ocular discharge (1), stromal abscess (1), uveitis (2), conjunctivitis, tearing/swelling/blepharospasm (5)), and other problems (6; septic carpus (1), canker/white line disease (1), splint bone removal (1), foreign body in hind foot (1), inguinal hernia surgery (1), hind limb abscess (1)).

**Relative Specificity**

**Non-GI horses.** The relative specificity of the assay using primer set 1 for samples # 1 to 5 varied from 26/30 (87%) to 30/30 (100%) (Table 4-7). Overall, 7/150 samples were classified as false-positive on real-time PCR, compared to bacteriological culture. The median threshold cycle ($C_T$) values for these 7 samples was 34.70 (1st quartile = 33.28, 3rd quartile = 35.29; range = 31.98 to 37.00).

Relative specificity of the assay using primer set 2 for samples # 1 to 5 varied from 29/30 (97%) to 30/30 (100%). Overall, 5/150 samples were classified as false-positive on real-time PCR, compared to bacteriological culture. The median $C_T$ for these 5 samples was 33.19 (32.34, 34.48; 32.33 to 35.31).

The relative specificity for the real-time PCR assay when using both primer sets 1 and 2 for samples # 1 to 5 varied from 26/30 (87%) to 30/30 (100%).

**GI horses.** The relative specificity of the assay using primer set 1 for samples # 1 to 3 varied from 36/41 (88%) to 42/43 (98%) (Table 4-8). Overall, 10/129 samples were classified as false-positive on real-time PCR, compared to bacteriological culture. The median $C_T$ values for these 10 samples = 35.39 (1st quartile = 26.93, 3rd quartile = 36.00; range = 22.03, 36.95).

Relative specificity of the assay using primer set 2 for samples # 1 to 3 varied from 36/42 (86%) to 41/43 (95%). Overall, 12/129 samples were classified as false-positive on real-time PCR, compared to bacteriological culture. The median $C_T$ for these 12 samples was 34.45 (1st quartile = 30.57, 3rd quartile = 36.09; range = 21.44, 36.70).

The relative specificity for the real-time PCR assay when using both primer sets 1 and 2 for samples # 1 to 3 varied from 36/43 (84%) to 41/43 (95%).

**Relative Sensitivity**

Relative sensitivity of the assay using primer sets 1 and 2 for samples # 1 to 7 = 20/20 (100%). Eighteen of 20 GI horses tested positive for the first time both by PCR and culture on the same sample number (Table 4-9). Two of 20 GI horses tested positive by PCR earlier, compared to culture (Table 4-9; horse No. 11 and horse No. 20).

**Positive and Negative Predictive Values**

**Non-GI horses.** For primer set 1, on the basis of a specificity of 28/30 (93%) for sample # 1 and sensitivity of 20/20 (100%), the positive and negative predictive values were estimated to be 20/22 (91%) and 28/28 (100%) respectively. For primer set 2, based on a specificity of 29/30 (97%) for sample # 1 and sensitivity of 20/20 (100%), the

positive and negative predictive values were estimated to be 20/21 (95%) and 29/29 (100%) respectively.

**GI horses.** For primer set 1, based on specificity of 42/43 (98%) for sample # 1 and sensitivity of 20/20 (100%), the positive and negative predictive values were estimated to be 20/21 (95%) and 42/42 (100%) respectively. For primer set 2, based on specificity of 41/43 (95%) for sample # 1 and sensitivity of 20/20 (100%), the positive and negative predictive values were estimated to be 20/22 (91%) and 41/41 (100%) respectively.

## Discussion

We have optimized and evaluated the diagnostic performance of a real-time PCR assay for detection of *Salmonella* spp in fecal specimens of hospitalized horses. The data presented also includes the side-by-side primer and probe optimization with both SYBR Green and TaqMan probe technology to confirm that no background fluorescence was generated that could produce false-positive results. Furthermore a bio-informatical analysis is presented that was performed against up-to-date bacterial sequence databases in order to assess assay specificity in silico. The assay includes enrichment of fecal samples in tetrathionate broth for 12-24 hours, extraction of DNA using a commercially available DNA kit (UltraClean fecal kit), and running of the real-time PCR assay. The two primer sets used in this PCR assay target the invasion A (invA) gene of *Salmonella.* The invA gene is located on Pathogenecity Island 1 of the *Salmonella* chromosome and is essential for epithelial invasion, and is present in all invasive strains of *Salmonella* and absent from closely related organisms such as *E. coli.*[191-193]

In the present study, all fecal or swab samples were enriched. Enrichment of fecal specimen prior to PCR amplification has been shown to improve sensitivity of a PCR assay for diagnosis of *Salmonella* spp in equine feces.[143] In a previous conventional PCR study in which enrichment was used prior to PCR amplification, PCR detected $10^0$ CFU of *S.* Enteritidis/g of feces,[143] and in another study, performed by the same author, where enrichment was not used, PCR detected $10^3$- $10^4$ CFU of *Salmonella* spp/g of feces.[7] In another study using real-time PCR to detect *Salmonella* spp in 911 fecal samples without enrichment and enriched (broth) samples, three of the 911 (0.3%) fecal samples without enrichment tested positive while 22 (2.4%) enriched broth samples tested positive.[10] In the present study, all fecal or swab samples were enriched in tetrathionate broth for 24 h at 37C as part of bacteriological culture procedures. The following day, part of the broth was sub-cultured on Hektoen Enteric Agar plates (for culture) and the remainder of the broth was aliquoted and stored at -80C and later used for *Salmonella* DNA extraction and real-time PCR testing.

In this study, the UltraClean® fecal kit had the highest sensitivity (lowest mean $C_T$ values) compared to the QIAamp® DNA Stool Mini Kit and DNeasy™ tissue kit when used to extract *Salmonella* DNA from spiked horse fecal samples that were enriched in tetrathionate broth. No $C_T$ values were reported for the DNeasy™ tissue kit indicating that this method of DNA extraction was not appropriate for extracting *Salmonella* DNA from horse fecal samples that were spiked and enriched. However when this experiment was re-run using samples in which PBS was spiked with *S*. Typhimurium or horse fecal samples that were spiked with *S*. Typhimurium and enriched, $C_T$ values were reported for spiked PBS but again no $C_T$ values were reported for spiked and

enriched fecal samples. These results suggest that horse fecal samples that are enriched may contain inhibitors of PCR which could not be removed using the DNeasy™ tissue kit. This suggestion was supported by results of the third primer set (universal bacteria primer) that was used as a control of the efficiency of DNA purification and as an indicator of fecal inhibition. No $C_T$ values were reported using the third primer set. When this experiment was re-run using samples in which PBS was spiked with *S*. Typhimurium or horse fecal samples that were spiked with *S*. Typhimurium and enriched, $C_T$ values were reported for spiked PBS but again no $C_T$ values were reported for spiked and enriched fecal samples. The finding in the present study that the DNeasy™ tissue kit could not extract *Salmonella* DNA from spiked horse fecal samples that were enriched differs from the finding in a previous real-time PCR study.[12] In that study, the relative sensitivity and specificity of a PCR assay when using three different commercially available DNA extraction kits to extract *Salmonella* DNA from enrichment broth was compared. Different sensitivities and specificities for the PCR assay were reported for the 3 kits: 100 and 98%; 80 and 100%; and 93 and 100%, and the DNeasy™ tissue kit had a sensitivity and specificity of 100 and 98% and was considered optimal.[12]

In the present study, a bioinformatics analysis was performed to investigate if the target gene, invA, was specific for *Salmonella enterica*. In addition, the histidine transport operon gene targeted in previous studies[8,11] that produced a high proportion of PCR false-positive results (26/152 or 17% and 76/105 or 72%, respectively) was investigated. Results showed that the invA gene had sequence alignment with one non-*Salmonella* organism only; *Photobacterium profundum*. This organism is a Gram-

negative bacterium found mainly in marine environments and was not considered biologically relevant to horses. However, results for the histidine transport operon gene revealed that this gene had sequence alignments with several non-*Salmonella* organisms including *Escherichia coli, Enterobacter spp, Shigella spp,* and *Pseudomonas spp.* Some of these non-*Salmonella* organisms are biologically relevant because they can be found in horses. These results may explain the high proportion of PCR false-positive results identified in previous studies.[8,11]

In the present study, the overall relative specificity of the real-time PCR assay when using primer set 1 for non-GI and GI-negative horses was 87 to100% and 88 to 98% respectively, compared to bacteriologic culture. A previous real-time PCR study[10] that targeted the invA gene and used the same primer set reported a specificity of 904/905 or 99% when fecal samples without enrichment were used and 889/905 or 98% when fecal samples with enrichment were used. Although the results for specificity when using enriched fecal samples appear comparable, there are major differences in both studies. In our study, enriched fecal samples (only) were tested by culture and PCR. In addition, a total of 5 fecal samples collected at 24-hour intervals from non-GI equine inpatients were examined. In contrast, in the previous study[10], the 905 horses used to assess specificity included horses with or without colic, and only one fecal sample was tested from each horses. It is possible that some of these horses with colic were infected with *Salmonella* and could not be detected based on one culture result only. The lack of a gold standard definition for true-negatives, and the use of horses that tested culture-negative on one fecal sample only can lead to misclassification bias which produces inconclusive results.

Overall relative specificity of the assay when using primer set 2 for non-GI and GI-negative horses was 97 to100% and 86 to 95% respectively. A previous real-time PCR study that targeted the invA gene and used the same primer set reported a comparable specificity estimate of 339/345 or 98%.[13] However, in that study, fecal samples used to estimate specificity were collected from horses and the environment of pens of a horse feedlot. The clinical status of study horses was not reported; therefore it is not clear if horses in that study had colic with or without clinical signs of salmonellosis. In addition, in that study, only one fecal sample was collected from each study horse. The lack of a gold standard definition for true-negatives, and the use of horses that tested culture-negative on one fecal sample only can lead to misclassification bias which produces inconclusive results. In the present study, these two potential limitations were addressed by using multiple fecal samples collected from horses classified as true-negatives (defined as horses without signs of GI disease (including colic, diarrhea, fever, or leucopenia) that tested *Salmonella*-negative on 5 fecal samples by bacteriological culture).

For non-GI horses, a total of five samples were classified as PCR false-positive results using primer set 1 and primer set 2, and two additional samples were classified as false-positive by primer set 1. For GI negative horses, a total of ten samples were classified as PCR false-positive results using primer set 1 and primer set 2, and two additional samples were classified as false-positive by primer set 2. The $C_T$ values for these PCR false-positive samples were relatively high when compared to the cut-off $C_T$ value (≤ 37) used in the present study. The relatively high $C_T$ values for these samples may be explained by presence of small quantities of initial template DNA (*Salmonella*) in

these samples. False-positive results can represent replication of very small initial numbers of *Salmonella* DNA in broth samples.[10]

In this study, positive and negative predictive values for the PCR assay were estimated for primer set 1 and 2. For primer set 1, the positive and negative predictive values when using non-GI horses were 91 and 100% respectively, and the positive and negative predictive values when using GI negative horses were 95 and 100% respectively. For primer set 2, the positive and negative predictive values when using non-GI horses were 95 and 100% respectively, and the positive and negative predictive values when using GI negative horses were 91 and 100% respectively. From a clinician and hospital surveillance perspective, a PCR assay with a negative predictive value of 100% is important. This value indicates that the probability that a horse is not shedding *Salmonella* following a PCR negative test is high or equal to one. These results suggest that the PCR assay evaluated in the present study is reliable in ruling out horses that are not shedding *Salmonella* and can be used as a diagnostic tool in a *Salmonella* surveillance program.

The overall relative sensitivity of the real-time PCR assay when using primer set 1 was 100%. The high sensitivity estimate when using primer set 1 is likely explained by the fact that culture-positive samples from GI horses classified as true-positive were used to assess sensitivity. In addition, results of analytical sensitivity which included 13 *Salmonella* isolates from five different serogroups were all positive using primer set 1. In a previous real-time PCR study[10] that targeted the invA gene and used the same primer set, a sensitivity of 100% was reported, although the assessment was limited to 6

horses only that were classified as *Salmonella* culture-positive, and 4 of 6 horses had signs of GI disease.

The overall relative sensitivity of the real-time PCR assay when using primer set 2 was 100%. The high sensitivity estimate when using primer set 2 is likely explained by the fact that culture-positive samples from GI horses classified as true-positive were used to assess sensitivity. In addition, results of analytical sensitivity which included 13 *Salmonella* isolates from five different serogroups were all positive using primer set 2. The sensitivity estimate in the present study was comparable to that reported in a previous real-time PCR study[13] that targeted the invA gene and used the same primer set; sensitivity was 28/28 or 100%. In that study,[13] however, the clinical status (GI or non-GI) of horses used in the evaluation was not reported.

This study had several strengths and a few limitations. The main strength of this study was the use of standard definitions for horses classified as true-negatives and true-positives to assess specificity and sensitivity respectively. By using horses classified as true-negatives and true-positives, this study addressed the sampling limitations observed in previous PCR studies. In addition, the present study used two different groups of horses to assess specificity; non-GI horses that tested culture-negative on five samples and GI horses that tested culture-negative on three samples. The use of GI horses that tested negative on three culture samples allowed the evaluation of the diagnostic performance of the PCR assay using a representative population that is typically targeted in most hospital *Salmonella* surveillance programs. Therefore our study findings can be extrapolated to hospitalized horses with and without signs of GI disease. A potential limitation of this study is reported specificity and

sensitivity estimates cannot be extrapolated to other hospital settings because of differences in study populations and PCR technology (type of PCR machines and materials).

On the basis of the relative specificity and sensitivity estimates, and positive and negative predictive values determined using horses classified as true-negatives and true-positives, we have evaluated and optimized a real-time PCR protocol using either primer set 1 or 2 that is reliable, with a relatively high specificity and sensitivity compared to bacteriological culture. This real-time PCR protocol can be used as a surveillance tool to detect *Salmonella* spp in fecal specimens of hospitalized horses. Considering its advantage of being rapid (reporting laboratory results approximately 48 hours later), compared to culture, this PCR protocol can be used as a screening test that may be confirmed by culture if a samples tests PCR-positive. In addition, culture would provide additional data (antibiogram and serotyping data) that are needed to establish if there is an epidemiological relationship between *Salmonella* cases or isolates, thus providing evidence that *Salmonella* infection or colonization is nosocomial in origin.

Table 4-1. Primer optimization matrix used to determine the minimum primer concentration that yielded the minimum $C_T$ and maximum $\Delta$Rn.

| Forward Primer (nM) | Reverse Primer (nM) | | |
|---|---|---|---|
| | 50 | 300 | 900 |
| 50 | 50/50 | 50/300 | 50/900 |
| 300 | 300/50 | 300/300 | 300/900 |
| 900 | 900/50 | 900/300 | 900/900 |

Table 4-2. A summary of nucleotide blast results of published individual primers and probes

| Target Gene | Query Template | Total number of hits | Total number of organisms | Number of *Salmonella* hits | *Salmonella* % max identity (score range) | Number of non-*Salmonella* hits | Non-*Salmonella* % max identity (score range) | Reference number |
|---|---|---|---|---|---|---|---|---|
| histidine transport operon | Fwd | 198 | 196 | 32 | 95-100 | 164 | 94-100 | 11 |
| | Rev | 241 | 232 | 30 | 100 | 202 | 90-100 | |
| invE | Fwd | 256 | 251 | 2 | 100 | 249 | 93-100 | 12 |
| | Rev | 622 | 622 | 31 | 100 | 591 | 100 | |
| | Probe | 431 | 362 | 54 | 100 | 308 | 100 | |
| Sip | Fwd | 835 | 827 | 27 | 100 | 800 | 100 | 12 |
| | Rev | 457 | 448 | 26 | 100 | 422 | 94-100 | |
| | Probe | 659 | 659 | 25 | 90-100 | 634 | 94-100 | |
| spaQ | Fwd | 382 | 382 | 51 | 95-100 | 329 | 94-100 | 12 |
| | Rev | 270 | 270 | 49 | 100 | 206 | 93-100 | |
| | Probe | 483 | 483 | 51 | 100 | 430 | 94-100 | |
| invA | Fwd | 227 | 227 | 74 | 100 | 149 | 94-100 | 13 |
| | Rev | 643 | 643 | 61 | 100 | 571 | 94-100 | |
| | Probe | 314 | 314 | 80 | 95-100 | 231 | 94-100 | |
| invA | Fwd | 146 | 146 | 59 | 96-100 | 16 | 91-100 | 10 |
| | Rev | 390 | 390 | 66 | 88-100 | 324 | 94-100 | |
| | Probe | 171 | 124 | 71 | 90-100 | 53 | 94-100 | |

Table 4-3. Nucleotide sequences of the two selected sets of primers and probes and universal bacteria primer and probe

| Target gene | Reagent | Sequence | Reference # |
|---|---|---|---|
| invA | Fwd primer 1 | CATTTCTATGTTCGTCATTCCATTACC | 10 |
| | Rev primer 1 | AGGAAACGTTGAAAAACTGAGGATTCT | |
| | Probe 1 | TCTGGTTGATTTCCTGATCGCACTGAATATC | |
| invA | Fwd primer 2 | AACTTCATCGCACCGTCA | 13 |
| | Rev primer 2 | TATTGTCACCGTGGTCCAG | |
| | Probe 2 | TCGGCATCAATACTCATCTGTTTACCG | |
| Univ Bact | Fwd primer | GGATGATCAGCCACACTGGA | 190 |
| | Rev primer | CCAATATTCCTCACTGCTGCC | |
| | Probe | CCCGTAGGAGTCTGGACCGTGTCTCA | |

Table 4-4. A summary of nucleotide blast results for the invA and histidine transport operon genes

| Target Gene | Query Template (bp) | Total number of hits | Total number of organism hits | Number of *Salmonella* hits | *Salmonella* % max identity (score range) | Number of non-*Salmonella* hits | Non-*Salmonella* % max identity (score range) |
|---|---|---|---|---|---|---|---|
| invA (1950bp) | 1 – 452 | 70 | 70 | 70 | 94-100 | 0 | None |
| | 402 – 853 | 71 | 71 | 70 | 92-100 | 1 | 93 |
| | 803 – 1254 | 66 | 66 | 66 | 90-100 | 0 | None |
| | 1204 – 1655 | 67 | 67 | 67 | 91-100 | 0 | None |
| | 1605 – 1950 | 60 | 60 | 60 | 92-100 | 0 | None |
| histidine operon (3700bp) | 1 – 841 | 40 | 40 | 31 | 91-100 | 9 | 77-85 |
| | 741 – 1581 | 33 | 33 | 28 | 90-100 | 5 | 81-88 |
| | 1481 – 2321 | 33 | 33 | 26 | 91-100 | 7 | 72-95 |
| | 2221 – 3061 | 100 | 74 | 26 | 94-100 | 48 | 73-85 |
| | 2961 – 3700 | 31 | 31 | 27 | 91-100 | 4 | 76-79 |

Table 4-5. Mean $C_T$ values for the DNeasy tissue kit, UltraClean fecal kit, and QIAamp DNA Stool Mini Kit for 3 trials performed in triplicates using Primer set 1.

| Trial | DNeasy Kit Mean $C_T$ | UltraClean Kit Mean $C_T$ | QIAamp Kit Mean $C_T$ |
|---|---|---|---|
| 1 | | 17.17 | 19.13 |
| 1 | | 17.59 | 18.81 |
| 1 | | 16.70 | 19.27 |
| 2 | | 16.25 | 18.80 |
| 2 | | 16.61 | 18.76 |
| 2 | | 16.07 | 18.51 |
| 3 | | 15.88 | 19.43 |
| 3 | | 16.12 | 19.51 |
| 3 | | 16.09 | 19.12 |

Table 4-6. Mean $C_T$ values for the DNeasy tissue kit, UltraClean fecal kit, and QIAamp DNA Stool Mini Kit for 3 trials performed in triplicates using Primer set 2.

| Trial | DNeasy Kit Mean $C_T$ | UltraClean Kit Mean $C_T$ | QIAamp Kit Mean $C_T$ |
|---|---|---|---|
| 1 | | 16.80 | 19.56 |
| 1 | | 17.29 | 19.02 |
| 1 | | 16.70 | 19.13 |
| 2 | | 15.85 | 18.32 |
| 2 | | 16.11 | 19.02 |
| 2 | | 15.64 | 18.26 |
| 3 | | 15.48 | 19.16 |
| 3 | | 15.88 | 19.74 |
| 3 | | 15.68 | 19.29 |

Table 4-7. Relative specificity of the real-time PCR assay determined using fecal samples from non-GI horses that tested culture-negative on five consecutive samples

| Fecal sample number | Specificity (%) Primer set 1 | Specificity (%) Primer set 2 |
|---|---|---|
| 1 | 28/30 (93) | 29/30 (97) |
| 2 | 26/30 (87) | 27/30 (90) |
| 3 | 30/30 (100) | 30/30 (100) |
| 4 | 29/30 (97) | 29/30 (97) |
| 5 | 30/30 (100) | 30/30 (100) |

Table 4-8. Relative specificity of the real-time PCR assay determined using fecal samples from GI horses that tested culture-negative on atleast three samples

| Fecal sample number | Specificity (%) Primer set 1 | Specificity (%) Primer set 2 |
|---|---|---|
| 1 | 42/43 (98) | 41/43 (95) |
| 2 | 38/42 (90) | 36/42 (86) |
| 3 | 36/41 (88) | 37/41 (90) |

Table 4-9. Bacteriological culture and real-time PCR results for fecal samples collected from GI horses that tested *Salmonella*-positive and were classified as true-positives (n=20)

| Horse | Samp #1 | Samp #2 | Samp #3 | Samp #4 | Samp #5 | Samp #6 | Samp #7 |
|---|---|---|---|---|---|---|---|
| 1 | - | - | - | - | - | - | + |
| 2 | + | + | - | | | | |
| 3 | + | + | | | | | |
| 4 | + | + | + | - | - | | |
| 5 | - | + | + | | | | |
| 6 | - | - | - | + | - | | |
| 7 | - | + | + | - | + | - | - |
| 8 | - | - | + | | | | |
| 9 | - | + | + | | | | |
| 10 | - | - | + | | | | |
| 11 | - | + | + | | | | |
| 12 | - | - | - | + | - | | |
| 13 | - | + | - | | | | |
| 14 | - | - | + | | | | |
| 15 | + | | | | | | |
| 16 | - | + | | | | | |
| 17 | | - | + | | | | |
| 18 | - | + | | | | | |
| 19 | - | + | | | | | |
| 20 | - | + | | | | | |

Samples that tested positive by real-time PCR using primer set 1 and 2 are highlighted in gray. Horse number 11 and 20 tested culture-negative and PCR-positive on sample number 1.

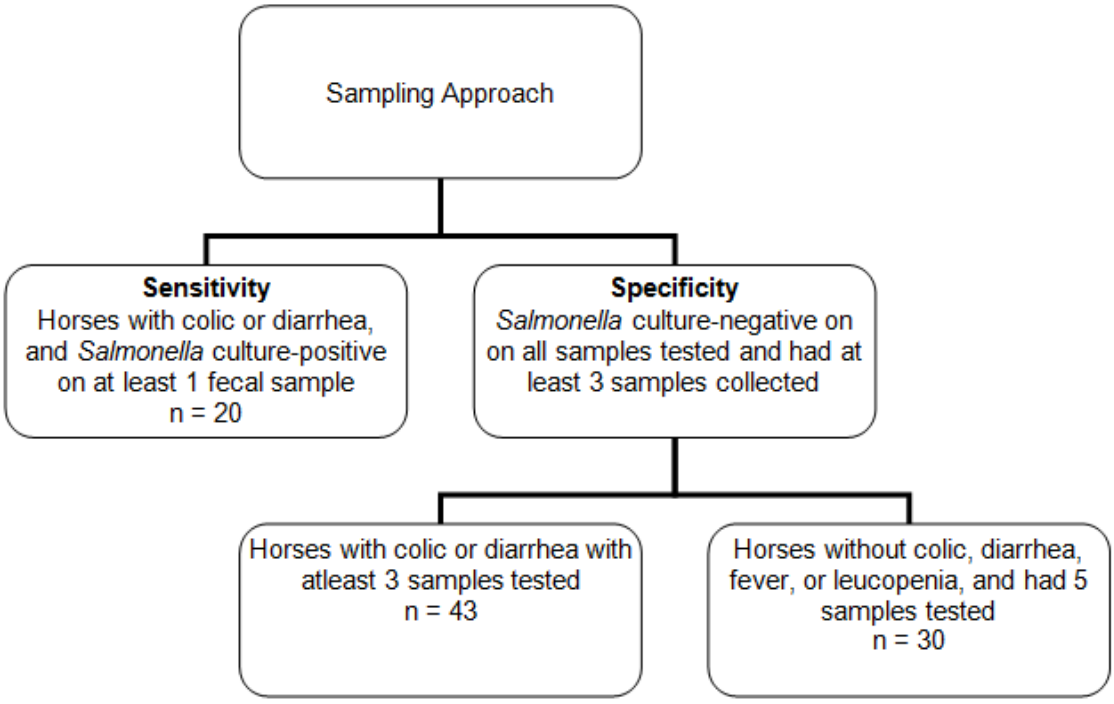


Figure 4-1. Sampling approach used to estimate the relative sensitivity and specificity of the real-time-PCR assay.

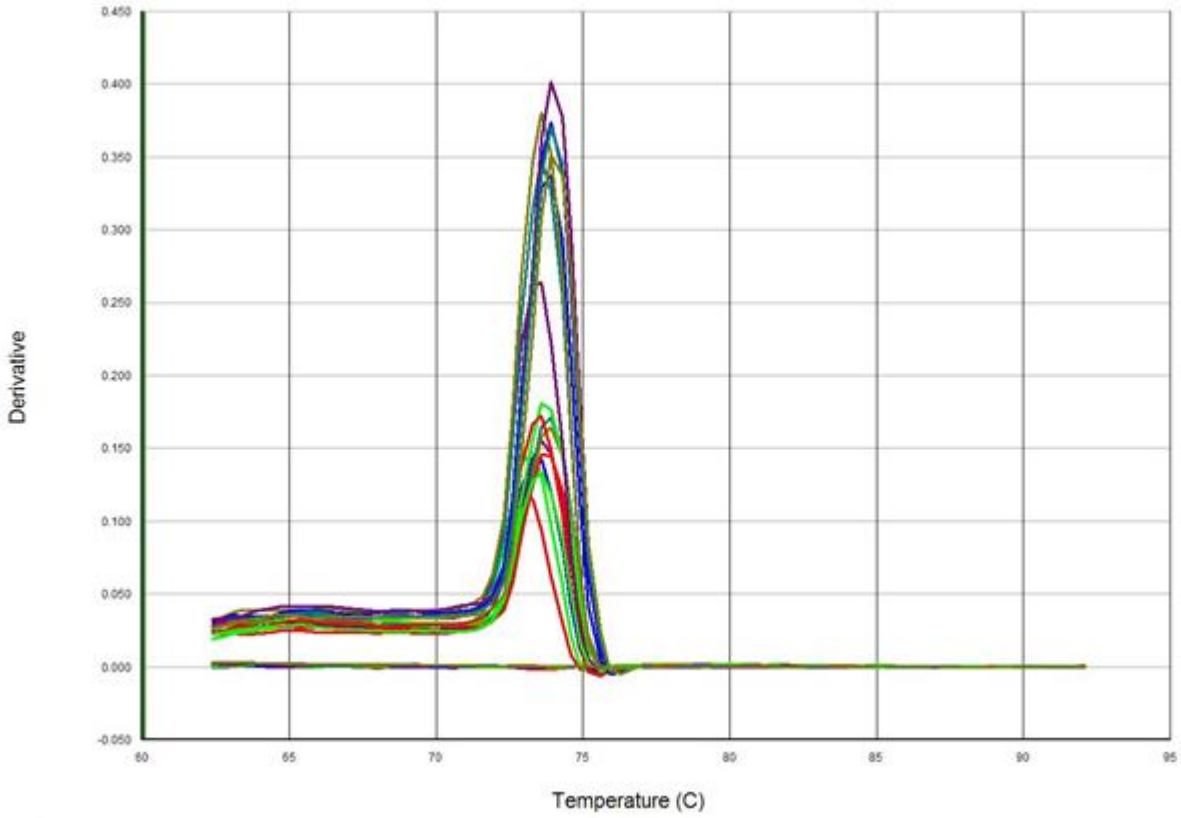


Figure 4-2. Dissociation curve analysis for primer set 1 using SYBR Green: showing one melting peak for the primer concentrations that were evaluated, indicating absence of non-specific primer binding.

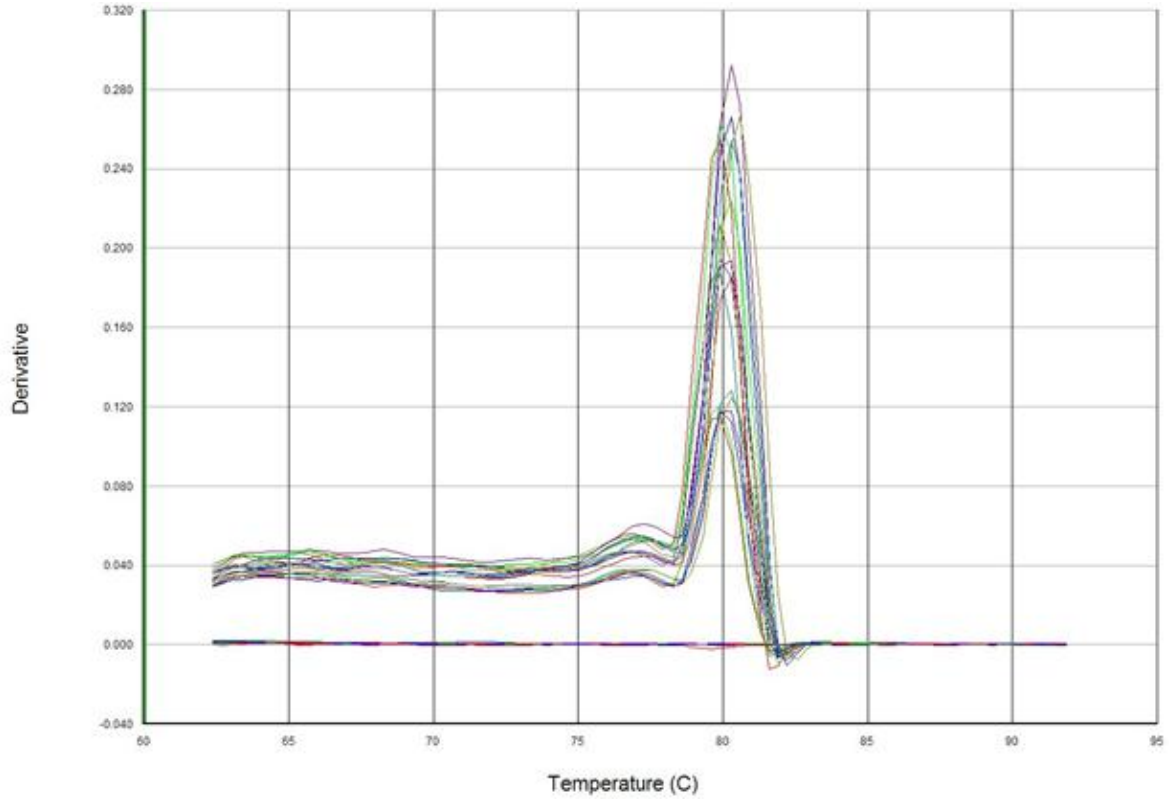


Figure 4-3. Dissociation curve analysis for primer set 2 using SYBR Green: showing one melting peak for the primer concentrations that were evaluated, indicating absence of non-specific primer binding.

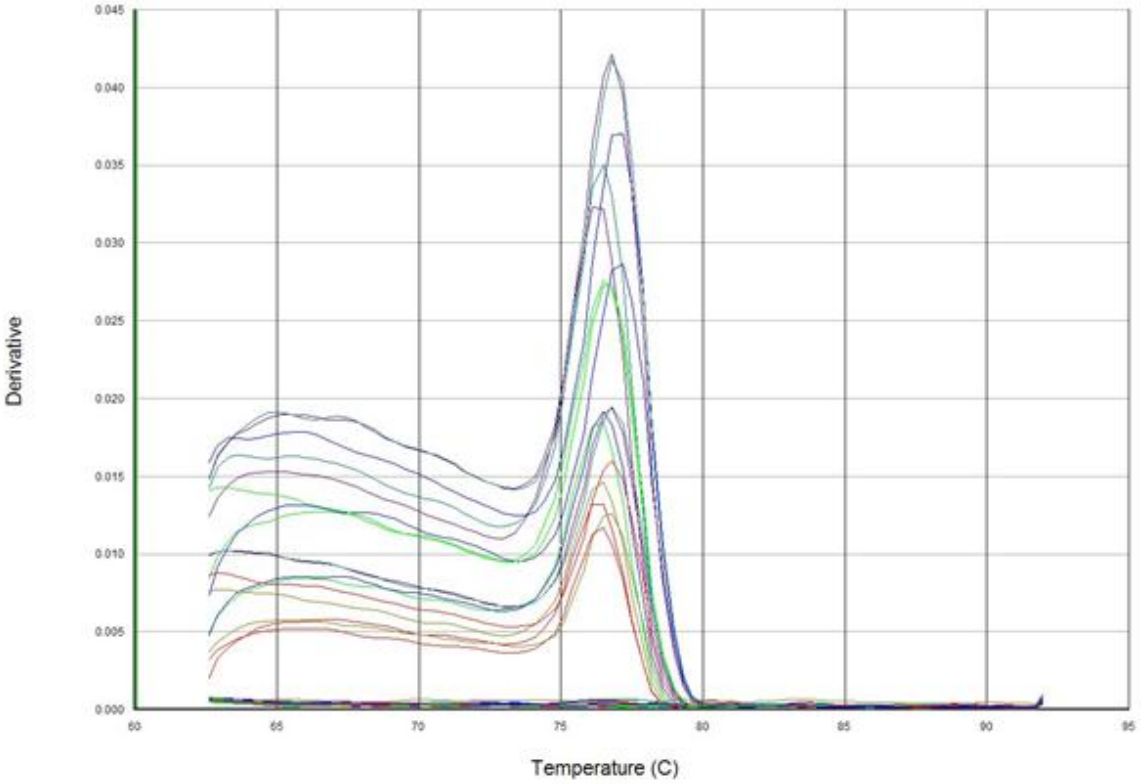


Figure 4-4. Dissociation curve analysis for the universal bacteria primer set using SYBR Green: showing one melting peak for the primer concentrations that were evaluated, indicating absence of non-specific primer binding.

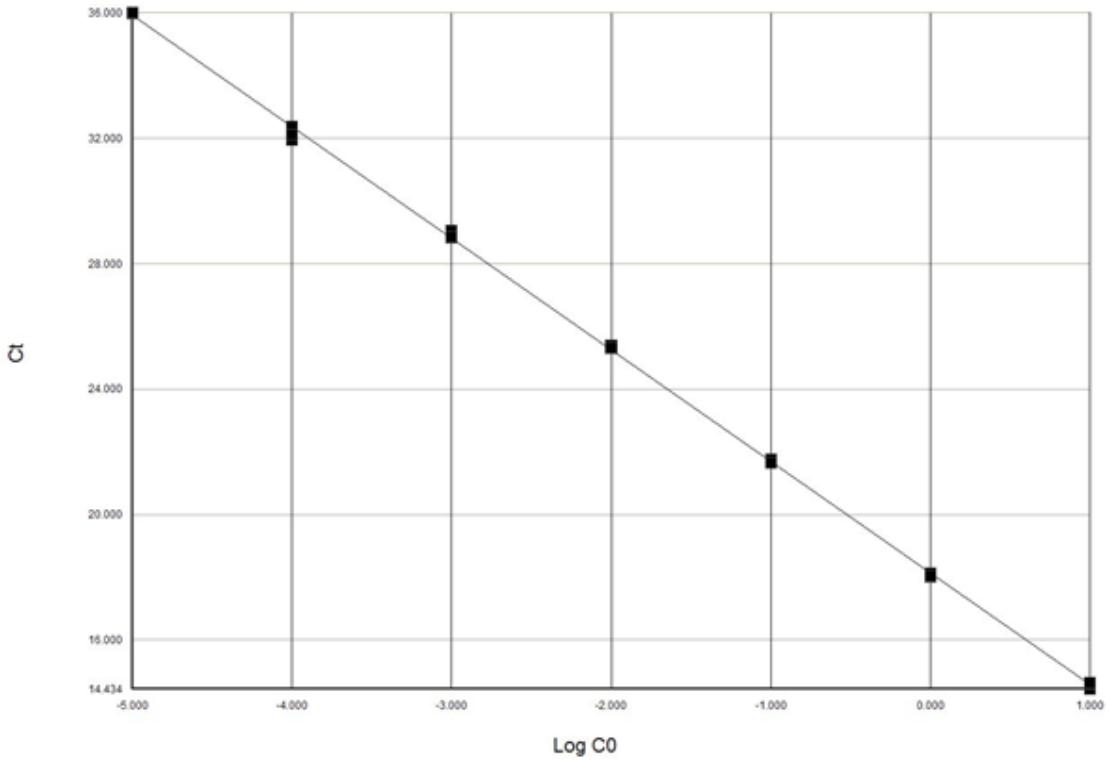

Figure 4-5. Standard curve for primer set 1

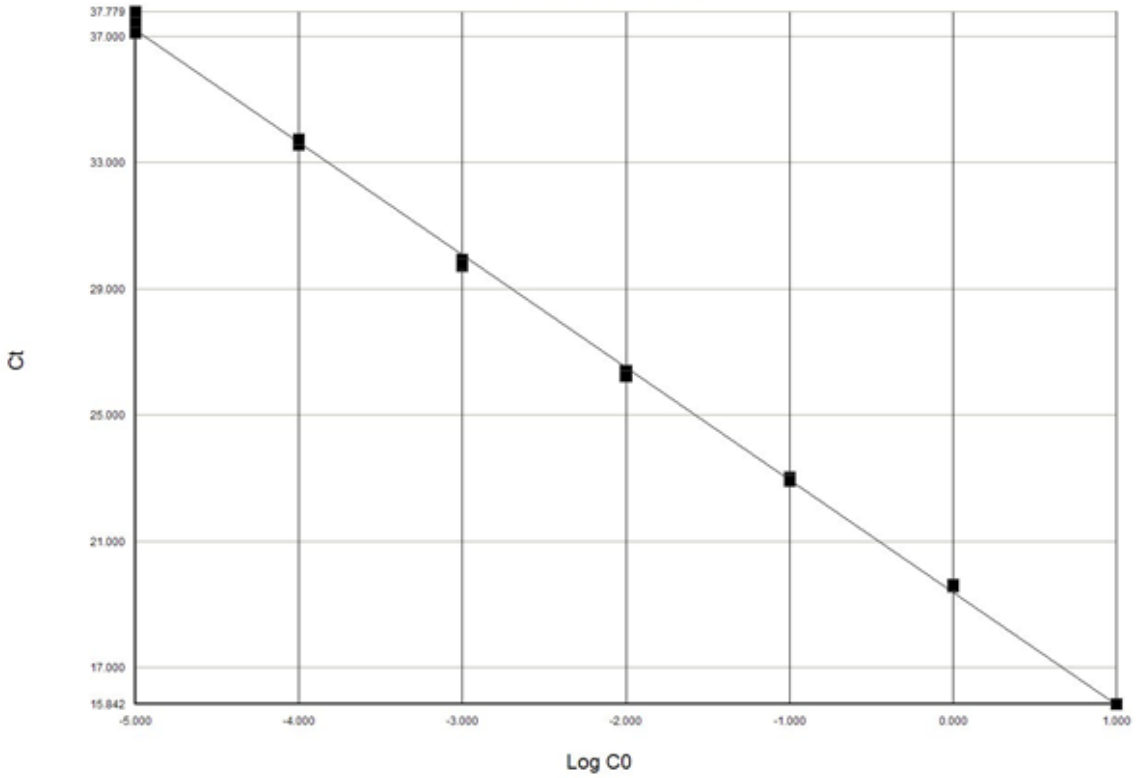

Figure 4-6. Standard curve for primer set 2

# CHAPTER 5
# AWARENESS AND RELEVANCE OF HOSPITAL SURVEILLANCE AND INFECTION CONTROL SERVICES AMONG REFERRAL VETERINARIANS AND CLIENTS

At a referral hospital, referring veterinarians (RDVMs) and clients/animal owners play a vital role in a hospital surveillance and infection control program. Veterinarians participate in the program by referring clients to a tertiary care hospital because of its specialized expertise and need for specialized care. In turn, clients bring high risk patients such as those with signs of gastrointestinal (GI) disease that may be targeted in a hospital surveillance and infection control program. At some hospitals, clients pay for costs related to surveillance and infection control procedures. At the UF LAH, horses that present with signs of GI disease are hospitalized for 3-4 days on average and 2 fecal samples are collected and submitted for bacteriological culture testing, resulting in a cost of approximately $56 ($28 per sample) that is paid by the client.

At a referral hospital, information about hospital surveillance and infection control and its importance to RDVMs and clients is promoted in various ways. At the UF LAH, educational efforts about the program include provision of an infection control brochure to all new clients to educate them about the infection control measures that are instituted at the hospital to optimize patient care. In addition, every time a patient tests positive for *Salmonella,* the attending clinician immediately updates the affected clients and RDVMs about the patient *Salmonella* shedding status and infection control measures that were implemented in the hospital for that patient. At the time of discharge, clients with *Salmonella*-positive horses are given a *Salmonella* fact sheet by the attending clinician. The purpose of the fact sheet is to educate the client about the potential risks of having a *Salmonella*-positive horse on a farm and measures that

should be implemented at the farm-level to reduce the risk of exposure to humans and other farm animals.

One element of an animal disease surveillance system is evaluation and feedback. Evaluation of the performance of a hospital surveillance program may involve assessing the frequency of nosocomial infections within a given period of time, frequency of environmental contamination with selected pathogens, compliance level by hospital clinicians and personnel, and level of awareness about the program among RDVMs and clients who refer or bring patients to the hospital. To have an efficient program, it is important that feedback is collected from all individuals involved in the program including clinicians, hospital personnel, RDVMs and clients. Considering the role played by RDVMs and clients, it is important to know the level of awareness and perceptions about the relevance of a hospital surveillance and infection program among this group. This information can be used to guide decision-making on hospital policy issues related to surveillance and infection control and for streamlining costs and efficiency of services provided to clients.

The objective of this study was to assess the awareness and relevance of a hospital surveillance and infection control program among referral veterinarians and clients who refer or send horses to a referral hospital for veterinary care.

## Materials and Methods

### Study Population

All clients (n= 3095) who sent at least one equine patient to the UF LAH for veterinary care during July 2007 through July 2011 and whose contact information (mail/email address) was available were considered for inclusion in the study. Contact information (mail/email address) for eligible clients was obtained from hospital records

for clients that brought equine patients to the UF LAH during July 2007 through July 2011.

All RDVMs (n= 567) who referred equine patients to UF LAH in the past and whose contact information (mail/email address) was available were considered for inclusion in the study. Contact information (mail/email address) for eligible RDVMs was obtained from a list of RDVM contacts kept by the UF LAH and only RDVMs that referred horses to the LAH in the past were eligible. Clients and RDVMs with incorrect or ineligible contact information were excluded. This study protocol was approved by the Institutional Review Board at the University of Florida (Protocol # 2011-U-0729).

**Study Design**

An electronic and mail survey was conducted to assess the awareness and relevance of the UF LAH surveillance and infection control program among referral veterinarians and clients who refer or send horses to the UF LAH for veterinary care. Because surveys generally have low response rates, a convenience sampling approach was used to select eligible RDVMs and clients, and all selected study participants were sent surveys.

Two separate 2-page questionnaires were designed for RDVMs and clients (Appendix A & B). The RDVM and clients surveys consisted of 15 and 14 questions respectively, most of which were closed ended, and both surveys required at most ten minutes to complete. The RDVM questionnaire was pretested by administering questionnaires to 100 RDVMs during the UF Veterinary Hospitals referring veterinarian appreciation day on June 25$^{th}$ 2011. Thirty five of 100 questionnaires were completed. Based on the pretest responses, questions in the survey instruments were revised to improve clarity. The client questionnaire was not pretested.

The electronic (email) survey was sent on September 9, 2011 to all enrolled RDVMs (n=242) and clients (n=483) that had email addresses. The electronic correspondence included an introductory email and a web-link to the survey. The introductory letter explained the purpose of the study and assured the respondents that identifying information and responses would remain confidential. A remainder email communication was sent 10-days later to urge the respondents to complete the survey. After the second email communication, data from all respondents were entered into an excel database for analysis. Personal identifying information (names) was requested in the electronic surveys and this was delinked by assigning codes to each respondent prior to data entry.

The mail survey was mailed out on November 21, 2011 to all enrolled RDVMs (n=225) and clients (2612) that did not participate in the electronic survey, or that did not have an email address but had a physical mailing address (and were never contacted during the time when the electronic survey was administered). A cover letter and a postage-paid return envelope were included in the mailing. The cover letter described the purpose of the study and assured the respondents that identifying information and responses would remain confidential. Respondents remained anonymous. To avoid duplication of respondents, identifying information (codes) was printed on each mail survey and used to identify the respondents during data analysis. One month was allowed for responses to be returned by mail. Remainder mail communication was not sent. Data for the respondents were entered into an excel database for analysis.

**Data Collection**

Briefly, the survey instrument for RDVMs focused on three major areas and respondents were asked questions on the following variables (Appendix A): (1) general

information: county in which practice is based; years in practice; type of clinical practice; animal species seen at practice; number of horses referred to the UF Hospital for veterinary care in the last two years; (2) awareness:  awareness that the UF LAH operates a surveillance and infection control program; awareness that the UF LAH has an infection control committee; awareness that the UF LAH has an infection control officer; awareness that horses presenting with signs of gastrointestinal tract disease are sampled and tested for diagnosis of *Salmonella* shedding during hospitalization; awareness that horses with diarrhea, fever and leucopenia, or that test positive to *Salmonell*a shedding at admission or during hospitalization are placed in isolation; awareness that every time there is evidence that a horse has potentially acquired a nosocomial *Salmonella* infection during hospitalization, enhanced infection control measures are implemented immediately; (3) relevance:  importance of being informed by a UF Hospital attending clinician when a horse (that you referred) tests positive to *Salmonella* shedding; importance of sampling and testing horses for *Salmonella* shedding; and importance of a referral hospital operating a surveillance and infection control program.

The survey instrument for clients focused on three major areas and respondents were asked questions on the following variables (Appendix B): (1) general information: county in which horse farm/premises is located; number of horses on premises; main use of horses on premises; number of horses brought to the UF Hospital for veterinary care in the last two years; (2) awareness:  awareness that the UF LAH operates a surveillance and infection control program; awareness that the UF LAH has an infection control committee; awareness that the UF LAH has an infection control officer;

awareness that horses presenting with signs of gastrointestinal tract disease are sampled and tested for diagnosis of *Salmonella* shedding during hospitalization; awareness that horses with diarrhea, fever and leucopenia, or that test positive to *Salmonell*a shedding at admission or during hospitalization are placed in isolation; awareness that every time there is evidence that a horse has potentially acquired a nosocomial *Salmonella* infection during hospitalization, enhanced infection control measures are implemented immediately; (3) relevance:  importance of being informed by a UF Hospital attending clinician when a horse (that you referred) tests positive to *Salmonella* shedding; importance of sampling and testing horses for *Salmonella* shedding; and importance of a referral hospital operating a surveillance and infection control program.

**Data Analysis**

Descriptive statistics were determined for each variable of interest. Frequency distributions were determined for the categorical variables (yes/no). Medians, first quartiles, and third quartiles were calculated for continuos variables (e.g., number of horses on farm, number of practice years). Comparisons between continuos variables were made using the wilcoxon rank sum test (e.g., number of years in practice for RDVMs and whether they found testing to be expensive (yes/no)). Comparisons between categorical variables were made using chisquare.

## Results

Overall, 92 of 567 (16%) surveys were returned by RDVMs and 594 of 3095 (19%) were returned by clients during November 2011 through January 2012. Electronic survey responses from 18/242 RDVMs and 95/483 clients were received. Mail survey responses from 39/225 RDVMs and 499/2612 clients were received. Following

pretesting of the RDVM questionnaire in the early phase of the study, 35/100 responses were received and these data were added to data from the electronic and mail surveys in the analysis.

Response trend for the mail surveys was as follows. Of the 39 mail responses from RDVMs, 2 responses were received one week after mailing, 22 responses after two weeks, 7 responses after 3 weeks, 5 responses after 4 weeks, and the remaining 3 responses were received between weeks 5-7. Of the 499 mail responses from clients, 51 responses were received one week after mailing, 269 responses after two weeks, 94 responses after 3 weeks, 33 responses after 4 weeks, and the remaining 52 responses were received between weeks 5-10.

**General Information for Referral DVMs**

Most RDVMs who responded to the survey had their practiced based in Marion county (17/92 or 22%) (Table 5-1). The median number of years in practice was 22 (first quartile = 10; third quartile = 30). The median number of horses referred by RDVMs to the UF LAH in the last two years was 5 (2, 10). Most RDVMs classified their practiced as ‘mobile services only’ (n = 47 or 51%).

**General Information for Clients**

Among clients who responded to this survey, the main location of horse farms in Florida was Marion County (69/594 or 12%) (Table 5-2). The main single use of horses on their premises was ‘pleasure’ (73/594 or 13%). The median number of horses on the premises was 5 (2, 10), and the median number of horses brought to the UF LAH in the last two years for veterinary care was 1 (1, 1).

**Awareness**

Among RDVMs and clients who responded to the survey, more RDVMs (68%) were aware that the UF LAH operates a surveillance and infection control program, compared to clients (39%) ($P < 0.05$) (Table 5-3).

**Relevance**

Almost all RDVMs (97%) and clients (97%) considered it important to be informed by the UF Hospital attending clinician when a horse (their patient) tested positive to *Salmonella* shedding at admission or during hospitalization (Table 5-4). Both RDVMs (99%) and clients (96%) considered testing of horses with colic or diarrhea for early detection of *Salmonella* shedding in feces upon admission and during hospitalization to be justified. However, more clients (25%) considered the cost of testing (~ $56) to be expensive, compared to RDVMs (11%) ($P < 0.05$). The number of horses owned by clients was similar among clients who considered the cost expensive (Median = 5 horses; 3, 12), compared to those that did not (median = 4 horses; 2, 10). The median number of years in practice was lower among RDVMs that found cost of testing expensive (median = 9 years; 5, 15), compared to RDVMs that did not (24; 12, 30) ($P = 0.01$).

Using a scale of 1 (not important) to 10 (very important), both RDVMs (median = 10; 1$^{st}$ quartile = 8; 3$^{rd}$ quartile = 10) and clients (median = 10; 8, 10) considered it very important that a referral hospital operates a surveillance and infection control program. Finally, most RDVMs (88%) and clients (82%) responded that if the UF LAH were to experience an outbreak of salmonellosis and was forced to close temporarily for cleaning and disinfection of hospital facilities, they would consider referring horses to the hospital again after re-opening.

## Discussion

In this study, a survey was conducted to assess awareness and relevance of a hospital surveillance and infection control program among referral veterinarians and clients who refer or send horses to a referral hospital for veterinary care. Overall, most (7 of every 10) RDVMs were aware that the referral hospital operates a surveillance and infection control program. In contrast, few clients (4 of every 10) were aware. Both RDVMs and clients indicated that it is very important that a referral hospital operates a surveillance and infection control program.

Among RDVMs and clients who responded to the survey, 7 of every 10 RDVMs were aware that the referral hospital operates a surveillance and infection control program, compared to only 4 of every 10 clients. Currently, educational efforts targeting clients at the UF LAH include provision of an infection brochure to new clients and a *Salmonella* fact sheet to clients with *Salmonella*-positive horses. The finding that only 4 of every 10 clients are aware of the program suggests that more education efforts are required.

Almost all RDVMs and clients considered it important to be informed by the hospital attending clinician when a horse tested positive to *Salmonella* shedding at admission or during hospitalization. In addition, both RDVMs and clients considered testing of horses with colic or diarrhea for early detection of *Salmonella* shedding in feces upon admission and during hospitalization to be justified. These findings indicate that RDVMs and clients understand the potential benefits to patient care of having a hospital surveillance program. In addition, these findings suggest that clinician-RDVM/client interaction may be an important avenue for creating awareness about the program.

More clients (2 to 3 of every 10) considered the cost of testing to be expensive, compared to RDVMs (1 of every 10). At the UF LAH, clients pay for hospital charges related to testing for surveillance purposes and for isolation stall fees. Cost of testing and isolation varies depending on duration of hospitalization. On average, horses that present with signs of gastrointestinal disease are hospitalized for 3-4 days and two fecal samples are collected and submitted for bacteriological culture testing, resulting in a cost of approximately $56 ($28 per sample). Isolation stall fees for patients that test *Salmonella*-positive or have diarrhea or fever and leucopenia are $115 per day. In this study, it is not known if the demographics of clients who considered cost of testing to be expensive were different from those who did not. Among RDVMs, number of years in practice (≤ 9 years) was associated with the response that cost of testing was expensive.

Both RDVMs and clients considered it very important that a referral hospital operates a surveillance and infection control program, and most RDVMs and clients responded that if the hospital were to experience an outbreak of salmonellosis and was forced to close temporarily for cleaning and disinfection of hospital facilities, they would consider referring horses to the hospital again after re-opening. This finding again suggests that RDVMs and clients appreciate the benefits of having a hospital surveillance and infection control program, and efforts that are aimed at optimizing patient care.

The results of this study were subject to a number of limitations. First, the response rates were low (16% for RDVMs and 19% for clients) and this may have resulted in non-response bias. In this study, follow up contact to solicit for responses

was only performed for electronic surveys but not mail surveys due to financial constraints. It is not known if the demographics of RDVMs and clients that responded were different from those who chose not to participate. It is also not known if a higher response rate in the present study would have provided different results.

In conclusion, this study revealed that (1) a high frequency of clients who responded to this survey were not aware that the referral hospital operates a surveillance and infection control program to reduce the risk of hospital-acquired infections caused by pathogens such as *Salmonella*; (2) most RDVMs and clients find the frequency of testing for *Salmonella* justified and not expensive; and (3) both RDVMs and clients considered it very important that a referral hospital operates a surveillance and infection control program.

Table 5-1. Survey responses from RDVMs: general information

| Variable | RDVMs N = 92 (100%) |
|---|---|
| In what county is your practice based? | |
| Marion | 17 (22) |
| Alachua | 7 (9) |
| Lake | 7 (9) |
| Hillsborough | 4 (5) |
| Other* | 43 (55) |
| What type of clinical practice do you work in? | |
| Mobile Services only | 47 (51) |
| Clinic & Mobile | 35 (38) |
| Clinic Services only | 7 (8) |
| Teaching/ Referral Hospital | 2 (2) |
| Other | 1 (1) |
| What animal species do you see at your practice? | |
| Equine Exclusive | 51 (55) |
| Large Animal, all species | 20 (22) |
| Mixed Practice | 17 (19) |
| Other | 4 (4) |
| How many years have you practiced veterinary medicine? | 22 (10, 30)** |
| In the last two years (eg, summer 2009 to summer 2011), approximately how many horses have you referred to the UF Hospital for veterinary care? | 5 (2, 10)** |

*Other = counties where the number of RDVMs < 4.
**Data presented as median (1st quartile, 3rd quartile).

Table 5-2. Survey responses from clients: general information

| Variable | Clients N = 594 (100%) |
|---|---|
| In what county is your horse farm/premises located? | |
| Marion | 69 (12) |
| Alachua | 41 (7) |
| Volusia | 31 (5) |
| Lake | 26 (4) |
| Duval | 26 (4) |
| Clay | 23 (4) |
| Brevard | 23 (4) |
| St. Johns | 23 (4) |
| Other* | 325 (55) |
| What is the main use of horses that you have on your premises? | |
| Pleasure | 73 (13) |
| Jumper/ Hunter | 44 (8) |
| Show/ Dressage | 39 (7) |
| Riding (Trail/ Hiking) | 28 (5) |
| Pleasure and Riding (Trail/Hiking) | 24 (4) |
| Pet | 20 (3) |
| Breeding | 18 (3) |
| Racing | 14 (2) |
| Breeding and Racing | 14 (2) |
| Other** | 311 (53) |
| How many horses do you have on your premises? | 5 (2,10)*** |
| In the last two years (eg, summer 2009 to summer 2011), approximately how many horses have you brought to the UF Hospital for veterinary care? | 1 (1, 1)*** |

*Other = the number of clients in a county < 23

** Other = the number of horses < 14

***Data presented as median (1st quartile, 3rd quartile).

Table 5-3. Survey responses from RDVMs and clients: awareness

| Variable | RDVMs N = 92 (100%) | Clients N = 594 (100%) |
|---|---|---|
| Did you know the UF LAH operates a surveillance and infection control program to reduce the risk of hospital-acquired infections caused by pathogens such as Salmonella? | | |
| No | 29 (32) | 358 (61) |
| Yes | 62 (68) | 227 (39) |
| **Variable** | **RDVMs N = 62 (100%)** | **Clients N = 227 (100%)** |
| Were you aware that the UF Large Animal Hospital has an infection control committee that meets quarterly (or more often) to assess the overall hospital infection control status? | | |
| No | 45 (75) | 162 (76) |
| Yes | 15 (25) | 52 (24) |
| Were you aware that the UF LAH has an infection control officer that coordinates day-to-day surveillance and infection control activities? | | |
| No | 39 (65) | 151 (71) |
| Yes | 21 (35) | 63 (29) |
| Did you know that horses presenting with signs of gastrointestinal tract disease are sampled and tested for diagnosis of Salmonella shedding at admission and during hospitalization? | | |
| No | 4 (7) | 87 (41) |
| Yes | 57 (93) | 128 (59) |
| Did you know that horses with diarrhea, fever and low white blood cell count, or that test positive to Salmonella shedding at admission or during hospitalization are placed in isolation? | | |
| No | 0 (0) | 61 (28) |
| Yes | 61 (100) | 154 (72) |
| Did you know that every time there is evidence that a horse has potentially acquired a hospital-acquired Salmonella infection during hospitalization, enhanced infection control measures are implemented immediately? | | |
| No | 7 (12) | 57 (27) |
| Yes | 54 (88) | 158 (73) |

Table 5-4. Survey responses from RDVMs and clients: relevance

| Variables | RDVMs N = 92 (100%) | Clients N = 594 (100%) |
|---|---|---|
| Is it important for you to be informed by the UF Hospital attending clinician when a horse (your patient) tests positive to *Salmonella* shedding at admission or during hospitalization? | | |
| No | 3 (3) | 16 (3) |
| Yes | 86 (97) | 570 (97) |
| On average, horses presenting with signs of gastrointestinal tract disease at the UF hospital are hospitalized for 5 days. This group of horses is sampled and tested for diagnosis of two fecal samples. The cost of testing to the client is $28 per sample or $56 for both samples? | | |
| Do you find this level of testing justified? | | |
| No | 1 (1) | 20 (4) |
| Yes | 90 (99) | 554 (96) |
| Do you find this cost expensive? | | |
| No | 81 (89) | 428 (75) |
| Yes | 10 (11) | 144 (25) |
| Some clients have expressed that they would not consider sending their horses to a referral hospital that does not operate a surveillance and infection control program because of the perceived risk of disease transmission. Do you feel the same way? On a scale of 1 (not important) to 10 (very important), how important is it for you that a referral hospital operates a surveillance and infection control program? | 10 (8, 10)* | 10 (8, 10)* |
| In the past, several veterinary hospitals have been forced to close temporarily for 1 to 3 months for cleaning and disinfection because of *Salmonella* outbreaks in horses or food animals. While we don't anticipate such an event to occur in our hospital, if the UF LAH were to experience an outbreak of salmonellosis and was forced to close temporarily for cleaning and disinfection of hospital facilities, would you consider referring horses to the hospital again after re-opening? | | |
| No | 5 (6) | 18 (3) |
| Yes | 80 (88) | 480 (82) |
| I am not sure | 6 (6) | 85 (15) |

*Data presented as median (1st quartile, 3rd quartile).

# CHAPTER 6
# SUMMARY AND RESEARCH CONCLUSIONS

This research work addressed four objectives. First, this research work provided a critical review and analysis of published studies that have contributed to the literature of epidemiology and infection control aspects of nosocomial *Salmonella* infections in hospitalized horses. The review was structured in three main parts: surveillance and infection control; epidemiological research; and a discussion of relevant infection control issues and areas of research that can further improve existing hospital surveillance and infection control programs. This critical review identified knowledge gaps in the epidemiology of *Salmonella* infections in hospitalized horses, some which were addressed in this research work.

Second, an investigation of the relationships between clinical signs, hematological and plasma chemistry parameters, and clinical procedures, and *Salmonella* shedding in GI horses with or without diarrhea was conducted. Study results indicated that among horses that tested positive for *Salmonella* ≤ 2 days after admission and had diarrhea, use of antimicrobials and anti-inflammatories before admission, high plasma triglycerides, low plasma sodium, low plasma protein, and high mean corpuscular hemoglobin concentration at admission were associated with *Salmonella* shedding. Among horses that tested positive for *Salmonella* ≤ 2 days after admission and without diarrhea, high plasma protein concentration and high plasma triglycerides at admission were associated with *Salmonella* shedding. Among horses without diarrhea that tested positive for *Salmonella* ≥ 4 days after admission, abdominal surgery and season were associated with *Salmonella* shedding. In conclusion, high plasma protein concentration, high plasma triglycerides, abdominal surgery and season are parameters that can be

considered for evaluation in hospital biosecurity standard operating procedures to identify equine GI inpatients at high risk of *Salmonella* shedding.

The third research objective was to assess the diagnostic performance of a real-time PCR assay for detection of *Salmonella* spp in feces of hospitalized horses, compared to bacteriological culture. Results indicated that the overall relative specificity of the PCR assay when using primer set 1 in horses without signs of GI disease that tested negative and horses with signs of GI disease that tested negative was 87 to100% and 88 to 98% respectively. The overall relative specificity of the PCR assay when using primer set 2 in horses without signs of GI disease that tested negative and horses with signs of GI disease that tested negative was 97 to100% and 86 to 95% respectively. The overall relative sensitivity of the assay when using primer set 1 or 2 in horses with signs of GI disease that tested positive was 100%. Based on the relative specificity and sensitivity estimates determined using horses classified as true-negatives and true-positives, this real-time PCR protocol (using primer set 1 or 2) is reliable, with a relatively high specificity and sensitivity compared to bacteriological culture. This real-time PCR protocol can be used as a surveillance tool to detect *Salmonella* spp in fecal specimen of hospitalized horses. Considering its advantage of being rapid (reporting laboratory results approximately 48 hours later), compared to culture, this PCR assay can be used as a screening test that may be confirmed by culture if a samples tests PCR-positive. In addition, culture would provide additional data (antibiogram and serotyping data) that are needed to establish if there is an epidemiological relationship between *Salmonella* cases or isolates, thus providing evidence that *Salmonella* infection or colonization is nosocomial in origin.

The fourth objective was to assess the awareness and relevance of a hospital surveillance and infection control program among referral veterinarians and clients who refer or send horses to a referral hospital for veterinary care. Survey results revealed that (1) most clients who responded to this survey were not aware that the referral hospital operates a surveillance and infection control program to reduce the risk of hospital-acquired infections caused by pathogens such as *Salmonella*; (2) Most RDVMs and clients find the frequency of testing for *Salmonella* justified and not expensive; and (3) both RDVMs and clients considered it very important that a referral hospital operates a surveillance and infection control program.

This research work addressed four important aspects that are relevant to the epidemiology, diagnostics, and surveillance and infection control of *Salmonella* infections in hospitalized horses. First, a critical review of the literature of the epidemiology and infection control aspects of nosocomial *Salmonella* infections in hospitalized horses was performed. This critical review identified several knowledge gaps, three of which were addressed in this research work. Second, an investigation of the relationships between clinical signs, hematological and plasma chemistry parameters, and clinical procedures, and *Salmonella* shedding in GI horses with or without diarrhea was performed. Findings from this investigation can be considered for use in current hospital surveillance programs to improve early detection of *Salmonella* shedding and permit rapid implementation of infection control measures. Third, an investigation was performed to assess the diagnostic performance of a real-time PCR assay for detection of *Salmonella* spp in feces of hospitalized horses, compared to bacteriological culture. Findings from this investigation revealed that the PCR protocol

that was evaluated was reliable, with a relatively high specificity and sensitivity compared to bacteriological culture. This real-time PCR protocol can be used as a surveillance tool to detect *Salmonella* spp in fecal specimen of hospitalized horses. Finally a survey was conducted to assess the awareness and relevance of a hospital surveillance and infection control program among referral veterinarians and clients. Findings from this survey can be used to justify the need for enhanced education efforts about the program among clients, guide decision-making on hospital policy issues related to surveillance and infection control, and streamline costs and efficiency of services provided to clients.

APPENDIX A
SURVEY FOR REFERRAL DVMS

S U R V E Y *…your feedback is appreciated!*

Please answer the questions in sections A, B, and C. This survey includes 15 questions and will take no more than 10 minutes of your time to complete.

Thank you for your participation!

**SECTION A: GENERAL INFORMATION**

1. In what county is your practice based? _____

2. How many years have you practiced veterinary medicine? [ ]

3. What type of clinical practice do you work in?
   Teaching/referral hospital [ ] Mobile services only [ ]
   Clinic services only [ ] Clinic & mobile [ ]
   Other (please specify)_____

4. What animal species do you see at your practice?
   Equine exclusive [ ] Bovine exclusive [ ]
   Large animal, all species [ ] Feline exclusive [ ]
   Small animal, all species [ ] Mixed practice [ ]
   Other (please specify) _____

5. In the last two years (eg, summer 2009 to summer 2011), approximately how many horses have you referred to the UF Hospital for veterinary care? [ ]

6. Did you know that the UF Large Animal Hospital operates a surveillance and infection control program to reduce the risk of hospital-acquired infections caused by pathogens such as *Salmonella*?
   No [ ] Yes [ ]

   If your answer to this question is No, please continue with question No. 12 in section C.

**SECTION B: AWARENESS**

7. Were you aware that the UF Large Animal Hospital has an infection control committee that meets quarterly (or more often) to assess the overall hospital infection control status?
   No [ ] Yes [ ]

8. Were you aware that the UF Large Animal Hospital has an infection control officer that coordinates day-to-day surveillance and infection control activities under the supervision of a hospital epidemiologist?
   No [ ] Yes [ ]

9. Did you know that horses presenting with signs of gastrointestinal tract disease are sampled and tested for diagnosis of *Salmonella* shedding at admission and during hospitalization?
   No [ ] Yes [ ]

10. Did you know that horses with diarrhea, fever and leukopenia, or that test positive to *Salmonell*a shedding at admission or during hospitalization are placed in isolation?
    No [ ] Yes [ ]

11. Did you know that every time there is evidence that a horse has potentially acquired a nosocomial *Salmonella* infection during hospitalization, enhanced infection control measures are implemented immediately (eg, footmats with disinfectant, use of gloves and gowns are mandatory on every large animal inpatient)?
    No [ ] Yes [ ]

**SECTION C: RELEVANCE**

12. Is it important for you to be informed by the UF Hospital attending clinician when a horse (that you referred) tests positive to *Salmonella* shedding at admission or during hospitalization?
    No [ ] Yes [ ]

13. On average, horses presenting with signs of gastrointestinal tract disease at the UF Hospital are hospitalized for 5 days. This group of horses is sampled and tested for diagnosis of *Salmonella* shedding at admission and an additional time during hospitalization for a total of two fecal samples. The cost of testing to the client is $28 per sample or $56 for both samples.

    Do you find this level of testing justified?
    No [ ] Yes [ ]

    Do you find this cost expensive?
    No [ ] Yes [ ]

14. Some clients have expressed that they would not consider sending their horses to a referral hospital that does not operate a surveillance and infection control program because of the perceived risk of disease transmission. Do you feel the same way? On a scale of 1 (not important) to 10 (very important), how important is it for you that a referral hospital operates a surveillance and infection control program?
    1[ ] 2[ ] 3[ ] 4[ ] 5[ ] 6[ ] 7[ ] 8[ ] 9[ ] 10[ ]

15. In the past, several veterinary hospitals in the US have been forced to close temporarily for 1 to 3 months for cleaning and disinfection because of *Salmonella* outbreaks in horses or food animals. While we don't anticipate such an event to occur in our hospital, if the UF Large Animal Hospital were to experience an outbreak of salmonellosis and was forced to close temporarily for cleaning and disinfection of hospital facilities, would you consider referring horses to the hospital again after re-opening?
    No [ ] Yes [ ] I am not sure [ ]

    If you have additional comments, please use the space below to share your feedback.

    | |
    |---|
    | |

    Thank you again for your participation!

APPENDIX B
SURVEY FOR CLIENTS

S U R V E Y *…your feedback is appreciated!*

Please answer the questions in sections A, B, and C. This survey includes 14 questions and will take no more than 10 minutes of your time to complete.

Thank you for your participation!

**SECTION A: GENERAL INFORMATION**

1. In what county is your horse farm/premises located? _____

2. How many horses do you have on your premises? [ ]

3. What is the main use of horses that you have on your premises?

| | | | | | |
|---|---|---|---|---|---|
| Pleasure | [ ] | | Pet | [ ] | |
| Racing | [ ] | | Show/dressage | [ ] | |
| Jumper/Hunter | [ ] | | Riding (trail, hiking) | [ ] | |
| Breeding | [ ] | | Retired | [ ] | |
| Roping | [ ] | | Other (please specify) | _____ | |

4. In the last two years (eg, summer 2009 to summer 2011), approximately how many horses have you brought to the UF Hospital for veterinary care? [ ]

5. Did you know that the UF Large Animal Hospital operates a surveillance and infection control program to reduce the risk of hospital-acquired infections caused by pathogens such as *Salmonella*?

   No [ ] Yes [ ]

   If your answer to this question is No, please continue with question No. 11 in section C.

**SECTION B: AWARENESS**

6. Were you aware that the UF Large Animal Hospital has an infection control committee that meets quarterly (or more often) to assess the overall hospital infection control status?

   No [ ] Yes [ ]

7. Were you aware that the UF Large Animal Hospital has an infection control officer that coordinates day-to-day surveillance and infection control activities under the supervision of a hospital epidemiologist?

   No [ ] Yes [ ]

8. Did you know that horses presenting with signs of gastrointestinal tract disease are sampled and tested for diagnosis of *Salmonella* shedding at admission and during hospitalization?

   No [ ] Yes [ ]

9. Did you know that horses with diarrhea, fever and low white blood cell count, or that test positive to *Salmonell*a shedding at admission or during hospitalization are placed in isolation?

No [ ] Yes [ ]

10. Did you know that every time there is evidence that a horse has potentially acquired a hospital-acquired *Salmonella* infection during hospitalization, enhanced infection control measures are implemented immediately (eg, footmats with disinfectant, use of gloves and gowns are mandatory on every large animal inpatient)?
    No [ ] Yes [ ]

**SECTION C: RELEVANCE**

11. Is it important for you to be informed by the UF Hospital attending clinician when a horse (your patient) tests positive to *Salmonella* shedding at admission or during hospitalization?
    No [ ] Yes [ ]

12. On average, horses presenting with signs of gastrointestinal tract disease at the UF Hospital are hospitalized for 5 days. This group of horses is sampled and tested for diagnosis of *Salmonella* shedding at admission and an additional time during hospitalization for a total of two fecal samples. The cost of testing to the client is $28 per sample or $56 for both samples.

    Do you find this level of testing justified?
    No [ ] Yes [ ]

    Do you find this cost expensive?
    No [ ] Yes [ ]

13. Some clients have expressed that they would not consider sending their horses to a referral hospital that does not operate a surveillance and infection control program because of the perceived risk of disease transmission. Do you feel the same way? On a scale of 1 (not important) to 10 (very important), how important is it for you that a referral hospital operates a surveillance and infection control program?
    1[ ] 2[ ] 3[ ] 4[ ] 5[ ] 6[ ] 7[ ] 8[ ] 9[ ] 10[ ]

14. In the past, several veterinary hospitals in the US have been forced to close temporarily for 1 to 3 months for cleaning and disinfection because of *Salmonella* outbreaks in horses or food animals. While we don't anticipate such an event to occur in our hospital, if the UF Large Animal Hospital were to experience an outbreak of salmonellosis and was forced to close temporarily for cleaning and disinfection of hospital facilities, would you consider referring horses to the hospital again after re-opening?
    No [ ] Yes [ ] I am not sure [ ]

    If you have additional comments, please use the space below to share your feedback.

    | |
    |---|
    | |

    Thank you again for your participation!

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# BIOGRAPHICAL SKETCH

Abel Ekiri received a degree in veterinary medicine from Makerere University, Kampala, Uganda in 2002. In 2006, he joined the University of Florida, Large Animal Hospital as the hospital Infection Control Officer. At the same time, he enrolled in graduate school and pursued Master of Science (MS) and Doctor of Philosophy (PhD) degrees with a concentration in epidemiology under the supervision of Dr. Jorge Hernandez. His MS and PhD research work focused on epidemiology, surveillance, and infection control of *Salmonella* infections in hospitalized horses. He received his MS degree in May 2008, and will complete the PhD in May 2012.