SEROTYPE DIVERSITY AND ANTIMICROBIAL RESISTANCE AMONG *SALMONELLA ENTERICA* ISOLATED FROM PATIENTS AT AN EQUINE REFERRAL HOSPITAL

A Thesis

by

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ABSTRACT

In horses, salmonellosis is a leading cause of life threatening colitis. Researchers have identified multidrug-resistant *Salmonella* among serotypes commonly associated with clinical disease in horses and humans. Few published studies have utilized whole genome sequencing (WGS) to evaluate *Salmonella* isolates from horses, though this approach is growing in application. Our aim was to evaluate the proportional morbidity attributed to phenotypic and genotypic antimicrobial resistance (AMR) patterns and serotypes of *Salmonella* from patients at an equine referral hospital in the southern United States.

A total of 255 *Salmonella* isolates were obtained from samples submitted to a clinical microbiology laboratory arising in patients with diagnoses of salmonellosis that were admitted to the hospital between January 8, 2007 and November 4, 2015. Phenotypic antimicrobial resistance profiles were determined by the Sensititre® system. WGS was used to confirm serotypes determined according to the Kauffmann White scheme and to determine antimicrobial resistance genes. Sequencing libraries were sequenced in the Illumina MiSeq platform. Phylogenetic analyses on the main serotypes were performed in Parsnp and visualized using FigTree.

The most common serotypes were Newport (18%), Anatum (14.1%) and Braenderup (11.4%). The majority of the isolates were pansusceptible 219, 10 were resistant to less than 3 antimicrobial groups, while 25 were multi-drug resistant (>3 antimicrobial groups). The most concerning group of resistance genes were

betalactamases (*bla*) such as *bla*$_{\text{CMY-2}}$, *bla*$_{\text{SHV-12}}$, *bla*$_{\text{CTX-M-27}}$ and *bla*$_{\text{TEM-1B}}$. The *qnr*B2 and *aac(6')-Ib-cr* genes were present in isolates with reduced susceptibility to ciprofloxacin. Additionally genes encoding resistance to gentamicin (*aph(3')-Ia*, *aac(6')-IIc*), streptomycin (*str*A and *str*B), sulfonamides (*sul*1), trimethoprim (*dfr*A), phenicol (*ca*tA), tetracyclines (*tet*(A) and *tet*(E)), and macrolides (*ere*(A)) were identified. The predominant replicon type was the conjugative plasmid I1 (10%) that often carries AmpC/ESBL betalactamases. Core-genome-based analyses revealed genetic associations among the strains that helped to rule in and rule out outbreaks.

The presence of AMR *Salmonella* in equine patients raises the risk of unsuccessful treatment and concern for potential zoonotic transmission to horse owners, attending veterinarians and hospital staff. Understanding the epidemiology of *Salmonella* in horses admitted to referral hospitals is important for the prevention, control, and treatment of salmonellosis in horses.

DEDICATION

I have always known that to get to the top you have to climb a long road, with obstacles, but when you reach the top of the journey it was simply worth it, and that if you trust, you can achieve even the unimaginable. I have never given up in my life when I have to fight, but even so, I could not have achieved it without the help of angels on earth who, in the most difficult moments gave me their hand and the strength to continue. This thesis is dedicated to my parents, especially my mom who, with her patience, support and warm love always believes and trusts in me and has driven me to fly to the highest skies, and who has made me understand that there are no limits to achieve your dreams, especially if you do it with your heart. To my lovely sisters Eylin, and Marce and my brother of heart Andres who gave me their support. To my loyal friends, who always give me their love and who are very proud of me. Thanks to Amelita, that little girl who will soon be sharing with us, and who in the days of maximum stress has managed to inspire me and give me joy. To Pilar, my beloved friend, who gave me all her support from the beginning, who has been my example, who showed me that with perseverance, enthusiasm, and dedication everything can be achieved. To Danny, my true and eternal love, who illuminated my life with his presence, and who through the clarity and transparency of his eyes showed me that love exists and that with love anyway is easier to walk, that efforts are worth it, that all things should always be done with great dedication and who has inspired me to be a better person with his love, support and advice.

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TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGEMENTS v

CONTRIBUTORS AND FUNDING SOURCES vii

TABLE OF CONTENTS viii

LIST OF FIGURES xi

LIST OF TABLES xv

CHAPTER I INTRODUCTION AND LITERATURE REVIEW 1

Overview 1
Etiology 1
Salmonella serotypes associated with horses 2
Clinical signs 3
Epidemiology 5
Risk factors at hospitals 6
Pathogenesis 7
Detection of *Salmonella* 8
Antimicrobial use and antimicrobial resistance 9
Antimicrobial resistance in *Salmonella* 10
Plasmids in *Salmonella* 15

CHAPTER II MATERIALS AND METHODS 18

Evaluation of the proportional morbidity attributed to each *Salmonella enterica* serotype 18
Sample set and isolates 18
DNA extraction for whole genome sequencing (WGS) 19
Whole genome sequencing using the Illumina MiSeq™ 20
Bioinformatics analysis of WGS data 21
Serotype analysis 22

Validation of serotypes through serotyping using the White-Kauffmann-Le Minor scheme and WGS 22
Plasmid analysis 22
Statistical Analysis 23
Evaluation of antimicrobial resistance patterns of *Salmonella* isolated from horses admitted to an equine referral hospital in the southern United States 23
Antimicrobial susceptibility testing 23
Controls 25
Bioinformatics analysis of WGS data 26
Distribution of temporally clustered cases of the major *Salmonella* serotypes throughout the years 26
Phylogenetic analysis 27

CHAPTER III RESULTS 28

Evaluation of the proportional morbidity attributed to each *Salmonella enterica* serotype 28
Breeds 28
Age 28
Complaints 30
Associations between age and clinical complaint 32
Associations between breed and clinical complaint 32
Serotypes 33
Associations between serotype and clinical complaint 34
Association between serotype and age of horse 34
Temporal trends of serotypes 35
Distribution of temporally clustered cases of the major *Salmonella* serotypes throughout the years 37
Plasmid analysis 39
Presence of plasmids between the serotypes 40
Evaluation of antimicrobial resistance patterns of isolated *Salmonella* from horses admitted to an equine referral hospital in the southern United States 44
Antimicrobial resistance patterns 44
Antimicrobial resistance genes 47
Beta-lactamase resistance genes 47
Quinolone resistance genes 49
Aminoglycoside resistance genes 50
Sulfonamide resistance genes 53
Folate pathway inhibitor resistance genes 54
Phenicol resistance genes 56
Tetracycline resistance genes 57
Macrolide resistance genes 59
Other resistance genes 60
Agreement between detected AMR genes and MICs values 62

Phylogenetic analysis 62
Geographical location of the phylogenetic clusters 73

CHAPTER IV DISCUSSION 79

Evaluation of the proportional morbidity attributed to each *Salmonella enterica* serotype 79
Evaluation of antimicrobial resistance patterns of isolated *Salmonella* from horses admitted to an equine referral hospital in the southern United States 86
Phylogenetic analysis 96
Salmonella Anatum 97
Salmonella Braenderup 98
Salmonella Newport 99

CHAPTER V CONCLUSIONS 100

REFERENCES 102

LIST OF FIGURES

Page

Figure 1. Antimicrobial resistance in bacteria. Material adapted from Tadvi, 2012 (55).....11

Figure 2. Nextera XT DNA library preparation workflow 21

Figure 3. Number of cases for each horse breed admitted to the hospital with differential diagnosis of salmonellosis. 29

Figure 4. Percentage of cases (out of 233) for each age category of horse admitted to the hospital with a differential diagnosis of salmonellosis. 30

Figure 5. Percentage of the most common complaints in horses admitted to the hospital with a differential diagnosis of salmonellosis between January 8, 2007 and November 4, 2015.....31

Figure 6. Percentage of presenting clinical complaints classified by affected system in horses admitted to the hospital with a differential diagnosis of salmonellosis between January 8, 2007 and November 4, 2015. 31

Figure 7. Frequency of detection of the main serotypes throughout the years of the study (2007-2015).....36

Figure 8. *Salmonella* Braenderup and *Salmonella* Newport case sequences by admission date in 2008. 37

Figure 9. *Salmonella* Braenderup case sequences per admission date in 2009.....38

Figure 10. *Salmonella* Anatum case sequences per admission date in 2015. 39

Figure 11. Number of detected replicons in *Salmonella* from horses admitted to the hospital with differential diagnosis of salmonellosis between January 8, 2007 and November 4, 2015.....40

Figure 12. Number of detected plasmid replicon types in the different *Salmonella* serotypes. 41

Figure 13. Number of detected plasmid replicon types in *Salmonella* Anatum.....42

Figure 14. Number of detected plasmid replicon types in *Salmonella* Typhimurium. 42

Figure 15. Number of detected plasmid replicon types in *Salmonella* Newport. 43

Figure 16. Number of detected plasmid replicon types in *Salmonella* Braenderup.....43

Figure 17. Heat map summary of serotypes, antimicrobial resistance phenotypes and AMR genotypes, among 36 *Salmonella* isolates exhibiting resistance to at least one antimicrobial. Also included are the corresponding Inc plasmid type (s). Blue and white squares denote the presence and absence of a specific feature, respectively. AUG2 = amoxicillin/clavulanic acid 2:1 ratio, AMP = ampicillin, AZI = azithromycin, AXO = ceftriaxone, CHL = chloramphenicol, CIP = ciprofloxacin, FIS = sulfisoxazole, FOX = cefoxitin, GEN = gentamicin, NAL = nalidixic acid, SXT = trimethoprim/sulfamethoxazole, STR = streptomycin, TET = tetracycline and XNL = ceftiofur. 46

Figure 18. Proportion of beta-lactamase genes detected in the *Salmonella* from patients at an equine referral hospital 48

Figure 19. Beta-lactamase genes detected in the main serotypes of strains of *Salmonella* from patients at an equine referral hospital. The numbers inside of the bars show the identification of the strains. 48

Figure 20. Relative proportion of quinolone genes (sums to 100%) detected in *Salmonella* from patients at an equine referral hospital in the southern United States. 49

Figure 21. Quinolone genes detected among the main serotypes of *Salmonella* isolated from patients at an equine referral hospital in the southern United States. The numbers inside of the bars show the identification of the strains. .50

Figure 22. Relative proportion of aminoglycoside genes (sums to 100%) detected in *Salmonella* from patients at an equine referral hospital in the southern United States. 51

Figure 23. Aminoglycoside genes detected among the main serotypes of strains of *Salmonella* from patients at an equine referral hospital. Numeric coding within each bar represent case numbers. The numbers inside of the bars show the identification of the strains. 52

Figure 24. Relative proportion of sulphonamide genes (sums to 100%) detected in *Salmonella* from patients at an equine referral hospital in the southern United States. 53

Figure 25. Sulfonamide genes detected among the main serotypes of strains of *Salmonella* from patients at an equine referral hospital in the southern

United States. The numbers inside of the bars show the identification of the strains. 54

Figure 26. Relative proportion of folate pathway inhibitors alleles (sums to 100%) detected in *Salmonella* from patients at an equine referral hospital in the southern United States. 55

Figure 27. Folate pathway inhibitors genes detected in the main serotypes of strains of *Salmonella* from patients at an equine referral hospital in the southern United States. The numbers inside of the bars show the identification of the strains. 55

Figure 28. Relative proportion of amphenicol genes (sums to 100%) detected in *Salmonella* from patients at an equine referral hospital in the southern United States. 56

Figure 29. Amphenicol genes detected in the main serotypes of strains of *Salmonella* from patients at an equine referral hospital in the southern United States. The numbers inside of the bars show the identification of the strains..... 57

Figure 30. Relative proportion of tetracycline genes (sums to 100%) detected in *Salmonella* from patients at an equine referral hospital in the southern United States. 58

Figure 31. Tetracycline genes detected in the main serotypes of strains of *Salmonella* from patients at an equine referral hospital in the southern United States. The numbers inside of the bars show the identification of the strains..... 58

Figure 32. Proportion of macrolide genes detected in *Salmonella* from patients at an equine referral hospital in the southern United States. 59

Figure 33. Macrolide genes detected in the main serotypes of strains of *Salmonella* from patients at an equine referral hospital in the southern United States. The numbers inside of the bars show the identification of the strains..... 60

Figure 34. Proportion of ansamycin alleles detected in *Salmonella* from patients at an equine referral hospital in the southern United States. 61

Figure 35. Ansamycin alleles detected in the main serotypes of strains of *Salmonella* from patients at an equine referral hospital in the southern United States. The numbers inside of the bars show the identification of the strains..... 61

Figure 36. Whole-genome SNP-based phylogenetic tree of 33 *Salmonella* Anatum and a *Salmonella* Anatum reference strain generated by Parsnp and visualized by FigTree. The branch lengths are expressed in terms of

changes per number of SNPs. The numbers in color show the bootstrap corresponding to the specific internal node. Strain names are marked with the colors red (reference), blue (MDR), green (resistant) and black (susceptible). Strain names are labeled with the year of admission of the patient to the hospital. Clusters are colored according to the phylogenetic group (clade). Cluster I includes: Sub-cluster I-a (pink), I-b (blue), I-c (purple) and I-d (green). Cluster II includes: Sub-cluster II-a (yellow) and II-b (red). The scale bar shows the estimated number of substitutions per SNP. 65

Figure 37. Whole-genome SNP-based phylogenetic tree of 27 *Salmonella* Braenderup and a *Salmonella* Braenderup reference strain generated by Parsnp and visualized using FigTree. The branch lengths are expressed in terms of changes per number of SNPs. The numbers in color show the bootstrap corresponding to the specific internal node. Strain names are marked with the colors red (reference), blue (resistant) and black (susceptible). Strains names are labeled with the year of admission of the patient to the hospital. Clusters are colored according to the phylogenetic group (clade). Cluster I includes: Sub-cluster I-a (cyan blue), I-b (purple), I-c (pink), I-d (yellow) and I-e is (green). Clade II (light violet). The scale bar shows the estimated number of substitutions per SNP. 69

Figure 38. Whole-genome SNP-based phylogenetic tree of 42 *Salmonella* Newport and *Salmonella* Newport reference strain generated by Parsnp. The branch lengths are expressed in terms of changes per number of SNPs. The tree was visualized using FigTree. Reference strain is marked with color red, blue stars (MDR) and black (susceptible). Strain names are labeled with the year of admission of the patient to the hospital. Clusters are colored according to the phylogenetic group (clade). Cluster I includes: Sub-cluster I-a (yellow) and I-b (cyan blue). Cluster II includes: Sub-cluster II-a (gray), II-b (rose), II-c (light green), II-d (emerald green), II-e (blue) and II-f (pink). The scale bar shows the estimated number of substitutions per SNP... 72

Figure 39. Location of genetically related cases of *Salmonella.* The size of the circle represents the number of cases. 75

Figure 40. Location of genetically related cases of *Salmonella* Anatum. The size of the circle reflects the number of cases in each location..... 76

Figure 41. Location of genetically related cases of *Salmonella* Braenderup. The size of the circle reflects the number of the cases in each location. 77

Figure 42. Location of genetically related cases of *Salmonella* Newport. The size of the circle reflects the number of cases. 78

LIST OF TABLES

Page

Table 1. Minimum Inhibitory Concentration range used in the sensititre CMV3AGNF plate.....25

Table 2. Frequency of *Salmonella* serotypes in horses admitted to the hospital with a differential diagnosis of salmonellosis between January 8, 2007 and November 4, 2015. 33

Table 3. Resistance patterns for the *Salmonella* strains isolated from equine samples submitted to the clinical microbiology laboratory between January 8, 2007, and November 4, 2015 that were tested for susceptibility to 14 antimicrobials. 45

Table 4. Genotype and phenotype comparison of 255 *Salmonella* strains obtain from equine samples submitted to the clinical lab between January 8, 2007, and November 4, 2015 63

Table 5. Equine cases of genetically related *Salmonella* Anatum from Cluster I and II by phylogeny. 67

Table 6. Equine cases of genetically related *Salmonella* Braenderup from Cluster I and II by phylogeny. 70

Table 7. Equine cases of genetically related *Salmonella* Newport from Cluster I and II by phylogeny. 74

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Overview

Salmonella is one of the most important foodborne pathogens in the world, estimated to cause 93.8 million human cases of gastroenteritis and leading to 155,000 deaths each year (1). Salmonellosis in horses is a leading cause of life threatening colitis. Salmonellosis produces economic losses related to treatment costs, morbidity, and mortality of valued horses (2).

Etiology

Salmonella enterica, first named as *Salmonella choleraesuis*, was discovered by Theobald Smith in 1885, where his first thought was that *Salmonella* was the cause of hog cholera. Later, it was shown that hog cholera was produced by a virus and *Salmonella* was an opportunistic bacterial pathogen. Although *Salmonella* was discovered by Smith, Daniel E. Salmon (Smith's chief), claimed credit for the discovery (3).

Salmonella are part of the family Enterobacteriaceae and are classified into serotypes (serovars) based on the lipopolysaccharide (O), flagellar protein (H), and occasionally the capsular (Vi) antigens (4). Salmonellae are Gram-negative rods, non-spore-forming, with diameters between 0.7 to 1.5μm and lengths of 2 to 5μm with a few exceptions, and mainly motile by flagellae. Salmonellae do not produce urease, oxidase,

or indole. Salmonellae are facultative anaerobic bacteria that can get energy from oxidation and reduction reactions using organic sources. The bacteria produce acid from glucose sources frequently with the production of gas (5). The *Salmonella* genus contains two species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* has 6 sub-species *(enterica, salamae, arizonae, diariazonae, houtonea and indica)* with more than 2,500 identified serotypes of *Salmonella enterica* (6). Serotyping is performed by phenotypic characterization of the O and H antigens through agglutination using the antigenic formulae of the *Salmonella* serovars (7). Molecular epidemiological studies using high-throughput genomic technologies (*Salmonella* genus project: http://www.sanger.ac.uk/Projects/*Salmonella*/) are helping to refine the classifications based on the evolution of the previous grouped *Salmonella* serotypes (8).

Salmonella **serotypes associated with horses**

Although the trends of dominant serotypes may vary across the years, the most common serotypes in clinical cases in horses in United States have been reported to be: Typhimurium (9), Newport, Javiana and Anatum. Newport, is one of the most reported serotypes related to outbreaks of nosocomial *Salmonella* infection in horses in the United States (10); however, there are other serotypes reported such as Infantis (11), Oranienburg, Agona (12) and Typhimurium (13). A national study conducted in 1998 (including 28 states) estimated the prevalence of fecal shedding in the equine population and identified 14 different serotypes, with the most frequent being Muenchen, Newport, Schwartzengrund and Typhimurium (14). There are host-restricted (HR) serotypes that

affect only one species, there are host-adapted (HA) serotypes that can cause severe systemic infection in various species, and there are un-restricted (UR) serotypes that can produce a self-limiting gastroenteritis in a broad range of species. In horses, the Abortusequi serovar is the only HR serotype that produces a systemic disease with the ability to proliferate in fetal tissues; however, the majority of the *Salmonella* in horses are UR serotypes such as Typhimurium, producing a persistent enteric colonization in the host, often without any major symptoms. UR serotypes have the ability to replicate because of their ability to adapt, while they can also develop a quick response from the immune system that can generate a faster clearance (15). Although HA serotypes are not common in horses, however the horses can become a vehicle in the distribution of these serotypes to other species. HA serotypes have more virulence factors and additional mechanisms of antimicrobial resistance that help them to survive (16).

Clinical signs

Horses with *Salmonella enterica* infections can exhibit a variety of clinical signs: from shedding bacteria in feces without apparent signs through to experiencing peracute death (17). Based on early experiments, there are four clinical syndromes described, as follows: 1) asymptomatic, 2) mild disease, 3) toxic enterocolitis, and 4) sepsis. Others symptoms include abortion and infectious cholangiohepatitis. Among asymptomatic horses, there have been two sub-categories recognized as: active carriers and silent or passive carriers. Active carriers are horses that exhibit fecal shedding of *Salmonella*. Passive carriers do not exhibit fecal shedding but can start to shed the bacteria after

surgery or other stressors (18). In mild disease, this syndrome had been described as a delayed onset of the disease but with rapid fecal shedding (17). Fever, depression, and anorexia occur within the first week followed by a short duration pf diarrhea (1-3 days). This syndrome has a low risk of mortality (8). In the case of toxic enterocolitis this is the typical, widely recognized clinical form of salmonellosis (19). Experimental cases have been characterized by fulminant diarrhea 2 or 3 days after experimental inoculation. Most horses start to shed the bacteria within 1-3 days after inoculation, and continuing to shed the bacteria if the horses remain alive (20). Horses are febrile, depressed, and anorexic. There is an increase in frequency of defecation and also the volume of feces because of high water content. Horses exhibit abdominal pain with increased heart rate, respiratory rate, and may exhibit injected mucous membranes. Upon abdominal auscultation, tympany may be heard. The horses can become bacteremic and laminitis is a common sequela (8). Finally, sepsis Presents in adult horses with profound depression. They are febrile and anorexic, and often develop ileus and gastric dilation (21). There is often no significant diarrhea and sometimes there is no fecal output. Tachycardia (80-90 beats/per min) is often present in affected animals. This syndrome has a high case fatality rate. *Salmonella* may be isolated from the liver, mesenteric lymph nodes and kidneys (8). Foals can present with neonatal septicemia, either with or without diarrhea (22, 23). Foals can also develop enterocolitis, or may develop (as sequelae) osteomyelitis, arthritis and omphalophlebitis. At necropsy, *Salmonella* can be isolated from multiple organs including the lungs (interstitial pneumonia) (8). Equine paratyphoid is caused by the host-restricted *Salmonella Abortus equi*, transmitted

primarily by the fecal-oral route and stallions can even transmit it through semen. The serotype remains present in the population due to subclinical carriers. This serotype has been not reported in recent years in United States and Europe, but has caused outbreaks in Japan (24). It causes abortion in mares at 7 to 8 months of gestation. Also, it has been associated with fistulous withers and orchitis. In foals, this serotype produces septicemia (8).

Epidemiology

The epidemiology of equine salmonellosis is complex. This is because a healthy animal can have a latent infection subsequently activated by stressful conditions such as overtraining, worming, early weaning, hot weather, transportation, hospitalization, or antimicrobial therapy, thus leading to the animal shedding *Salmonella* and possibly exhibiting clinical signs of gastrointestinal and other diseases (9). The successful spread and propagation of *Salmonella* depends on the infective dose, age of the host, immune response and additional conditions (25). Outbreaks usually develop in locations with large groups of horses such as breeding farms, racetracks, or veterinary hospitals (26). *Salmonella* shedding from horses is a potential zoonosis with public health consequences, because the organism has the possibility to spread to humans who are in direct and indirect contact with infected animals. In horses, *Salmonella* mortality depends on the animal's age and other predisposing factors like immune system status and the serotype involved (18). The likelihood and magnitude of fecal shedding of bacteria are associated with antimicrobial treatment and stressful situations like

transportation, competition, general anesthesia or surgery, concurrent illness, season, administration of drugs, and hospitalization (27). Outbreaks of nosocomial infections caused by *Salmonella* among patients in veterinary hospitals have been well described (13). In veterinary medical teaching hospitals there is an additional potential economic hazard that results from closure or restriction of admissions, causing economic losses, often affecting the reputation of hospitals, and interrupting educational and clinical activities (10).

Risk factors at hospitals

Several studies were aimed at trying to identify exposure factors that can influence policies to prevent and control *Salmonella* transmission in the hospital environment (8). In two studies of hospitalized horses, it was found that the odds of nosocomial *Salmonella* Saintpaul infection were higher in patients with colic, receiving parental antimicrobials and in horses intubated with nasogastric tubes (28, 29). Abdominal surgery has been identified as a risk factor for nosocomial *Salmonella* infection in horses (30). Animals treated with antimicrobials before surgery may be at higher risk for salmonellosis or *Salmonella* shedding. One hypothesis is that the normal intestinal flora competes with *Salmonella* in its ecological niche and treatment with antimicrobial drugs may disrupt these complex bacterial population dynamics. Another hypothesis is that surgical stress may generate severe alterations of host-defense mechanism in horses (30). One study reported that foals with gastrointestinal disease more readily shed *Salmonella* during hospitalization (31). There are 4 possible

explanations: 1) foals are more susceptible to colonization with *Salmonella* after parturition due to the lack of microflora (32), 2) foals are immune-incompetent, depending on maternal immunoglobulins to generate protection against the bacteria (33), 3) after foaling the infected mares can become asymptomatic shedders and infect the foals (23), and 4) coprophagia is common, especially in foals between 1 to 2 months after birth (34).

Pathogenesis

The main mode of transmission of *Salmonella* is via the fecal-oral route; rarely, airborne transmission has also been recognized (35). Non-specific and specific host defenses are in place to mitigate infection of *Salmonella*. Following ingestion, the majority of the bacteria die because the organism cannot survive the gastric environment and salivary bactericidal enzymes. However, if sufficient bacteria arrive in the stomach ($\geq 10^8$), some of the organisms can survive and will reach the small intestine and colon. Other barriers to *Salmonella* infection, found in the gut include: intestinal proteases, lysozymes, antimicrobial peptides, bile salts, complement and phagocytes, as well as the other bacteria that comprise the gut microbiome (36). In the gut, *Salmonella* adhere to the microfold (M) cells. This is the first target of the bacteria since there is a lack of physical barrier (e.g., glycocalyx and mucus); however, later they can invade other cells like goblet cells and enterocytes (37). Entrance into the host cells allows the pathogen to evade host defenses and invade target tissues. Fimbriae are pattern-recognition receptors that activate signaling pathways in macrophages by binding on the cell surface or within

the phagosome (38). Virulence and host interactions are possible due the presence of particular proteins encoded by genes found in the large clusters on the bacterial chromosome on pathogenicity islands (SPI). *Salmonella* have 16 pathogenicity islands distributed among the different species, subspecies and serotypes, each encoding different virulence factors needed for different phases of pathogenesis. However, almost all *Salmonella* contain the following common five pathogenicity island: SPI 1 necessary for bacterial invasion of the intestinal epithelial cells (intestinal phase), SPI 2, 3, and 4 required for bacterial growth and survival inside the host cells (systemic phase), and SPI 5 products that mediate intestinal inflammation and fluid secretion. There are additional genes that are extrachromosomal on plasmids and can promote bacterial growth, prolonged survival in the host and antimicrobial resistance (39).

Detection of *Salmonella*

The diagnosis of salmonellosis is made via appropriate clinical findings; however, asymptomatic cases may occur. Confirmation by laboratory culture through isolation of the organism from a clinical specimen is required (40). Isolation of the bacterium is the most commonly used confirmative test to detect the presence of *Salmonella*. When used, bacterial isolation is a highly sensitive diagnostic method via the use of selective enrichment and selective plating agar (41). Serial bacterial cultures are recommended to decrease the possibility of failing to detect *Salmonella* in patients by shedding low concentrations of organisms, or intermittently (17). Further characterization of the *Salmonella* causing disease typically includes serotyping and

antimicrobial susceptibility testing. This characterization helps to establish the appropriate treatment regimen and improves the ongoing surveillance of the organism (8). PCR detection is faster than bacterial isolation and may provide rapid results, which helps to implement earlier infection control measures; however, this technique has the potential for misclassification (false positives) of horses classified negative after 7 serial samples (8). The specificity and sensitivity of the real-time PCR increases if the target gene is the *spa*Q (42) or *inv*A (43). In this new era, 'omics' technologies like whole genome sequencing (WGS) are useful to make available a fast, cost-effective, and high throughput method that substitutes for many phenotypic and genotypic assays and provides an enormous set of analyzable data (44).

Antimicrobial use and antimicrobial resistance

The most common antimicrobials used to treat salmonellosis in horses are ceftiofur, enrofloxacin, and gentamicin (45, 46). However, the use of antimicrobials to treat *Salmonella* is controversial because such treatments may favor the persistence of the organism in the intestines following recovery. Additionally, the intestinal protective flora is affected by broad-spectrum antimicrobials; as such, their use may increase the selection of antimicrobial-resistance associated with commensal bacteria (45) and may contribute to overgrowth of undesirable toxigenic bacteria such as *Clostridium difficile* (47).

Antimicrobial resistance (AMR) is a great challenge for animal and human health, and while there are several studies exploring this area in horses, there remains a

great necessity to more carefully evaluate the temporal trends and associations of antimicrobial resistance among *Salmonella* serotypes in horses in order to identify strategies to prevent infection and control outbreaks in the hospital environment. In a recent retrospective study in United States, *Salmonella* from equine specimens submitted to a diagnostic center were found to be more resistant to ceftiofur and gentamicin than to enrofloxacin (48). Isolates from sick horses submitted to the National Veterinary Service Laboratories (NVSL) tended to have increased resistance year after year to beta-lactams, phenicols, aminoglycosides, sulfisoxazoles, tetracyclines and quinolones (49). Furthermore, some serotypes tend to harbor more resistance genes than others, as was the case of an MDR *Salmonella* Newport (8 antimicrobials) that harbored and expressed an ESBL gene ($bla_{\text{SHV-12}}$) identified in an outbreak in a large animal hospital with many patients affected and with high case fatality (50). Although there are many studies that focus on outbreaks caused by *Salmonella* in horses, there are few studies that study the trends of resistance to antimicrobials of *Salmonella* isolated from equine teaching veterinary hospitals, thus further taking into account the additional risks to public health.

Antimicrobial resistance in *Salmonella*

Successful treatment is aided by rapid diagnosis and the ability to choose an effective antimicrobial for patients with invasive *Salmonella* infections. Recently, evaluation of antimicrobial resistance in bacteria isolated from horses has started to receive increased attention; however, until now the studies have often been focused on methicillin-resistant *Staphylococcus aureus*. Clinically significant resistance is now

being reported in Gram-negative members of the *Enterobacteriaceae* family (51). *Salmonella* is recognized as an important carrier of genes encoding for antimicrobial resistance and easily can transfer its resistance by plasmids to other resident *E. coli* (52).

The antimicrobial classes each have different modes of action that disrupt the normal functioning of the bacteria. The main modes of actions are: disruption of cell wall synthesis, inhibition of DNA/RNA synthesis, inhibition of protein synthesis, or interference with another important metabolic pathway (53). Bacteria can acquire resistance to an antimicrobial via a chromosomal mutation, inductive expression of a latent chromosomal gene or by exchange of genetic material (Figure 1). The main antimicrobial resistant mechanisms are classified in three categories: 1) alteration of the target site, 2) exclusion of the antimicrobial from the cell (reducing permeability or through efflux pumps), and 3) production of antimicrobial inactivating enzymes (54).

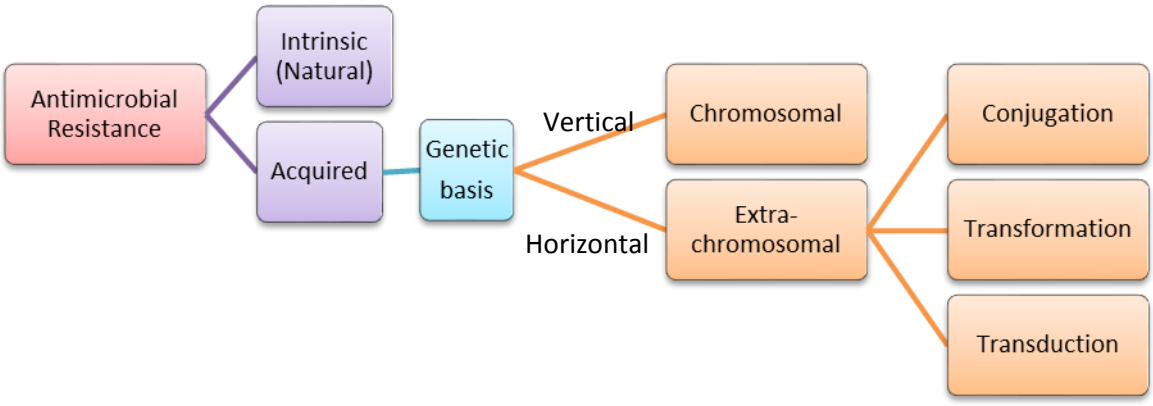

Figure 1. Antimicrobial resistance in bacteria. Material adapted from Tadvi, 2012 (55).

Chloramphenicol is a broad spectrum antimicrobial that inhibits the microbial protein synthesis specifically joining to the 50S subunit at the ribosome (56). Enzymatic inactivation by CATs enzymes is a well know mechanism but also there are other resistance mechanisms reported; for example, permeability barriers (loss of porins) (57), phosphotransferases inactivation, target site with mutations, and efflux pumps systems (58). CATs can inactivate chloramphenicol, thiamphenicol and azidamfenicol; however, florfenicol is not inactivated due to a different chemical structure. The A-1 chloramphenicol acetyl transferase (A-1 CAT) add 2 acetyl groups to the antimicrobial causing its inactivation (56).

All the antibiotics of the Beta-lactam family have a beta-lactam nucleus in their structure. Penicillins and derivatives, beta-lactam inhibitors, cephalosporins, carbapenems and monobactams are part of this antimicrobial family (59). The mechanism of action is by binding to the penicillin-binding proteins (PBPs) and inhibit the cell wall production in the bacteria interfering with the linking of peptidoglycans and stopping terminal transpeptidation. In general, the enzymes that hydrolyze the beta-lactam family are classified based in their activity into: 1) **narrow** conferring resistance only to penicillins and cephalosporins), 2) **moderate**, 3) **broad** and 4) **ESBLs** (Extended Spectrum Beta-lactamases) conferring resistance to penicillins, cephalosporins of first, second and third generation, aztreonam, but not to carbapenems and they are inhibited by inhibitors (60).

Ampicillin (and amoxicillin) inhibits the enzymes that synthesize pedtidoglycan and inducing autolytic enzymes associated to the membrane. Ampicillin resistance is by

beta-lactamases such as TEM-1 and SHV-1, though these enzymes are inhibited by the synergistic activities of clavulanic acid (61).

Cephalosporins and cephamycins can inhibit the bacterial cell wall synthesis similarly as penicillin (59). The cephalosporins are classified by generations based on general features of the antimicrobial action (62). First generation (cephalotin and cefazolin) with modest activity against gram negative bacteria; these cephalosporins are inhibited by TEM and AmpC enzymes (63). Second generation (cefoxitin, cefotetan, and cefmetazole) have better activity against gram negative, but less than third generation. Third generation (cefotaxime, ceftazidime, ceftiofur and cefoperazone) are less active against gram positive instead they are very active against Enterobacteriaceae. The CTX enzyme can hydrolyze cefotaxime, and they are inhibited effectively in its following order, first by tazobactam, second by clavulanate and third by sulbactam. Fourth generation (cefepime) have a broad expectrum of activity compared with third generation and is more stable against hydrolysis mediated by plasmid and chromosomal beta-lactamases (59). The continued selective pressure to these antimicrobials resulted in selection of plasmids that carried mutants of the enzymes TEM and SHV, becoming responsible of the hydrolysis of third and sometimes fourth generation cephalosporins (64). High levels of resistance to extended-spectrum cephalosporins is mainly due to extended spectrum beta-lactamases (ESBLs) encoded by $bla_{\text{CTX-M-15}}$ and $bla_{\text{SHV-12}}$ genes and also by plasmid mediated AmpC beta-lactamase enzymes as CMY, DHA, ACC-1 (65).

The mode of action of trimethoprim and sulfamethoxazole is by inhibition of phases in the folate metabolism and inhibition of the DNA synthesis. Modification of targets by dihydrofolate reductase and dihydropteroate synthase respectively produce resistance to trimethoprim and sulfamethoxazole (56).

Fluoroquinolones (FQ), like ciprofloxacin, have become the first–line drug to treat *Salmonella* in adult humans. However, treatment failures have been associated with reduced susceptibility to ciprofloxacin (0.125-1 ug/mL) in some bacteria (66). Nalidixic acid resistance is an early indicator of ciprofloxacin resistance. The increase in the use of ciprofloxacin has led to increased resistance to it (65). Quinolone resistance-determining region (QRDR) mutations in the A subunit of DNA gyrase and presence of plasmid-mediated quinolone resistance genes *qnr* and *aac(6')-lb-cr* develop quinolone resistance in the bacteria. Presence of mutations in many genes, reduced membrane permeability, active efflux pump, and/or presence of *qnr* genes are associated with high level resistance to ciprofloxacin (67).

The increase in fluoroquinolone resistance and the contraindication for use of ciprofloxacin in children due to its effects on cartilage development has led to increased use of third-generation cephalosporins like ceftriaxone. Fluoroquinolone and ESBL resistance remains a main problem in the effective treatment of bacterial infections in both human and animals (68).

Aminoglycosides are broad spectrum antimicrobials, their mechanism of action is by inhibition of protein synthesis disintegrating the bacterial cell membranes (69). There are different recognized mechanisms of resistance to aminoglycosides as follows:

1) Active efflux (70), 2) Decreased permeability (71), 3) Change in the ribosome (72) and 4) The most common by aminoglycoside-modifying enzymes that inactivate the aminoglycosides (73).

Tetracyclines are antimicrobials with broad spectrum, relative safe, with low cost, becoming the second antimicrobial in use after penicillin (60). Different modes of action for tetracycline are described. The first mode described was the interaction with the ribosomes and blocking protein synthesis (74). Later was discover that some tetracycline derivatives are not good interacting with the ribosomes, instead of that, the interaction is with the bacterial membrane (75). Tetracycline resistance mechanism are three: 1) efflux pumps reliant on energy, 2) ribosomal protection proteins and 3) or enzymatic inactivation. In gram negative bacteria *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(I), *tet*(J), *tet*(Y), *tet*(30) and *tet*(31) genes are recognized as exclusive (76).

Plasmids in *Salmonella*

Bacteria carry extra-chromosomal self-replicating genetic elements called plasmids. The most frequent serotypes associated with infections in humans and farm animals commonly harbor plasmids. Some serotypes have specific virulence plasmids. *Salmonella* often also harbor additional plasmids that encode resistance to antimicrobials, and still other plasmids with unknown function (77, 78).

The plasmids can be classified by 1) functionality as virulence, antimicrobial resistance or unknown function; or classified by 2) incompatibility groups accordingly to

their bacterial replication and maintenance (79). IncI, producing I pili; IncN, N3-related; IncF, producing F pili; and IncP, RP4-related plasmids among others (78). At this time, 27 different Inc groups among *Enterobacteriaceae* are recognized by the plasmid section of the National Collection of Type Cultures including – very importantly for *Salmonella* – IncF (FII to FVII) and IncI (I1, Iγ, I2) (80). Although PCR-based replicon typing (PBRT) can be used to detect the replicons of the majority of plasmid families in *Enterobacteriaceae,* this scheme has some limitations because the grouping is built only on plasmids from the old recognized Inc groups and could not identify novel replicons. A more accurate approach is to characterize the replicon according to its full length sequence; as of late 2017, more than 800 plasmids have been identified. Currently, more than 100 resistance plasmids have been typed and classified accordingly to their specific families (80).

The majority of the plasmids are conjugative, conferring resistance to different antimicrobials. The most common location of the antimicrobial resistance genes is within transposons that can be transfer from plasmids to chromosome and from chromosome to plasmid (77). While plasmids are most important for the storage and spread of genetic information, they can also carry integrons as is the case of IncF and IncL/M plasmids that carried Class 1 integrons (81). Antimicrobial resistance is routinely shared among a complementary group of conjugative plasmids, transposons and integrons. New cassettes can be incorporated by the integron to transfer by a conjugative plasmid to a host bacterium, and thereafter can be integrated into the host chromosome by transposition (77). The very important and clinically relevant

association between resistance plasmids and virulence genes has been previously described (81).

In summary, although *Salmonella* can produce severe disease in horses, relatively few serotypes of *Salmonella* are capable of producing clinical disease. Treatment for *Salmonella* is currently limited to antimicrobials like beta-lactams, quinolones and gentamicin. Recent studies demonstrate incremental increases in the resistance of *Salmonella* to additional antimicrobials which impedes the effective treatment of salmonellosis and other important diseases, both in horses and in humans.

This research was performed to estimate the diversity of *Salmonella* isolated from patients at an equine referral hospital in the southern United States. The objective was to contribute further to developing a better understanding of the causative strains associated with clinical salmonellosis, its transmission and its association with antimicrobial resistance.

CHAPTER II

MATERIALS AND METHODS

Evaluation of the proportional morbidity attributed to each *Salmonella enterica* serotype

Sample set and isolates

The sample set included 255 *Salmonella* strains derived from the first clinical sample collected and submitted to the clinical microbiology laboratory from any patient admitted to the veterinary medical teaching hospital with a differential diagnosis of salmonellosis between January 8, 2007 and November 4, 2015. Samples were initially inoculated into tetrathionate broth and incubated overnight at 37°C prior to DNA extraction – or else culture on XLT-4 and MacConkey agar. Prior to 2010, samples were only subjected to culture; after 2010, patient samples were first tested by PCR to spaQ gene which encode for the surface presentation of antigens protein SpaQ (transmembrane) (82) and then cultured if positive by PCR. Tetrathionate broths that tested positive by direct PCR were subsequently streaked onto XLT-4 and MacConkey agar. Hydrogen sulfide producing colonies from XLT-4 and lactose negative colonies from MacConkey agar were subcultured to trypticase soy agar supplemented with 5% sheep blood and grown overnight at 37°C. Isolates were then tested for oxidase production. Oxidase negative isolates were then presumptively identified as *Salmonella* based on production of characteristic biochemical reactions when grown on triple sugar

iron agar (TSI), lysine iron agar (LIA), Christensen's urea agar, tryptophan broth and agglutination with polyvalent antisera. These isolates were sent to the National Veterinary Services Laboratory (NSVL) in Ames, Iowa for serotyping according to the White-Kauffmann-Le Minor scheme. Some isolates were preserved in skim milk at 10% and others in cryopreservation beads at -80°C for further analysis.

DNA extraction for whole genome sequencing (WGS)

In the present study, *Salmonella* DNA from stored isolates was extracted using the QIAamp 96 DNA QIAcube HT Kit ™ (Qiagen, Valencia, CA) in the QIAcube HT ™ instrument. One *Salmonella* colony per isolate from a fresh culture was suspended into 5 ml of Trypticase Soy Broth (Difco, Becton Dickinson, Franklin Lakes, NJ) and incubated overnight at 37°C. From the overnight culture, 1 ml was pipetted into a 1.2 ml micro-collection tube and centrifuged at 4,000 rpm for 15 minutes at room temperature. The supernatant was removed and the pellet was re-suspended in a mixture of ATL buffer and DX reagent. One tube of small pathogen lysis beads was combined with the suspension to break the cells using the Qiagen TissueLyser system ™ (Qiagen, Valencia, CA) at 25 Hz, for 5 minutes. The tubes were briefly centrifuged and 40 µl of Proteinase K was added to each tube. The tubes were incubated at 56°C for 1 hour at 900 rpm in the ThermoMixer ™ (Eppendorf, Hauppauge, NY) and then a heat shock was done for 10 minutes at 95°C. The suspension was cooled at room temperature and 4 µl of RNAse A was added to the suspension. The samples were placed on the QIAcube HT for DNA extraction using the program Gram- QCHT *Salmonella* & *E. coli* Microtube (woAL).

DNA quality was measured by 260/280 absorbance ratio on the Omega Fluostar microplate reader (BMG LABTECH, Cary, NC). DNA quantity was measured with a Quant-iT™ Pico Green® ds DNA Assay kit (ThermoFisher Scientific) and the DNA was stored at -20C until further use.

Whole genome sequencing using the Illumina MiSeq™

Whole-genome sequencing (WGS) was performed using the Illumina MiSeq™ platform (Illumina, San Diego, CA) to determine serotypes and plasmid replicons of the *Salmonella* strains. Libraries for 32 *Salmonella* DNA samples were multiplexed using the Illumina Nextera XT kit following the manufacturer instructions using the Qiagility™ robot (Qiagen, Valencia, CA) to set up the reactions and the thermocycler (Eppendorf) for amplification. The quality and quantity of the libraries were checked on the Fragment Analyzer™ instrument (Advanced Analytical, Ankeny, IA, USA). The libraries were run on the Miseq (automated DNA sequencer) using the MiSeq Reagent Kit v3 (Illumina, San Diego, CA) with paired-end 2 x 300 base pairs reads. A comprehensive work flow of the library preparation is showed in Figure 2.

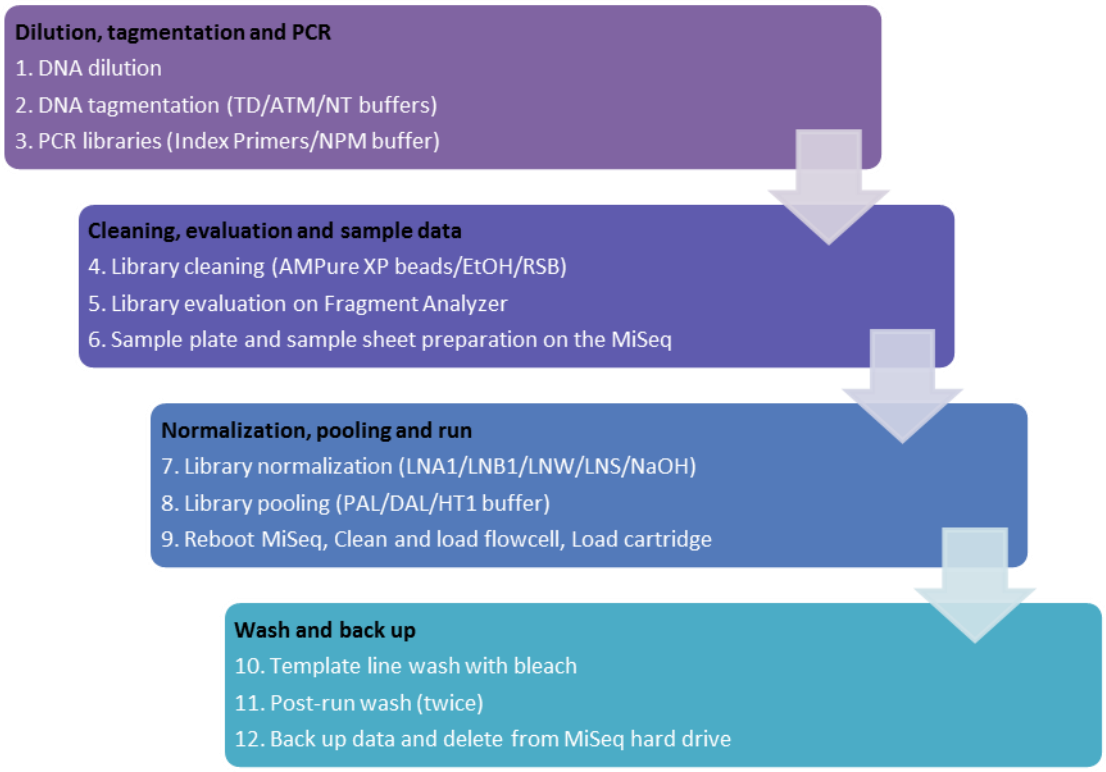

Figure 2. Nextera XT DNA library preparation workflow

Bioinformatics analysis of WGS data

Raw sequence data (fastq files for reverse and forward ends) were obtained from the Illumina MiSeq runs and used for further analysis after the sequencing run was complete. *De novo* genome assembly was performed by the Velvet software package using SRST2 (a short read sequence typing pipeline) (83) on the Illumina BaseSpace™ platform, by Spades software package using PATRIC (Pathosystems Resource Integration Center) (84) and by CGE (Center for Genomic Epidemiology) website (85).

Serotype analysis

The SeqSero pipeline was used for serotype identification (86). On the web-based tool, raw sequence reads derived from the Illumina MiSeq were uploaded. The somatic (O) group was determined by analysis of *wzx* and *wzy* genes and by analysis of the *rfb* cluster; meanwhile, flagellar (H) phases were determined by analysis of *fliC* and *fljB* genes combined in the same H antigen database. Mapping was run 3 times for the final serotype call.

Validation of serotypes through serotyping using the White-Kauffmann-Le Minor scheme and WGS

All *Salmonella* were previously sent to the National Veterinary Services Laboratories (NSVL) and serotyped using the White-Kauffmann-Le Minor scheme; those results were compared with the results obtained by the SeqSero pipeline and any notable differences and potential biases explored.

Plasmid analysis

Genomes were analyzed through SRST2 on the Illumina Basespace platform using the PlasmidFinder database (87) for the determination of plasmid types. In addition, PlasmidFinder from the database of the Center for Genomic Epidemiology (CGE) (88) was used to perform additional comparative analyses.

Statistical Analysis

The epidemiological unit for analysis was the isolate (individual serotype) recovered from the first sample per patient admission to the hospital. Information from each isolate was recovered from the database of the clinical laboratory and hospital database and saved as a spreadsheet in Excel format. The data were imported to STATA® software version 14.0 (StataCorp, College Station, TX) and were used to create variables to perform descriptive analyses (including graphics such as bar and pie charts, and statistics such as proportional prevalence and 95% confidence intervals). Determination of the association (odds ratios) between the clinical signs of salmonellosis and the main serotypes were evaluated using likelihood-based chi-square or Fisher exact tests.

Evaluation of antimicrobial resistance patterns of *Salmonella* isolated from horses admitted to an equine referral hospital in the southern United States

Antimicrobial susceptibility testing

The determination of the antimicrobial susceptibility of *Salmonella* was performed using the broth microdilution method via the Sensititre® system (TREK, Thermo Scientific Microbiology, Oakwood Village, OH) to identify the MIC (Minimum Inhibitory Concentration). This test was performed using the National Antimicrobial Resistance Monitoring System (NARMS) custom plate CMV3AGNF that has 14 antimicrobials among 9 classes of antimicrobials (Table 1).

The guidelines of surveillance from NARMS were followed to generate comparable data procedures and interpretations with representative antimicrobial families as beta-lactam including ampicillin, amoxicillin/clavulanic acid, cefoxitin, ceftiofur and ceftriaxone; quinolones such as nalidixic acid and ciprofloxacin; folic acid inhibitors like sulfisoxazole and trimethoprim/sulfamethoxazole; aminoglycosides like streptomycin and gentamicin were part of the observation. Lastly, azithromycin, chloramphenicol and tetracycline also were evaluated. The Sensititre® system is a simple, practical and quantitative method that measures the MIC and provides clinical interpretative criteria (resistant, intermediate or susceptible) based on the Clinical Laboratory Standards Institute (CLSI) breakpoints (89).

The isolates were streaked onto Trypticase Soy Agar (TSA) with 5% sheep blood agar and incubated at 37 °C for 18 hours. The colonies were suspended in 4 ml of sterilized water and adjusted to 0.5 McFarland standard. 50 µl of the culture suspension was transferred into 11 ml of Mueller-Hinton broth and 50 µl of suspension was inoculated onto the plates using the Sensititre® Automated Inoculation Delivery System (TREK). Plates were incubated at 37 °C for 18 hours, then read on Sensititre OptiRead™ instrument (TREK, Thermo Scientific Microbiology, Oakwood Village, OH). The results were processed in the SWIN software (TREK, Thermo Scientific Microbiology) and were interpreted accordingly to CLSI (89). Intermediate isolates were reclassified as susceptible for the purpose of epidemiological analysis. Isolates resistant to three or more classes of antimicrobials were considered multidrug-resistant (MDR).

Controls

Escherichia coli ATCC 25922, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212 (American Type Culture Collection, Manassas, VA) were used as quality control (QC) strains for susceptibility testing. During each laboratory processing run, the QC strains were run with each new batch of plates, or during an extended break in processing.

Table 1. Minimum Inhibitory Concentration range used in the sensititre CMV3AGNF plate.

Antimicrobial	Abbreviation	Range	Breakpoint
Ampicillin	AMP	1 - 32	≥ 32
Amoxicillin/Clavulanic Acid	AUG2	1/0.5 - 32/16	≥ 32 / 16
Azithromycin	AZI	0.12 - 16	≥ 32
Cefoxitin	FOX	0.5 - 32	≥ 32
Ceftiofur	XNL	0.12 - 8	≥ 8
Ceftriaxone	AXO	0.25 - 64	≥ 4
Chloramphenicol	CHL	2 - 32	≥ 32
Ciprofloxacin	CIP	0.015 - 4	≥ 1
Gentamicin	GEN	0.25 - 16	≥ 16
Nalidixic Acid	NAL	0.5 - 32	≥ 32
Streptomycin	STR	2 - 64	≥ 64
Sulfisoxazole	FIS	16 - 256	≥ 512
Tetracycline	TET	4 - 32	≥ 16
Trimethoprim/Sulphamethoxazole	SXT	0.12/2.4 - 4/76	≥ 4 / 76

Bioinformatics analysis of WGS data

Resistance genes

The genomes were analyzed for presence of AMR genes through the ResFinder tool using the SRST2 application in the Illumina BaseSpace platform and via the Center for Genomic Epidemiology webpage (88).

Statistical analysis

The data were imported into STATA® software version 14.0 (StataCorp., College Station, TX) in Trek SWIN (csv) format. Intermediate resistance was collapsed into susceptible when binary encoded resistance was portrayed. The presence or absence of a known antimicrobial resistance gene or genes was compared with the interpretation of resistant or susceptible to a given corresponding antimicrobial. Measure of agreement between the phenotypic and genotypic results was made using kappa analysis (90).

Distribution of temporally clustered cases of the major Salmonella *serotypes throughout the years*

The distribution of cases was analyzed according to the main *Salmonella* serotypes by the day of admission of the patient by each year in order to identify temporal clusters of cases that can suggest potential outbreaks and detect possible nosocomial transmission.

Phylogenetic analysis

In this study, phylogenetic analysis was conducted in three of the most common serotypes that presented temporal clusters of cases according to the admission date of the patient to the hospital (*Salmonella* Anatum, *Salmonella* Braenderup, and *Salmonella* Newport) to explore the epidemiology of possible outbreaks or potential circulating strains at the hospital.

Assembled genomes (contigs) from the *Salmonella* genome of the three main serotypes were used to construct the massive core-genome alignments into Parsnp in the Harvest package (91). Parsnp is useful in intraspecific genome analysis (outbreaks analysis). The phylogenetic tree was visualized with FigTree (92).

Phylogenetic trees were created and analyzed by each major serotype along with a complete genome reference and subsequently with the information available per case (year of admission, antimicrobial pattern, presenting complaint, age of the patient, and farm location).

CHAPTER III

RESULTS

Evaluation of the proportional morbidity attributed to each *Salmonella enterica* serotype

The 255 *Salmonella* strains isolated between January 8, 2007 and November 4, 2015 were analyzed and 98.4 % were isolated from fecal samples and 1.6 % (4) were isolated from blood.

Breeds

The most common horse breed among the 30 different breeds admitted to the hospital with a differential diagnosis of salmonellosis was Quarter Horse 51.8% (132), followed by Thoroughbred 9.4% (24), American Paint 7.4% (19), and then Arabian 6.7% (17) (Figure 3).

Age

The mean age of the horses based on 233 records was 7 years with a range of newborn through 24 years (Figure 4). Horses were classified into 4 groups – according to age – for further analysis, as follows: a) Foals (less than 1 year), b) Juvenile (1 - 2 years), c) Adult (greater than 2 years, less than 20 years) and d) Seniors (greater than or equal to 20 years).

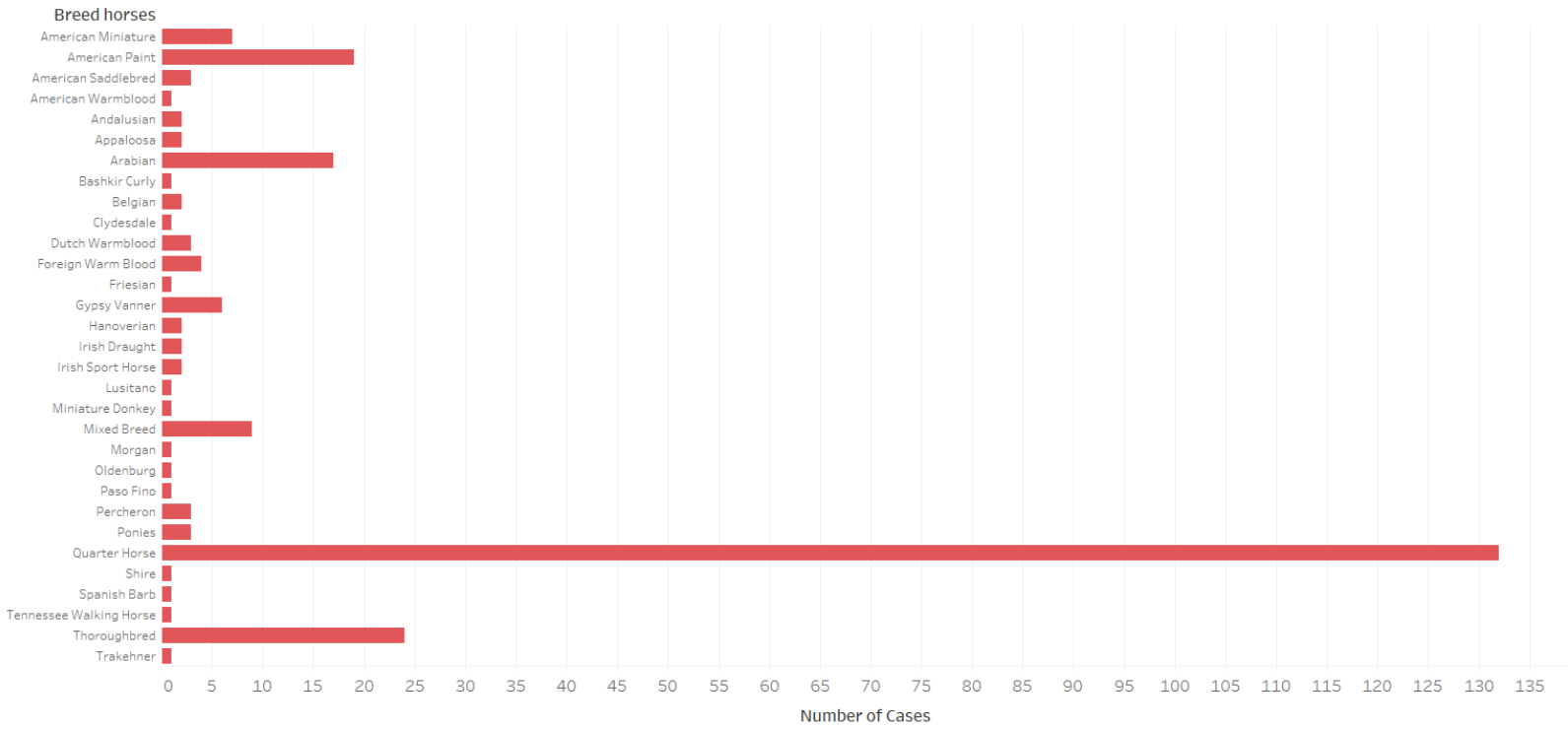

Figure 3. Number of cases for each horse breed admitted to the hospital with differential diagnosis of salmonellosis.

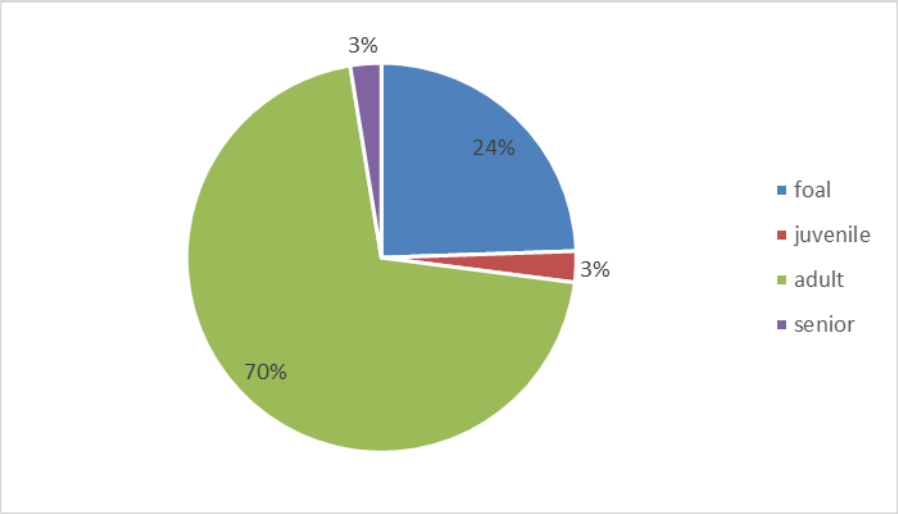

Figure 4. Percentage of cases (out of 233) for each age category of horse admitted to the hospital with a differential diagnosis of salmonellosis.

Complaints

The most common complaints in horses were colic 47.4 % (121), followed by diarrhea 21.6 % (55), and then other general symptoms 20.4% (52) such as fever, dehydration, weight loss or anorexia. Some of the cases came directly from necropsy 2.8 % (7) that were either dead on arrival or else were euthanized (Figure 5). The complaints were grouped according to the system affected for further analysis (Figure 6).

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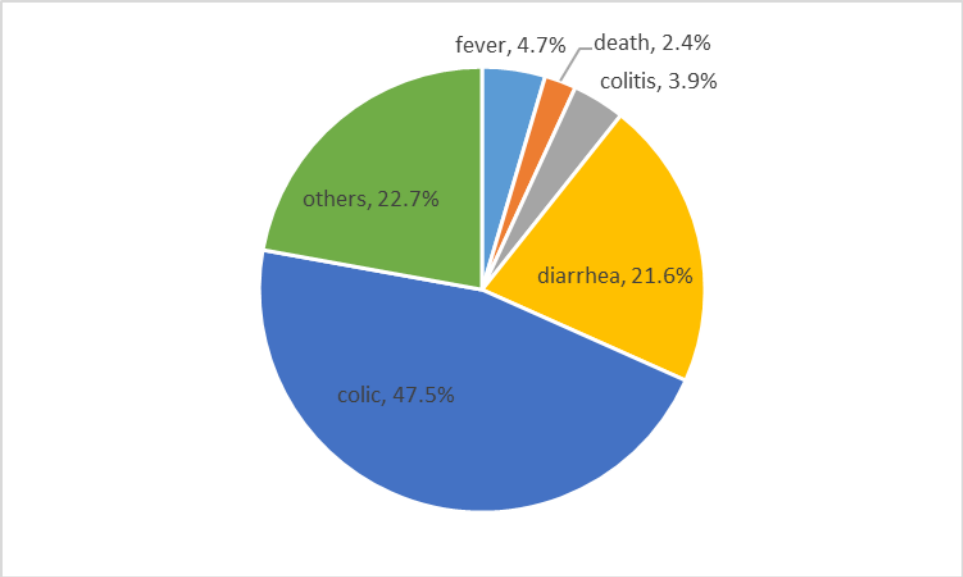

Figure 5. Percentage of the most common complaints in horses admitted to the hospital with a differential diagnosis of salmonellosis between January 8, 2007 and November 4, 2015.

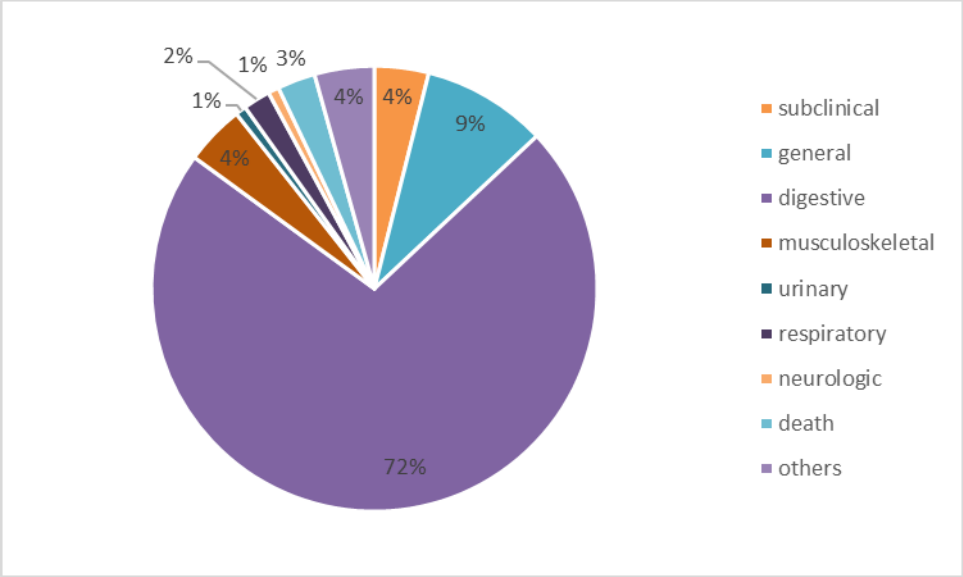

Figure 6. Percentage of presenting clinical complaints classified by affected system in horses admitted to the hospital with a differential diagnosis of salmonellosis between January 8, 2007 and November 4, 2015.

Associations between age and clinical complaint

From the horses positive to *Salmonella* admitted to the hospital, the odds of fever complaint were significantly ($P = 0.033$) higher (8.6 times as high) in juvenile horses, compared with the odds of horses from other age (Odds ratio = 8.6; 95% CI = 0, 44.6). Upon looking at associations with more specific symptoms we found that the odds of diarrhea complaint were significantly ($P < 0.0001$) higher (4.5 times as high) in foals compared with the odds of older horses (Odds ratio = 4.5; 95% CI = 2.32, 8.76). The odds of colic complaint were significantly ($P < 0.0001$) higher (7.29 times as high) in adults compared with the odds of horses in other age (Odds ratio = 7.29; 95% CI = 3.66, 14.48). The odds of musculoskeletal complaint were significantly ($P = 0.0001$) higher (9.41 times as high) in foals compared with the odds of older horses (Odds ratio = 9.41; 95% CI = 2.59, 33.93). No statistically significant associations were found between urinary and respiratory complaints and horse age groups.

Associations between breed and clinical complaint

From the horses positive to *Salmonella* admitted to the hospital, the odds of colic complaint were significantly ($P = 0.0017$) higher (3.38 times as high) in American Paint breed compared with the odds of other breeds (Odds ratio = 3.38; 95% CI = 1.22, 9.29). The odds of respiratory signs were significantly ($P < 0.0001$) higher (23.14 times as high) in mixed breed compared with the odds of other breeds (Odds ratio =23.14; 95% CI = 4.01, 139.3).

Serotypes

Among 255 *Salmonella* isolates, 46 different serotypes were identified by the White-Kauffmann-Le Minor scheme and SeqSero; while, 4% (10 strains) did not match when comparing both techniques. Due that in –silico serotyping platforms are based in the antigenic gene carried but not necessarily expressed by the isolate (93), for the ten problematic strains it was used the most confident result gave it by White-Kauffmann-Le Minor scheme. The most common serotype recovered was Newport 18 % (46), followed by Anatum 14% (37), Braenderup 11.4% (29), Infantis 7.8 % (20), Javiana 5.5% (14), Typhimurium 5.5% (14), Rubislaw 5.1% (13) and Montevideo 2.4% (6), the remaining percentage (30.3%) was comprised of 38 additional serotypes (Table 2).

Table 2. Frequency of *Salmonella* serotypes in horses admitted to the hospital with a differential diagnosis of salmonellosis between January 8, 2007 and November 4, 2015.

Serotype	Frequency	Percent (%)
111_51:g,z51:-	1	0.39
4,12:i:-	3	1.18
6,7:-:e,n,z	1	0.39
6,7:k:-	1	0.39
Agona	4	1.57
Altona	1	0.39
Anatum	37	14.51
Bareilly	1	0.39
Bovismorbificans	1	0.39
Braenderup	29	11.37
Carrau	1	0.39
Cerro	4	1.57
Freetown	2	0.78
Fresno	1	0.39
Gaminara	3	1.18
Give	4	1.57
Havana	2	0.78
Hvittingfoss	2	0.78

Table 2. Continued

Serotype	Frequency	Percent (%)
Infantis	20	7.84
Javiana	14	5.49
Kentucky	3	1.18
Kiambu	2	0.78
Manhattan	1	0.39
Mbandaka	3	1.18
Meleagridis	2	0.78
Minnesota	1	0.39
Mississippi	2	0.78
Montevideo	6	2.35
Muenchen	2	0.78
Muenster	3	1.18
Newport	46	18.04
Norwich	1	0.39
Oranienburg	4	1.57
Paratyphi	2	0.78
RoughO:b:1,5:	1	0.39
RoughO:gms:	1	0.39
Rubislaw	13	5.1
Ruiru	1	0.39
Saintpaul	2	0.78
Senftenberg	1	0.39
Tennessee	1	0.39
Thompson	3	1.18
Typhimurium	14	5.49
Typhimurium Var	5	1.96
Untypeable	1	0.39
Weslaco	2	0.78
Total	**255**	**100**

Associations between serotype and clinical complaint

From the horses positive to *Salmonella* admitted to the hospital, the odds of subclinical presence of salmonellosis were not significantly ($P = 0.058$) higher (3.61 times as high) in *S.* Braenderup compared with the odds of other serotypes (Odds ratio = 3.61; 95% CI = 1.22, 9.29). The odds of digestive (complaints affecting the digestive

system) were significantly (P = 0.013) lower (0.27 times) in *S.* Typhimurium compared with the odds of other serotypes (Odds ratio = 0.27; 95% CI = 0.09, 0.78), meaning that other serotypes had 3.7 times the odds of digestive complaints as S. Typhimurium. The odds of the signs associated with musculoskeletal system were significantly (P = 0.0015) higher (13.37 times as high) in *S.* Typhimurium compared with the odds of other serotypes (Odds ratio = 13.37; 95% CI = 3.59, 50.76). The odds of respiratory system signs were not significantly (P = 0.2318) higher (4.96 times as high) in *S.* Rubislaw compared with the odds of other serotypes (Odds ratio = 4.96; 95% CI = 0, 36.71). None of the serotypes were statistically significantly associated with urinary, nervous system or general complaints.

Association between serotype and age of horse

From the horses positive to *Salmonella* admitted to the hospital, the odds of serotype *S.* Braenderup presence were significantly (P = 0.0014) higher (3.94 times as high) in foals compared with the older horses (Odds ratio = 3.94; 95% CI = 1.59, 7.71). The odds of serotype *S.* Typhimurium presence were significantly (P = 0.0014) higher (5.58 times as high) in foals compared with the older horses (Odds ratio = 5.58; 95% CI= 1.83, 16.98).

Temporal trends of serotypes

The frequency of detection of the main serotypes varied across the years (Figure 7). *Salmonella* Anatum, *S.* Braenderup and *S.* Newport, were the most common

serotypes throughout the years of the study; however, the cases of *S.* Newport and *S.* Braenderup decreased in 2015 while *S.* Anatum increased during the same year. *Salmonella* Javiana was present at a low frequency throughout the years, similar to *S.* Infantis, although the latter was entirely absent in 2013. *Salmonella* Typhimurium was present at a low frequency in most of the years, increasing in 2008, but absent again in 2011 and 2014. *Salmonella* Rubislaw was present in most of the years in low frequency but with 3 cases in 2009 and 2012. Of the top 8 serotypes by frequency, *Salmonella* Montevideo had the lowest frequency and it was not present in the years 2010 and 2014. 38 additional serotypes not represented in Figure 7 were also present through the years of the study but at a lower frequency.

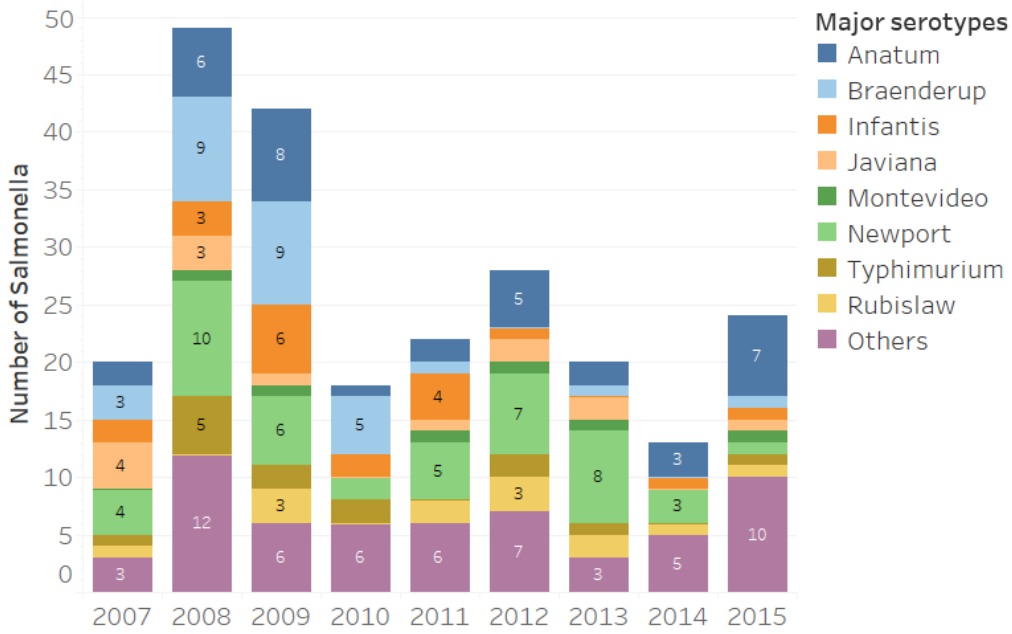

Figure 7. Frequency of detection of the main serotypes throughout the years of the study (2007-2015)

Distribution of temporally clustered cases of the major Salmonella *serotypes throughout the years*

There was no cluster of serotypes in 2007; however, *S.* Braenderup was highly prevalent in the first part of the 2008 year (8 closest cases between March and June) with 1 further case on the 3rd of November. Meanwhile, *S.* Newport was highly prevalent in the second part of the same year with 10 closely clustered cases between July and December (Figure 8).

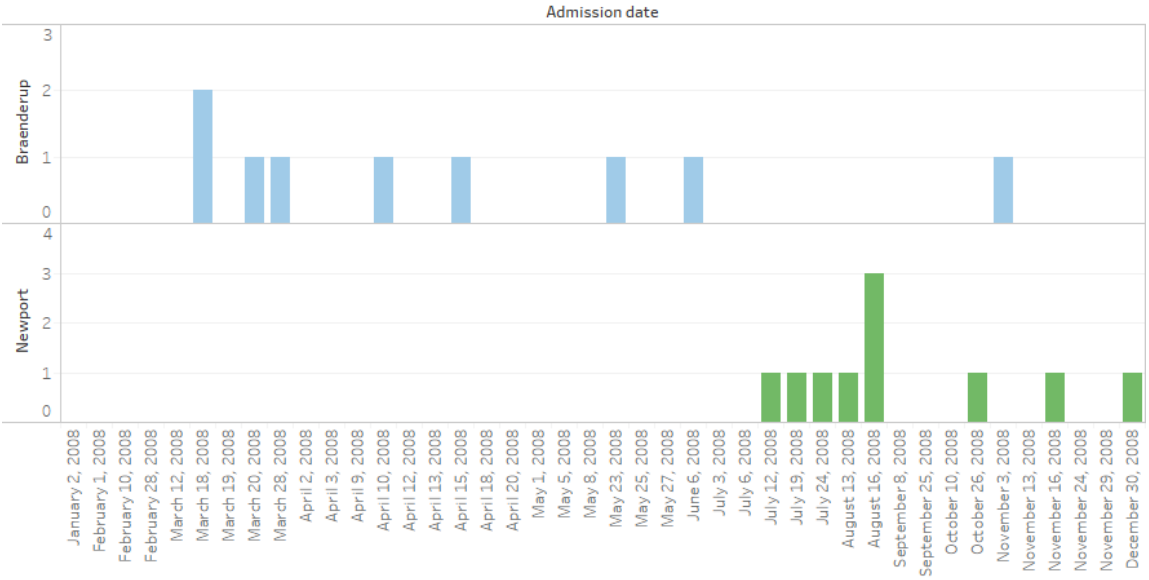

Figure 8. *Salmonella* Braenderup and *Salmonella* Newport case sequences by admission date in 2008.

S. Braenderup was present with a maximum of 5 cases during two dates in February of 2009. *Salmonella* Anatum also was present with its 2 closest cases in April

and July. *Salmonella* Infantis was present in May, September and October (with its 3 closest cases) (Figure 9).

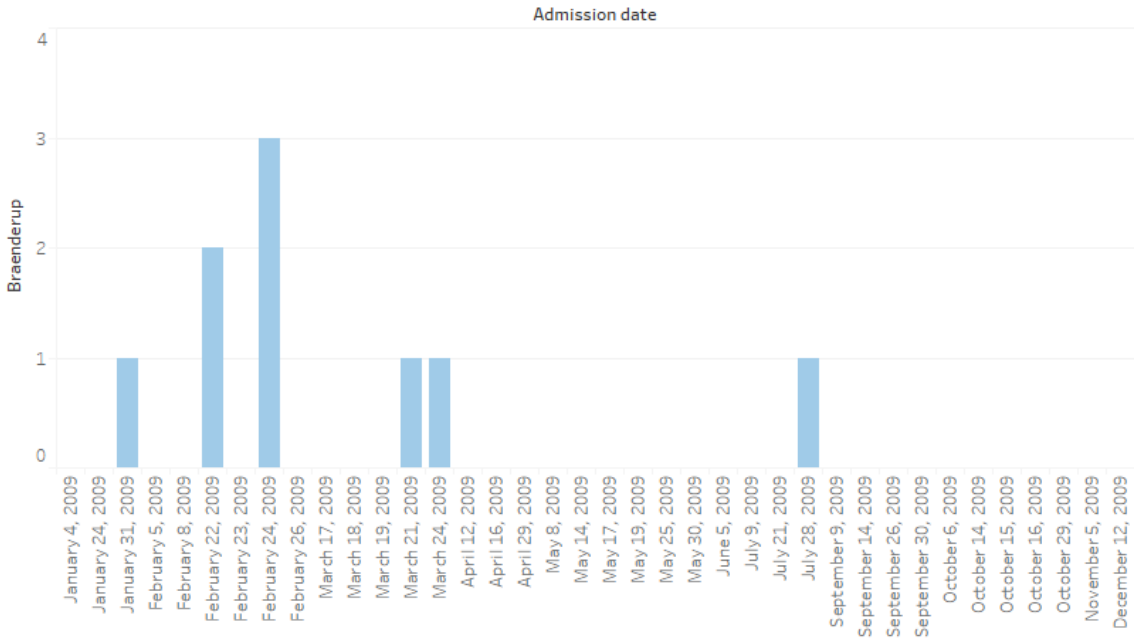

Figure 9. *Salmonella* Braenderup case sequences per admission date in 2009.

In 2010, *S.* Braenderup was present in April and May with 3 close cases, followed by 2 additional cases on October 11th 2010. During 2011, *S.* Infantis presented 3 close cases in November, while *S.* Newport was present with 2 cases occurring within September and October. In 2012, *S.* Newport was the serotype that presented as an apparent temporal cluster with 2 close cases in September, 3 cases in October, and 2 cases between November and December. From the 8 major serotypes found in the study, *S.* Newport was the most common in 2013 with three close cases occurring in September (out of 7 cases in the year). In 2014, *S.* Newport was present between 26 and 109 days

after New Year (3 cases). In 2015, *S.* Anatum was present between April and May (6 cases) and 1 case occurred in August (Figure 10).

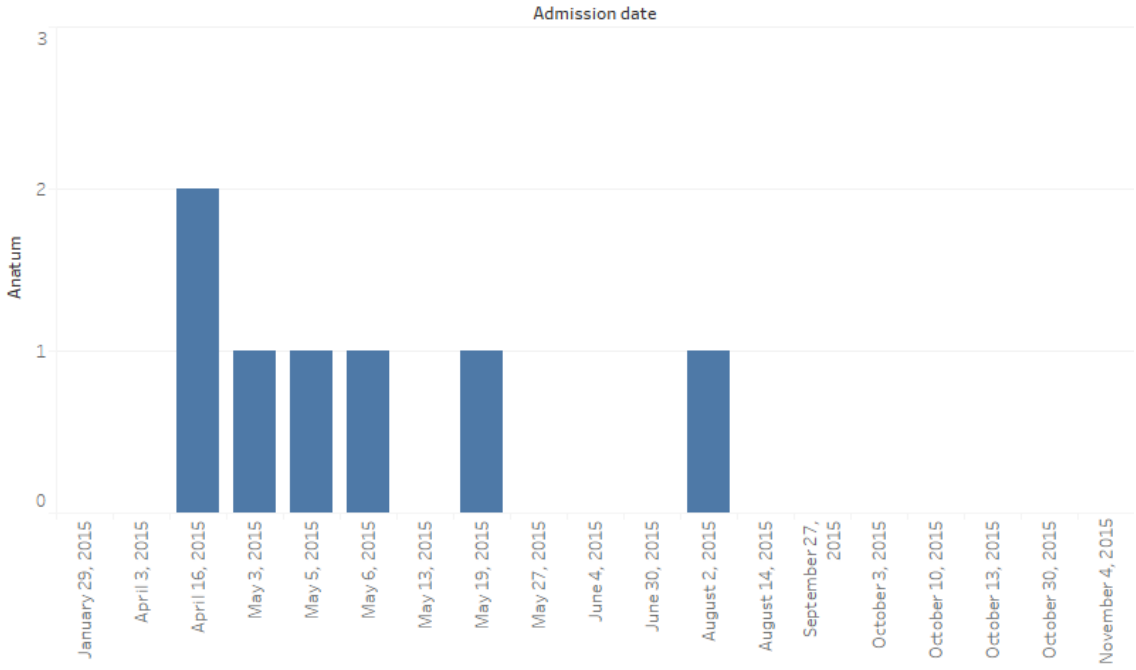

Figure 10. *Salmonella* Anatum case sequences per admission date in 2015.

Plasmid analysis

Fifteen different replicon types were identified (A/C2, COL, FIA, FIB, FII, FIIS, HIR, HI2, I1, I2, Q, R, U, X, and Y); meanwhile, the predominant type was I1 at ~10% present in 25 isolates out of the total (Figure 11). Out of 255 isolates, 96 (38%) of the *Salmonella* isolates where identified as harboring at least one plasmid.

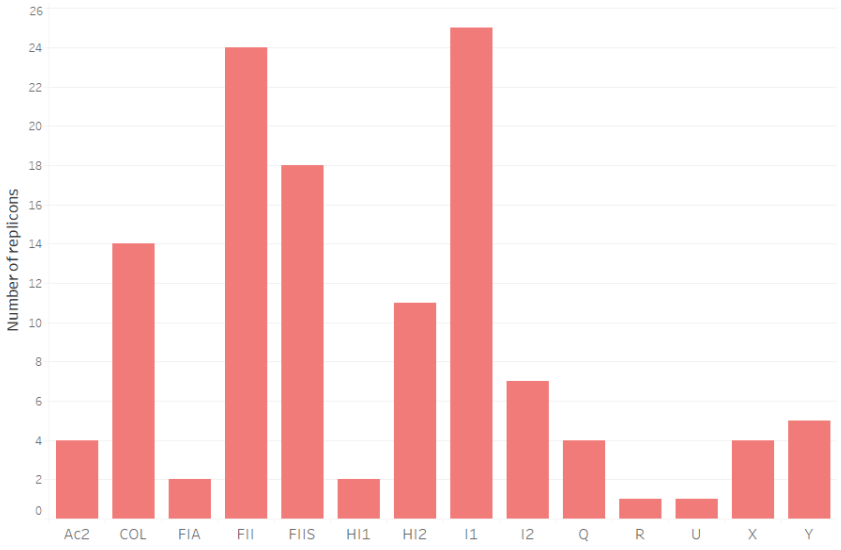

Figure 11. Number of detected replicons in *Salmonella* from horses admitted to the hospital with differential diagnosis of salmonellosis between January 8, 2007 and November 4, 2015.

Presence of plasmids between the serotypes

The highest prevalence of plasmids was detected in *Salmonella* Anatum (31/21), *S.* Typhimurium (20/10), *S.* Newport (14/10), S, Braenderup (12/10), *S.* Rubislaw (9/7), *S.* Javiana (9/8), *S.* Agona (8/4) and *S.* Typhimurium var 5 (7/3) (Figure 12). Interestingly, the most prevalent replicon type in *S.* Anatum was I1 (11) among 10 different replicon types that were found (Figure 13). The most prevalent replicon types in *S.* Typhimurium were FIB (9) and FIIS (9), among 4 different replicon types (Figure 14). The most common replicon types in *S.* Newport were COL (3) and FII (3) among 9 different replicon types (Figure 15). The most prevalent replicon types in *S.* Braenderup were I1 (6), and Y (4) among 4 different replicon types (Figure 16).

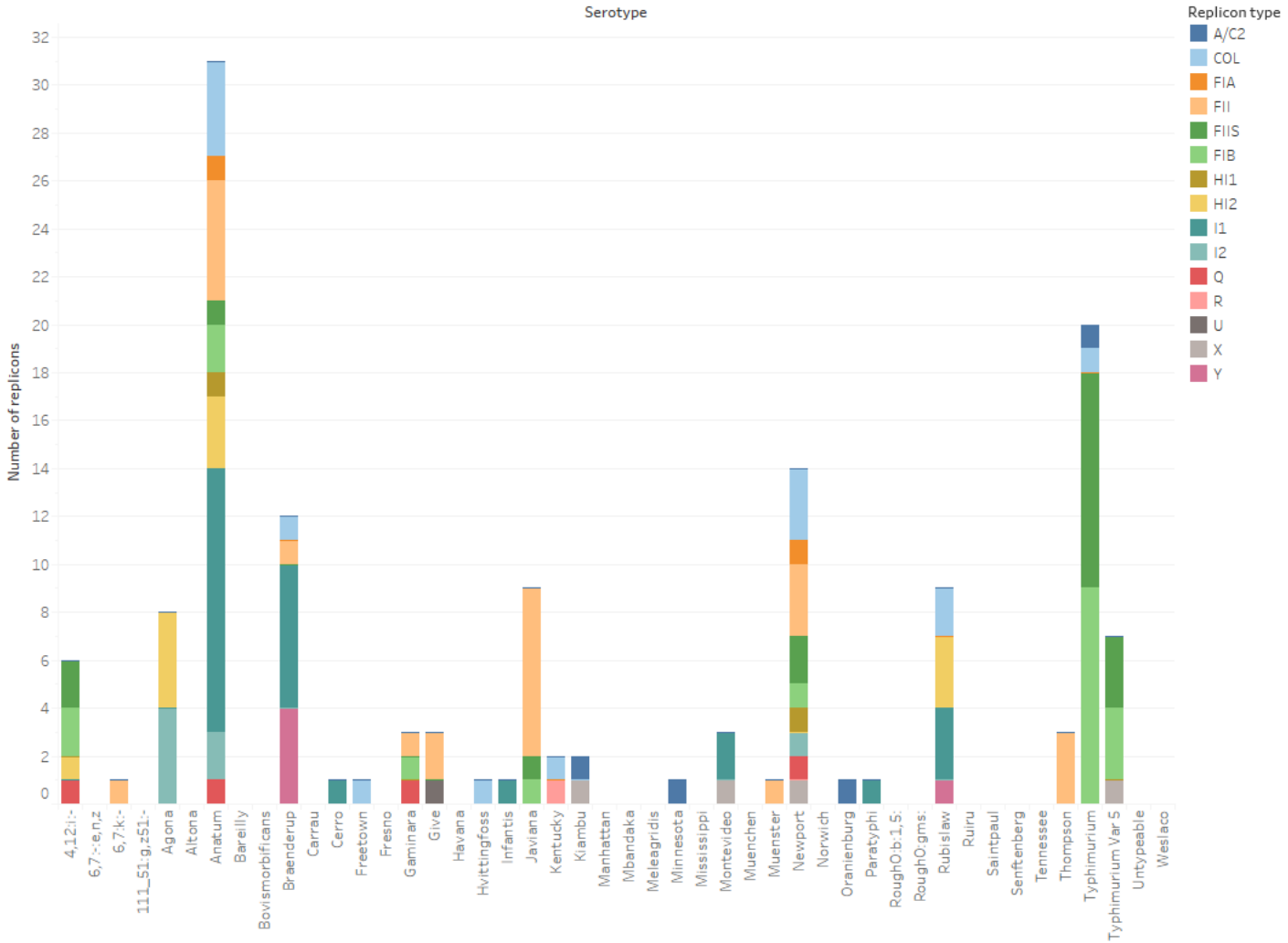

Figure 12. Number of detected plasmid replicon types in the different *Salmonella* serotypes.

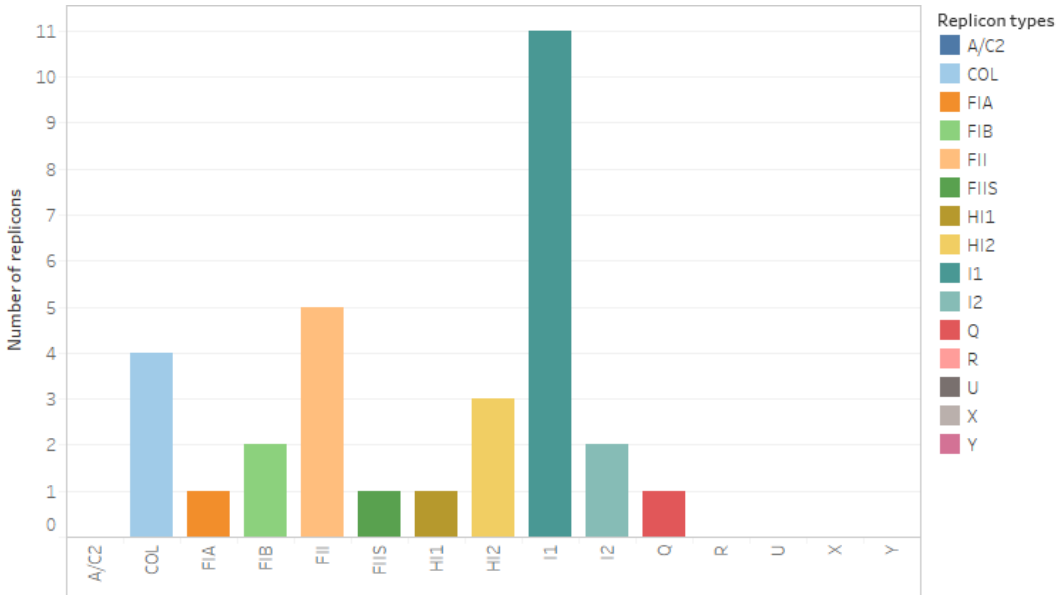

Figure 13. Number of detected plasmid replicon types in *Salmonella* Anatum.

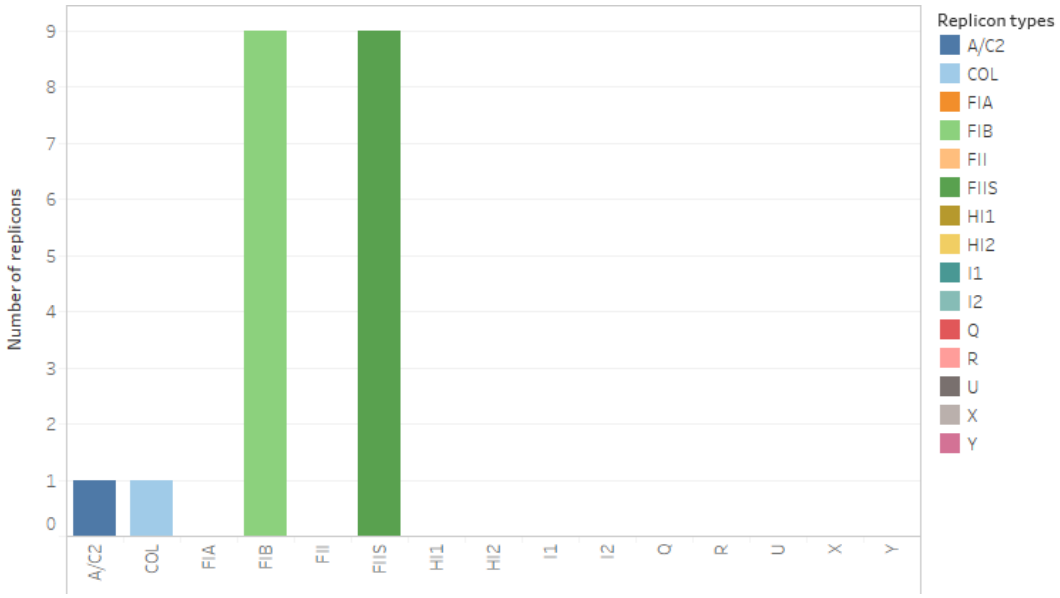

Figure 14. Number of detected plasmid replicon types in *Salmonella* Typhimurium.

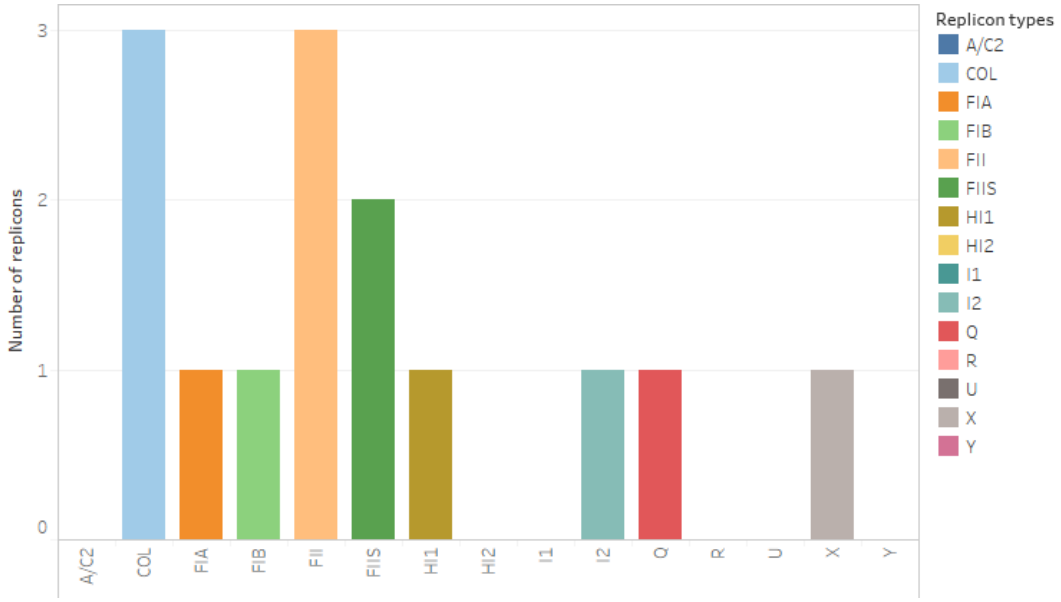

Figure 15. Number of detected plasmid replicon types in *Salmonella* Newport.

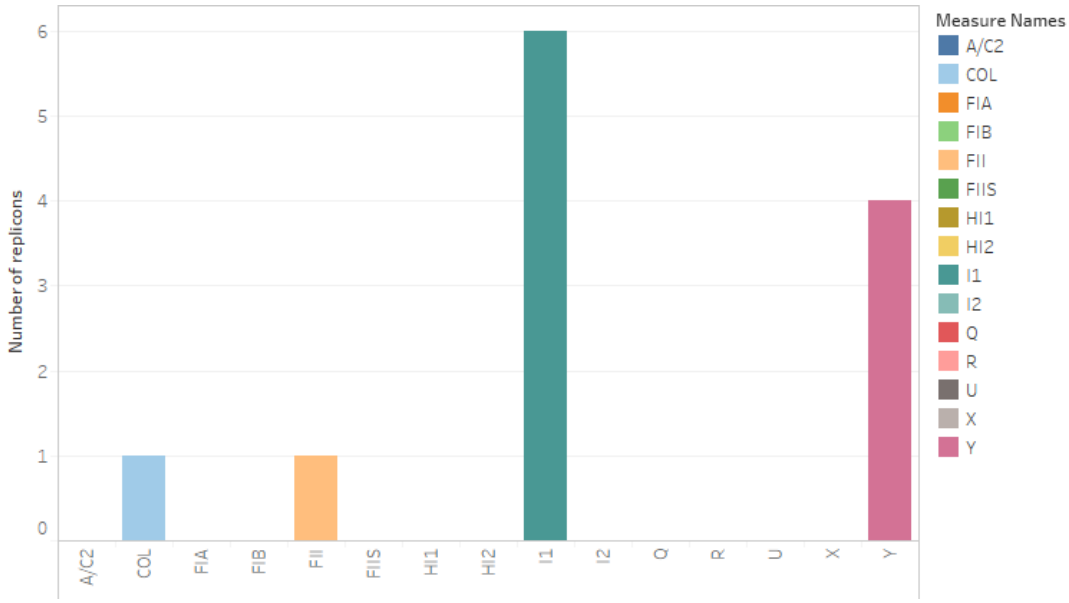

Figure 16. Number of detected plasmid replicon types in *Salmonella* Braenderup.

Evaluation of antimicrobial resistance patterns of isolated *Salmonella* from horses admitted to an equine referral hospital in the southern United States

Antimicrobial resistance patterns

The minimum inhibitory concentration (MIC) was determined for each of 255 *Salmonella* strains across all antimicrobials in the NARMS Gram-negative panel. The proportion of tested isolates that were resistant to any individual antimicrobial ranged from a low of 0% (ciprofloxacin) to a high of 10.2% (sulfisoxazole). Out of 255 strains, 219 (85.9%) were pan-pansusceptible (i.e., susceptible to all the antimicrobials), 10 (3.9 %) were resistant to less than 3 groups of antimicrobials and 26 (10.2%) were classified as multidrug resistant - MDR (resistant to 3 or more classes of antimicrobials). Among isolates exhibiting resistance to at least one antimicrobial, a total of 20 distinct patterns of antimicrobial resistance was found among 14 different serotypes. *S.* Anatum presented 8 different patterns of resistance with 5 being MDR strains, followed by Rubislaw (3 MDR), Braenderup (2 MDR strains) and Newport (1 MDR) (Table 3).

Out of 255 strains, 19 (7.4 %) of the *Salmonella* did not harbor any AMR genes, while 236 (92.5 %) carried at least a single gene. A ‘heat map’ of serotype and plasmid replicon types among 36 antimicrobial-resistant *Salmonella* isolates are illustrated in Figure 17.

Table 3. Resistance patterns for the *Salmonella* strains isolated from equine samples submitted to the clinical microbiology laboratory between January 8, 2007, and November 4, 2015 that were tested for susceptibility to 14 antimicrobials.

Resistance pattern	Serotype	% (no.) of resistant isolates
AMP-XNL-AXO-CHL-GEN-STR-FIS-TET-SXT	4,12:i:- Anatum Rubislaw	33 (1/3)[a] 3 (1/37) 15 (2/13)
AMP-AXO-CHL-GEN-NAL-STR-FIS-TET-SXT	Anatum	3 (1/37)
AMP-AZI-XNL-AXO-CHL-GEN-STR-FIS-TET-SXT	Agona	100 (4/4)
AMP-AZI-GEN-NAL-STR-FIS-TET-SXT	Anatum	3 (1/37)
AMP-AZI-GEN-NAL-TET-SXT	Anatum	3 (1/37)
AMP-CHL-GEN-STR-FIS-TET-SXT	Newport	2 (1/46)
AMP-CHL-GEN-FIS-TET	Anatum	3 (1/37)
AMP-XNL-AXO-GEN-STR-FIS-TET-SXT	Rubislaw	8 (1/13)
AMP-XNL-AXO	Braenderup	3 (1/29)
AUG2-AMP-FOX-XNL-AXO-CHL-GEN-STR-FIS-TET	Kiambu Oranienburg	50 (1/2) 25 (1/4)
AUG2-AMP-FOX-XNL-AXO-CHL-STR-FIS-TET	Braenderup Typhimurium	3 (1/29) 7 (1/14)
AUG2-AMP-FOX-XNL-AXO-GEN-FIS-SXT	Braenderup Rubislaw	14 (4/29) 8 (1/13)
FIS-TET-SXT	Infantis	5 (1/20)
STR-FIS-TET	Muenster	33 (1/3)
STR-FIS	Newport	2 (1/46)
FIS	Newport	2 (1/46)
GEN	Anatum	3 (1/37)
STR	Anatum Kentucky	3 (1/37) 33 (1/3)
TET	Anatum Kentucky Montevideo	5 (2/37) 33 (1/3) 17 (1/6)

[a]Number of *Salmonella* exhibiting a resistance pattern /Total number of *Salmonella* per serotype

None of the isolates were resistant to ciprofloxacin. AUG2 = amoxicillin/clavulanic acid 2:1 ratio, AMP = ampicillin, AZI = azithromycin, AXO = ceftriaxone, CHL = chloramphenicol, CIP = ciprofloxacin, FIS = sulfisoxazole, FOX = cefoxitin, GEN = gentamicin, NAL = nalidixic acid, SXT = trimethoprim/sulfamethoxazole, STR = streptomycin, TET = tetracycline and XNL = ceftiofur.

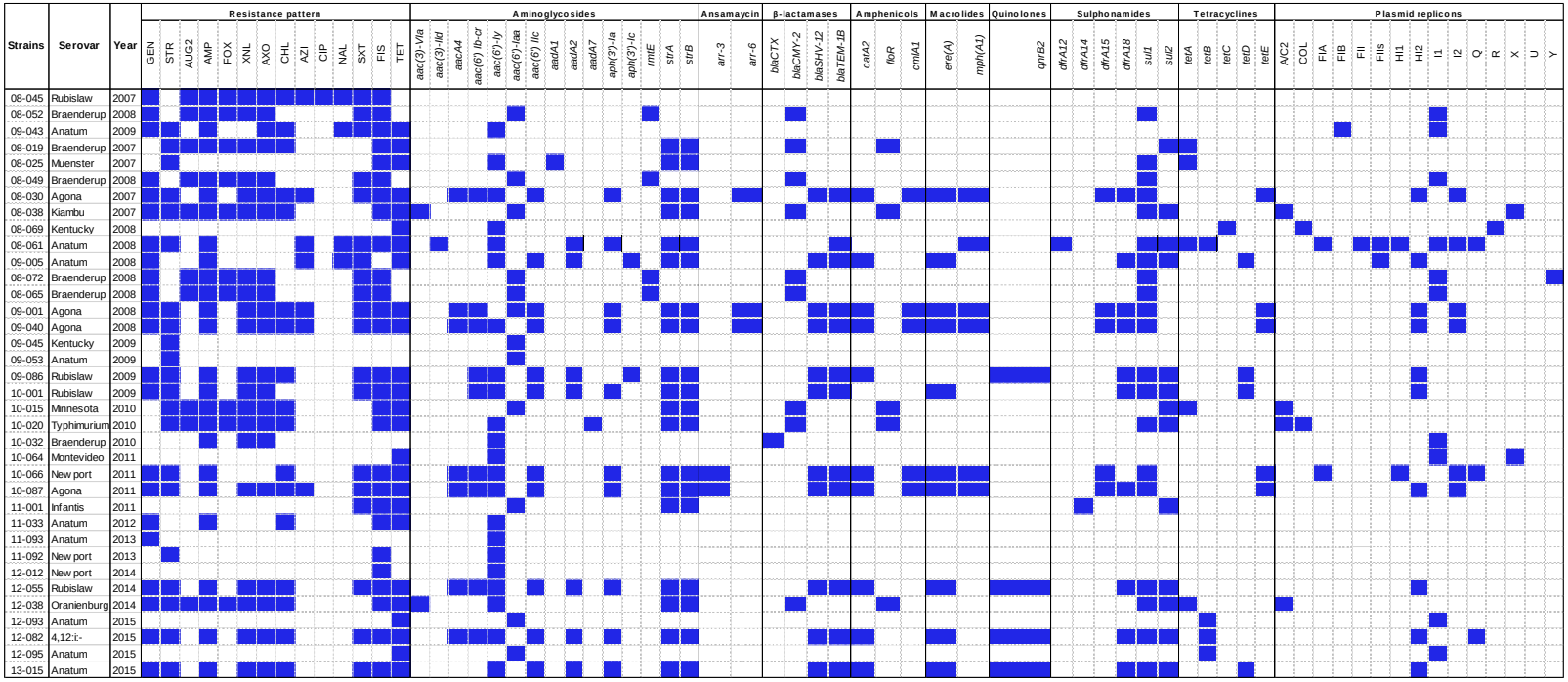

Figure 17. Heat map summary of serotypes, antimicrobial resistance phenotypes and AMR genotypes, among 36 *Salmonella* isolates exhibiting resistance to at least one antimicrobial. Also included are the corresponding Inc plasmid type (s). Blue and white squares denote the presence and absence of a specific feature, respectively. AUG2 = amoxicillin/clavulanic acid 2:1 ratio, AMP = ampicillin, AZI = azithromycin, AXO = ceftriaxone, CHL = chloramphenicol, CIP = ciprofloxacin, FIS = sulfisoxazole, FOX = cefoxitin, GEN = gentamicin, NAL = nalidixic acid, SXT = trimethoprim/sulfamethoxazole, STR = streptomycin, TET = tetracycline and XNL = ceftiofur.

Antimicrobial resistance genes

A total of 38 different resistance genes were identified and most of them were associated with clinical resistance with a few associated with decreased susceptibility to at least one of the 14 tested antimicrobials.

Beta-lactamase resistance genes

A total of four different genes encoding beta-lactamase enzymes were identified. Out of 255 strains, *bla*$_{\text{TEM-1B}}$ (encoding resistance to aminopenicillins and 1st generation cephalosporins) was found in 5% of *Salmonella* isolates, *bla*$_{\text{SHV-12}}$ (an extended-spectrum betalactamase ESBL of significant concern encoding resistance to third and fourth generation cephalosporins) was found in 4%, *bla*$_{\text{CMY-2}}$ (a AmpC cephamycinase encoding resistance to second and third generation cephalosporins) was found in 4%, and *bla*$_{\text{CTX-M-27}}$ (another ESBL gene of great concern) was found in 0.4% (Figure 18). Among the serotypes, the cephamycinase *bla*$_{\text{CMY-2}}$ gene was detected mainly in *S.* Braenderup, whereas *bla*$_{\text{SHV-12}}$ and *bla*$_{\text{TEM -1B}}$ were detected mainly among other serotypes like *S.* Agona and Rubislaw (Figure 19).

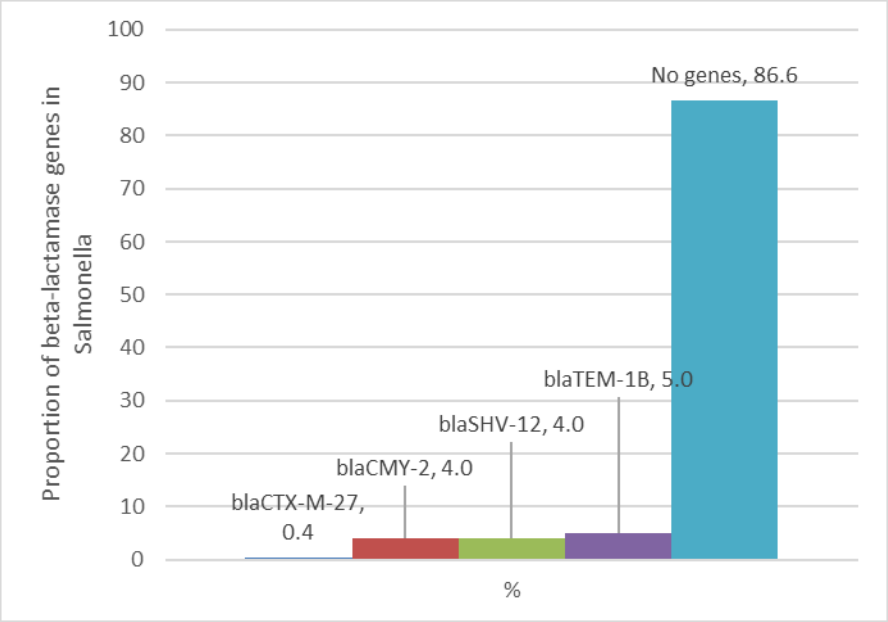

Figure 18. Proportion of beta-lactamase genes detected in the *Salmonella* from patients at an equine referral hospital

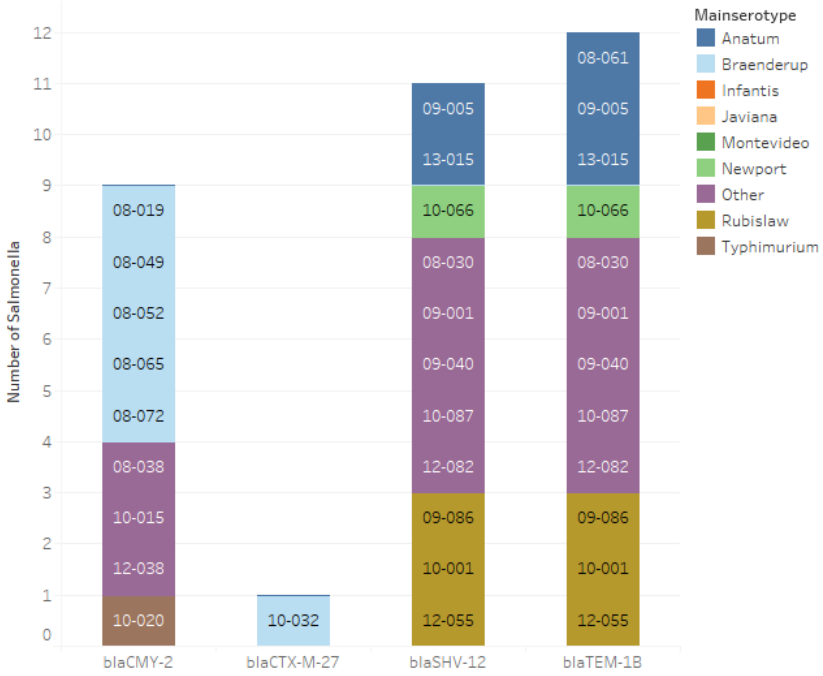

Figure 19. Beta-lactamase genes detected in the main serotypes of strains of *Salmonella* from patients at an equine referral hospital. The numbers inside of the bars show the identification of the strains.

Quinolone resistance genes

Out of 255 strains, 2 different genes encoding quinolone resistance were identified *aac(6')-lb-cr* (3%) and *qnr*B2 (2%) (Figure 20). For the latter, the resistance phenotype is typically that of reduced susceptibility, but below the breakpoint MIC value established for clinical resistance. Among the serotypes, the *aac(6')-lb-cr* gene was detected mainly in *S.* Rubislaw and Agona, whereas the *qnr*B2 was detected also among *S.* Rubislaw isolates (Figure 21).

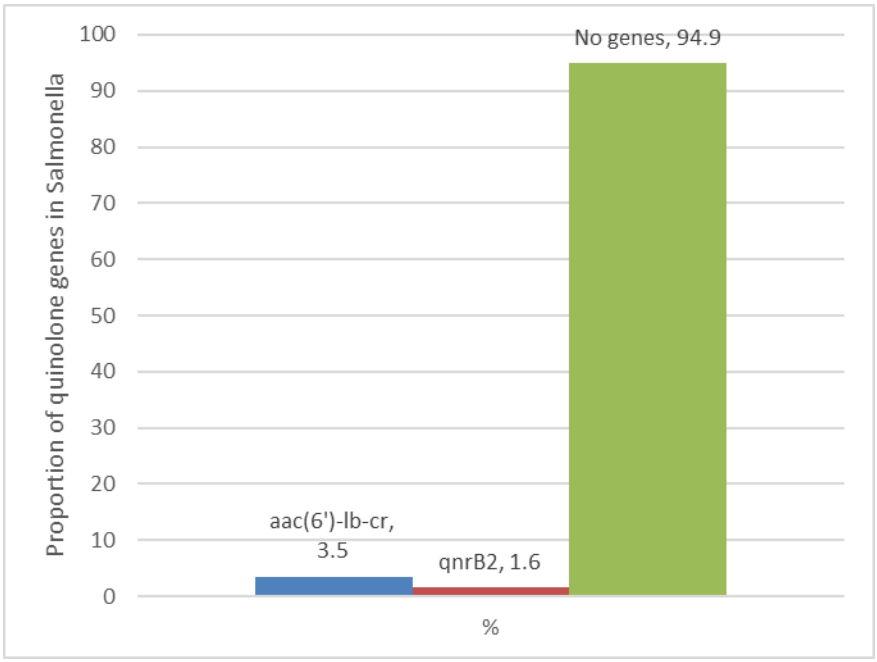

Figure 20. Relative proportion of quinolone genes (sums to 100%) detected in *Salmonella* from patients at an equine referral hospital in the southern United States.

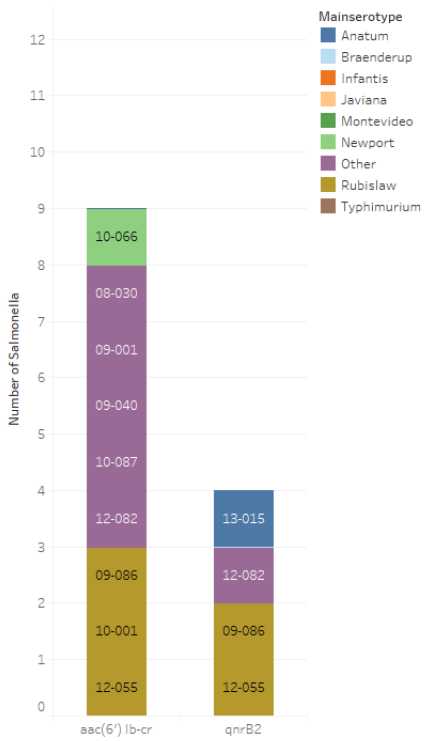

Figure 21. Quinolone genes detected among the main serotypes of *Salmonella* isolated from patients at an equine referral hospital in the southern United States. The numbers inside of the bars show the identification of the strains.

Aminoglycoside resistance genes

Although the most common aminoglycosides genes were *aac(6')-Iaa* and *aac(6')-Iy*, these genes did not appear to confer phenotypic resistance to aminoglycosides or any other class of antimicrobial. Out of 255 strains, the most common aminoglycoside resistance genes from 15 different genes detected were *strA* (7.1%), *strB* (7.5%) both encoding streptomycin resistance and *aac6-IIc* (4.4%) encoding gentamicin resistance (Figure 22). Among the serotypes, the *aac(3')-IId* gene encoding gentamicin resistance was detected only in *S. Anatum*, *aac(3')-VIa* encoding gentamicin resistance was detected in both *S.* Kiambu and *S.* Oranienburg, *aac(6')-IIc* was detected mainly in *S.* Rubislaw, *S.* Agona and *S.* Anatum, *aacA4* encoding

tobramycin and amikacin and kanamycin resistance was detected mainly in *S.* Agona, *aadA1* encoding streptomycin resistance was detected in *S.* Muenster, *aadA2* also encoding streptomycin resistance was detected mainly in *S.* Rubislaw and S. Anatum, *aadA7* encoding streptomycin resistance was detected only in *S.* Typhimurium, *aph(3')-la* encoding gentamicin resistance was detected mainly in *S.* Agona, *aph(3')-lc* encoding gentamicin resistance was detected in *S.* Anatum and *S.* Rubislaw, *rmtE* encoding gentamicin resistance was detected only in *S.* Braenderup, *strA* was detected mainly in *S.* Rubislaw and Agona, and *strB* gen and was detected also in *S.* Rubislaw (Figure 23).

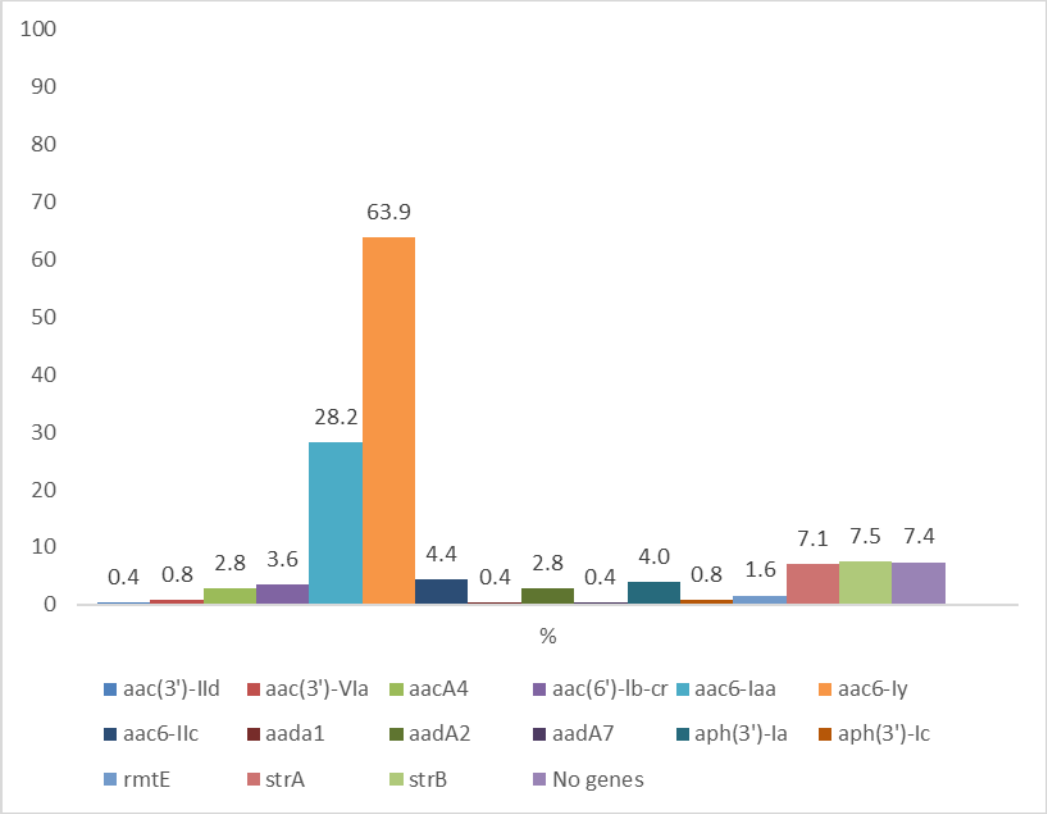

Figure 22. Relative proportion of aminoglycoside genes (sums to 100%) detected in *Salmonella* from patients at an equine referral hospital in the southern United States.

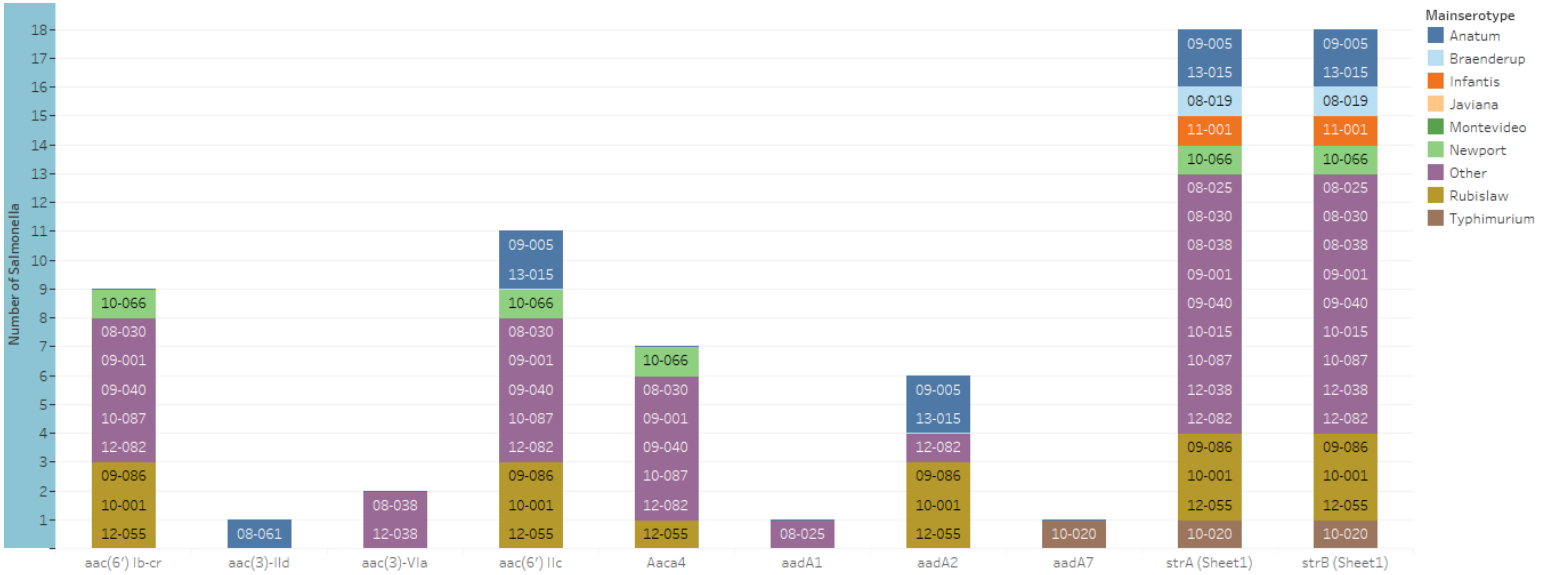

Figure 23. Aminoglycoside genes detected among the main serotypes of strains of *Salmonella* from patients at an equine referral hospital. Numeric coding within each bar represent case numbers. The numbers inside of the bars show the identification of the strains.

Sulfonamide resistance genes

Out of 255 strains, the *sul* gene was found with two different variations encoding for sulfonamide resistance (*sul1* (8%) and *sul2* (5%)) (Figure 24).

Among the serotypes, the *sul1* gene was detected mainly in *S.* Braenderup and the *sul2* was mainly detected among *S.* Rubislaw (Figure 25).

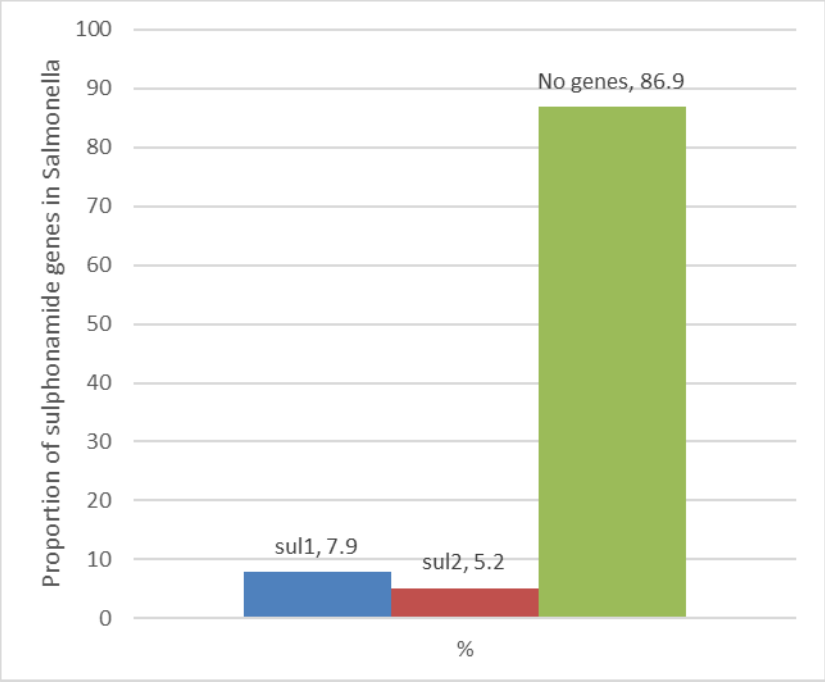

Figure 24. Relative proportion of sulphonamide genes (sums to 100%) detected in *Salmonella* from patients at an equine referral hospital in the southern United States.

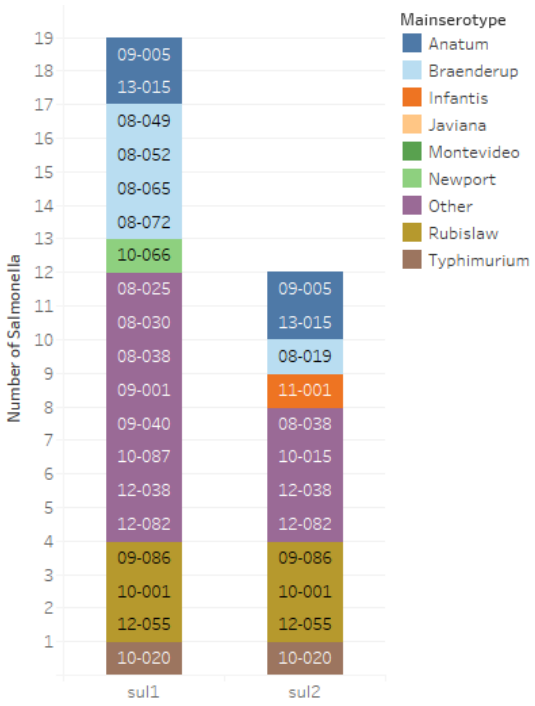

Figure 25. Sulfonamide genes detected among the main serotypes of strains of *Salmonella* from patients at an equine referral hospital in the southern United States. The numbers inside of the bars show the identification of the strains.

Folate pathway inhibitor resistance genes

Among trimethoprim / sulfamethoxazole resistant strains, a single gene family with four variants encoding for trimethoprim resistance were identified as *dfrA18* (3.9%), *dfrA15* (1.9%), *dfrA12* (0.4%) *and dfrA14* (0.4%) (Figure 26). Among the serotypes, *dfrA18* and *dfrA15* alleles were detected mainly in *S.* Agona, *dfrA14* in *S.* Infantis and *dfrA12* in *S.* Anatum (Figure 27).

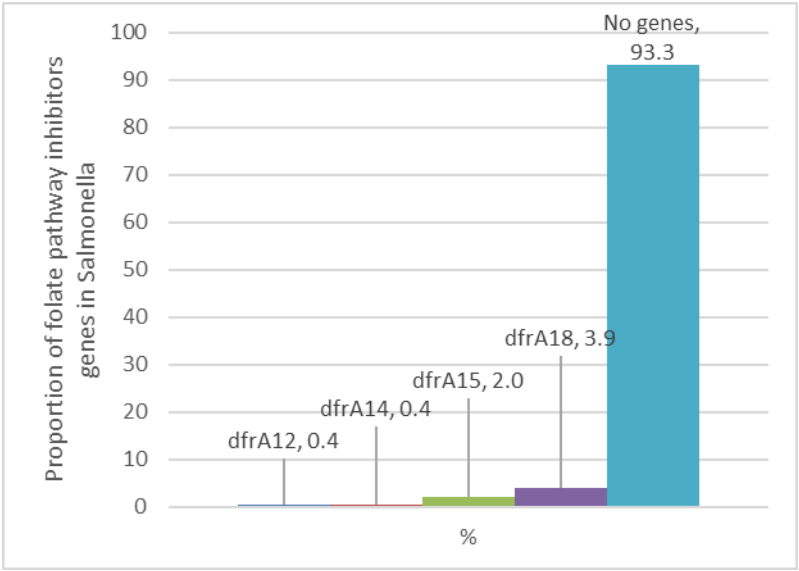

Figure 26. Relative proportion of folate pathway inhibitors alleles (sums to 100%) detected in *Salmonella* from patients at an equine referral hospital in the southern United States.

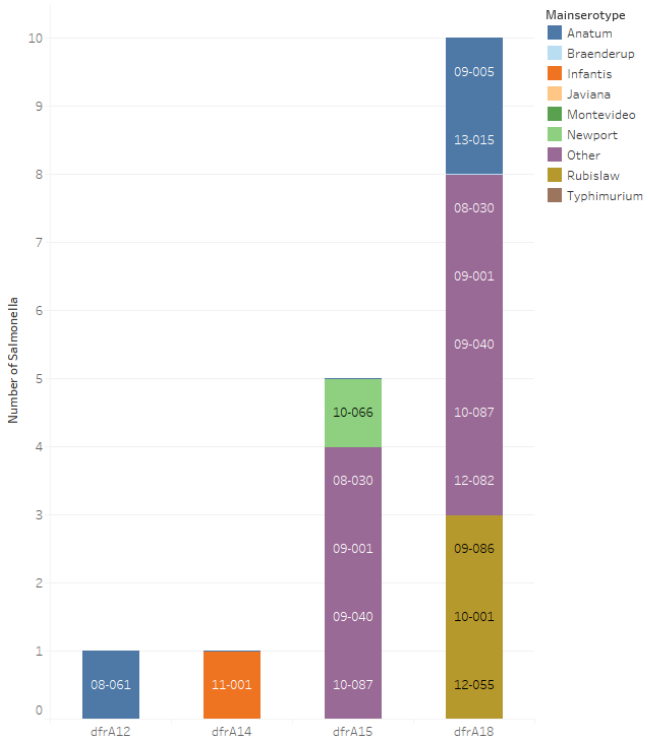

Figure 27. Folate pathway inhibitors genes detected in the main serotypes of strains of *Salmonella* from patients at an equine referral hospital in the southern United States. The numbers inside of the bars show the identification of the strains.

Phenicol resistance genes

Out of 255 strains, 3 different genes encoding phenicol resistance were identified as *cat*A2 (4.1%), *flo*R (2.0%) and *cml*A1 (2.0%) (Figure 28). Among the serotypes, *cat*A2 and *cml*A1 were detected mainly in Agona while *flo*R was detected among a variety of different serotypes (Figure 29).

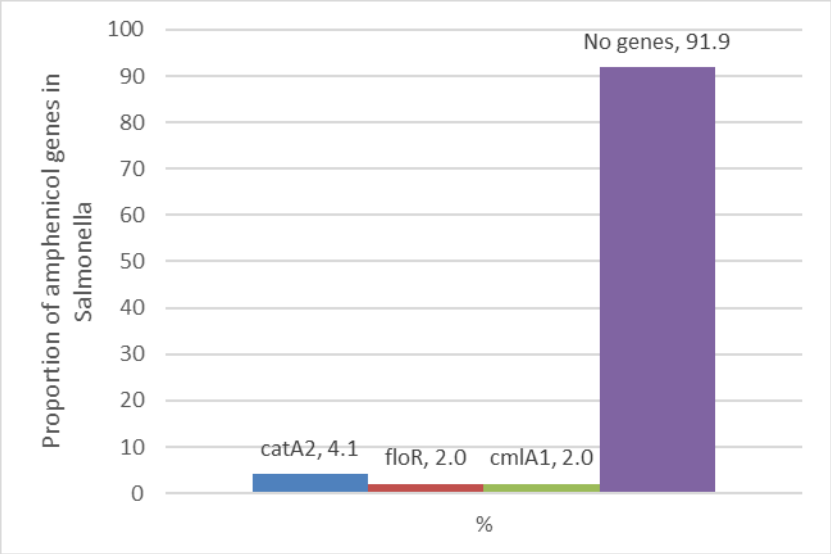

Figure 28. Relative proportion of amphenicol genes (sums to 100%) detected in *Salmonella* from patients at an equine referral hospital in the southern United States.

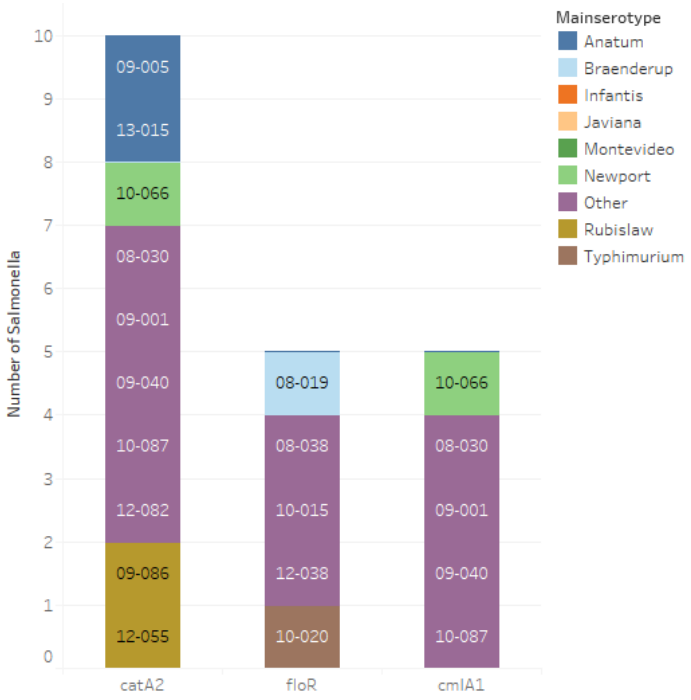

Figure 29. Amphenicol genes detected in the main serotypes of strains of *Salmonella* from patients at an equine referral hospital in the southern United States. The numbers inside of the bars show the identification of the strains.

Tetracycline resistance genes

Out of 255 strains, 5 different tetracycline resistance genes were identified as follows: *tet*(A) (1.9%), *tet*(B) (2.4%), *tet*(C) (0.4%), *tet*(D) (1.6%) and *tet*(E) (2.4%) (Figure 30). Among the serotypes, *tet*(A) was detected in multiple serotypes, *tet*(B) was detected mainly in *S.* Anatum *tet*(C) was detected only in *S.* Kentucky, *tet*(D) was detected in *S.* Rubislaw and S. Anatum, and *tet*(E) was detected mainly in *S.* Agona (Figure 31).

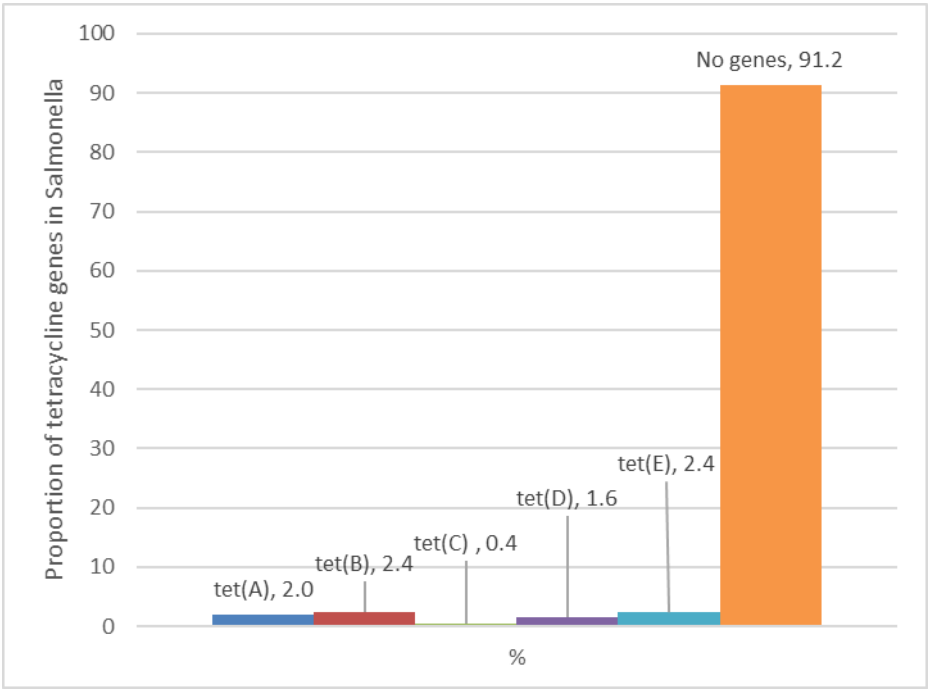

Figure 30. Relative proportion of tetracycline genes (sums to 100%) detected in *Salmonella* from patients at an equine referral hospital in the southern United States.

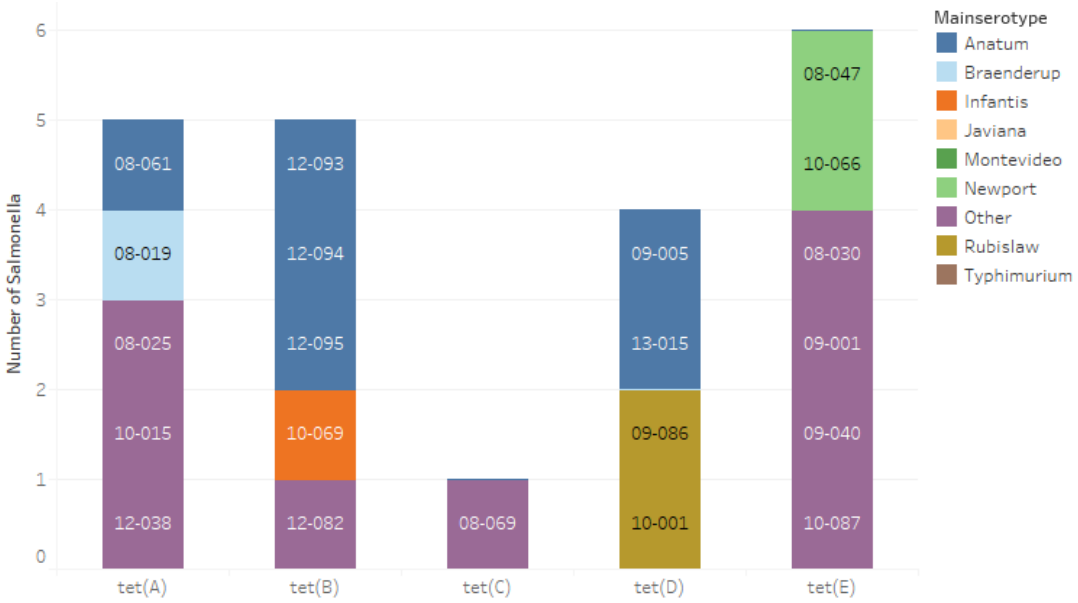

Figure 31. Tetracycline genes detected in the main serotypes of strains of *Salmonella* from patients at an equine referral hospital in the southern United States. The numbers inside of the bars show the identification of the strains.

Macrolide resistance genes

Out of 255 strains, 2 different genes encoding macrolide resistance were identified as *ere*(A) (3.9%) encoding for erythromycin and *mph*A (2.8%) encoding for azithromycin (Figure 32).Among the serotypes, *ere*(A) and *mph*A were mainly detected among *S.* Agona (Figure 33).

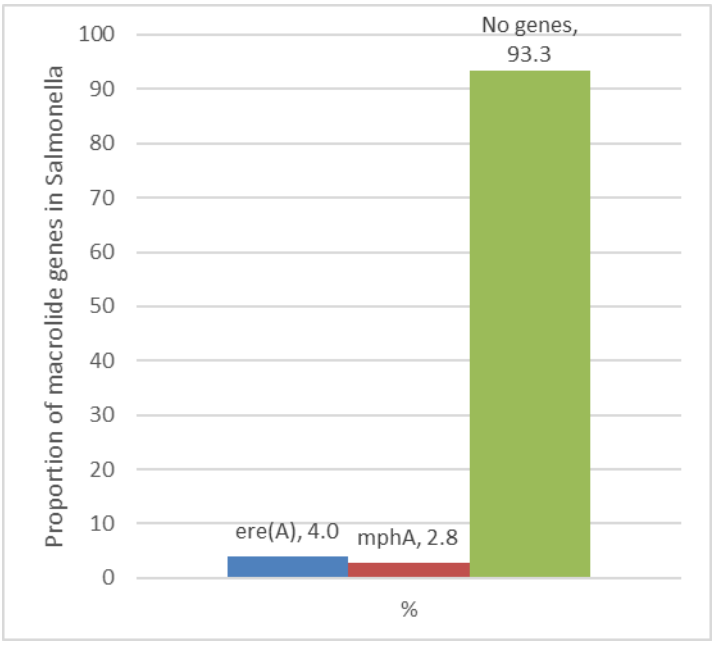

Figure 32. Proportion of macrolide genes detected in *Salmonella* from patients at an equine referral hospital in the southern United States.

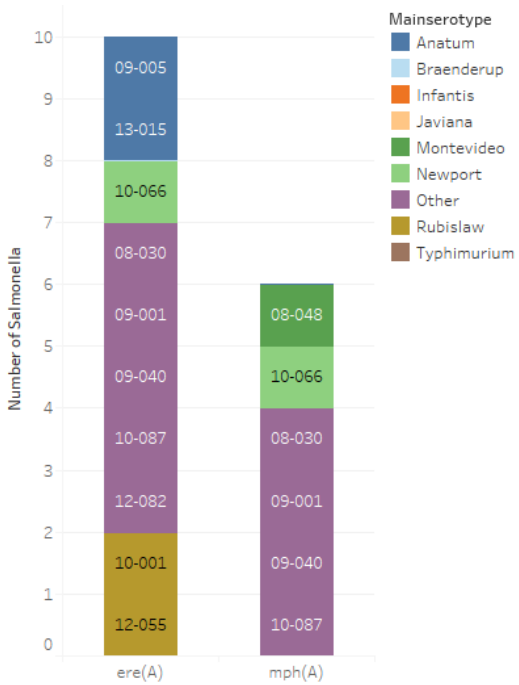

Figure 33. Macrolide genes detected in the main serotypes of strains of *Salmonella* from patients at an equine referral hospital in the southern United States. The numbers inside of the bars show the identification of the strains.

Other resistance genes

Among ansamycin resistant strains, 2 different alleles encoding ansamycin resistance were identified as *arr-3* (0.8%) and *arr-6* (1.2%) encoding for rifampin (Figure 34). Among the serotypes, *arr-3* and *arr-6* were mainly detected among *S.* Agona (Figure 35).

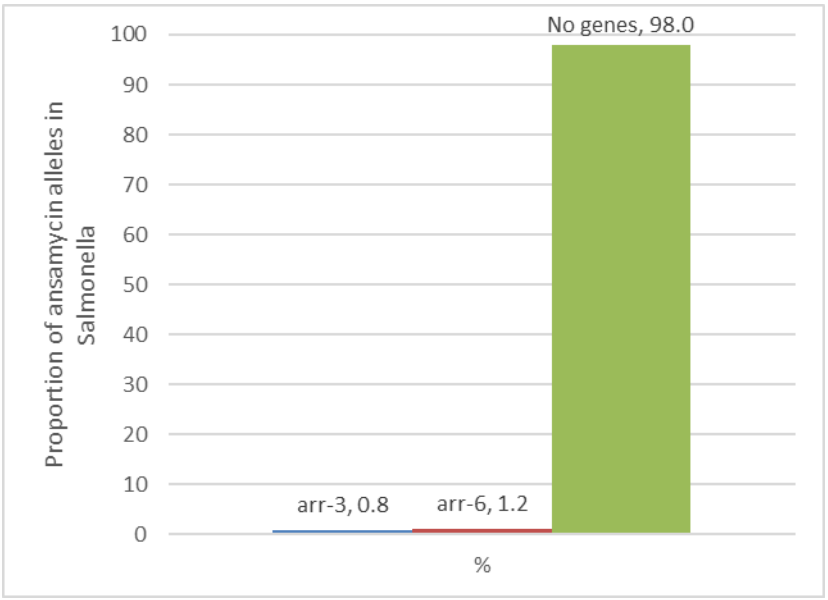

Figure 34. Proportion of ansamycin alleles detected in *Salmonella* from patients at an equine referral hospital in the southern United States**.**

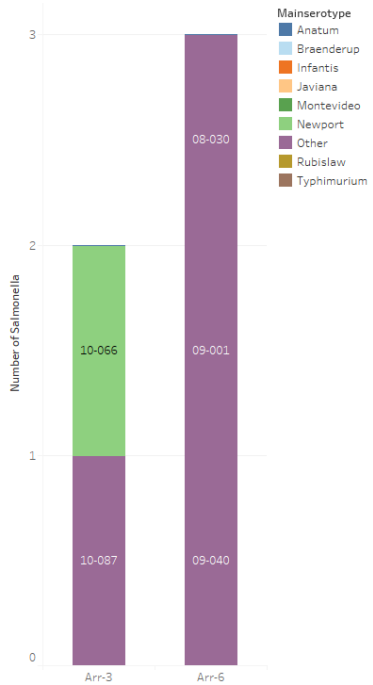

Figure 35. Ansamycin alleles detected in the main serotypes of strains of *Salmonella* from patients at an equine referral hospital in the southern United States. The numbers inside of the bars show the identification of the strains.

Agreement between detected AMR genes and MICs values

The agreement between presence of AMR genes identified by the ResFinder tool and the resistance phenotype by Sensititre system are shown in Table 4. Aminoglycosides, beta-lactams, cephems, penicillins, folate pathway inhibitors, phenicols and macrolides had substantial agreement (Kappa = 0.61-0.94) when comparing phenotypic and genotypic results. Tetracyclines presented good agreement (Kappa = 0.76), and when considering the intermediate MIC of ciprofloxacin, it presented moderate agreement (Kappa=0.48) with the presence of quinolone resistance genes (encoding reduced susceptibility, but not clinical resistance). Nalidixic acid had very poor agreement (Kappa=-0.02) when comparing the phenotypic resistance and the presence of quinolone genes.

Phylogenetic analysis

A whole-genome single nucleotide polymorphism (SNP)-based tree was constructed using Parsnp 1.2 and visualized with FigTree. The analysis was conducted only with the most frequent serotypes (*Salmonella* Anatum, Braenderup and Newport) with presence of clusters between the days of admission of the patients to the hospital in the years of the study to investigate a possible genetic association between the isolates that may be indicative of an outbreak in the hospital.

Table 4. Genotype and phenotype comparison of 255 *Salmonella* strains obtain from equine samples submitted to the clinical lab between January 8, 2007, and November 4, 2015

Antimicrobial agent	No. of test results				Agreement	Kappa
	Resistant phenotype		Susceptible phenotype			
	Genotype: resistant	Genotype: susceptible	Genotype: resistant	Genotype: susceptible		
Aminoglycoside						
Gentamicin	18	4	3	230	97.25	0.8222
Streptomycin	17	4	2	232	97.65	0.8373
Beta-lactam/beta-lactam inhibitor						
Amoxicillin/clavulanic acid	9	1	0	245	99.61	0.9453
Cephems						
Cefoxitin	9	1	0	245	99.61	0.9453
Ceftiofur	19	1	3	232	98.43	0.8962
Ceftriaxone	19	1	3	232	98.43	0.8962
Penicillin						
Ampicillin	22	3	0	230	98.82	0.9297
Folate pathway inhibitor						
Sulfisoxazole	22	5	1	227	97.65	0.8670
Trimethoprim/sulfamethoxazole	13	6	0	236	97.65	0.8004
Macrolide						
Azithromycin	5	1	2	246	98.43	0.7064
Phenicol						
Chloramphenicol	14	2	1	238	98.82	0.8970
Quinolone						
Ciprofloxacin*	4	2	6	249	96.86	0.4848
Nalidixic Acid*	0	3	10	242	94.9	-0.0184
Tetracycline						
Tetracycline	18	7	3	227	96.08	0.7612

* Analysis using MIC (minimum inhibitory concentration) classified for reduced susceptibility

Salmonella Anatum

We analyzed 37 genomes; however, the phylogenetic tree was constructed with a subset of 31 genomes (Figure 36). Measurement of the maximal unique exact matches shared by two sequences by the MUM index >0.01 were excluded from the analysis (91). The complete genome of *Salmonella enterica* subsp. *enterica* serotype Anatum strain USDA-ARS-USMARC-1765 (NZ_CP014659.2) was used as a reference to assemble the phylogenetic tree; this strain is from a human salmonellosis case and was part of a study that compared the genomes of *Salmonella* Anatum arising from human and bovine sources (94). There were 2 main clusters: Cluster (I) and Cluster (II), with two cases from 2014 grouped outside of the two main clusters. Cluster (I) could be further sub-divided into four sub-clusters (I-a to I-d). Sub-cluster I-a contained 3 cases from 2008, 2011 and 2012. Two of the 3 cases were adult horses and one was a foal; all presented with colic. Interestingly, in both cases from adults *S.* Anatum were pan-susceptible, while the foal case was MDR (AMP-CHL-GEN-FIS-TET) detected by annotation genes in the PATRIC platform. Sub-cluster I-b contained 2 cases from two adults with a presenting complaint of colic and the *Salmonella* was pansusceptible in both cases. Sub-cluster I-c contained 6 cases and grouped with the reference strain; 3 isolates were from adult horses, one from a juvenile, one from a foal and other without reported age; 3 cases had a complaint of colic and one horse was euthanized; 2 isolates were MDR and 4 isolates were pansusceptible. Sub-cluster I-d contained 5 cases from April and May of 2015 and all the horses were adults; however, the complaints were different; one case was presented with colic and two cases presented with a dental complaint. Two isolates

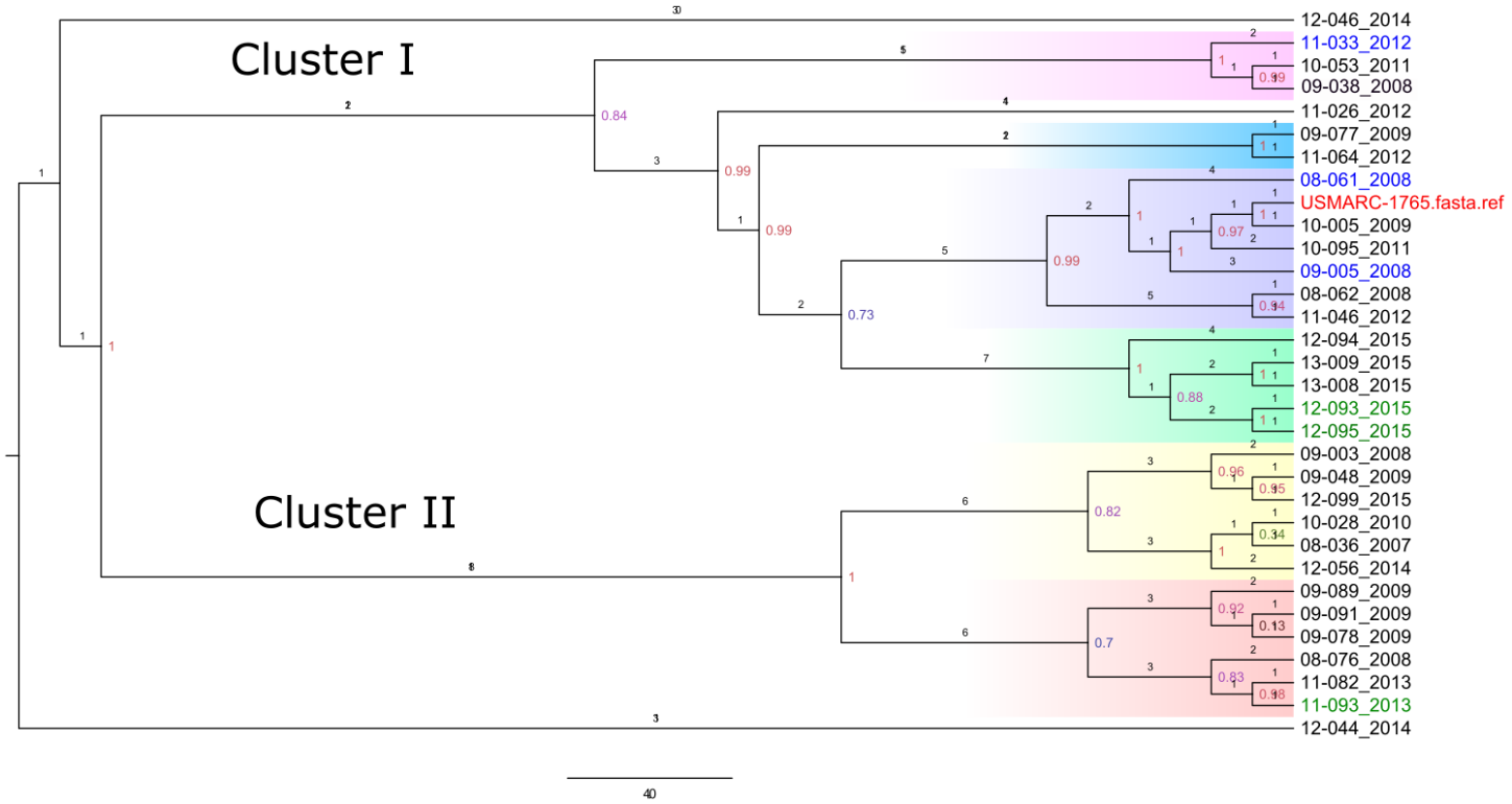

Figure 36. Whole-genome SNP-based phylogenetic tree of 33 *Salmonella* Anatum and a *Salmonella* Anatum reference strain generated by Parsnp and visualized by FigTree**.** The branch lengths are expressed in terms of changes per number of SNPs. The numbers in color show the bootstrap corresponding to the specific internal node. Strain names are marked with the colors red (reference), blue (MDR), green (resistant) and black (susceptible). Strain names are labeled with the year of admission of the patient to the hospital. Clusters are colored according to the phylogenetic group (clade). Cluster I includes: Sub-cluster I-a (pink), I-b (blue), I-c (purple) and I-d (green). Cluster II includes: Sub-cluster II-a (yellow) and II-b (red). The scale bar shows the estimated number of substitutions per SNP.

had tetracycline resistance; while the others strains were pansusceptible.

Cluster II contained 2 sub-clusters (II-a, and II-b). Cluster II-a (6 cases) were from 2007, 2008, 2009, 2010, 2014 and 2015. Four cases were adult horses, one was a foal and one was without reported age. Four cases were presented as colic and two had diarrhea. All the strains were pan-susceptible. Cluster II-b (6 cases) from 2008, 2009 and 2013 included four adults and two foals. Most of the strains were pan-susceptible; however, one was gentamicin resistant. Detailed information concerning the cases per each cluster is shown in Table 5.

Salmonella **Braenderup**

A phylogenetic tree was constructed using data from 27 sequenced *S.* Braenderup (Figure 37). The complete genome of *S.* Braenderup Sal_JBP_2011K-0222 from the United States Centers for Disease Control and Prevention (CDC) was obtained through personal communication with Dr. Henk den Bakker from the University of Georgia; this was used as a reference to analyze the phylogenetic tree.

Table 5. Equine cases of genetically related *Salmonella* Anatum from Cluster I and II by phylogeny.

									Aminoglycosides								Beta-lactams		Amphenicol	Macrolide	Folate pathway inhibitor				Tetracycline		
Cluster	Sub-cluster	Vial	Breed	Admission date	Discharge date	Age	Complaint	Pattern	*aac(3)-IId*	*aac(6')-Iaa*	*aac(6')-Iy*	*aac(6') IIc*	*aadA2*	*aph(3')-Ic*	*strA*	*strB*	*blaSHV-12*	*blaTEM-1B*	*catA2*	*mph(A1)*	*dfrA12*	*dfrA18*	*sul1*	*sul2*	*tetA*	*tetB*	*tetD*
I	I-a	09-038	Mixed Breed	11/29/2008	12/3/2008	Adult	colic	Pansusceptible			■																
I	I-a	10-053	Hanoverian	2/17/2011	2/28/2011	Adult	colic	Pansusceptible			■																
I	I-a	11-033	Quarter Horse	5/15/2012	5/24/2012	Foal	colic	AMP-CHL-GEN-FIS-TET			■																
I	I-b	09-077	Quarter Horse	4/12/2009	4/15/2009	Adult	colic	Pansusceptible			■																
I	I-b	11-064	Quarter Horse	12/17/2012	12/19/2012	Adult	colic	Pansusceptible			■																
I	I-c	08-061	Quarter Horse	2/1/2008	2/4/2008	Adult	colic	AMP-AZI-GEN-NAL-STR-FIS-TET-SXT	■	■	■							■		■	■		■	■	■	■	
I	I-c	08-062	Arabian	2/28/2008	2/28/2008	Foal	general signs	Pansusceptible			■																
I	I-c	09-005	Shire	5/23/2008	5/28/2008	Adult	colic	AMP-AZI-GEN-NAL-TET-SXT			■	■	■	■	■	■	■	■	■	■		■	■	■			■
I	I-c	10-005	Quarter Horse	11/5/2009	11/7/2009	Adult	colic	Pansusceptible		■																	
I	I-c	10-095	American Paint	10/15/2011	10/24/2011	Juvenile	diarrhea, fever	Pansusceptible			■																
I	I-c	11-046	Thoroughbred	10/1/2012	10/1/2012	NR	euthanasia	Pansusceptible			■																
I	I-d	12-093	Quarter Horse	5/3/2015	5/7/2015	Adult	lethargic, down, fever	TET		■																■	
I	I-d	12-094	Quarter Horse	5/6/2015	5/10/2015	Adult	colic	Pansusceptible			■																
I	I-d	12-095	Quarter Horse	5/5/2015	5/17/2015	Adult	epistaxis	TET		■																■	
I	I-d	13-008	Mixed Breed	4/16/2015	4/16/2015	Adult	dental	Pansusceptible			■																
I	I-d	13-009	Mixed Breed	4/16/2015	4/16/2015	Adult	dental	Pansusceptible			■																
II	II-a	08-036	Quarter Horse	9/27/2007	10/1/2007	NR	colic, dehydrated, colitis, diarrhea	Pansusceptible			■																
II	II-a	09-003	Foreign Warm Blood	5/27/2008	6/2/2008	Foal	colic	Pansusceptible			■																
II	II-a	09-048	Quarter Horse	2/8/2009	3/18/2009	Adult	colic	Pansusceptible		■																	
II	II-a	12-056	Appaloosa	9/9/2014	9/10/2014	Adult	colic	Pansusceptible		■																	
II	II-a	12-099	Quarter Horse	5/19/2015	5/23/2015	Adult	diarrhea	Pansusceptible		■																	
II	II-a	10-028	Thoroughbred	8/25/2010	8/28/2010	Adult	dehydration	Pansusceptible			■																
II	II-b	08-076	Irish Sport Horse	4/13/2008	5/14/2008	Foal	fetlock arthroscopy	Pansusceptible			■																
II	II-b	09-078	Quarter Horse	4/16/2009	4/29/2009	Foal	diarrhea	Pansusceptible		■																	
II	II-b	09-089	Quarter Horse	7/21/2009	7/21/2009	Adult	colic	Pansusceptible		■																	
II	II-b	09-091	Quarter Horse	7/9/2009	9/13/2009	Adult	general signs	Pansusceptible			■																
II	II-b	11-082	Arabian	7/8/2013	7/24/2013	Adult	colitis	Pansusceptible			■																
II	II-b	11-093	Quarter Horse	9/16/2013	9/30/2013	Adult	colic	GEN			■																

There were 2 main clusters: Cluster (I) and Cluster (II). Cluster (I) contained strains from 2007 to 2015 and could be further sub-divided into five sub-clusters (I-a to I-e). Sub-cluster I–a contained 6 cases from 2009, four foals presented with diarrhea and two adults were boarding without signs; the strains had the same pan-susceptible pattern and these cases where coming from a known outbreak on the same ranch. Sub-cluster I–b contained two cases in foals, and one in adult – the adult was boarding. The strain from one foal had the ESBL gene *bla*$_{\text{CTX-M-27.}}$ Sub-cluster I–c contained one case in an adult and one in a foal. Sub-cluster I–d was formed by three cases in adults presenting with colic. Sub-cluster I-e contained 5 cases from 2008, four with the same antimicrobial resistance pattern (AUG2-AMP-FOX-XNL-AXO-GEN-FIS-SXT) and with the same presence of antimicrobial resistance genes (*rmtE*, *bla*$_{\text{CMY-2}}$ and *sul1*), suggesting that it was a clonal group; however, the single isolate from Case 08-056 was pan-susceptible and had 4 nucleotide substitutions per site more than the multi-drug resistant strains. Cluster (II) contained 6 cases from 2007 (1 case), 2008 (2 cases) and 2009 (3 cases). Three adults and three foals; two with diarrhea, two with colic, one dystocia and one surgery. Detailed information of the cases per each cluster is shown in Table 6.

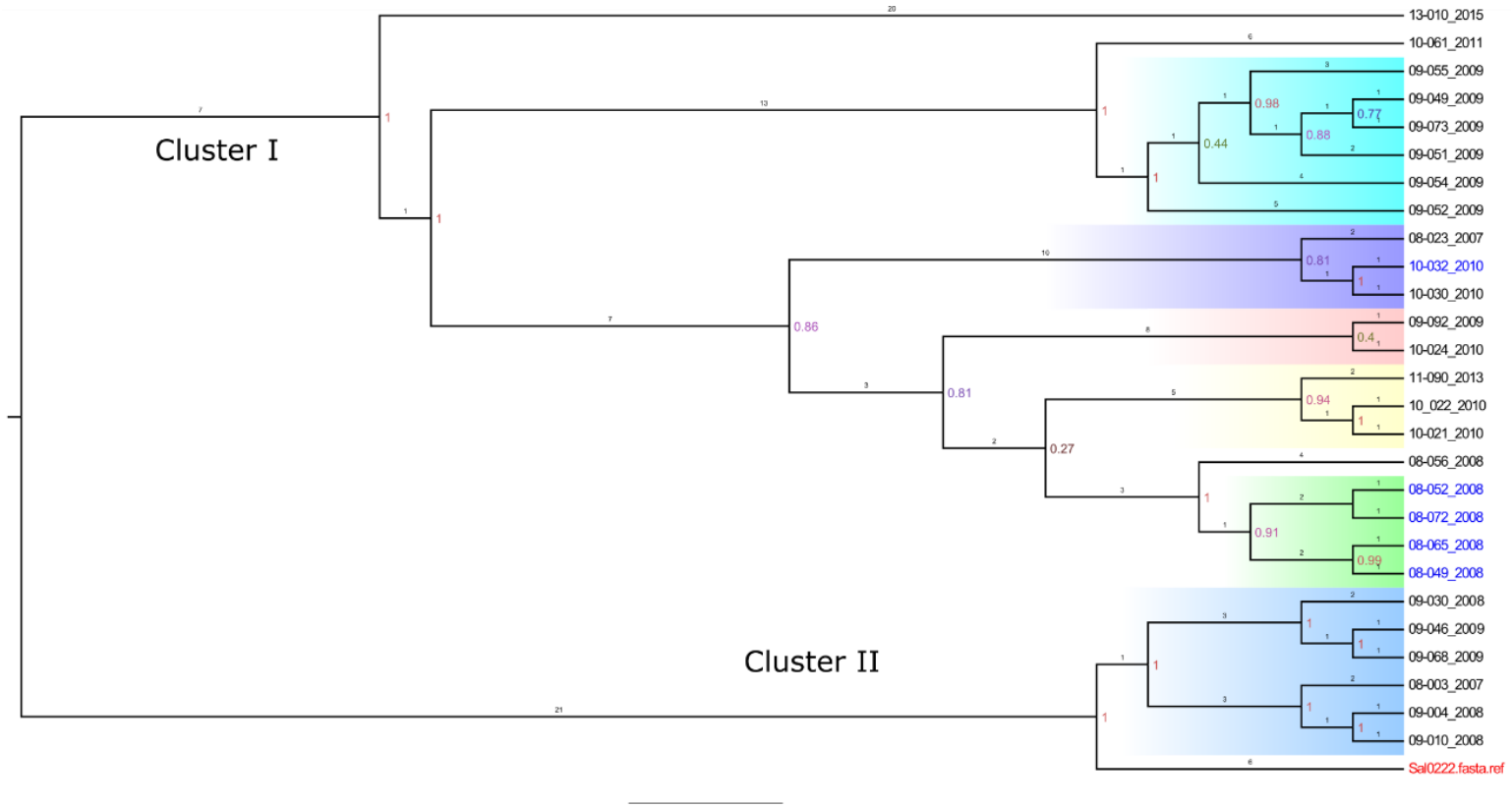

Figure 37. Whole-genome SNP-based phylogenetic tree of 27 *Salmonella* Braenderup and a *Salmonella* Braenderup reference strain generated by Parsnp and visualized using FigTree. The branch lengths are expressed in terms of changes per number of SNPs. The numbers in color show the bootstrap corresponding to the specific internal node. Strain names are marked with the colors red (reference), blue (resistant) and black (susceptible). Strains names are labeled with the year of admission of the patient to the hospital. Clusters are colored according to the phylogenetic group (clade). Cluster I includes: Sub-cluster I-a (cyan blue), I-b (purple), I-c (pink), I-d (yellow) and I-e is (green). Clade II (light violet). The scale bar shows the estimated number of substitutions per SNP.

Table 6. Equine cases of genetically related *Salmonella* Braenderup from Cluster I and II by phylogeny.

									Aminoglycosides			Beta-lactams		Folate pathway inhibitors
Cluster	Sub-cluster	Vial	Breed	Admission date	Discharge date	Age	Complaint	Pattern	*aac(6')-Iaa*	*aac(6')-Iy*	*rmtE*	*blaCMY2*	*blaCTX-M-27*	*sul1*
I	I-b	08-023	Dutch Warmblood	6/26/2007	7/12/2007	Foal	lameness	Pansusceptible						
I	I-e	08-049	Thoroughbred	4/10/2008	4/11/2008	Adult	respiratory - poss. strangles	AUG2-AMP-FOX-XNL-AXO-GEN-FIS-SXT						
I	I-e	08-052	Irish Draught	3/28/2008	4/16/2008	Juvenile	colic	AUG2-AMP-FOX-XNL-AXO-GEN-FIS-SXT						
I	I-e	08-056	Quarter Horse	3/18/2008	3/20/2008	Foal	diarrhea	Pansusceptible						
I	I-e	08-065	Quarter Horse	3/20/2008	3/27/2008	Foal	diarrhea	AUG2-AMP-FOX-XNL-AXO-GEN-FIS-SXT						
I	I-e	08-072	Quarter Horse	3/18/2008	3/22/2008	Foal	bloody and watery diarrhea	AUG2-AMP-FOX-XNL-AXO-GEN-FIS-SXT						
I	I-a	09-049	Thoroughbred	2/24/2009	2/28/2009	Foal	diarrhea	Pansusceptible						
I	I-a	09-051	Thoroughbred	2/22/2009	3/23/2009	Foal	diarrhea, lethargic, dehydration	Pansusceptible						
I	I-a	09-052	Thoroughbred	2/24/2009	3/9/2009	Foal	diarrhea	Pansusceptible						
I	I-a	09-054	Thoroughbred	2/22/2009	3/23/2009	Adult	boarding	Pansusceptible						
I	I-a	09-055	Thoroughbred	2/24/2009	3/1/2009	Adult	boarding	Pansusceptible						
I	I-a	09-073	Thoroughbred	3/24/2009	4/6/2009	Foal	watery diarrhea, dehydration	Pansusceptible						
I	I-c	09-092	Quarter Horse	7/28/2009	8/5/2009	Adult	colic	Pansusceptible						
I	I-d	10-021	American Paint	4/18/2010	4/24/2010	Adult	colic	Pansusceptible						
I	I-d	10-022	Quarter Horse	5/14/2010	5/25/2010	Adult	colic	Pansusceptible						
I	I-c	10-024	Quarter Horse	5/29/2010	6/3/2010	Foal	diarrhea	Pansusceptible						
I	I-b	10-030	Gypsy Vanner	10/11/2010	10/25/2010	Adult	boarding	Pansusceptible						
I	I-b	10-032	Gypsy Vanner	10/11/2010	10/25/2010	Foal	salmonellosis	AMP-XNL-AXO						
I	I-d	11-090	Gypsy Vanner	9/11/2013	9/11/2013	Adult	colic	Pansusceptible						
II		08-003	Arabian	1/8/2007	1/24/2007	Adult	colic	Pansusceptible						
II		09-004	Dutch Warmblood	5/23/2008	5/29/2008	Foal	watery diarrhea, fever, not eating	Pansusceptible						
II		09-010	Foreign Warm Blood	6/6/2008	6/6/2008	Foal	surgery	Pansusceptible						
II		09-030	Quarter Horse	11/3/2008	11/8/2008	Adult	colic	Pansusceptible						
II		09-046	Quarter Horse	1/31/2009	2/25/2009	Foal	diarrhea	Pansusceptible						
II		09-068	Quarter Horse	3/21/2009	3/22/2009	Adult	dystocia, possible ruptured rectum	Pansusceptible						

Salmonella Newport

The *S.* Newport phylogenetic analysis used 48 genomes; however, the final phylogenetic tree included only 42 genomes (Figure 38). Genomes with MUMi distance >0.01 were excluded from the analysis. The complete genome of *Salmonella enterica* subsp. *enterica* serotype Newport strain 0007-33 (accession number: NZ_CP013685.1) was used as a reference to analyze the phylogenetic tree; this strain is from a bovine gastroenteritis case collected by University of Pennsylvania *Salmonella* Reference Center (95). There were 2 main clusters: Cluster I with 2 subclusters and Cluster II with 6 subclusters. Subcluster I-a contained 2 strains from 2 adult horses from 2011 and 2012. Subcluster I-b contained 8 cases from different years, including three adults, two foals, one senior horse and two with ages not reported; four had signs of colic, one with colitis, one with diarrhea and one foal that was lame in its front legs. Cluster (II) contained 6 sub-clusters. Sub-cluster II-a included two cases: one adult, and one without age reported; the first case was presenting with diarrhea and weight loss, while the other presented with fever and problems in the lungs. Sub-cluster II-b contained two cases: one foal with colic and one adult with possible choke.

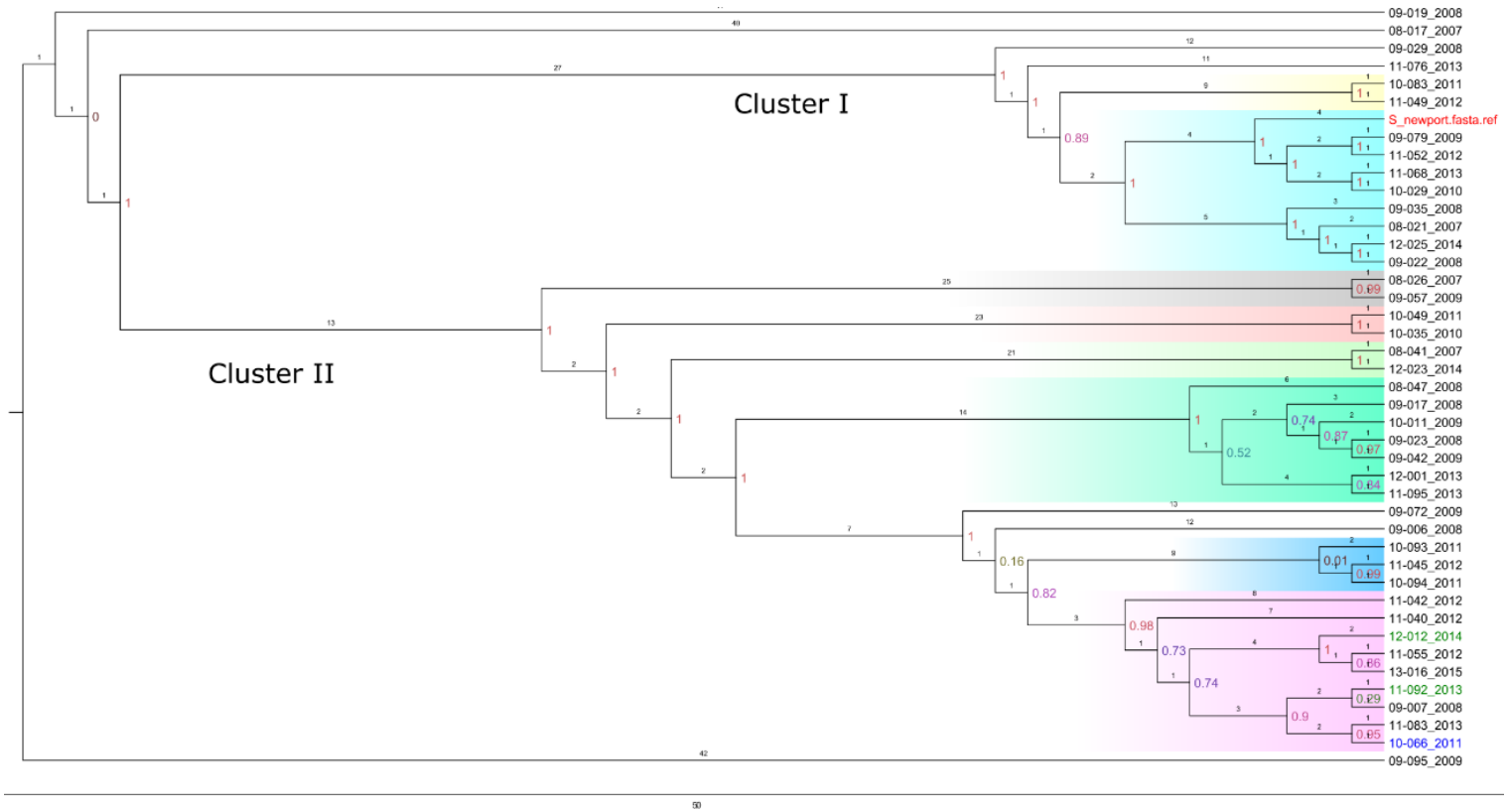

Figure 38. Whole-genome SNP-based phylogenetic tree of 42 *Salmonella* Newport and *Salmonella* Newport reference strain generated by Parsnp. The branch lengths are expressed in terms of changes per number of SNPs. The tree was visualized using FigTree. Reference strain is marked with color red, blue stars (MDR) and black (susceptible). Strain names are labeled with the year of admission of the patient to the hospital. Clusters are colored according to the phylogenetic group (clade). Cluster I includes: Sub-cluster I-a (yellow) and I-b (cyan blue). Cluster II includes: Sub-cluster II-a (gray), II-b (rose), II-c (light green), II-d (emerald green), II-e (blue) and II-f (pink). The scale bar shows the estimated number of substitutions per SNP.

In Sub-cluster II-c both cases had colic. Sub-cluster II-d contained 7 cases, 6 were adults, 5 presented with colic (one of these with presence of stomach ulcers), and one with colitis and one was a case in a patient that was receiving a diagnostic test (ultrasound). Sub-cluster II-e contained 3 cases, 2 from 2011 and 1 from 2012, the patients were adults with colic complaint. The last sub-cluster is the II-f and contained 9 cases: 7 from adults, 1 from a foal and one without reported age. Four cases presented with colic, one with chronic diarrhea, one with a mass in the stomach and 3 with general signs. From these cases, one strain was resistant to sulfisoxazole; one strain was resistant to streptomycin and sulfisoxazole; and one harbored and exhibited MDR to 7 antimicrobials (with 16 antimicrobial resistance genes detected). Detailed information of the cases per each cluster is shown in Table 7.

Geographical location of the phylogenetic clusters

The cases in the clusters were roughly geo-located by GPS coordinates (so as to protect the identity of the owner by zip code) into a map to find common locations and potential outbreaks. Via mapping it was noticed that Salmonella Anatum and S. Braenderup presented some grouping of cases in the same location as we saw in the phylogenetic analysis (Figure 39).

Salmonella Anatum had 4 cases came from the same location. In general, the cases of Anatum were grouped by location (Figure 40). Furthermore, Salmonella Braenderup had 6 cases associated with the same source (Figure 41). In contrast, the cases of S. Newport were very scattered as can be seen in the map (Figure 42).

Table 7. Equine cases of genetically related *Salmonella* Newport from Cluster I and II by phylogeny.

									Aminoglycosides							Ansamycin	Beta-lactams		Amphenicol		Macrolide		Folate pathway inhibitor		Tetracycline
Cluster	Sub-cluster	Vial	Breed	Admission date	Discharge date	Age	Complaint	Pattern	*aac(6')-Iaa*	*aacA4*	*aac(6')-Ib-cr*	*aac(6')-Iy*	*aac(6')-Ic*	*aph(3")-Ia*	*strA*	*strB*	*arr-3*	*blaSHV-12*	*blaTEM-1B*	*catA2*	*cmlA1*	*ere(A)*	*mph(A1)*	*dfrA15*	*sul1*	*tetE*
I	I-a	10-083	American Paint	8/27/2011	9/3/2011	Adult	colic	Pansusceptible	■			■														
I	I-a	11-049	Quarter Horse	10/7/2012	10/8/2012	Adult	diarrhea	Pansusceptible	■			■														
I	I-b	08-021	Miniature Donkey	7/11/2007	7/11/2007	NR	colic	Pansusceptible				■														
I	I-b	09-022	Quarter Horse	8/16/2008	8/19/2008	NR	colitis	Pansusceptible				■														
I	I-b	09-035	Mixed Breed	11/16/2008	11/19/2008	Adult	colic	Pansusceptible				■														
I	I-b	09-079	American Miniature	5/8/2009	5/8/2009	Adult	colic	Pansusceptible				■														
I	I-b	10-029	Gypsy Vanner	9/27/2010	10/7/2010	Foal	diarrhea	Pansusceptible				■														
I	I-b	11-052	Quarter Horse	10/12/2012	10/31/2012	Adult	fever	Pansusceptible				■														
I	I-b	11-068	Thoroughbred	1/18/2013	1/26/2013	Senior	colic	Pansusceptible				■														
I	I-b	12-025	Quarter Horse	4/19/2014	4/25/2014	Foal	decreased suckle reflex, lame in front legs	Pansusceptible				■														
II	II-a	08-026	Quarter Horse	8/8/2007	8/15/2007	NR	intermitent diarrhea, now drawn up, weight loss	Pansusceptible				■														
II	II-a	09-057	Mixed Breed	2/26/2009	3/12/2009	Adult	fever, possible fluid in lungs	Pansusceptible	■																	
II	II-b	10-035	Quarter Horse	11/15/2010	11/22/2010	Foal	colic	Pansusceptible	■																	
II	II-b	10-049	Quarter Horse	1/14/2011	2/3/2011	Adult	possible choke	Pansusceptible				■														
II	II-c	08-041	Andalusian	12/6/2007	12/14/2007	NR	toxic insult to liver-enzymes up, colic	Pansusceptible				■														
II	II-c	12-023	Quarter Horse	4/9/2014	4/9/2014	Adult	colic	Pansusceptible				■														
II	II-d	08-047	Quarter Horse	12/28/2007	12/28/2007	NR	colic	Pansusceptible	■																	
II	II-d	09-017	Clydesdale	8/13/2008	8/17/2008	Adult	colitis	Pansusceptible				■														
II	II-d	09-023	Quarter Horse	8/16/2008	8/25/2008	Adult	colic	Pansusceptible	■																	
II	II-d	09-042	American Paint	12/30/2008	1/13/2009	Adult	colic	Pansusceptible				■														
II	II-d	10-011	Spanish Barb	12/12/2009	12/16/2009	Adult	colic	Pansusceptible				■														
II	II-d	11-095	Arabian	9/25/2013	9/27/2013	Adult	stomach ulcers, colic	Pansusceptible				■														
II	II-d	12-001	Quarter Horse	9/16/2013	9/16/2013	Adult	ultrasound	Pansusceptible				■														
II	II-e	10-093	American Miniature	9/29/2011	9/29/2011	Adult	colic	Pansusceptible	■																	
II	II-e	10-094	American Paint	10/11/2011	10/20/2011	Adult	colic	Pansusceptible	■																	
II	II-e	11-045	Thoroughbred	9/24/2012	9/24/2012	Adult	colic	Pansusceptible				■														
II	II-f	09-007	Belgian	7/19/2008	7/21/2008	Adult	fever	Pansusceptible	■																	
II	II-f	10-066	Quarter Horse	6/5/2011	6/12/2011	Adult	mass in stomach	AMP-CHL-GEN-STR-FIS-TET-SXT		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
II	II-f	11-040	Quarter Horse	8/18/2012	8/25/2012	Adult	low protein level	Pansusceptible				■														
II	II-f	11-042	Thoroughbred	9/4/2012	9/21/2012	Adult	colic	Pansusceptible				■														
II	II-f	11-055	Foreign Warm Blood	10/29/2012	12/18/2012	Adult	colic	Pansusceptible				■														
II	II-f	11-083	Quarter Horse	7/18/2013	8/1/2013	Adult	colic	Pansusceptible	■																	
II	II-f	11-092	American Miniature	9/21/2013	10/1/2013	Foal	chronic diarrhea	STR-FIS				■														
II	II-f	12-012	American Paint	1/26/2014	2/9/2014	Adult	colic	FIS				■														
II	II-f	13-016	Paso Fino	9/27/2015	9/27/2015	NR	dehydration	Pansusceptible				■														

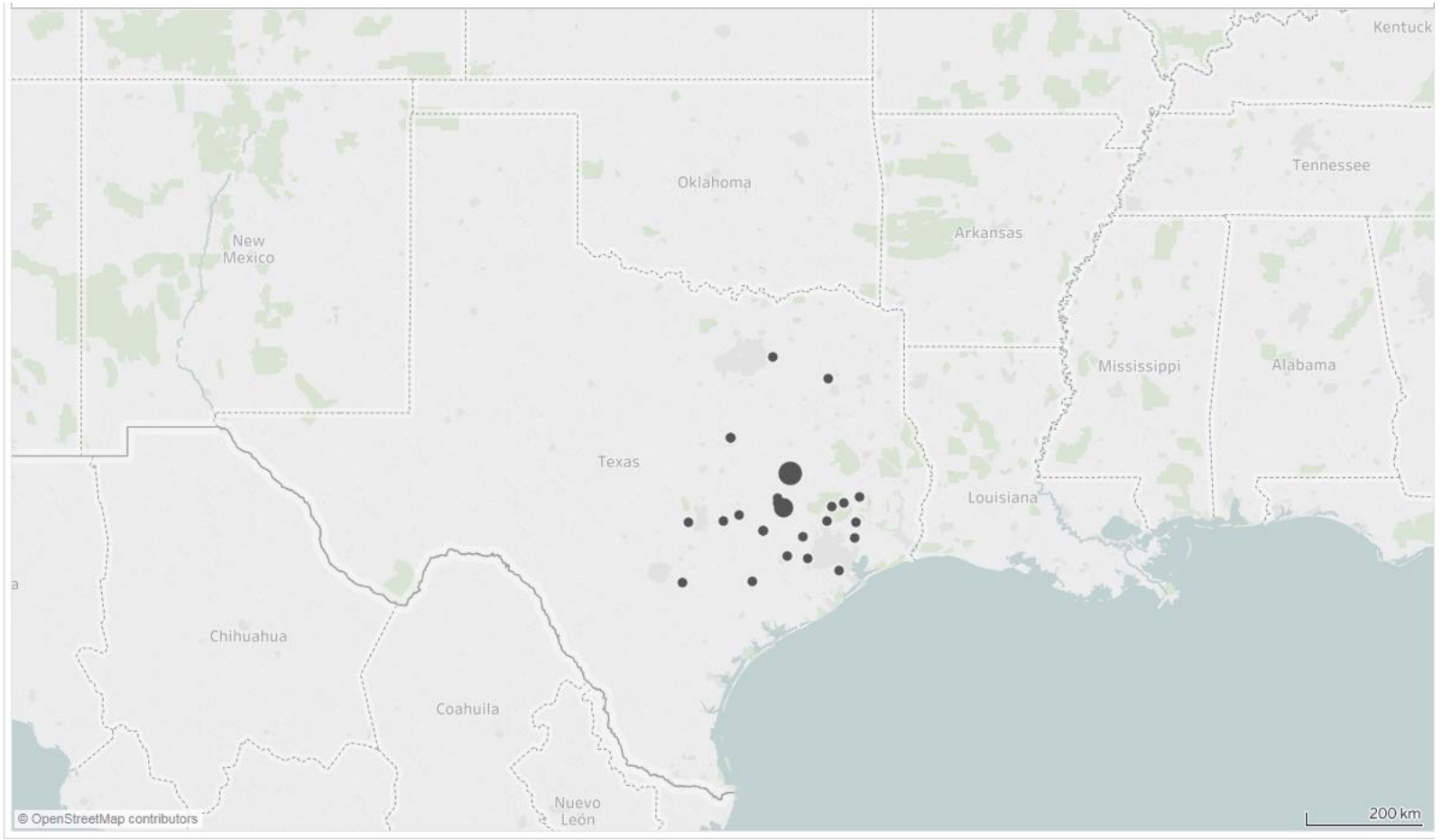

Figure 39. Location of genetically related cases of *Salmonella.* The size of the circle represents the number of cases.

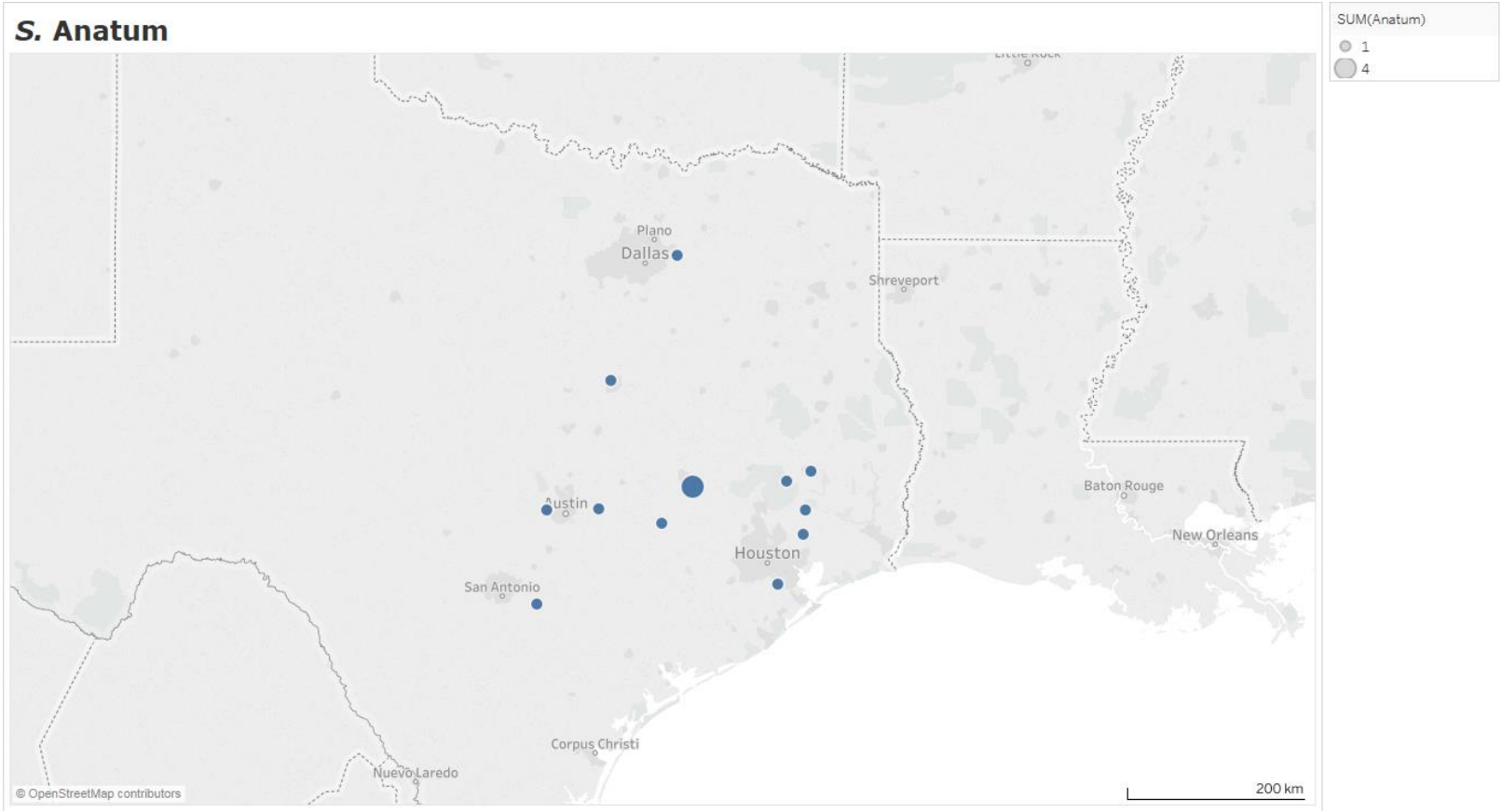

Figure 40. Location of genetically related cases of *Salmonella* Anatum. The size of the circle reflects the number of cases in each location.

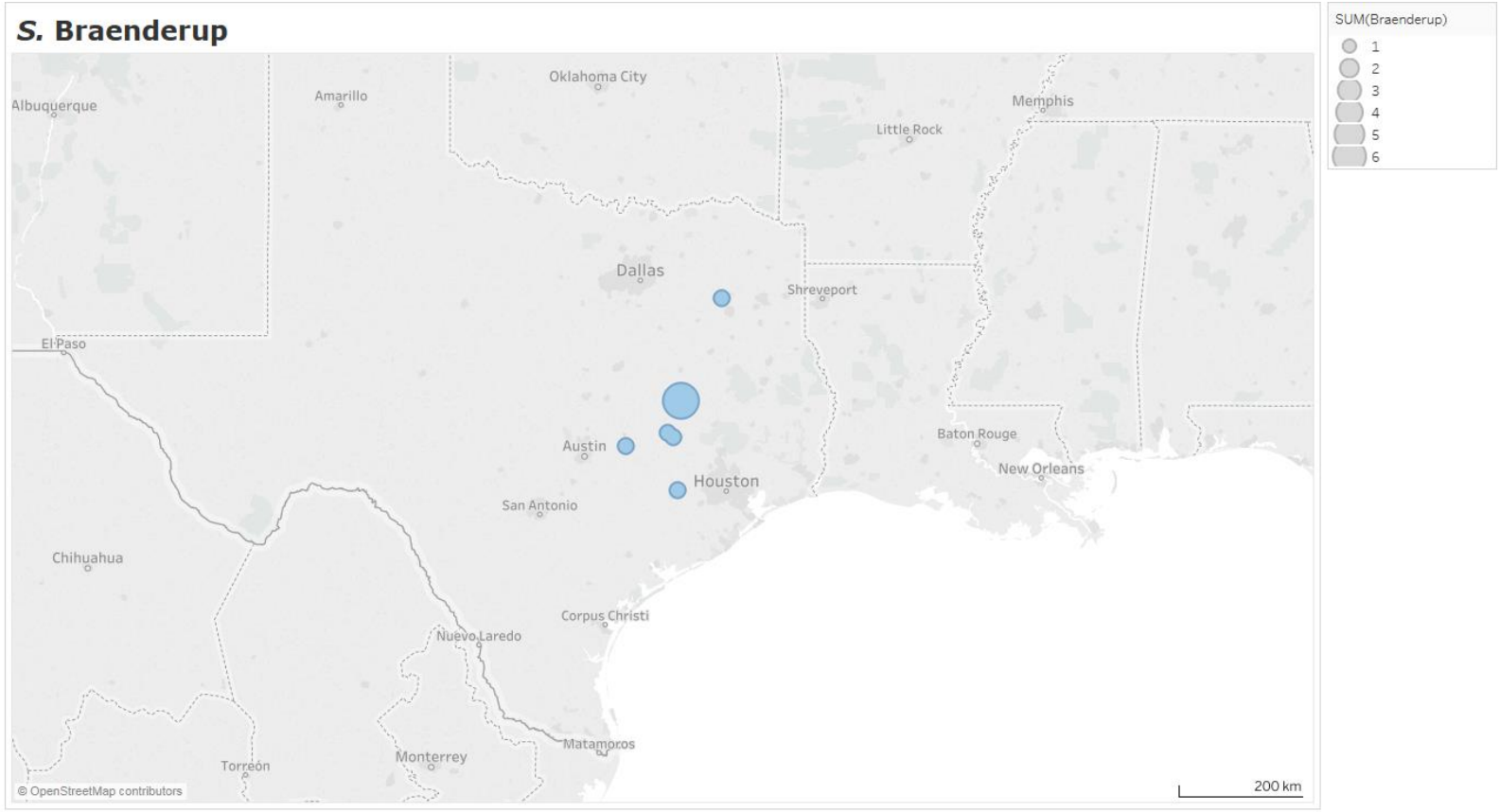

Figure 41. Location of genetically related cases of *Salmonella* Braenderup. The size of the circle reflects the number of the cases in each location.

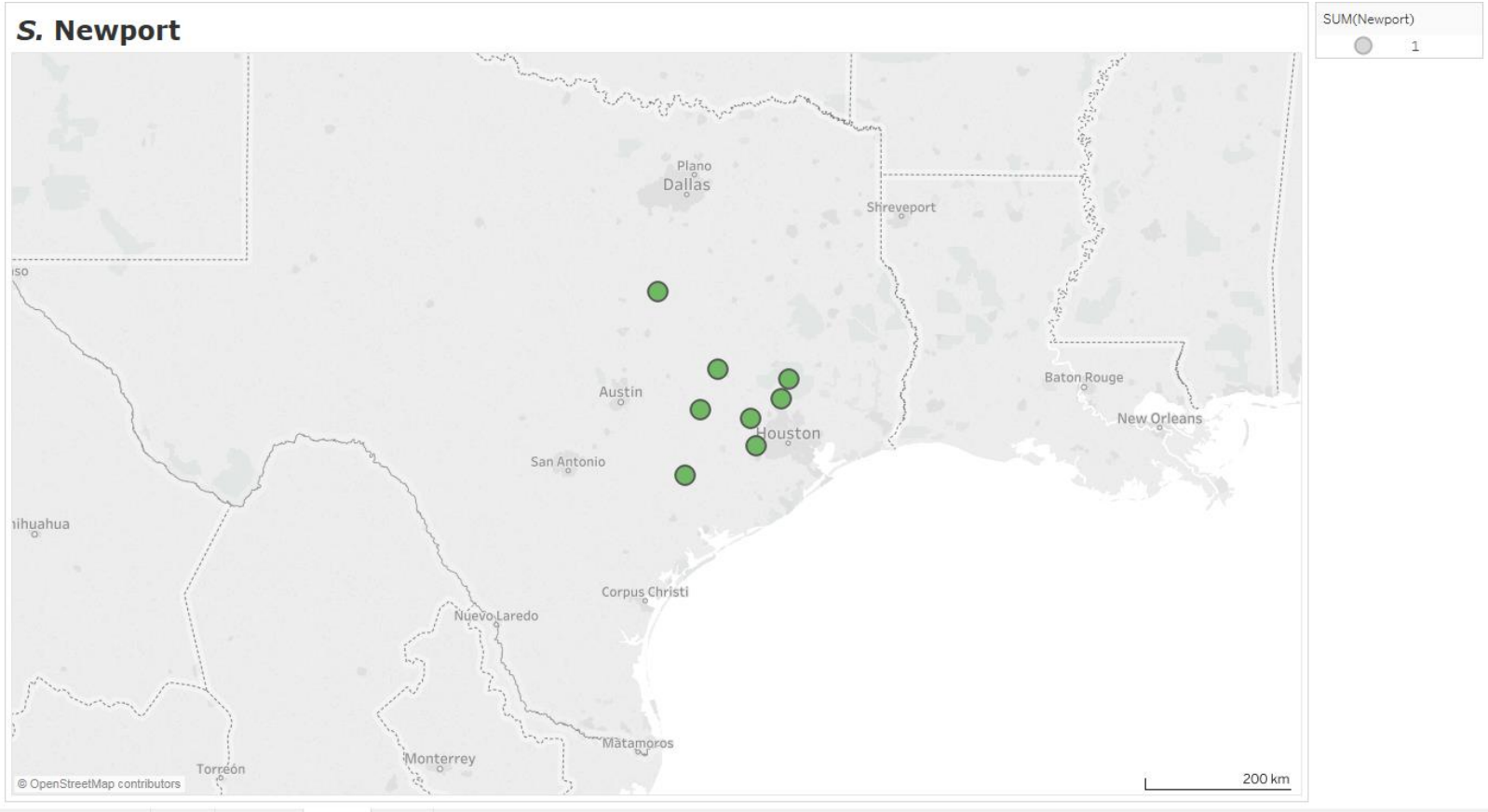

Figure 42. Location of genetically related cases of *Salmonella* Newport. The size of the circle reflects the number of cases.

CHAPTER IV

DISCUSSION

Evaluation of the proportional morbidity attributed to each *Salmonella enterica* serotype

Monitoring changes in the trends of prevalence of *Salmonella* in horses is useful for identification of possible nosocomial or other common-source outbreaks, and to improve the knowledge in diagnosis, treatment and epidemiology of the disease (8). Although *Salmonella* is often found as an asymptomatically shed organism in the feces of horses, some factors like age, immune system status and serotype can result in high morbidity and even mortality in this species. Although there have been studies published on specific *Salmonella* outbreaks in equine hospitals (10), and some studies on clinical cases in the northeastern United States, to our knowledge there are no published studies on the proportional morbidity of *Salmonella* cases in horses in the southern United States. This area of the United States is of major importance to the equine community. As one example, Texas has the highest population of horses (978,822) in the U.S. (96). The present study analyzed *Salmonella enterica* recovered from 255 samples collected over a 9-year period (2007-2015) among horses admitted to an equine referral hospital located in the southern region of the U.S. This study was unable to estimate the prevalence of *Salmonella* in asymptomatic horses, nor to estimate the incidence of salmonellosis in the equine population. However, among admitted cases with a differential diagnosis of salmonellosis and a confirmed isolate of *Salmonella enterica,*

we have been able to establish the proportional morbidity attributed to each serotype and explore aspects of the resistance phenotype, plasmid profiles, and phylogenetic relatedness of strains of the same serotype. In doing so, we are able to explore the relative frequency of potential field versus hospital outbreaks versus random cases admitted to the hospital.

The breeds of the horses in this study reflected reasonably well the demographic population in the United States, with Quarter Horses representing the highest number of horses (3,288,203) followed by Thoroughbreds (1,291,807) (96), as reported in similar studies (50). The mean age of the patients was 7 years and was similarly reported in other studies of horses with *Salmonella* (50).

Findings in this study demonstrate that the signs of colic and diarrhea were associated with patients with diagnostic of salmonellosis. Studies have shown that horses with colic are more susceptible to acquiring and shedding *Salmonella*, compared with other hospitalized equine patients (97); in addition, colic can be a presenting sign of colitis. Diarrhea is considered a traditional sign of salmonellosis (50). The syndrome "toxic enterocolitis" is characterized by fulminant diarrhea, between 2 or 3 days after inoculation (98). In this research, general signs (fever, dehydration, loss of weight, and anorexia, between others) were more often associated with juvenile horses; these signs are part of a milder GI syndrome that can develop in the first week post-inoculation once the horses start rapid fecal shedding; however, it can also be associated with toxic enterocolitis syndrome (8). Peracute sepsis can occur with general symptoms, where the most common complications are the ileus and gastric dilation with this syndrome usually

being fatal (8). Studies in Florida (U.S.) associated younger horses with risk factors like undergoing surgery, or remaining in larger groups (herds) with the increased risk of exposure to pathogens when compared with solitary individuals (99). In our study, symptoms as diarrhea was associated with foals and colic was associated with adults; this was expected, especially because diarrhea is the most common sign of salmonellosis and colic is associated with shedding of *Salmonella*. Diarrhea in foals is the second most common symptom of salmonellosis and specifically chronic diarrhea in foals is the third most common following after an acute episode (23). Chronic diarrhea can have a poor prognosis if there is no improvement within 4 to 6 weeks (17). The musculoskeletal signs (bone infections, fetlock problems, and swollen joints) were more often associated with foals in our dataset, suggesting that these animals may have been developing septicemia and consequently developing osteomyelitis (22).

The American Paint breed, when infected with *Salmonella*, was more often associated with presenting signs of colic; more generally, other studies have reported that Thoroughbred horses are more likely to exhibit colic (not *Salmonella* specific) than Quarter Horse, Paint, or Appaloosa breeds (14). There are other studies that demonstrated that breed is not a risk factor for salmonellosis (100). The respiratory signs possible as a first cause of admission to the hospital was more associated with mixed breed than with others breeds in the study maybe due to few cases reported with these symptoms.

Identification of *Salmonella* serotypes helps to focus *Salmonella* outbreak investigations and track them to their sources (e.g., food, environment, human, animals)

(101). Increment of cases of a specific serotype might make infection control officers suspicious of a potential outbreak. The classification of isolates into serotypes is mainly accomplished using the White-Kauffmann-Le Minor (WKL) scheme. However, with advancements in whole genome sequencing (WGS), and improved bioinformatics tools great advances have taken place to help determine pathogen relatedness and also to generate information about the characteristics of the organisms (e.g., serotype, virulence, antimicrobial susceptibility) (102).

Results showed that serotyping and SeqSero (in silico *Salmonella* serotype prediction) were very consisting, founding difference between both techniques in only 4% (10 strains). In this study, the most common among the 46 different *Salmonella* serotypes was Newport, as also described by others (31, 48); furthermore, *S.* Newport remains one of the most frequently identified serotypes in humans in the U.S. (103). In another study by Vetro (2004), among clinical cases in horses the second most common *Salmonella* serotype was Newport (11%) (99). In our study, the second most prevalent serotype was *S.* Anatum, followed by *S.* Braenderup, *S.* Infantis, *S.* Javiana and *S.* Typhimurium; these findings agree with the most frequent serotypes in horses (8). Although *S.* Braenderup was found among the main 16 serotypes in a study with 106 cases of salmonellosis, in horses in Florida at a veterinary teaching hospital (99), and is one of the 10 most common serotypes among human cases in the U.S, it has generally been reported in low percentages (<1%) in other animals (104).

There was a significant association between *S.* Braenderup and the likelihood of presentation as a subclinical presence of salmonellosis. This finding could be explained

by the fact that *S.* Braenderup in horses is an unrestricted (UR) serotype, often without presence of severe clinical signs. Nevertheless, this finding indicates the potential risk of zoonotic transmission to humans in contact with the asymptomatic infected horses (105). In this study, *S.* Typhimurium was associated with enteric disease and it is known that *S.* Typhimurium can be highly pathogenic in horses (106). It is also classified as a UR serotype with the ability to invade many different hosts (105). *S.* Typhimurium was also associated with musculoskeletal signs in our study, which agreed with Platt who found that *S.* Typhimurium was associated with septicemia and consequently osteomyelitis (107). This could be another potential risk factor to develop an outbreak with these UR serotypes in hospitals, due to the fact that horses with exhibiting signs of colic usually are not placed in isolation (50). In the hospital from which the isolates in this study were collected, the standard practice is to isolate horses with diarrhea or horses with three or more clinical signs consistent with salmonellosis (that is, other than diarrhea but including also fever and neutropenia). The presence of *Salmonella* Rubislaw could be due to possible source of contaminated surface waters as was reported in a previous study (108, 109).

In an study in 2010, the Arabian breed was found as one of the best predictors of *Salmonella* shedding in horses with acute colic and lacking typical salmonellosis signs (50); however, there have been no studies published that are appropriate for predicting of *Salmonella* shedding for specific serotypes or by breed (including ours, since we start with 100% of cases harboring *Salmonella*). In our study, salmonellosis in Arabian horses was associated with *S.* Infantis and *S.* Javiana and those serotypes are reported as being

the most frequent in clinical cases in horses (8). *Salmonella* Braenderup was more often associated with Thoroughbred horses. There are few reports of *S.* Braenderup clinical cases in horses, nor association with horse breeds, and the reason of the unreported presence of this serotype could be due to its subclinical presence; however, *S.* Braenderup has been commonly reported in beef cattle (110) and horses that are co-located with beef cattle on pastures or in dry lots might be at risk of exposure to this serotype. Two of the most prevalent serotypes in our study (*S.* Typhimurium and *S.* Braenderup) were significantly associated with increased risk of isolation from foals and adults.

Seasonality is an important variable to evaluate with the prevalence of *Salmonella* serotypes because some studies reported association between seasonality with higher incidence of salmonellosis in hospitalized horses (13). In our research, *Salmonella* Braenderup (typically, subclinical) was present at the beginning of the spring season of 2008, and then *S.* Newport was more detected in the middle of the summer until the end of 2008; this may be consistent, knowing that the summer season has been associated with high shedding of *Salmonella* in horses (13, 27). One reason is that as enteric bacteria *Salmonella* are better able to multiply at higher ambient temperatures with inter-generational times of 20 minutes, and the growth rate increases even further at 30°C and above (111). In our study, *S.* Braenderup was present in the spring of year 2009 similar to 2008. In 2010, *S.* Braenderup was presented in spring and also in fall season; one hypothesis for this observation could be that there is more contamination with flies in this season (112), or increased agricultural activities. Perhaps counter-

intuitively, cooler wet weather can contribute to the survival of the organism for long periods of time (113). *S.* Newport was present in the summer of 2010 until the end of the year, similar to 2008. *S.* Newport was present from spring to fall in 2013. In 2014, *S.* Newport was present from the beginning of the year until spring. However, the trends changed in 2015, where *S.* Anatum exhibited the highest proportional prevalence from spring to summer seasons. These results demonstrate high variability of the seasonality of serotypes and the finding of specific serotypes throughout the year.

Although many of the serotypes of *Salmonella enterica* subspecies *enterica* may not have plasmids, plasmids are often associated with those serotypes that cause clinical disease in humans and farm animals (e.g., Abortusovis, Cholerasuis, Dublin, Gallinarum, Pullorum and Typhimurium) (77). These strains usually have specific virulence plasmids, but also can harbor plasmids that more generally can transfer resistance to antimicrobials.

In our study, plasmid incompatibility replicon type IncI was the most frequently identified plasmid and this agrees with previous findings concerning *Salmonella* (77). IncI1 was the predominant plasmid especially in *Salmonella* Anatum. IncI1 has been recognized for harboring antimicrobial resistance genes, particularly ESBLs (114, 115). Our study showed the highest presence of plasmid IncF present in the main serotypes: *S.* Anatum, *S.* Typhimurium, *S.* Newport and *S.* Braenderup. The IncF plasmid is very common in *Salmonella* isolates, usually carrying beta-lactamases and *acc(6')-lb-cr* genes. On the other hand, this family also is very common in the intestinal flora of humans and animals, even so without the presence of antimicrobial resistance genes

(116). The IncHI plasmid type was present mainly in *S.* Anatum and *S.* Newport. The IncCOL plasmid was mainly detected in *Salmonella* Anatum and Newport. Bacteria use this plasmid to produce bactericidal proteins known as colicins which help to kill (or, suppress) other competing bacteria. Furthermore, they are frequently competent for plasmid transfer (117). The IncA/C2 plasmid type was detected in low frequency in all the serotypes, and was more often detected in *S.* Typhimurium and *S.* Rubislaw. This plasmid has been related with the *sul2*-containing resistance island ARI-B (118). As well it is a helper in the mobilization of the *Salmonella* Genomic Island 1 (SGI1) family that contains integrative mobilizable elements that contain different combinations of AMR (Antimicrobial Resistance) in a complex class 1 integron to recipients that lack SGI1 (119). *Salmonella* Newport harbors the X plasmid type, which has been associated with some of the most relevant and dispersed resistance genes ($bla_{\text{NDM-1}}$, $bla_{\text{KPC-2}}$ and $bla_{\text{CTX-M-15}}$) (87). IncQ in *S.* Anatum and *S.* Newport, has been associated with *strA*, *strB* and *sul2* genes in *E. coli* (87). Further characterization is necessary to evaluate the specific genes in the corresponding plasmids and that information would be indispensable to improving the epidemiologic surveillance in *Salmonella* strains, in addition to tracing the spread and evolution of the antimicrobial resistance.

Evaluation of antimicrobial resistance patterns of isolated *Salmonella* from horses admitted to an equine referral hospital in the southern United States

Increasingly resistant bacterial isolates can generate high rates of morbidity and mortality that consequently increase costs to the human and animal health systems due to

failure of treatments, extended disease duration, additional diagnostic tests, and the use of more expensive drugs (120).

Although there are a lot of government institutions working on programs of surveillance for antimicrobial resistance (CIPARS, ESVAC, DANMAP, MARAN, GermVet, ITAVARM), there are few that provide antimicrobial resistance surveillance in horses (121). It has been shown that antimicrobial resistant bacteria could be transferred between horses and humans (122). For this reason, it is imperative to identify sources of *Salmonella* and antimicrobial resistance transmission where humans and horses come into contact (such as referral hospitals), to subsequently give guidelines for the correct use of antimicrobials, and to measure the potential risks to public health (48).

The present study analyzed data over a 9 year period (2007-2015) including 255 *Salmonella* isolated from patients of an equine referral hospital in the southern United States. All isolates were tested for antimicrobial susceptibility to each of the 14 antimicrobials according to National Antimicrobial Resistance Monitoring System (NARMS) guidelines. Furthermore, each isolate was analyzed using whole genome sequencing (WGS) for detection of antimicrobial resistance genes (ARG). The use of WGS will help to enhance our knowledge on ARG circulating in *Salmonella* from horses and their environment. In addition this information, such knowledge can help to identify resistance hazards and will improve control strategies to mitigate the risks (123). In general, it was found that most of the *Salmonella* isolates were pan-susceptible to all 14 antimicrobials; however, they were more often resistant to sulfisoxazole 10.59% (27), ampicillin 9.8% (25) and tetracycline 9.8% (25) than to newer classes of antimicrobials.

These results are somewhat consistent in that the most common antimicrobials used in horses in the United States are gentamicin, potentiated sulfonamides and doxycycline (124).

In a recent study in *Salmonella* from horses at w University Hospital, it was found that the isolates were more resistant to amoxicillin-clavulanic acid, ampicillin, cefazolin, cefoxitin, ceftiofur, chloramphenicol and tetracycline than to other classes (48). Similar results had been reported in a study in Netherlands with 232 *Salmonella* from horses with resistance to tetracycline (53%) and ampicillin (34%) (125). *Salmonella* recovered from horse diagnostic samples at four state veterinary diagnostic laboratories (AZ, MO, NC, and TN) had higher resistance to ampicillin, chloramphenicol and sulfamethoxazole (126).

The resistant *Salmonella* were multidrug resistant (MDR: resistant to greater than two classes of antimicrobial) in 10.2% of isolates. These MDR *Salmonella* usually were harboring plasmids that could confer resistance to multiple antimicrobials, and furthermore many could have multidrug efflux pumps (127). Our results showed that the prevalence of resistant *Salmonella* was variable among the 12 different resistant serotypes. The most resistant serotype with 8 different resistance patterns and 5 MDR strains was *S.* Anatum, followed by *S.* Rubislaw and then *S.* Braenderup. Different results were reported by Cummings in 2016, where the most resistant serotypes were *S.* Newport, *S.* Oranienburg and *S.* Typhimurium (48). The most resistant serotypes in horses at a hospital in Florida (U.S.), were *S.* Java, Typhimurium var. Copenhagen,

Javiana and Newport (99). In a study of horses in the Netherlands (2002) the most resistant serotype was *S.* Typhimurium (125).

Although laboratory-based, phenotype criteria still dominate the epidemiology of antimicrobial resistance, new genome scale tools are becoming a routine part of the laboratory analysis (128). Whole genome sequencing (WGS) can help to improve the comparison among isolates, allowing evaluation of different antimicrobials at the same time and possibly refining interpretative criteria, however, there are still some issues to consider as the requirements needed of minimum sequence data quality standards and good understanding of the genetic context to make interpretations (123).

We found that most of the *Salmonella* isolates carried at least one antimicrobial resistance gene (per ResFinder), including more specifically two aminoglycoside genes: *aac (6')-Iaa* and *aac (6')-Iy*, however those genes have reached their limit of evolution. Those genes no longer appear to encode resistance to aminoglycosides, consistent with the small evolution of AAC(6') proteins (129). The retention of the *aac(6')-Iy* gene evolution suggests a potential cellular function that differ from aminoglycoside resistance. Studies have suggested that the usefulness of this gene is production of enzymes involved in carbohydrate transport or metabolism endogenous and specific to *Salmonella* (130).

In general, our results show that acquired phenotypic resistance in *Salmonella* presented almost perfect agreement with the presence of known antimicrobial resistance genes. High congruence was present between MICs (especially above the clinical breakpoint) and the presence of known resistance genes, with agreement in 96% of

cases. There were discrepancies between phenotypic and genotypic interpretations for some antimicrobials; mainly, quinolones, aminoglycosides, and tetracyclines.

Nalidixic acid and ciprofloxacin were the representative quinolones tested. There were 3 isolates that had a resistant (R) phenotype to nalidixic acid but a susceptible (S) genotype. A similar situation was detected in 2 isolates with an R phenotype to ciprofloxacin but an S genotype. For these isolates one hypothesis could be that the phenotypic resistance to these quinolones was conferred by a DNA topoisomerase mutation and was not generated by *qnrB2* or *aac (6')-lb-cr* genes and that could not be detected by the analysis through the Resfinder tool; that is, because this web server only curates horizontally acquired resistance genes and does not include resistance mediated by single- or double-stage mutations of genes (131). There were four *Salmonella* with an R genotype to nalidixic acid but an S phenotype; however, the same isolates had a ciprofloxacin R genotype presenting moderate agreement using ciprofloxacin intermediate MICs (0.12 to 0.25 ul/ml). Isolates with Plasmid-Mediated Quinolone Resistance (PMQR) (e.g., *qnr*B) do not usually show clinical resistance to ciprofloxacin or nalidixic acid, unless Quinolone Resistance-Determining Regions (QRDR) mutations or additional PMQR genes are present (123).

It was previously reported that an *E. coli* form a clinical case was resistant to ciprofloxacin, but susceptible to nalidixic acid. The authors demonstrated that at least one of two mechanisms of resistance were present: 1) decrease of the OM (Outer membrane) permeability or 2) alteration of the DNA gyrase (132). The results in ciprofloxacin illustrate that WGS is a very efficient tool to detect decreased

susceptibility to some antimicrobials. It is suggested that fluoroquinolones are the antimicrobials of choice to treat salmonellosis in adult humans because these antimicrobials are lipid soluble and *Salmonella* are facultative intracellular pathogens. Oral use of ciprofloxacin is not recommended in horses because it can cause colitis and it is not recommended to use in foals because it affects cartilage development, similar to children (133). This antimicrobial is included on the list of critically important antimicrobials for humans (134); therefore, its use should be restricted to cases that have few other options for treatment.

Tetracyclines is consider highly important antimicrobial for human medicine (135). Tetracycline resistance genes (*tet*(A), *tet*(B), *tet*(C), *tet*(D) and *tet*(E)) were present in *Salmonella*. One study in *E. coli* from non-clinical horses samples found *tet*(A), *tet*(B), and *tet*(C) genes in the bacteria (136). The common tetracycline genes reported for *Salmonella* are *tet*(A), *tet*(B), *tet*(C), *tet*(D) and *tet*(G). On the other hand, *tet*(E) was previously reported in *Aeromonas*, *Providencia*, *Pseudomonas*, *Serratia*, and *Vibrio* (76) and often present in aquatic environments (137). The *tet*(E) gene also has been detected in LFE (lactose-fermenting Enterobacteriacea) isolated from sewage treatment plants in the United States and China (138).

Tetracycline also presented some discrepancies: 7 isolates had an R phenotype with an S genotype and 3 isolates with an R genotype harbored an S phenotype. Previous work has suggested that this phenomenon could be due to low antimicrobial use that can result in carriage of tetracycline resistance pseudogenes that remain phenotypically susceptible when compared with high antimicrobial use (139). The genotype resistant

isolates that were phenotypically susceptible may harbor pseudogenes. Pseudogenes are coding sequences that have lost their ability to be expressed because they have been inactivated by mutations, including non-sense substitutions and frame-shifts, truncation by deletion, or else rearrangements (140). However, more work is needed to determine whether these mutations prevent gene expression (139). To confirm this hypothesis, it would be necessary to correlate the use of antimicrobials with the phenotypic and genotype antimicrobial profiles of the bacteria. Another explanation for the R phenotype and S genotype isolates could be that it could well be an unidentified resistance mechanism in the genome. Those disagreements should disappear with the discovery of new antimicrobial resistance mechanism and repeated *in silico* analyses (123).

In our study, beta-lactamase genes ($bla_{\text{TEM-1B}}$), plasmid mediated mobilized AmpC genes ($bla_{\text{CMY-2}}$) and extended-spectrum beta-lactamases (ESBLs) ($bla_{\text{SHV-12}}$ and $bla_{\text{CTX-M-27}}$) genes were found, with good agreement with phenotypic susceptibility. When $bla_{\text{TEM-1B}}$ was found in the presence of an ESBL, the latter phenotype dominated, conferring resistance to third generation cephalosporins that would otherwise not be seen. The presence of these plasmid-borne genes in *Salmonella* from equine patients at a referral hospital is alarming because these genes can be transmitted easily to other bacteria and distributed to other animals and to humans. Although some years ago ESBL genes were quite rare among *Salmonella* from human infections in the United States (141), a recent surveillance in all 50 states and the District of Columbia found in nontyphoidal *Salmonella* numerous ESBLs, containing $bla_{\text{SHV-12}}$, $bla_{\text{SHV-30}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-55}}$ and two $bla_{\text{CTX-M-6}}$ genes (142).

An outbreak associated with *Salmonella* Newport MDR-AmpC at another veterinary teaching hospital involved a high case fatality in hospital patients due to failures in the ICP (Infection Control Program) (50). In 2003, an ESBL-producing *S. enterica* serotype Newport MDR-AmpC with *bla*$_{\text{TEM-1b}}$ and *bla*$_{\text{SHV-12}}$ genes from an outbreak in equines was reported for the first time and led to the closure of a veterinary teaching hospital for 3 months (143). Interestingly, in our study almost all of the isolates of *Salmonella* with *bla*$_{\text{SHV-12}}$ (an ESBL) contained the *bla*$_{\text{TEM-1b}}$ gene as well. Carriage of beta-lactamase resistance in these isolates is not restricted to the United States. In Argentine in 2010, there was a reported case of infection with ESBL-producing *Salmonella* Typhimurium with *bla*$_{\text{CMY-2}}$ gene in a race horse with diarrhea (144). In a retrospective study in Germany *bla*$_{CTX\text{-}M\text{-}15}$ was detected in *E. coli* and *Salmonella* from animals, and *bla*$_{CTX\text{-}M\text{-}15}$ was detected in *Salmonella* Typhimurium (145). The presence of ARG may result in failure of the treatment for salmonellosis, especially in foals. In foals, it is recommended to use extended-spectrum cephalosporins or ampicillin-sulbactam, alone or in combination with an aminoglycoside (gentamycin or streptomycin) (133). In this study, we found resistance to ampicillin and potentiated amoxicillin (clavulanic acid) with the presence of beta-lactamase genes; these findings have important implications as the presence of these genes may be associated with treatment failure.

Presence of aminoglycoside resistance genes in *Salmonella* from horses has been previously reported. Similar to other studies, we found *aadA1*, *aadA2*, producing resistance to streptomycin (126), and *aadA7*, *str*A and *str*B were also detected. Presence

of gentamicin resistance genes (*aac(3')-IId, aac(3')-VIa, rmtE, aac(6')-IIc, aph(3')-Ia* and *aph(3')-Ic)* were detected in *Salmonella*. These results are important because aminoglycosides are categorized as critically important for use in horses (146). Aminoglycosides are used to treat septicemia, and different digestive, respiratory and urinary diseases. Moreover, aminoglycosides are critical to treat infections with *Pseudomonas aeruginosa*. Enterobacteriaceae and *Pseudomonas* spp. can share AMR genes (147).

Genes producing resistance to sulfonamides (*sul1* and *sul2*) and trimethoprim/sulfas (*dfr*A) were found. Zhao, et al. reported presence of *dfr*A1 in *Salmonella* Anatum from horses (126). Meanwhile, *S.* Typhimurium recovered from clinical cases in Netherlands carried *dfr*A1, *dfr*A or *dfr*A14 (148). Sulfonamides are also on the list of medically important antimicrobials in animals and human (149). This family of antimicrobials are used to treat bacterial, coccidial, and protozoal infections (146).

Chloramphenicol resistance was present in *Salmonella*. This finding agrees with Cummings et al. who found chloramphenicol resistance at 51.5% in their isolates (48). In our study, phenicol resistance phenotype and genotype exhibited the highest agreement (98.8%). The presence of phenicol resistance genes (*cat*A2, *flo*R and *cml*A1) was detected. The *cat* gene, responsible for enzymatic resistance, was the predominant phenicol resistance gene among our isolates. These data support that chloramphenicol acetyltransferases are often the cause of resistance to chloramphenicol as reported by others (150). The *flo*R gene is a common genetic determinant responsible for florfenicol

resistance in Gram-negative bacteria, including *E. coli* from animals (Cloeckaert, Baucheron et al. 2000). Phenicol resistance was reported in *Salmonella* Typhimurium DT104 from a clinical horse case with the *flo*R gene associated with SGI1 (*Salmonella* Genomic Island 1) (148).

In this study, *Salmonella* occasionally carried *mph*A and *ere*(A). The *mph*A gene has been associated with resistance to azithromycin among *E.coli* and *Shigella* (151), while *ere*(A) has been reported in *Citrobacter, Enterobacter, Escherichia, Klebsiella, Pantoeae, Pseudomonas, Proteus, Serratia, Stenotrophomonas, Vibrio* and *Staphylococcus* (152). In horses, macrolides are not used in the treatment of *Salmonella* because they can induce acute colitis by disruption of the microflora (153, 154), and although macrolides are not currently used commonly to treat non-typhoidal *Salmonella*, the presence of these genes may generate major public health consequences because *E. coli, Shigella* and *Salmonella* Typhi can exchange plasmids (151).

Antimicrobial therapy for *Salmonella* in horses generally should be restricted to cases of septicemia and osteomyelitis, and other severe extra-intestinal involvement (155). Antimicrobial therapy may cause persistence of the bacteria in the intestine following recovery and prolonged shedding of the organism. Also, antimicrobial therapy may increase resistance among commensal bacteria and lead to the overgrowth of toxigenic bacteria such as *Clostridium difficile*. It is important to emphasize that antimicrobial treatment must ideally be established according to the results of the antimicrobial susceptibility of the specific isolate; or in cases that require immediate intervention to prevent death, it is possible to use the historic antimicrobial susceptibility

records at the hospital, farm or geographic region to improve so-called empirical therapy (8).

Phylogenetic analysis

Improvement and new capabilities in sequencing technologies have allowed for new applications in clinical microbiology and epidemiology. Decreasing the cost of sequencing may allow for WGS to be the new standard for bacterial strain typing and allow for improved epidemiological investigation of outbreaks. Molecular epidemiology based on WGS is developing rapidly. Before the WGS era, pulse-field gel electrophoresis (PFGE) was the gold standard for epidemiological investigations (156). However, PFGE cannot separate high closely related strains because that difference does not have effect in the electrophoretic mobility of a restriction fragment (157). Usually, outbreak isolates have very little diversity and require extensive genomic methods to differentiate and categorize the isolates (158). There are currently two methods to analyze the phylogeny: 1) core genome SNP based analysis, and 2) MLST based on whole genomes, pangenomes, or core genomes. Core genome SNP analysis is more sensitive for discrimination of very closely related isolates to a level not previously achievable by sub-genomic typing tools (e.g., PCR-based MLST and PFGE) because of their limited resolution (159).

SNP-based comparison methods are effective to analyze genomes with low numbers of SNPs and inversions, insertions, and deletions, and can be used to trace evolutionary processes during extended outbreaks (91).

To the best of our knowledge, ours is a pioneering study using WGS to analyze *Salmonella* genomes from clinical cases of horses from a referral hospital over an extended period of time. Phylogenetic analysis of the presumptively epidemiologically related *Salmonella* Anatum, *Salmonella* Braenderup, and *Salmonella* Newport isolates were conducted.

Salmonella *Anatum*

Two cases in Sub-cluster I-c from 2008 were very closely related with differences in four SNPs; one had 12 resistant genes with 7 different plasmids detected, while the other presented 13 resistant genes with 2 plasmids. Both MDR cases arose from different farm locations; however, they arrived at the hospital in the same month. This results are suggestive of a potential common source in the hospital. Four cases out of five in Sub-cluster I-d from 2015, came from the same farm; 2 of them carried *tetB* genes. These results suggested that these horses were coming to the hospital with the infection. In Sub-cluster II-b, the 3 cases from 2009 came from different farms, further suggesting a possible nosocomial transmission.

These finding reinforce that it is necessary to implement measures to control the spread of these MDR *Salmonella*. Deeper research is needed to explore possible outbreaks in the hospital and identify common sources of this specific serotype.

Salmonella *Braenderup*

Between the period of March and April of 2008, four identical genomes from Sub-cluster I-e of MDR S. *Braenderup* (i.e., resistant to 8 antimicrobials) and with the same three resistant genes (*rmtE*, bla_{CMY-2} and sul_1) that could be transmitted by the same plasmid replicon type (I1) suggest a possible outbreak at the hospital. An acknowledged outbreak of *S.* Braenderup in 2009 was confirmed by phylogenetic analysis in our study. This outbreak included mares (2) and their foals (4) coming from the same external facility. From Sub-cluster I-b in 2010, an MDR *S.* Braenderup was isolated from a foal that was genetically closely related to a pansusceptible isolate. Moreover, an unusual ESBL gene (bla_{CTX-27}) was present in this MDR strain and likely carried on an IncI1 plasmid. These two cases both came from a mare and its foal. These findings could point to acquisition of the plasmid either from the flora of the dam or another common environmental source.

Salmonella *Newport*

In 2008, Sub-cluster II-d included three closely genetically related cases; however, there was not enough information to associate with the origin of the outbreak. In 2011, Sub-cluster II-e presented cases closely related between September and October and while it seems possible that those cases were coming from the same location, we did not have information available to confirm.

In general, *Salmonella* Newport in our study were very susceptible to the antimicrobials; however, some strains belong to Sub-cluster II-f across different years were resistant – one of them was MDR with 8 resistant genes detected (*aac(6')-lb-cr*, *aac(6')-IIc*, *aph(3')-Ia*, *strA*, *strB*, $bla_{\text{SHV-12}}$, $bla_{\text{TEM-1B}}$, *ere*(A), *mph*A1, *dfrA15* and *sul1)* carried on 4 plasmids (FIA, H1, 12 and Q). In general, the closely related *Salmonella* genomes did not come from the same ranch; however, it is necessary to research more about possible common sources of this frequent and widely dispersed (geographically and temporally) serotype.

CHAPTER V

CONCLUSIONS

This study enhances our understanding of *Salmonella* epidemiology among horses admitted to referral hospitals with a differential diagnosis of salmonellosis. Our analysis provides a contextual framework necessary to make conclusions regarding likely infective sources of *Salmonella* in horses presenting to the hospital and potential action plans to prevent and control the dissemination of the bacteria. The pursuit of mitigation strategies include: improved communication, isolation of animals, restriction of contact with suspicious animals (including emphasis on routine hygiene following animal contact), restriction of movements inside the hospital, improvement of the use of protective equipment for personal, environmental and animal sampling, and thorough cleaning and disinfection are some of the most important biosecurity strategies to avoid future outbreaks at a veterinary referral hospital. Biosecurity measures are recommended also at the level farm as many of these outbreaks clearly trace back to a common farm source.

Horses shedding antimicrobial-resistant *Salmonella* remain a potential hazard to public health. Easy dispersion and a short period of incubation of *Salmonella*, the presence of asymptomatic horses and multiple mechanisms of resistance to antimicrobials increase the risk of acquiring the bacteria and the potential failure of antimicrobial treatments in humans. Antimicrobial resistance genes can limit the potential treatment of salmonellosis, especially in cases where the use of antimicrobials

(e.g., septicemic cases) is essential. In cases where antimicrobial treatment is recommended, individual bacteriological and antimicrobial susceptibility testing remains clinically relevant, especially in guiding therapy. Minimizing and optimizing the antimicrobial therapy in horses can be achieved by developing and following judicious use guidelines. Continued monitoring of antimicrobial resistance can be used to guide recommendations for antimicrobial use in veterinary medicine (including stewardship programs) and assessing the potential risks to human. Whole genome sequencing (WGS) can be valuable in discriminating between closely related isolates when suspecting an outbreak and for monitoring persistence of a particular strain within the hospital environment**.** WGS is helpful in the investigation and monitoring of trends over time of the antimicrobial resistance genes, continued submission of complete genome sequences to globally representative whole genome databases (e.g., NCBI) will improve comparisons and detection of outbreaks across hospitals and region. Continued development of robust genomics and bioinformatics capabilities is necessary to rapidly generate genomics–based data that can be useful to detect, prevent and control the dispersion of pathogenic microorganisms and their antimicrobial resistance.

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