POPULATION DYNAMICS OF ENTERIC SALMONELLA IN RESPONSE TO ANTIBIOTIC USE IN FEEDLOT CATTLE

A Dissertation

by

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ABSTRACT

The various uses of antibiotics in feedlot cattle have been a concern as a potential source of antibiotic resistant *Salmonella* infections in humans. A 26-day randomized controlled longitudinal field trial was undertaken to assess the effects of injectable ceftiofur crystalline-free acid (CCFA) versus in-feed chlortetracycline (CTC) on the temporal dynamics of *Salmonella enterica* subsp. *enterica* in feedlot cattle. Two replicates of 8 pens (total of 176 steers) received one of 4 different treatment regimens. All, or one, out of 11 steers were treated with CCFA on day 0 in 8 pens, with half of the pens later receiving three 5-day regimens of CTC. We isolated *Salmonella* from fecal samples, and antimicrobial susceptibility was assessed. *Salmonella* in the feces were quantified with probe real-time qPCR targeting *invA* gene and by direct spiral plating on brilliant green agar. Whole-genome sequencing was performed for all *Salmonella* isolates to analyze serotype, resistance genotype, MLST, and to explore the phylogenetic relations of the isolates.

The mean *Salmonella* prevalence was 75.0% on day 0, and most isolates were pansusceptible to 14 antibiotics. Both CCFA and CTC reduced the overall prevalence of *Salmonella*; however, these treatments increased the proportion of multi-drug resistant (MDR) *Salmonella*. Ceftriaxone and tetracycline resistant *Salmonella* were detectable in day 0 samples, suggesting that resistant *Salmonella* existed in the population before antibiotics use. The quantity of resistant *Salmonella* remained at approximately 10^3 CFU / gram of feces throughout the study. Significantly ($P < 0.05$) more animals were detected with resistant *Salmonella* following antibiotic treatments. Among the six serotypes detected, all *S.* Reading isolates were MDR and

carrying an IncA/C2 plasmid, suggesting a strong association between serotype and resistance type. The *S.* Reading isolates consisted of 2 phylogenetic clades with differential selection by CCFA versus CTC (alone). Our study demonstrated that the selection pressures of a 3rd generation cephalosporin and of CTC during the cattle feeding period selects for antibiotic resistant *Salmonella* and increases the proportion of cattle carrying resistant *Salmonella*, even after the treatment period ends. Further investigations are needed to assess whether an extended feeding period of 150 days provides a sufficient 'wash-out' period for the gut microbiota to return to normal status.

DEDICATION

In memory of my best friend Fumiki Ui.

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CHAPTER I

INTRODUCTION

1.1 Background

1.1.1 Antibiotic resistance

Antimicrobial resistance is a global public health problem [1, 2]. Bacterial infections with resistance to last-resort antimicrobials, such as carbapenems and colistin, are increasing and can be life threatening due to the lack of additional treatment options [3, 4]. In 2015, the World Health Organization (WHO) announced a Global Action Plan to combat antimicrobial resistance and to address the problem using a "One Health" approach in collaboration with the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE) [5]. It is estimated that 60% of all antimicrobials produced are utilized in animal agriculture for growth promotion, disease prevention, disease control, or treatment [6]. The use of antimicrobials in food producing animals is alleged to be one of the major sources of antimicrobial resistant bacteria transmitted to humans [7, 8].

Several critically important antimicrobials for humans health have been prohibited for extra-label use and disease prevention purposes in food-animal production in several European countries and in the United States to address the issue of antimicrobial resistance [9]. Such strategies have had conflicting results. For example, in Denmark, since 1998 the total mass of antimicrobials has been reduced by banning antimicrobial use for disease prevention and growth promotion purposes in food animal production; however, the total use of antimicrobials for treating sick animals has been increasing [10, 11]. Consequently, it is not yet clear whether the cases of antimicrobial resistant bacterial infections have decreased in humans through more restrictive use of antimicrobials in food animals [12]. Due to the complex nature of this issue, it

is important to investigate the best and most effective intervention strategies to reduce the further expansion of antimicrobial-resistant bacteria [13-15].

1.1.2 Antibiotics use in feedlot operations in the United States

The United States is the top beef producing country in the world, with an output of more than 12 million tons of beef per year [16]. Total annual beef consumption in the United States is around 24.8 billion pounds, averaging 67 pounds per person, and comprising about 40% of meat consumption in the country [17]. The high demand for beef is met by feedlot operations, where weaned calves from cow-calf operations are assembled and fed high-energy rations to reach the necessary weight and carcass characteristics to be sent to slaughter. The sizes of feedlots in the United States range from less than 1,000 head (small commercial feedlot) to more than 16,000 head of cattle (large commercial feedlot). The largest feedlots comprise 60% of the U.S. fed-cattle market [17].

Antimicrobials are important for the prevention, control, and treatment of disease and are necessary to maintain healthy cattle in the feedlot. Metaphylactic (control) and prophylactic (prevention) use of antimicrobials are two of the primary methods to treat animals, which leads to mass consumption of antimicrobials, rather than individualized treatment. The most common disease problem in feedlots is bovine respiratory disease (BRD). BRD is caused by an infection with bacterial and/or viral pathogens along with complex environmental and host factors, such as transportation stress and comingling with calves from different sources. Metaphylactic treatment of calves upon arrival to the feedlot is a common practice for the control and treatment of BRD, for at-risk and sick calves, respectively. Several antibiotics are approved by the U.S. Food and Drug Administration (FDA) for the control and treatment of BRD, including labeled indications for ceftiofur crystalline-free acid (CCFA) and chlortetracycline (CTC). There is little research

concerning the effects of CCFA and CTC on the gut microbiota in cattle. The effects of antimicrobials on commensal and pathogenic bacteria residing in the cattle microbiota, such as non-typhoidal *Salmonella* and Shiga-toxin producing *E. coli* (e.g., O157:H7), are a public health concern because these bacteria are common foodborne pathogens and can cause life-threatening infections in humans.

1.1.3 Foodborne non-typhoidal Salmonella

Non-typhoidal *Salmonella*, the causative agent of most cases of salmonellosis in the developed world, was the leading cause of death (215 out of 552 (39%) of all deaths) in the U.S. from 1996 to 2005 attributed to foodborne pathogens, according to the data from FoodNet [18]. Children under 5 years old and adults over 65 years old are at the highest risk [18, 19]. An estimated 1.2 million cases, 23,000 hospitalizations and 450 deaths occur due to non-typhoidal *Salmonella* infections in the United States annually [19-21]. In the United States, from 2008 to 2012, 30% of foodborne outbreaks and 45% of illnesses were caused by *Salmonella* [22].

Clinical salmonellosis in humans is typically self-limiting and affected individuals recover within 5-7 days. However, in severe cases involving invasion of the host by the bacterium, such as bacteremia and meningitis, hospitalization and antimicrobial treatments are often necessary. At present, the primary choices of antimicrobials are third-generation cephalosporins (e.g., ceftriaxone) and fluoroquinolones (e.g., ciprofloxacin). Due to the potential toxicity of fluoroquinolones to cartilage development as reported in animal models [23] and in a child [24], ceftriaxone is the primary choice of treatment for infants, children, pregnant women, and immunocompromised people. Ceftriaxone has been classified as a critically important antibiotic for human use according to the WHO [25], According to the Centers for Disease Control and Prevention (CDC) Threat Report (2013), 3% of human-derived non-typhoidal

Salmonella isolates are resistant to ceftriaxone [20]; further, approximately 9.8% of *Salmonella* were multidrug resistant in 2014 [26]. Chicken and eggs are the most common source of *Salmonella*; however, *Salmonella* infections have been attributed to beef consumption. *Salmonella* commonly reside in the cattle gut microbiota; however, most are subclinical and cause no measurable negative effects on the animal host.

1.1.4 Ceftiofur and chlortetracycline

Ceftiofur is a beta-lactam antibiotic used in dairy cattle, beef cattle, pigs, poultry and horses. Ceftiofur is the only third-generation cephalosporin that is approved for treatment of BRD in cattle in the U.S.; however, a fourth-generation cephalosporin, cefquinome, is approved for this purpose in Europe [27]. Ceftiofur was first approved in 1988 by the FDA in an inconvenient powdered form that required reconstitution as an injectable liquid; later, a ready-to-use (RTU) injectable formulation was introduced in the 1990s, and finally a long-lasting ceftiofur crystalline-free acid (CCFA; Excede®, Zoetis) formulation was approved in 2003.

Ceftiofur is in the same antibiotic class as ceftriaxone which is used for the treatment of salmonellosis in humans and classified as a critically important antibiotic. A significant increase in cephalosporin resistant *Salmonella* was seen after 1997 and although fluctuating annually, an overall decreasing tendency has been reported since 2010 by the National Antimicrobial Resistance Monitoring System (NARMS) [26]. The beta-lactamase gene, $bla_{\text{CMY-2}}$ is most often responsible for ceftiofur resistance in commensal and pathogenic bacteria of livestock, such as *Escherichia coli* and *Salmonella*, in the United States, which typically exhibit cross-resistance to ceftriaxone and a nearly identical minimum inhibitory concentration [28-30]. In 2012, the FDA prohibited certain extra-label uses of ceftiofur in food-producing animals in an effort to reduce cephalosporin resistant *Enterobacteriaceae* [9]. A study in Denmark showed that the voluntary

ban of cephalosporins in its pig production system reduced the prevalence of extended-spectrum cephalosporinase producing *Escherichia coli* [11]. Several observational studies have been conducted concerning the effects of ceftiofur use on the emergence, spread and propagation of antimicrobial resistant *E. coli* and *Salmonella* in feedlot cattle; however, no randomized controlled studies have been reported.

Because of its broad-spectrum activity and product safety, chlortetracycline (CTC; Aureomycin®, Zoetis, Kalamazoo, Michigan) has a long history of use as an in-feed antibiotic for disease prevention and growth promotion purposes. However, all label claims of antibiotics for growth promotion in livestock disappeared as of January 1st, 2017 in the United States and the use of CTC in feed now is mandated under the Veterinary Feed Directive (VFD) which requires veterinarian's supervision [31]. Chlortetracycline is used for the control and treatment of BRD in feedlot cattle. The NARMS 2015 Integrated Report showed that approximately 26.5% of *Salmonella* from cattle and 50% from retail ground beef were resistant to tetracycline [32]. Tetracycline resistant isolates are often also co-resistant to ampicillin, chloramphenicol, streptomycin, and sulfonamides (in the penta-resistant ACSSuT phenotype) [33].

A previous study from our research group showed that chlortetracycline treatment by itself reduced phenotypic ceftiofur resistant *E. coli* in feedlot cattle [34]. In contrast, a follow-up study by Kanwar *et al*., showed an increase of ceftiofur resistant non-type specific (NTS) *E. coli* following CTC treatment, often in cattle previously treated with ceftiofur [35]. These conflicting results may be due to different populations of tetracycline resistant *E. coli*. Or, the priming of the *E. coli* populations with CCFA may have changed the population that the CTC was impacting. The tetracycline resistant *E. coli* from the Platt *et al*. study were likely to have primarily harbored the *tet*(B) gene, while *E. coli* in the Kanwar study were primarily carrying the *tet*(A) gene

following prior CCFA selection of the IncA/C plasmid bearing *E. coli* that carry both *tet*(A) and *bla*$_{CMY-2}$ genes. The *tet*(B) gene is the most detected tetracycline gene in Gram-negative bacteria coded on chromosomes [36]. Additionally, at the protein level TetA and TetB are not compatible proteins. We investigated the population dynamics of *Salmonella* in the same cattle population as used in the Kanwar study. The population dynamics of *Salmonella* in cattle feces following antibiotic treatment have not been previously reported.

1.2 Study objectives

The objectives of this study were to determine the effects of ceftiofur and chlortetracycline use on the temporal dynamics of multidrug-resistant *Salmonella* in feedlot cattle. The prevalence and quantity of *Salmonella* were determined and compared across treatment groups and days using broth enrichment and direct culture on brilliant green agar (BGA) and via a quantitative real-time probe PCR (qPCR). The quantity of antibiotic resistant *Salmonella* was determined by plating on BGA containing tetracycline (16 mg/L) and ceftriaxone (4 mg/L). We compared the antibiotic resistant phenotypes and genotypes using antibiotic susceptibility testing and whole-genome sequencing (WGS), respectively, comparing among days and treatment groups and their interactions. Additionally, serotypes and plasmids were determined by WGS and the phylogenetic relations of the isolates among the days and by treatments were explored. Finally, we assessed the utility of WGS in determining antimicrobial resistance profiles and serotypes in comparison to standard serological serotyping methods and antimicrobial susceptibility testing.

CHAPTER II

LITERATURE REVIEW

2.1 Antibiotic resistance as public health issue

Antimicrobial resistance has been a public concern for decades, since the first antibiotic, penicillin, was discovered by Alexander Fleming in 1928. Penicillinase, the enzyme that digests penicillin and causes penicillin resistance in bacteria, was discovered shortly afterward in 1940. Resistance mechanisms to antibiotics have been discovered anywhere from 2 to 16 years after the introduction of the antibiotic into the market [1, 37]. Antibiotic resistant mechanisms have been discovered in bacteria isolated from permafrost or isolated caves where no human activities have been recorded, nor antibiotics have been used [38-40]. Antibiotic resistance is a basic system that bacteria have developed as a defense mechanism for their survival. However, when soil samples from the 1940s, which were never exposed to antibiotics, were compared with samples from the 1970s, more than 15 times the number of tetracycline resistance genes were detected from the 1970s soil sample [41]. This provides evidence that the recent problem involving the emergence and widespread dissemination and propagation of antibiotic-resistant bacteria in humans, animal populations, and environments is largely the result of selective pressure from antibiotics derived from human activities [41-43]. Antibiotics have saved millions of human and animals lives from mortality due to bacterial infections for over 80 years [44].

2.2 Feedlot operations and antibiotic use

2.2.1 Feedlot operations

Feedlot cattle operations are designed to raise weaned calves to an acceptable size, carcass grade, and weight for slaughter [45]. In the United States, the cattle and calve industry is worth

$76.4 billion and the feedlot cattle industry is worth $36.4 billion [46]. The feedlot cattle industry accounts for 26% of total U.S agricultural sales [46]. Beef cattle operations are divided into multiple stages in the United States starting from cow-calf operations that produce calves and sell to other producers (Figure 1) [47]. Seedstock breeders maintain herds for purebred breeding stock [47]. Calves from cow-calf operations are weaned at 6 to 10 months of age and may be sent to background-stocker operations or else directly to feedlots. The purpose of background-stocker operations is to raise calves that are too small to enter the feedlots, often on a maintenance ration. Calves are grazed and fed an additional high roughage ration and at 12 to 18 months of age, calves then are moved to feedlots or finishing operations to be fattened for slaughter. Typically, cattle spend 4 to 6 months in feedlots and finishing operations and are fed high-energy rations until they reach a weight between 1,100 to 1,300 pounds. Once the cattle reach the desired weight and carcass grade, they are sent to the slaughterhouse [47].

Feedlot operations are a style suited to raising a large number of cattle at a faster rate by utilizing limited land efficiently. The use of feedlots started before the U.S. Civil War when grain farmers were trying to sell excess commodities and to maintain workers year-round; therefore, they started to sell grains to feed food animals. Later, they discovered that feeding grains to food animals increased the growth rate of the animals. Nowadays, beef cattle feedlot operations are located mainly in the high plains of the United States in the Texas Panhandle, Oklahoma, western Kansas, eastern Colorado, and western Nebraska [45]. Not coincidentally, this region also provides access to a very large aquifer (Ogallala) of water.

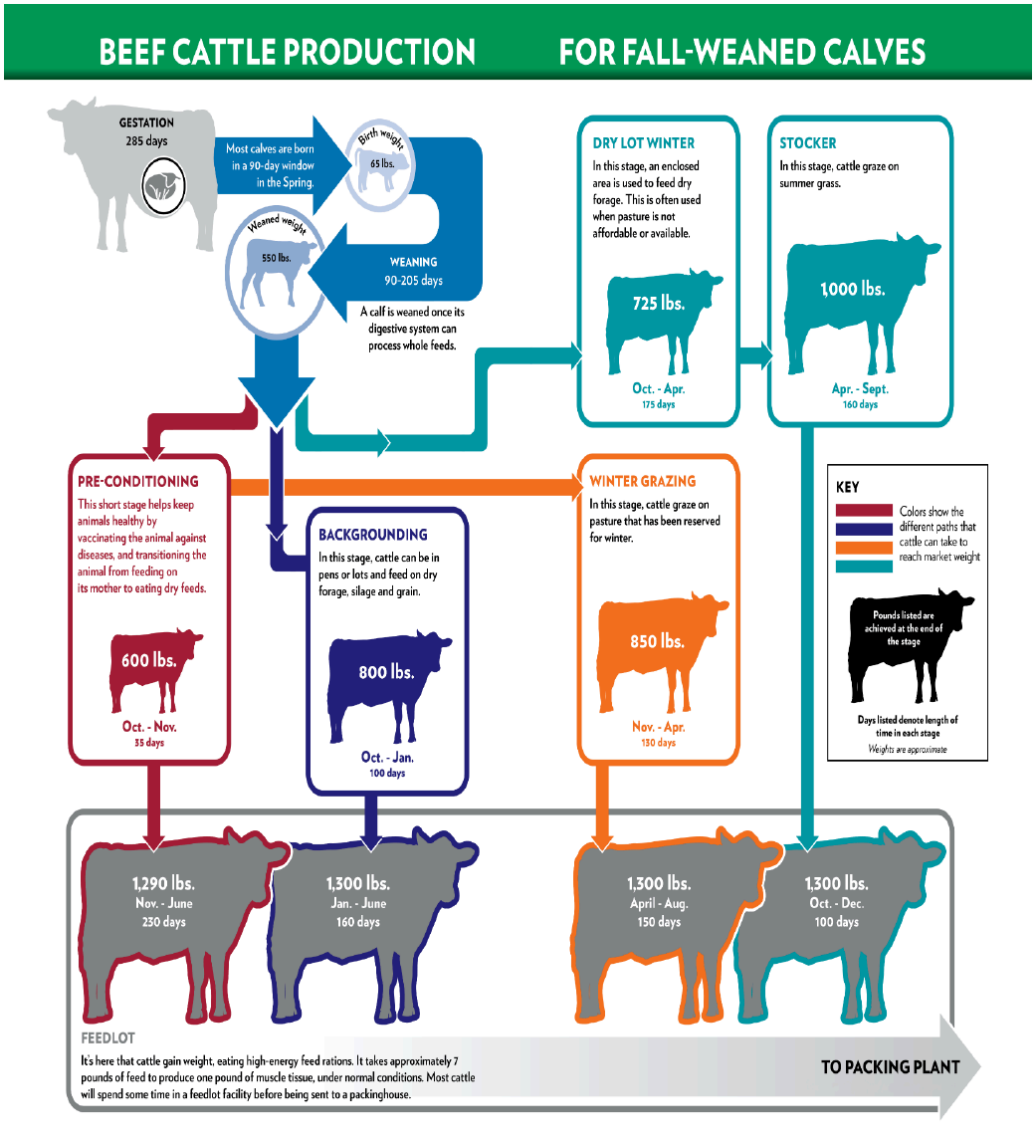


Figure 1. Beef cattle operation system

(Adapted from Overview of the United States Cattle Industry June 2016 [47])

Cattle are assembled from different sources and taken to the feedlot. Due to weaning, shipping, climatic and mingling stresses, they often are susceptible to bovine respiratory disease (BRD) or “shipping fever”. BRD is one of the most common diseases that feedlot operators

encounter [45, 48]. Economic losses due to BRD are estimated to be around $1 billion in the United States and more than $3 billion worldwide [49, 50]. The National Animal Health Monitoring System (NAHMS) 2011 Feedlot study reported that approximately 16.2% of cattle were affected by BRD [45]. BRD is a complex disease that is caused by a number of bacterial pathogens including *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni,* and *Mycoplasma bovis*, in combination with a number of viruses [51]. Bacteria responsible for BRD are mostly commensal bacteria of the upper respiratory tract that become opportunistic pathogens by expressing virulence factors when the host is under stress and immune conditions change [52]. Coinfection with viruses can suppress the host immune response and initiate replication of the bacterial pathogen in the upper respiratory tract, and eventually the bacteria reach the lung [52].

The risk of BRD in cattle is highest upon arrival to the feedlot because of the stress from transportation, weaning, bad weather, and comingling of cattle from different sources in the same pen. Diagnosing BRD accurately is a challenging task for pen riders, who check the cattle daily. Diagnosis often is based on visual observations, such as depression, loss of appetite, and coughing. This is further complicated by the fact that cattle and other ruminants have evolved to conceal their illness to deter predators [50]. Therefore, prevention of BRD infection is critical in the feedlot. The NAHMS Feedlot 2011 study reported that 96.6% of feedlots vaccinated cattle against viral pathogens that cause BRD, such as bovine viral diarrhea (BVD) virus, infectious bovine rhinotracheitis (IBR) virus, and bovine herpes virus 1. Two thirds of feedlots vaccinated against bacterial agents such as *Mannheimia haemolytica* and *Pasteurella sp.* upon arrival. The effectiveness of vaccination is inconsistent, but several articles provide evidence that vaccination

reduces the morbidity and mortality risk associated with BRD compared with unvaccinated cattle [53, 54].

2.2.2 Antibiotic use in feedlot operations

Metaphylactic treatment is one of the most common antibiotic practices used to control BRD [55]. Metaphylaxis is defined as " the timely mass medication of a group of animals with an antibiotic to eliminate or minimize an expected outbreak of disease"; meanwhile, it is referred to as "disease control" by the U.S. FDA [45]. Several injectable antibiotics are labeled for both the treatment and control of BRD, such as oxytetracycline, tilmicosin, tulathromycin (and other macrolides), florfenicol, several fluoroquinolones and ceftiofur [50]. According to the NAHMS Feedlot 2011 study, the top 3 injectable antibiotics to prevent or minimize BRD were tilmicosin (57.6% of feedlots), tulathromycin (45.3%), and ceftiofur (39.7%) and metaphylactic use of antibiotics to decrease morbidity and mortality from BRD was reported at 59.4% of feedlots [45].

Choice of antibiotics for the metaphylaxis often is based on several factors including history of BRD at the feedlot, source of the cattle, cost-effectiveness, and the effectiveness of antibiotics [45]. Injectable antibiotics are precise and easier for the feedlot operators to handle and often a one-time injection is all that is required, especially when using a longer-acting formulation of the drug. The effectiveness of metaphylaxis for BRD is proven for a number of different antibiotics [50, 56-60]. Metaphylactic use of oxytetracycline reduced morbidity but did not affect mortality attributed to BRD in a Canadian feedlot [59]. Treatment with tulathromycin reduced recurrence for BRD compared with tilmicosin treated cattle [56]. Cattle that received metaphylactic treatment with ceftiofur crystalline-free acid (CCFA) on arrival to the feedlot also had lower mortality and morbidity compared with those did not receive treatment [57]. Recent concern

regarding the rise of antibiotic resistant bacteria is also the case for bacteria that cause BRD [49, 51, 61]. Overall resistance rates in of *M. haemolytica*, *P. multocida*, and *H. somni* remained low between 1994-2002 at the Oklahoma Animal and Disease Diagnostic Laboratory. However, resistance increased in *M. haemolytica* against florfenicol, spectinomycin, tilmicosin, and erythromycin, and to spectinomycin, erythromycin, and tetracycline in *P. multocida* [61, 62]. For another of the BRD pathogens, *Mycoplasma bovis*, that were collected from Canadian feedlot cattle that received oxytetracycline metaphylaxis were largely susceptible to the antibiotics tested except for tilmicosin. Further, genetic diversity of the isolates was not affected by metaphylaxis treatment [59].

In-feed and in-water antibiotics are other convenient method for mataphylaxic treatment. Oral antibiotics are incorporated either into the water or feed for disease treatment, prevention, and (previous to 2017) for enhanced productivity (subtherapeutic dose). Ionophores are the most common antibiotics fed throughout the feeding period (90% of cattle in feedlot) to enhance production efficiency and for the control of coccidiosis; however, they are not considered to be medically important for human medicine. Tylosin is another common in-feed antibiotic that 31.0% of feedlots used for control liver abscess [45]. Chlortetracycline (CTC) is one of the most common in-feed antibiotics. It has been reported that 71.7% of feedlots use CTC for disease prevention and control [45]. In-feed CTC treatment for 5-days upon arrival reduced morbidity and the use of critically important antibiotics in next 120 days [63]. One study showed that metaphylactic treatment by CTC at subtherapeutic concentrations did not result in any benefits in reducing BRD in cattle derived from a single source, which generally are considered as low-risk of BRD [60].

One of the issues with antibiotics provided in feed is that the amount of antibiotic available and consumed may be inconsistent, especially if the cattle lack appetite. The FDA prohibited the extra-label use of the medically important antibiotics fluoroquinolones (danofloxacin and enrofloxacin) in 1997 and cephalosporins (ceftiofur, but not cephapirin) in 2012. Additionally, the FDA announced the final rule of Veterinary Feed Directive (VFD), which requires involvement of veterinarians to use medically important antibiotics in-feed or water for food animals as of January 1, 2017. This includes CTC and tylosin but not ionophores, because ionophores are not considered to be medically important antibiotics. Even though there are efficacy differences between antibiotics, overall, metaphylaxis is an effective method to reduce morbidity from BRD. As summarized here, antibiotic treatment is necessary for disease treatment, prevention and control in cattle feedlots; however, the emergence of antibiotic resistant bacteria including *Salmonella* has become a worldwide public health issue that poses a threat to the *status quo* [12].

After the wide dissemination of antibiotic resistant bacteria in human populations was revealed, uncontrolled use of antibiotics in food animals began to be blamed for the expansion of resistant bacteria responsible for human infections [7, 43, 64, 65]. It is estimated that around 60% of medically important antimicrobials sold in the United States in 2015 were used in food-animals [6]. On the other hand, it is not a simple process to investigate the connection between antibiotic use in the food-animal production system and its subsequent effects on human health [65]. For example, it is necessary to investigate whether the use of antibiotics in animals increases the number of resistant bacteria in animals (and for how long after treatment ceases), how resistant bacteria are transferred to carcasses, to meat products, and to prepared food, and

the effects of environmental contamination with antibiotics and resistant bacteria on surface and ground water and row crops such as leafy greens and other vegetables and fruits [65].

2.2.3 Resistant bacteria in human and animal populations

Through the development of advanced molecular techniques, we are gaining a deeper and broader perspective on antibiotic resistance. In 2012, Mather *et al*. used novel ecological and epidemiological methods to compare the phenotypic resistance profiles of *Salmonella* Typhimurium DT104 isolates derived from cattle and human populations collected from 1990 to 2004 in Scotland [66] Later genotypic resistance profiles and phylogenetic relations of the same isolates were compared [67]. Although some similar resistance profiles were found to be shared among cattle and humans, uniquely distinguishable phenotypic resistance profiles in the cattle and human populations were observed and maintained within the respective populations. Among the similar resistance profiles, more profiles were observed earlier in the human population.

A similar study was conducted on *Salmonella* isolates derived from cattle and humans from 2004 to 2011 in Washington State by Afema *et al*. [68]. The phenotypic antimicrobial resistance profiles of the dominant serotypes (Typhimurium, Newport, and Montevideo) were not shared among cattle and human-derived isolates. Isolates derived from the human population had more diverse phenotypes than the cattle isolates. The bacterial population that enters the human population through food animals are those from healthy animals and not the animals exhibiting clinical symptoms. Most *Salmonella* infected cattle do not typically show clinical symptoms. These results suggest that among the resistant *Salmonella*, even though some are shared between human and cattle populations, many unique isolates circulate only within their own species.

2.3 **Salmonella *description***

2.3.1 Salmonella *taxonomy*

Karl Joseph Eberth isolated a bacillus bacterium, suspected to be the cause of typhoid fever, from the spleen and mesenteric lymph nodes of a patient who died from the disease. The bacterium was later discovered to be *Salmonella* Typhi. Theobald Smith isolated a bacillus from a hog with hog cholera, which was considered the likely pathogen causing the disease in 1885; instead, this bacterium turned out to be *S*. Choleraesuis and not the causative agent of hog cholera which turned out to be a virus. Unfairly, *Salmonella* was named after Smith's chief scientist, Daniel Elmer Salmon, a veterinarian at the United States Department of Agriculture (USDA) who claimed the credit [69].

Salmonella belongs to the *Enterobacteriaceae* family and is Gram-negative bacillus with flagella for motility. Two *Salmonella* species are known: *Salmonella enterica* and *Salmonella bongori. Salmonella enterica* is further classified into 6 subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houteniae* (IV), and *indica* (VI). Most of the human cases of salmonellosis are caused by serotypes of *Salmonella enterica* subsp. *enterica* (referred to as *Salmonella enterica* henceforth; or abbreviated further to *Salmonella* and its serotype). The host of the other subspecies are cold-blooded animals such as reptiles. *Salmonella enterica* is a facultative anaerobe and biochemically tests positive for dulcitol, sorbitol, L(+)-tartrate, γ-glutamyltransferase, mucate, and additionally is lysed by phage O1 [70]. Biochemically, *Salmonella enterica* are also lactose negative, oxidase negative and catalase positive. For β-glucuronidase, it is only positive in subsp. *diarizonae* in general and different reactions are given for different serotypes in other subspecies [70]. *Salmonella* can survive dehydration and low temperatures, but not extremely high temperatures [71]. They can adapt down to pH 3.3 [72].

2.3.2 Host relation of Salmonella

Within the serotypes of *Salmonella enterica,* non-typhoidal *Salmonella* is the major food-borne pathogen causing salmonellosis, while *Salmonella* Typhi (*S.* Typhi), *S*. Paratyphi A, *S.* Paratyphi B, and *S.* Paratyphi C are typically pathogenic only to humans and cause variations of typhoid fever. Three types of host specificities are known for *Salmonella* serotypes; host restricted (*S*. Typhi to human, *S*. Abortusequi to horse), host unrestricted (*S.* Typhimurium, *S.* Enteritidis), and host adapted which may cause severe illness (*S*. Dublin to cattle, *S*. Choleraesuis to pigs) [73]. The symptoms of salmonellosis are nausea, diarrhea, fever, abdominal pain, and vomiting with symptoms presenting 12 to 72 hours after ingesting contaminated food or water. Infectious doses vary from $<10^1$ to 10^9 CFU in humans; however, this varies by serotype. *Salmonell*a often is shed intermittently. Some animals infected with host-adapted and host-restricted serotypes become high-shedders or super-shedders. These are defined as individuals shedding over 10^8 CFU / gram feces in mice with *S*. Typhimurium infection, or 10^3-10^5 CFU / gram feces in cattle with *S*. Dublin infection [74].

2.3.3 Salmonella *infection*

Salmonella have adaptations that allow them to evade and escape from the host immune system. *Salmonella* infect the host cell by utilizing the specialized antigen-sampling membranous epithelial (M) cells of the host intestine epithelium and translocation of proteins within the Type III Secretion system encoded by the *Salmonella* pathogenicity Island 2 (SPI-2) (Figure 2) [75]. Invasion by *Salmonella* induces an inflammatory response in the host, in which macrophages, neutrophils, and lymphocytes migrate to the site of invasion. Following the host immune response, more than 90% of *Salmonella* are destroyed by phagocytic cells [76]; however, *Salmonella* can also survive within the engulfed macrophages and escape the host immune

response. Further, *Salmonella* can disseminate through the lymphatic system and cause systemic infection. From the neutrophils that migrate into the intestinal lumen, reactive oxygen species (ROS) are released which target the invading bacteria. However, *Salmonella* can utilize tetrathionate, a respiratory electron acceptor produced by the oxidation of thiosulfate, which is the byproduct of the release of ROS [77]. Therefore, inflammation caused by a *Salmonella* infection can be advantageous to *Salmonella* and they utilize the tetrathionate to outgrow the other competing microbes in the intestine [78]. *Salmonella* Typhimurium infection itself can disrupt the microbiota by causing acute enteritis and diarrhea; further, they can increase their invasiveness requiring SPI1 and SPI2 type 3 secretion systems in mice [79]. Alterations are often caused by host's diarrheal response rather than the presence of pathogen itself. In published studies, the host lost *Eubacterium rectale/Clostridium coccoides* 1 week after *Salmonella* infection. However, the microbiota recovered to normal after 1 month after the date of infection [79].

The interaction between antibiotic use, host microbiota, and *Salmonella* has been studied previously in mice [80, 81]. A study showed that *Salmonella* can use the indole produced by *E. coli* to increase its antibiotic tolerance by mediating oxidative stress response and phage shock response [82]. Recent studies have shown that antibiotic administration can reduce diversity of microbiota and induce diseases in human and mice [44, 80, 81]. Antibiotic administration decreased the complexity of healthy microbiota and induced elevation of host derived fucose and sialic acid levels in the gut lumen of mice [80, 81]. Ng *et al.* used gnotobiotic mice that carry only *Bacteroides thetatiotaomicron* (*Bt*) as a simplified model microbiota mimicking the disrupted microbiota after antibiotic treatment. *Bt* encodes sialidase which is required to consume the liberated sialic acid, while *Salmonella* Typhimurium lacks the sialidase but have the

catabolic pathway (*nan* operon). Under the presence of *Bt*, *Salmonella* can catabolize the liberated sialic acid and further colonize the host [80, 81]. These studies have shown that *Salmonella* exhibit tolerance under stressed conditions caused by antibiotic treatments. Further, they can take advantage of host responses against their infection itself and changes to the microbiota to proliferate under such conditions. Because of the complexity of microbiota in ruminants compared with mice and humans, these previous results are unlikely to be directly applicable to cattle. In the current study, we explore the effects of antibiotics on the quantity of susceptible and resistant *Salmonella* in fecal populations by performing qPCR from total community DNA and by direct spiral plating.

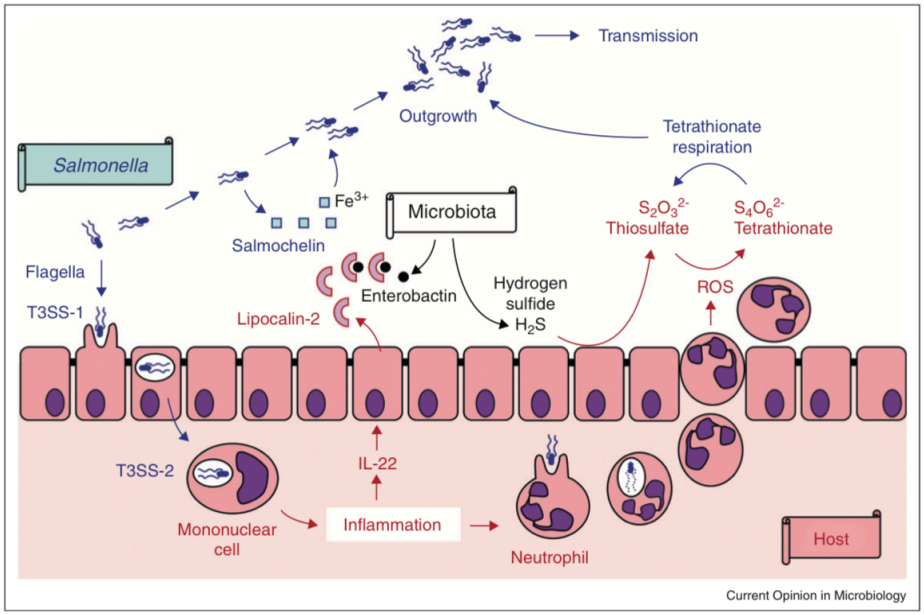


Figure 2. Schematic illustration of *Salmonella* infection (Adapted from Thiennimitr *et al.* [78])

2.4 Methods for identification and characterization of **Salmonella**

2.4.1 Isolation methods for Salmonella

Enrichment procedures and selective broths and media are necessary for the isolation of *Salmonella* from samples containing abundant microbes, such as feces and soils. These selective broths and agars were developed based on the biochemical specifications of *Salmonella*, and were designed to inhibit the growth of other organisms, while promoting the growth of *Salmonella*. In the current study, tetrathionate broth and Rappaport-Vassiliadis (RV) broth were used, respectively, followed by plating onto brilliant green agar (BGA). Tetrathionate broth is composed of sodium thiosulfate, calcium carbonate, pancreatic digest of casein, peptic digest of animal tissue, and bile salts. Additionally, it is necessary to add iodine-iodide solution immediately before adding the samples, in order to initiate the formation of tetrathionate. *Salmonella* carry tetrathionate reductase, which allow *Salmonella* to use the tetrathionate as a respiratory electron acceptor, produced during inflammation (Figure 2), to grow and outcompete other coliforms [77, 78].

The bile salts included in the tetrathionate broth inhibit the growth of Gram-positive bacteria and other coliforms. Pancreatic digest of casein and peptic digest of animal tissue provide nutrients for the *Salmonella* growth. Calcium carbonate absorbs toxic materials produced during the amplification. Rappaport-Vassiliadis broth is composed of soy peptone, sodium chloride, potassium phosphate, magnesium chloride, and malachite green. Malachite green inhibits the growth of other organisms. Magnesium chloride increases the osmotic pressure of the broth, which promotes the growth of *Salmonella* because they can survive dehydrating conditions when compared with other intestinal bacteria [83, 84]. Together with malachite green the low pH (5.2 ± 0.2), allows for the selection of *Salmonella*. Several selective agars are available for the

identification and isolation of *Salmonella*, including BGA, xylose lysine deoxycholate agar (XLD agar), xylose lysine tergitol-4 agar (XLT agar), *Salmonella-Shigella* agar (SS agar), and Hektoen enteric agar.

In our study, BGA was used for the isolation of *Salmonella*, which was first reported by Kristensen in 1925 and modified by Kauffman in 1935. BGA is composed of brilliant green dye to suppress the growth of Gram-positive bacteria and other Gram-negative bacilli, including *S.* Typhi and *S.* Paratyphi. Lactose, sucrose, and yeast extract are included to provide nutrients for *Salmonella* growth. The color of uninoculated BGA is brownish-orange due to the addition of phenol-red dye. The growth of *Salmonella* changes the surrounding agar to pink with the change of pH level (original pH is 6.9 ± 0.2). *Salmonella* colonies are identified by a transparent, light red-pink coloration. Lactose-fermenting bacteria such as *E. coli* colonies grow as yellow-green colonies due to acid production by lactose/sucrose fermentation and are therefore easy to distinguish from *Salmonella* on BGA. The concerns of using enrichment methods are the possibility of selecting only certain serogroups or serotypes, which thus may bias the serotype results [85, 86]. A few *Salmonella* do not carry the gene encoding tetrathionate reductase, and thus may not proliferate in tetrathionate broth.

Gorski examined the bias caused by enrichment processes in *Salmonella* isolation [86]. The growth of *Salmonella enterica* isolates from 4 serogroups, B, C1, C2, and E, including 13 serotypes, and 19 isolates in RV broth and Rappaport-Vassiliadis soya peptone broth (RVS) were compared. RV broth is routinely used by the U.S. FDA following their Bacterial Analytical Methods manual [87]. Gorski prepared 8 sets of mixtures containing 4 strains from each serogroup having similar growth kinetics. She compared the ratio of these 4 isolates after 24-hour enrichment at 35 °C in tryptic soy broth (TSB), and then enriched in RV or RVS broths at

42 °C for 24 hours, and finally plated on XLD agar. Even though TSB is a non-selective broth, there was clearly biased growth. However, the dominant serogroups seen in RV and RVS were not necessarily reflecting the dominant strains after TSB enrichment. In one mixture, serogroup C1 was approximately 10% after TSB enrichment but increased to 90% after RV enrichment. In the same mixture, serogroup B was dominant after RVS enrichment. In the other 4 mixtures, serogroup E was not dominant in TSB culture but become dominant after RV enrichment. The ratio of serogroups after RVS enrichment were more equivalent and similar to that of TSB than after RV enrichment; however, serogroups C2 and E were more dominant in 6 of the 8 tested groups after RVS enrichment.

Gorski [86] also examined the bias caused by the background microbiota of cattle feces by mixing these *Salmonella* strain mixtures with cattle fecal samples that were not detected as having *Salmonella*. RVS had a more equivalent distribution of serogroups compared with RV, similar to those without background microbiota. However, in some mixtures, serogroup B grew better than *Salmonella* only mixture, which suggests the presence of fecal microbiota may favor the growth of serogroup B. Serogroup B consists with serotypes that are highly pathogenic to humans including *S*. Typhimurium. The bias caused by an enrichment process will affect interpretation of results of surveillance, but less so during outbreaks, and clinical diagnosis where a single strain is expected to dominate. The fact that serogroup B strains were less dominant after RV enrichment, which is the routine broth used in most surveillance systems, may be problematic since they are more pathogenic. On the other hand, RV broth has been used as a standard broth because of the balance between growth of desired bacteria and suppression of competing bacteria such as *Proteus*. Gorski's study also showed that the dominance of serogroup

C2 and E is consistent with dominance of *S*. Newport and *S*. Kentucky in surveillance as shown by the CDC.

A similar concern was raised by Singer *et al*. that enrichment may bias the results of current surveillance system [85]. In fact, many healthy food animals are not infected with a single serotype, but multiple serotypes and at different ratios [88]. Therefore, it is important to consider the methods used in the surveillance system, outbreak analysis, and experimental studies. Caution is needed when comparing the results from different studies. Conducting multiple enrichment using different broths, or not conducting any enrichment may reduce such bias. Additionally, next-generation sequencing may help to overcome this culture bias by extracting the total genomic DNA from microbiota and examine the *Salmonella* serotypes as they are found *in situ* [89].

2.4.2 Typing of Salmonella

2.4.2.1 Traditional serotyping

Traditionally, *Salmonella enterica* has been classified based on serotypes. *Salmonella* consists of diverse serotypes; however, around 80% of cases of salmonellosis in humans in the U.S. are accounted for by 20 serotypes of *Salmonella enterica*. Within *Salmonella enterica* species, there are approximately 2,500 serotypes and 1,500 serotypes within subspecies *enterica*, which are classified following the White-Kauffman-Le Minor scheme. The White-Kauffmann-Le Minor scheme is curated by the Pasteur Institute, a World Health Organization (WHO) collaborating center [70]. *Salmonella* serotypes are determined by the expression of the somatic (O) and flagellar (H) antigens. The O-antigen is formed by chains of oligosaccharides on the lipopolysaccharide outer-membrane (LPS) [90]. This sugar chain is composed of 3 to 8 different combinations of core oligosaccharides, which are determined by the *rfb* gene cluster [90, 91].

There are seven known pairs of O-antigens that are common between *Salmonella* and *E. coli*. Recent discoveries are of *E. coli* O11 / *S. enterica* O16 and *E. coli* O21 / *S. enterica* O38 [92]. Therefore, when serotyping is performed by the agglutination of antisera, misclassification may occur.

H-antigens are biphasic and *Salmonella* express either the phase-1 or phase-2 antigen to escape the host immune system via the phase variation system; in turn, these are coded by *fli*C and *flj*B genes [93]. The expression of *flj*B gene is controlled by a repressor *fli*A, which is known for its inverted sequencing [93]. The majority of *Salmonella* serovars can express both antigens (diphasic), some serovars have a 3rd flagellar antigen, and some serovars have only one phase (monophasic) [94]. Serotyping based on O-antigens and H-antigens has been the standard method for classification of *Salmonella* for the past 75 years. Currently, serotypes are typically named after the location where the first isolate derived. The serotype is not italicized, and the first letter of the name is capitalized (e.g. *S.* Typhimurium). Traditional serotyping is conducted via the antisera-antigen reaction. It is rapid for determining common serotypes, but labor intensive and time consuming for rare serotypes; further, good quality antisera is expensive and with a limited shelf-life. In the United States, clinical isolates often are sent to the USDA, National Veterinary Services Laboratories (NVSL) for expert reference laboratory serotype determination.

2.4.2.2 Whole genome sequencing (WGS) based serotyping

Through the advancement of WGS technologies, England and the United States have transitioned to WGS based serotyping methods [95]. Currently, two *in silico* serotyping methods to determine serotypes via the *rfb* gene clusters encoding O-antigens, flippase (*wzx*), polymerase (*wzy*) and H-antigens (*fli*C and *flj*B) have been developed: SeqSero and SISTR (*Salmonella in*

Silico typing resource) [96, 97]. In addition to serotypes, SISTR determines multi-locus sequence type (MLST), ribosomal MLST (rMLST), and core genome MLST (cgMLST) using 330 genes to increase the specificity. The accuracy and possibility using WGS based *in silico* serotyping to replace traditional serotyping were reviewed by Yachison *et al*. [98]. All three methods, SeqSero, SISTR, and MLST have high match rates of 94.8, 88.2%, and 88.3% with traditional serotyping. However, all methods have the possibility of errors. For example, in monophasic *Salmonella*, where the second flagellar antigen is not expressed phenotypically, the gene may still be present. As a result, inconsistency occurs between traditional antisera serotyping and *in silico*-based serotyping. SeqSero accepts both FASTQ and FASTA file formats, while SISTR accepts only FASTA files from draft assemblies.

Accepting the FASTQ file format is advantageous because of the assessment of the sequencing quality and quick return of serotype. Additionally, FASTQ based raw reads use *wzx* and *wzy* for O group identification, which have more discriminatory power than using the *rfb* gene clusters that are available in FASTA and used in SISTR. The *rfb* gene clusters can end in two separate contigs after assembly. SeqSero overcomes this problem by further flanking the *rfb* cluster into four contigs and BLASTing with the *rfb* database, whereas SISTR often returns false results [96, 97]. Yachison *et al* discussed that the *rfb* gene clusters which determine the O-antigen, can be fragmented and removed during library preparation using Illumina Nextera XT library preparation kits [98]. This problem can be overcome by using different library preparation kits or else skipping the filtering procedure. Since assembly is required for SISTR, a certain level of assembly quality is required and genes encoding the O-antigen and H-antigen may be fragmented into separate contigs; thus, incorrect serotypes may be returned.

Based on our study, submitting raw FASTQ files from Illumina runs have higher return rates of serotypes in SeqSero, further described in Chapter V. Yachison *et al*. only used FASTA files as input files for the comparison of SeqSero and SISTR, and may have obtained better results in SeqSero by using FASTQ files as input files. In spite of the advancement of bioinformatics methods, traditional serotyping remains a continuously important method and resource in countries where WGS is too expensive to conduct due to equipment and reagent costs. Additionally, WGS requires bioinformatics and computing resources. In such cases, international collaboration on WGS can be a potential solution to allow for uniformity of the methods and meaningful comparisons between countries. Such collaborations are important, especially given food-borne pathogens are spread through travelers and imported/exported foods.

2.4.2.3 Multi-locus sequence types (MLST)

Multi-locus sequence types (MLST) are classified based on the sequence of 7 housekeeping genes. Housekeeping genes are amplified by PCR (450-500bp) and allele sequences of each fragment are determined and assigned distinct allele numbers. The pattern of the 7-integers defines its allelic profile and a sequence type (ST) is assigned to a strain. The variation within these housekeeping genes are sufficient enough to provide many alleles per locus and differentiate 20 billion multi-locus genotypes with an average of 30 alleles per locus specific to each bacterial species (see for example: MLST.net; http://beta.mlst.net/Instructions/default.html).

The seven housekeeping genes designated for *Salmonella* are *aro*C, *dna*N, *hem*D, *his*D, *pur*E, *suc*A, and *thr*A. As of April 2018, 4,127 MLST profiles are defined on the PubMLST website. Through the development of whole-genome sequencing (WGS) technology, MLST can now be conducted *in silico*. WGS has allowed for using more than the 7 housekeeping genes for MLST classification. Seven housekeeping genes, ribosomal MLST (rMLST), core-genome

MLST (cgMLST) or whole-genome MLST (wgMLST) have been developed for MLST based typing [97, 99-101]. STs are further classified into eBurst Groups (eBGs), which Achtman *et al*. defined as a group of STs that share six of the seven alleles that define a ST [99, 102]; however, eBGs can be user-defined. Most of the serotypes with multiple STs are classified into the same eBGs, while polyphyletic serotypes are in separate eBGs even by cgMLST. The rMLST method is more conserved than methods using housekeeping genes, and Achtman *et al*. found eBGs were similar between MLST and rMLST. Achtman *et al*. recommended replacing traditional serotyping with MLST for epidemiological studies because MLST reflects more of the genetic relatedness. Despite a broad belief that serotyping reflects genetic relatedness, that may not always be true.

It has been shown that horizontal gene transfer (HGT) and homologous recombination are important processes for the evolution and serotype diversity of *Salmonella* [103]. *S*. Heidelberg and *S*. Typhimurium share the same *fliC* alleles (Phase I flagellar antigen) but they are genetically very distinct [99, 104]. *S*. Lubbock and *S*. Mbandaka belong to the same ST 413 and have high genetic similarity, but the *fliC* gene of *S*. Lubbock is the same as *S*. Montevideo, suggesting a homologous recombination has occurred between *S.* Mbandaka and *S*. Montevideo [105]. Homologous recombination happens between two DNA regions that have homologous sequences. Therefore, serotyping does not necessarily reflect the evolutional relationships between the isolates.

In addition, many serotypes have been discovered to be polyphyletic, in that even though they share the same O- and H- antigen formula, they have multiple evolutionary origins; including *S*. Newport, *S*. Kentucky, and *S.* Montevideo [106-110]. The polyphyletic serotypes, *S.* Newport and *S*. Kentucky have 49 STs and 5 STs, respectively. A detailed study on *S*. Newport

lineages showed 3 groups of *S.* Newport I, II, and III. The 3 groups have host, antibiotic resistance, and geographic differences [106]. It has been shown that the diversity within the Newport serotype accumulated by a mixture of mutation and homologous recombination. Sangal *et al*. have tested the ratio of recombination vs. mutation (R/M per site) in *S*. Kentucky, Enteritidis, Newport and Typhimurium. In *S*. Enteritidis and Typhimurium, the R/M per site ranged from 0.81 to 1.69, respectively. Meanwhile, *S.* Kentucky was >10.0, which suggests that recombination plays an outsized role in the diversity of that serotype. *S*. Newport was not as extreme as these serotypes, which ranged 7.08 to 8.14 in Newport-II. *S*. Enteritidis had a more clonal structure than *S.* Newport. The authors discussed that the diversity by serotypes may be due to their differences in response to phage infection, such as CRISPR systems, and defense mechanisms against genetic exchange. In case of the *S*. Newport example above, they have distinct geographical distribution, as Newport-I is found mainly in humans in Europe while Newport-II and -III are found across host species in North America [106]. Newport-II were more associated with animals and Newport-III isolates were associated with humans in North America. Therefore, even if a *S*. Newport outbreak happens at the same time in the United States, these isolates may have consisted of Newport-I and Newport-II, and possibily from different sources.

Susceptibility to antibiotics was also significantly different between the lineages. In Newport-II and III, 60 to 80 % were pan-susceptible, whereas 40% of Newport-I were MDR. Approximately 30% of Newport-II were of the MDR-AmpC phenotype. To summarize, even within the same serotypes, there are distinct differences between the lineages. Determining the lineage of the polyphyletic serotypes is essential, especially in outbreaks to identify the source and distribution of the isolates. It is also essential to determine the lineages when examining the

dissemination of antibiotic resistant bacteria since antibiotic resistant profiles tend to be distinct between the lineages. Sangal *et al.* [106] also showed that MLST based eBG designations were distinct between the lineages, further illustrating the discriminatory power of MLST over traditional serotyping.

2.4.2.4 Pulsed-field gel electrophoresis (PFGE)

In addition to traditional serotyping, molecular typing of *Salmonella* is conducted in outbreaks to increase the discriminatory power of bacterial isolation and comparisons with contemporaneous and historical collections [111]. Pulsed-field gel electrophoresis (PFGE) has been the gold standard for typing of *Salmonella* in outbreaks by the Centers for Disease Control and Prevention (CDC). PFGE classifies *Salmonella* through the "fingerprinting" of the bacteria, which is performed through digestion of the DNA by specified restriction enzymes (i.e: *Xba*I) [112]. The PFGE type is classified based on the restriction pattern as designated by PulseNet methods. This technology is discriminative and especially useful in outbreak cases to cluster the bacteria and distinguish outbreak related and non-related strains. A study showed that PFGE is more discriminative than 3-gene-based MLST schemes and serotyping; however, in a few cases, two serotypes were classified into the same PFGE pattern [113]. Another study reported that PFGE was able to discriminate outbreak strains related to 5 serotypes attributed to Roma tomatoes occurring in Pennsylvania and surrounding states in 2004 [112]. The PulseNet database is maintained by the CDC to compare PFGE patterns and is now adopted worldwide as PulseNet International [114]. In an era of international food trade and travel, PulseNet International is helping to network national and regional laboratories to track the spread of global food-borne infections.

Even though PFGE has strong discriminatory power, there are still several disadvantages. Some strains cannot be typed by PFGE, and a single nucleotide change in a restriction site may change the PFGE banding pattern. Additionally, PFGE is time-consuming, labor intensive, and requires specific equipment, and thus may not ideal for labs with small budgets or small staffing capacity. Despite the multiple-day effort to create the banding pattern, this pattern provides no additional genomic information; further, no virulence or antibiotic resistance genes can be identified. Similar to serotyping and MLST, a shift towards WGS based typing for molecular typing has been occurring in national laboratories around the world including the United States [115]. In fact, a number of studies have shown that WGS have a higher resolution for *Salmonella enterica* classification than PFGE [116-120]. The U.S. FDA has been developing the GenomeTrakr Network with regional and national labs from multiple U.S. states and countries [121]. As of 2017, more than 100,000 *Salmonella* genomes have been collected in the database. It is expected that GenomeTrakr will function as PulseNet in the near future and help to harmonize the methodologies and bioinformatic analysis pipeline for rapid and accurate identification of outbreak bacterial strains among regions and countries.

2.4.2.5 Phylogenetic relationships of *Salmonella*

Multiple methods of phylogenetic analyses are available for whole-genome based analysis. Using the sequence data from outbreak cases, pan-genome single-nucleotide polymorphism (SNP), core-genome SNP, and MLST methods have been explored in multiple studies [102, 116, 119, 122, 123].

The core-genome is characterized by the genes that are shared among strains at the same taxonomic level; i.e., genus, species, subspecies, serotype. As the taxonomic rank goes higher, the pool of core-genome gets smaller and vice versa [122, 124]. The pan-genome contains the

core-genome and accessory genes, such as phages, plasmids, transposons, and *Salmonella* pathogenicity islands (SPIs) [125, 126]. The size of the pan-genome increases as more genomes are included in the analysis, often because more accessory genes are included. A study by Laing *et al*. reported the sizes of strict core-genome and pan-genomes of *Salmonella enterica* subspecies ranging between 1.5Mbp and 25.3Mbp, respectively, among 4939 genomes including all 6 subspecies and 28 *S. enterica* subsp. *enterica* serotypes downloaded from the National Center for Biotechnology Information (NCBI) GenBank [127]. Conserved core genes of 3.2Mbp were found in 96% of the genomes. Within *S*. *enterica* subsp. *enterica*, 404 *Salmonella* species-specific regions were found. *S*. Enteritidis had the highest average number of species-specific regions and *S*. Typhi had the most diverse genome among the tested serotypes, which is consistent with the previous study by Leekitcharoenphon *et al*. [124]. Fu *et al*. defined 3,846 genes as the core-genome of *S*. Typhimurium, while the *Salmonella enterica* subsp. *enterica* core-genome was 2,882 genes [122, 124].

Based on these core-genomes and the pan-genome, phylogenetic trees were constructed in the studies by Laing *et al*. and Leekitcharoenphon *et al*. The concordance between the phylogenetic trees constructed by the core-genome SNPs and presence/absence among the pan-genome were high in both Laing *et al*. and Leekitcharoenphon *et al*. studies. The inclusion of the accessory genome (pan-genome tree) increased the bootstrap values in many of the branches; however, a pan-genome tree requires high-quality assembly and computer power [124]. Overall, phylogenetic trees constructed with the core-genomes were valid for evolutionary analysis. Interestingly, core-genome SNP analysis showed 3 novel *Salmonella enterica* subspecies in addition to 6 existing subspecies by including 297 representative genomes from ribosomal eBGs in subspecies *enterica* and 593 isolates from ribosomal ST in *S. enterica* and *S. bongori* [102].

A WGS analysis classified *Salmonella enterica* subsp. *enterica* into at least 2 lineages [107, 108, 124]. A study by den Bakker *et al*. and Timme *et al*. showed 2 lineages: clade A and clade B in subspecies *enterica*. Most of the serotypes in clade A were negative for β-glucuronidase, SPI-18, and CdtB-islet, while clade B serotypes were positive for these genes [107]. Clade A was divided into 3 subclades consisted of a Typhi clade, an Agona clade, and the remainder of the clade A serovars. This result is consistent with the other study by Timme *et al*. [108]. Timme *et al*. divided clade B into 4 subclades based on strong bootstrap values [108]. Further, den Bakker *et al*. found clades A and B differed based on the presence of fimbrial operons, which suggests differences in their adhesion abilities. Other differences were in the genomic regions involved in nitrogen and carbon metabolism. The β-glucuronidase operon was found in clade B but not in clade A. The authors discussed that this may be associated with vertebrate host adaptation, since β-glucuronides are found in the gall bladder and gut of vertebrate hosts [107]. These phylogenetic analyses are useful tools to reveal the evolution and the host adaptation of *Salmonella* by analyzing SNP differences between the isolates or clades and identifying important gene differences.

In many outbreaks, high discrimination between isolates within a serotype is necessary because the isolates are mostly clonal except for outbreaks associated with multiple strains. A number of retrospective studies have reported the discriminatory power of WGS-based phylogenetic tree construction by SNPs, presence/absence of genes in pan-genome, and k-mer phylogenetic trees utilizing outbreak strains [109, 117-119, 122, 123, 128]. Among several methods for the genome comparison, SNP analysis has been shown to have high discriminatory power in *S*. Typhimurium, *S*. Enteritidis, and *S*. Montevideo, which are common outbreak serotypes. Both reference genome-based and *de novo* genomic variance-based methods showed

the same tree structure in a study by den Bakker *et al*. [129]. To acquire high-quality SNPs, a minimum sequence depth of 20x is required in *Salmonella* [117, 123]. Further, high-quality scores of the reads and removal of recombination sites and indels is necessary [117, 118, 123, 129].

Leekitcharoenphon *et al*. showed that a pan-genome tree with presence/absence of genes failed to form accurate clusters in outbreak strains; however, the pan-genome tree formed meaningful clusters for the phage types. They discussed that this may be because prophages were included in the pan-genome-based analysis. In the same study, the nucleotide difference tree (ND tree) and SNP tree were able to cluster the outbreak isolates at 100% accuracy in *S.* Typhimurium. Very small numbers of SNP differences between the isolates are found in most outbreak studies, typically ranging from 2 to 12, while one outbreak had 3 to 30 SNP differences [118, 123]. Further, no SNP differences or else temporal associations were observed in these outbreaks even during a 27-month long outbreak [118]. As of now, SNP-based phylogenetic tree construction is one of the most commonly used and accurate methods to classify the isolates in an epidemiological outbreak study.

Recently, the MLST-based tree construction method has also been used. A recent study from the research group of Acktman *et al.* compared the analysis of cgMLST (3,002 Loci) and wgMLST (21,065 Loci) with core-genome SNP analysis [102]. Core SNP and cgMLST classified outbreak and non-outbreak strains in a similar structure correctly, while wgMLST classified strains similar to that of PFGE, which was classified incorrectly at the time of an outbreak in 2005. Even though the authors recommend using cgMLST over core-genome SNPs, and MLST over traditional serotyping, determining the best phylogenetic methods may change as more data are collected and the methods mature. Pearce *et al*. also compared a *Salmonella*

Enteritidis outbreak in Europe by cgMLST and SNP analysis, and showed high congruence between the two methods [130]. The cgMLST method may be more reproducible and easier to implement across laboratories since it is a gene-to-gene based analysis with less variability [130].

Fast, accurate, and easy to execute methods are preferred for bioinformatic analyses. Reproducibility and comparable data are necessary for comparison of analyses across laboratories and research groups. Since the population of users with bioinformatics skills, knowledge, and training are limited and as the sequencing technique is becoming more and more affordable, user-friendly tools are required to support the growing sequencing community. Web servers from Center for Genomic Epidemiology at the Technical University of Denmark, PATRIC from University of Chicago, and the Galaxy project are resources available for researchers or scientists who are developing bioinformatics skills.

2.4.3 Antimicrobial susceptibility testing and genotyping of resistance genes in Salmonella

2.4.3.1 Phenotypic resistance

Antimicrobial susceptibility testing is commonly performed on bacterial culture to determine the minimum inhibitory concentration (MIC) of each of several antibiotics. The MIC is the lowest concentration of an antimicrobial where the growth of a bacterial isolate is inhibited. Common methods are by agar disk diffusion and broth micro-dilution. The microbroth dilution system is a commercialized and partially automated system developed and marketed by TREK® Sensititre® that is routinely used in diagnostic laboratories. The acquired MIC data are utilized to determine the appropriate treatment regimens for clinical patients. The MIC is compared to breakpoints established by Clinical and Laboratory Standards Institute (CLSI), which determines susceptible, intermediate, and resistance phenotypic breakpoints in addition to the MIC values [131]. The epidemiological cutoff value (ECOFF in European Committee on Antimicrobial

Susceptibility Testing (EUCAST)) is different from breakpoints, in that they are upper limit values that distinguish a wild-type population of bacteria from the population with acquired resistant traits that exhibit higher MIC values [132]. When the breakpoint is not set for a particular bacterium and antimicrobial combination, consensus interpretive criteria set by the U.S. National Antimicrobial Resistance Monitoring System (NARMS) are used. In our study as is common in other studies, we also rely on these consensus breakpoints [35, 133]. Even though antimicrobial susceptibility testing is widely used and breakpoint values are established, inconsistency in breakpoints between researchers and changes in the CLSI interpretive criteria over time causes difficulty in interpreting the results among published articles. Additionally, antimicrobial susceptibility testing is labor intensive and time-consuming. Thus, ensuring that the original MIC values are recorded and maintained is essential.

2.4.3.2 Genotypic resistance

Genotypic resistance data acquired by WGS matches with the phenotypic resistance data with high accuracy for most antibiotics in foodborne pathogens [134-136]. Among *Salmonella*, 8,872 out of 8,960 isolates had genotypic and phenotypic agreement (99.0%) in a study by McDermott et al [136]. Overall sensitivity was 98.8%, in which phenotypic resistance was set as the gold standard. Sensitivities of 90.0% to 100.0% were reported for most of the antibiotics including aminoglycosides, ampicillin, cephalosporins, sulfisoxazole, azithromycin, chloramphenicol, ciprofloxacin, and tetracycline. The sensitivity of trimethoprim-sulfamethoxazole and nalidixic acid were 86.4% and 86.7%, respectively [136]. Streptomycin had 98.8% sensitivity but a specificity of 90.8%, which was the lowest specificity of the antimicrobials tested. The disagreement for streptomycin occurred in isolates that were phenotypically susceptible but carried resistance genes. The MIC for streptomycin is not defined

by CLSI because streptomycin is not intended to be used for the treatment of enteric bacteria. The NARMS consensus breakpoint is >= 64 mg/L, while the MIC values of many isolates carrying resistance genes falls in the range of 32 mg/L [137]. Therefore, recently the consensus breakpoint of streptomycin has been changed to >= 32 mg/L in NARMS and EUCAST for *Salmonella*.

Chromosomal mutations in quinolone resistance-determining regions (QRDRs) of *gyr*A, *gyr*B, *par*C, and *par*E genes and plasmid-mediated quinolone resistance (PMQR) genes are common resistance mechanisms for quinolones. Even if the bacteria carry *qnr* genes on plasmids, additional chromosomal mutations in QRDR are needed for bacteria to be phenotypically resistant to nalidixic acid or ciprofloxacin based on CLSI breakpoints.

It is important to investigate both acquired resistance genes and chromosomal mutations to determine the genotype of the bacterium. Databases including ResFinder, ARG-ANNOT, and CARD are major databases for the detection of acquired resistance genes [138-142]. Recently, PointFinder has become available in the Center for Genomic Epidemiology (CGE) web server at the Danish Technical University (DTU), which identifies chromosomal mutations encoding resistance to quinolones, polymyxins, rifampicin, macrolides, and tetracyclines [143].

In accordance with the recent advancement in sequencing technology, there is an effort by NARMS to develop genotypic cutoff values [132]. The authors compared over 20,000 MIC values with resistance determinants from WGS for 1,738 non-typhoidal *Salmonella* to establish "genotypic" cutoff values (GCVs) for 13 antimicrobials against *Salmonella*. GCV is defined as "the highest MIC of isolates in a population devoid of known acquired resistance mechanisms." [132]. In the study by Tyson *et al*., only 0.36% of mismatch between phenotypic MIC values and GCVs were found. In amoxicillin-clavulanic acid (AMC), MIC values were compared among the

isolates without resistance genes, isolates carrying beta-lactamase genes (ESBLs: *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM-1}}$) but not predicted to confer resistance to AMC and isolates with expected resistance genes (*bla*$_{\text{CMY-2}}$). Interestingly, MIC distribution clearly separated into 3 groups in that isolates with MIC < 2 mg/L were all without genes, between 4 to 16 mg/L were carrying ESBL genes, and isolates with *bla*$_{\text{CMY-2}}$ genes were >= 32 mg/L. All the isolates with ESBL genes had slightly higher MIC values than isolates lacking any beta-lactamase encoding genes, but not so elevated as to be phenotypically resistant per CLSI. The authors proposed to set the GCVs for AMC at < 2 mg/L with a note that isolates with MIC values >= 32mg/L may have distinct resistance genes.

The benefits of GCVs are to set the breakpoints for drugs that do not have established breakpoint or cutoff values by CLSI or EUCAST. The limitation of this study that the authors did not compare the MIC values of isolates with multiple resistance genes, which would have higher MIC values, with those isolates with a single resistance gene. Even though phenotypes and genotypes have high concordance resistance genes are not always expressed, or else may have variants that result in lost ability to confer resistance. In the former case, only an exposure to antibiotics may trigger the expression of the resistance gene. Also, as different countries or regions are distributed with different resistance genes, it is possible to have different GCVs among countries. Comparison of more MIC and genotypic information will help further to establish the GCVs, which may eventually lead to a situation where it is no longer needed to conduct antimicrobial susceptibility testing from culture. A comprehensive study comparing the presence of resistance genes and MIC breakpoints provided by CLSI and EUCAST has not been previously reported.

2.5 **Salmonella *epidemiology and antibiotic resistance in humans and cattle***

2.5.1 Salmonella *epidemiology in humans*

Approximately 1.2 million cases and 450 deaths attributed to *Salmonella* are estimated to occur every year in the United States [19]. *Salmonella* can colonize or infect a wide range of hosts, such as reptiles, poultry, wild animals, pigs, cattle, horses, humans, fish, dogs and cats. Human infections often are caused by consumption of undercooked meat, raw eggs, contaminated vegetables, spices, person-to-person contact (fecal-oral), often exacerbated during international travel, or via direct contact with infected animals. It is not recommended to treat mild salmonellosis with antibiotics because empirical treatment may not be effective against all likely *Salmonella* strains, and this may allow the resistant *Salmonella* population to expand. Symptoms of salmonellosis are diarrhea, fever, abdominal cramp, and vomiting. Usually the infection is self-limiting and patients recover within 4-7 days with oral rehydration or intravenous fluids. However, in severe cases in adults fluoroquinolones or azithromycin may be used for treatment. Infants, children, and immunocompromised people may require antibiotic treatment with cephalosporins such as in cases of bacteremia and meningitis.

When severe cases of salmonellosis are caused by antibiotic-resistant *Salmonella*, there are fewer therapeutic options and treatment failures may occur. Medalla *et al*. estimated the incidence of resistant *Salmonella* infections as 0.51/100,000 person-years for ceftriaxone and ampicillin resistance [144]. In 2014, 72% of *S*. Typhi were resistant to nalidixic acid, 5% were resistant to ciprofloxacin, and 11% were MDR [145]. Among Paratyphi A, 80% were resistant to nalidixic acid. Since typhoid fever is treated with antibiotics such as quinolones, this is problematic due to reduced treatment choices and may lead to treatment failure. Importantly, human infection with these serotypes is not associated with animals or food.

Among the 831 foodborne outbreak cases recorded in 2012, 106 were caused by *Salmonella* according to the CDC. The food sources of *Salmonella* infection included vegetables, eggs, poultry, beef, and pork, among others. Beef is attributed to have caused 7.3% of the *Salmonella* infections from 1998 to 2008 [146]. Multiple retail meat associated outbreaks have been reported by the CDC. From 2011 to 2013, outbreaks related to ground beef were reported every year; however, no deaths were reported. A beef outbreak from 2011 caused by *S.* Typhimurium resulted in 20 infections and 8 hospitalizations. Isolates recovered from leftover food implicated beef and both these and the human isolates were of an MDR phenotype. A beef outbreak from 2012 was caused by *S.* Enteritidis and infected 46 people resulting in 12 hospitalizations. The company that sold the product recalled 29,339 pounds of fresh ground beef. An outbreak from 2012 caused by *S.* Typhimurium infected 22 people who ate a raw ground beef dish called kibbeh. All the isolates were pan-susceptible; in the end, the company recalled 500 pounds of ground beef.

2.5.2 Antibiotic resistant Salmonella *in humans*

The National Antimicrobial Resistance Monitoring System (NARMS) is a collaborative program run by the U.S. FDA, CDC, and USDA. The surveillance system launched in 1996 with testing of non-typhoidal *Salmonella* and *E. coli* O157:H7 isolated from humans. The animal component was launched in 1997 with testing of regulatory *Salmonella* isolates from chicken, turkey, swine, and cattle.

A retrospective study of 2,149 *Salmonella* isolated from humans by the CDC showed that the proportion of antimicrobial resistant *Salmonella* increased during the period from1948 to 1995 [147]. Among all *Salmonella*, 20% of the isolates were resistant to more than one antibiotic and there was increase in MDR isolates since 1950. They also found a significant increase in

resistance to older drugs such as ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline [147]. The top 5 serotypes were *S.* Enteritidis, Typhimurium, Newport, Heidelberg, and Saintpaul. *Salmonella* Typhimurium often exhibited resistance to more antibiotics than the other serotypes, while *S.* Enteritidis was typically the least resistant. In *S.* Typhimurium, tetracycline and streptomycin resistance rose from 0% to 42.6% from pre-1960 to post-1989. MDR isolates increased from 0% to 16.9%. Isolates with resistance to tetracycline was the most common phenotype observed, especially together with streptomycin or sulfamethoxazole resistance. No resistance to ceftiofur, ceftriaxone, and ciprofloxacin was detected [147]. However, this study only contained isolates up to 1995, when cephalosporins were not yet widely used in food-animals.

A powdered cephalosporin requiring reconstitution (NAXCEL®) was first approved in 1988. Later, a sterile suspension form (EXCENEL®) was approved in 1998 by the FDA for food animal use. More recent NARMS data, including 24,903 non-typhoidal *Salmonella* isolates, reported the same distribution of serotypes as the study mentioned above; however, the proportion of MDR isolates decreased between 1996 to 2009, which was largely attributed to the decline in *S.* Typhimurium isolates [148]. In contrast to the study from 1948 to 1995, isolates with resistance to ceftriaxone increased from 0.2% in 1996 to 3.4% and resistant or intermediate susceptibility to ciprofloxacin increased from 0.4% to 2.4%.

The most recent data from the 2015 NARMS Integrated Report show a decline in tetracycline and streptomycin resistance from 52.1% to 18.7% in *S.* Typhimurium from 1996 to 2015, respectively [32]. However, there was an increase in nalidixic acid resistance from 0.3% to 4% and ceftiofur resistance from 0% to 4%. Interestingly, *S.* Dublin isolates are showing a markedly significant increase in ceftiofur resistance, which was 0% until 2003 versus 66.7% as of 2015 in

human isolates. Tetracycline and streptomycin resistance were around 25% in 1998 and increased to 83.3% in 2015 in *S*. Dublin. *S*. Dublin is a host-adapted serotype commonly found in cattle. Human cases involving this serotype are rare; however, when they do occur they tend to cause severe illness in humans often resulting in bloodstream infections, which require antibiotic treatment and hospitalization.

2.5.3 Salmonella *epidemiology and antibiotic resistance in feedlot cattle*

The prevalence of *Salmonella* in feedlot cattle varies by region, season, and method of sampling [149-153]. Prevalence at the feedlot level, pen level, and sample level were 60.3%, 35.6%, and 9.1%, respectively in a USDA APHIS 2014 study [154]. Southern regions have a higher prevalence than northern regions and a higher prevalence is found in the summer months compared to the winter [149, 155, 156]. Higher temperatures may be more suitable for the survival and propagation of *Salmonella* outside the host. One study showed that the thermal environment is associated with fecal shedding of *Salmonella* in dairy cattle, which suggests that thermal stress can be a factor for increasing the prevalence in southern regions or during the summer months more generally [156].

Prevalence of *Salmonella* in cattle at slaughter has been studied extensively, typically because *Salmonella* contamination of meat products often occurs at the slaughterhouse. Samples collected from hides, lymph nodes, or carcasses at harvest tend to have a higher prevalence of *Salmonella* than in the same animals' feces [157-160]. Barham *et al*. showed that transportation of the cattle from farm to the abattoir is a potential stressor and causes animals to shed more *Salmonella*. In fact, the prevalence of *Salmonella* before transportation was 6% and 18% on hides and in feces at the feedlot. The prevalence increased to 89% on hides and 46% in feces upon arrival at the abattoir [157]. Fluckey *et al*. also observed a similar increase on hides from

before to after shipping cattle to a commercial abattoir [158]. *Salmonella* was identified in 33.9% of the fecal samples and on 37.3% of the hides before shipping; however, at the abattoir, *Salmonella* was identified from 84.2% of hides [158].

A study by Kunze *et al*. reported that the prevalence of *Salmonella* at abattoirs recovered from hides was 69.6%, while feces was 30.3% [159]. This may be due to stress shedding of *Salmonella* and cross-contamination of the external surface of multiple animals during the transport [158]. The concentration of *Salmonella* was also higher on hides (1.82 $\log_{10}$/100cm^2) compared with feces (0.75 $\log_{10}$/g feces). However, Kunze *et al*. discussed the likelihood of bias caused by the sampling size of hides versus feces (1,000cm^2 vs. 150g feces), where approximately 6 times a difference in dilution factors may occur. In addition, *Salmonella* within fecal samples may not uniformly distributed. To compare more accurately the prevalence of *Salmonella* between hides and feces before and after transportation, it is important to sample from multiple hides and enough fecal material. Due to inconsistent methods and difficulties of back calculation between hides and feces, it may not be appropriate to directly compare the concentration of *Salmonella* between hides and feces.

Most often, *Salmonella* infections in cattle are subclinical, but the *Salmonella* shed by subclinical cattle can infect cattle within the same pen or herd. *Salmonella* Typhimurium, Newport, and Dublin are serotypes that are commonly pathogenic for cattle. Additionally, these serotypes are commonly MDR and can cause severe salmonellosis in humans, as mentioned above [160-163]. The recent increase in *S*. Dublin with resistance to cephalosporins in humans is a very serious concern [164]. Although the serotypes reported from feedlot cattle vary, the most common serotypes reported are *S*. Typhimurium, Anatum, Agona, Kentucky, Montevideo, Mbandaka, Muenster, Cerro, and Newport [150, 151, 153, 159].

Approximately 70% of *Salmonella* isolated from feedlot cattle feces are pan-susceptible to the panel of antibiotics tested in many studies [150, 153, 165, 166]. The most common antibiotic resistance reported is to tetracycline (21.7%) and then to sulfisoxazole (12.4%) [150, 153, 159]. Resistance to 3rd generation cephalosporins are reported to be around 0.5% [152] and rarely, *Salmonella* with reduced susceptibility to nalidixic acid was reported [166]. Often serotypes and antibiotic resistance profiles are associated in *Salmonella* isolates [159, 167]. In the cattle population, ceftiofur resistant *Salmonella* Newport isolates rapidly increased in the United States from 1998 to 2003. This increase in resistance was more than any other reported resistance in food-animals, according to CDC. This rise in *S.* Newport with resistance to ceftiofur coincided with the approval of ceftiofur in liquid form, EXCENEL® in 1998 and later the longer-acting formula, EXCEDE® in 2003 for use in cattle (Figure 3).

A study in humans, retail meat, and food animal-derived ceftriaxone-resistant non-typhoidal *Salmonella* from 1996 to 2013 showed that 2.9% of human-derived isolates were resistant to ceftriaxone [168]. Among the total isolates tested from different sources, 26.2% of retail chicken, 9.0% of ground turkey, and 13.5% of ground beef-derived isolates were ceftriaxone resistant. The common ceftriaxone-resistant serotypes were Newport (40%), Typhimurium (26%), and Heidelberg (12%). Although ceftriaxone-resistant *S.* Newport increased in humans and cattle, peaking around 2001 in humans and 2005 in cattle, the percentage of ceftriaxone resistance isolates was not correlated. In addition, *S.* Newport was not frequently detected in retail ground beef; however, resistance pattern profiles correlated significantly with isolates from humans and cattle [168]. On the other hand, antimicrobial resistance among *S.* Typhimurium isolates from cattle and chickens was higher than in human isolates [168]. Ceftriaxone resistance in *S.* Typhimurium correlated significantly between humans and cattle, while no correlation was

found between human and chicken isolates. Further, no correlation was found between retail meat and human or animal isolates. Resistant *S*. Heidelberg has recently been rising and ceftriaxone resistance is highly correlated between isolates from humans, chicken, and turkey in that serotype [168].

Although the study by Iwamoto *et al*. [168] only showed correlations between ceftriaxone-resistant *Salmonella* in food-animals and humans, and did not establish any causality, it provides evidence that food-animals may be a source of ceftriaxone-resistant *Salmonella* in humans or perhaps even vice versa. The data derived from NARMS including the study by Iwamoto *et al*. and others listed in this chapter need to be interpreted with caution because the isolates used in NARMS study may be biased by the differing sampling methods. For example, the human isolates were collected through passive surveillance at the state level until 2003. The human isolates also were only collected from clinical samples and do not including healthy individuals. Clinical samples derive from patients who are sick enough to visit the hospital and are more likely to have received antibiotic prescriptions or have taken antibiotics by themselves. Isolates from such patients are therefore more likely to be resistant. This can bias the distribution estimate of antibiotic resistant bacteria. To estimate overall distribution of antibiotic resistant bacteria among the healthy population, it is necessary to include healthy human derived isolates, though current dogma suggests that humans are not typically asymptomatic carriers of *Salmonella*. The sampling program of food-animal isolates is part of the USDA in-plant HACCP monitoring and changed in 2006 from a random selection of slaughter establishments to those in non-compliance. These biases have to be taken into consideration in evaluating NARMS data, though recently the sampling has further changed to that of an active surveillance using cecal sampling [169].

Since the partial ban on extra-label use of cephalosporins in 2012 [170], the NARMS Integrated Report from the USDA shows that ceftriaxone resistance in *Salmonella* has declined from all nonhuman sources including beef cecal, dairy cecal, and other cattle samples (HACCP); this may be because of the improvements in the judicious use of antibiotics [164]. The decline of *Salmonella* isolates with cephalosporin resistance genes has also been observed in retail chickens and ground turkey, as reported in the FDA portion of the NARMS study [32]. However, in retail ground beef *Salmonella* isolates carrying cephalosporin resistance genes increased from 2012 until 2014 and then declined in 2015 [32]. In the human derived isolates, ceftriaxone resistant *Salmonella* has been declining since 2003, according to the CDC [171]. The differences on detection of resistant *Salmonella* isolates among USDA, FDA, and CDC might be explained by the variation in detected serotypes of *Salmonella*.

Host specificity, resistance phenotype and genotype, and pathogenicity differ among serotypes of *Salmonella.* For example, the recent increase of cattle derived resistant *Salmonella* is likely explained by the increase of MDR *S.* Dublin, which is a cattle-adapted serotype. Since *S.* Dublin infection in human tends to be severe, this may be problematic. On the other hand, increased resistance in ground beef samples may be due to rare serotypes that are not highly pathogenic to humans, and thus may not be problematic. Data from CDC derived from clinical cases which may be from severe cases often are narrowed to serotypes that are pathogenic to humans. For example, monophasic *S.* Typhimurium tends to be MDR and pathogenic to humans, and therefore may more often be more detected from human isolates. As mentioned previously, in a polyphyletic serotype like *S.* Newport, host and antibiotic resistance differs within the serotype. Although the NARMS data will show the tendency of antibiotic resistant populations in

each section, which are food animal, retail meat, and human, careful investigation per serotype is always necessary.

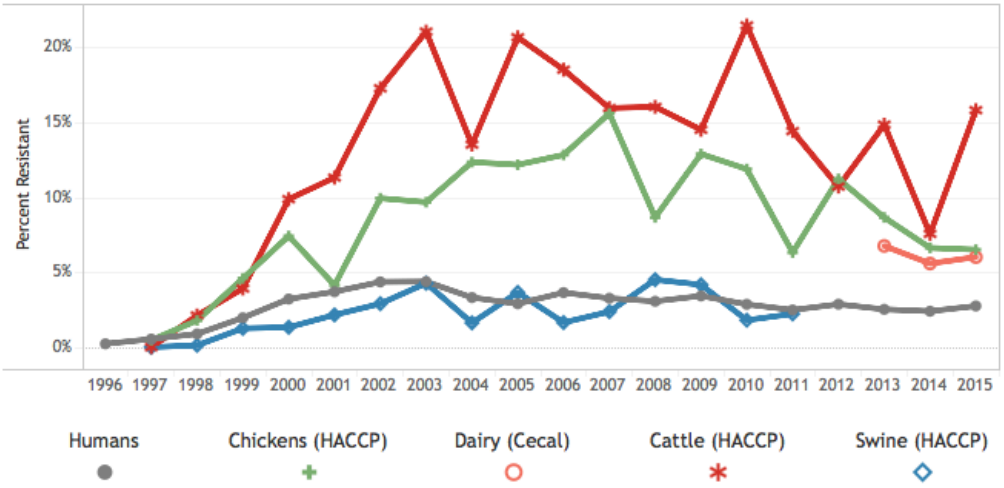


Figure 3. Ceftriaxone-resistant *Salmonella* from food-animals (2015 NARMS Integrated Report [32])

2.5.4 Comparison of resistant bacteria in human and animal populations

Through the development of advanced molecular techniques, we are gaining a broader perspective on antibiotic resistance. In 2012, Mather *et al*. used novel ecological and epidemiological methods to compare the phenotypic resistance profiles of *Salmonella* Typhimurium DT104 isolates derived from cattle and human populations collected from 1990 to 2004 in Scotland [66]. Although some similar resistance profiles were found between cattle and humans, unique distinguishable phenotypic resistance profiles in the cattle and human populations were observed and maintained within the population. Among the similar resistance profiles, more profiles were observed earlier in the human population. A similar study was conducted on *Salmonella* isolates derived from cattle and humans from 2004 to 2011 in

Washington State by Afema *et al*. [68]. The phenotypic antimicrobial resistance profiles of the dominant serotypes (Typhimurium, Newport, and Montevideo) were not shared among cattle and human-derived isolates. Isolates derived from the human population had more diverse phenotypes than the cattle isolates. They also observed that non-clinical cattle were carrying less diverse resistant bacteria than clinical cattle. The bacteria that enter the human population through food animals more often are those from healthy animals and not the animals exhibiting clinical symptoms. Most *Salmonella* infected cattle do not show clinical symptoms. These results suggest that among the resistant *Salmonella*, even though some are shared between human and cattle population, unique isolates are circulating mostly within their own species.

2.6 Control of Salmonella

As of the time of writing, *Salmonella* is not considered as an adulterant by the USDA Food Safety and Inspection Service (FSIS) [172]. If *Salmonella* are isolated from raw meat, the product can still be sold legally in the market. It is highly recommended to properly cook each food item to reach the recommended temperature to kill *Salmonella* (e.g. 62.8°C for raw meat, 71.1°C for ground meat and egg dishes, 73.9°C for poultry) [173]. Manufacturers voluntarily recall a contaminated batch if *Salmonella* are detected in multiple tests. On the other hand, *Salmonella* detected in ready-to-eat meats are considered an adulterant and legal action (such as issuance of a recall notice) can be taken by the FDA. That may happen, for example, when ready-to-eat meat is not cooked properly or a spice was rubbed on that was contaminated with *Salmonella*.

The FSIS implemented the 1996 Pathogen Reduction/HACCP Systems Final Rule, which required establishments that slaughtered animals or prepared meat to meet *Salmonella* performance standards [174]. The performance standards of *Salmonella* were set to the

prevalence of 7.5% for ground beef. Standards for market hogs, cows, and steers were discontinued in 2011. The standard for ground chicken was set to 44.6% in 2011 and in 2015 it was proposed to reduce the standard to 25%. By setting these standards, meat and poultry producers adopted various internal testing programs to reduce *Salmonella* contamination. Product recalls because of bacterial contamination negatively impact the producers financially and commercially; therefore, reducing the risk of contaminationis a priority for industry. In the outbreak case of Foster Farms related to *S.* Heidelberg from chicken in 2013 to 2014, a 5-year-old child suffered serious brain damage from *Salmonella* infection. The lawsuit filed against Foster Farms returned a verdict of $6.5 million with 30% of the fault attributed to Foster Farms and 70% to the family who improperly handled the chicken (https://www.businesswire.com/news/home/20180312005082/en/Pritzker-Hageman-Wins-6.5-Million-Verdict-Landmark).

A petition was prepared by a consumer group in 2014 in order to declare four antibiotic-resistant *Salmonella* serotypes (*S*. Hadar, Heidelberg, Newport, and Typhimurium) as adulterants. The petition was rejected by the USDA FSIS because there was not enough scientific evidence that antibiotic resistant *Salmonella* are more heat resistant and more virulent than susceptible strains of *Salmonella* [172]. As *Salmonella* are often subclinical and commonly exist in food-animals, recalling all meat contaminated with *Salmonella* may not be the best solution from an industry stand-point. From a scientific stand-point, determining the serotypes and antibiotic resistance profiles of *Salmonella* is more laborious than simply detecting *Salmonella*. It takes approximately one week to isolate *Salmonella* using standard enrichment and culturing techniques. Declaring only certain serotypes and antibiotic resistance profiles as adulterants is also not realistic, because different pathogenic serotypes emerge in humans (e.g.

increase of *S*. Dublin and monophasic *S*. Typhimurium) and antibiotic resistance is not necessarily linked to pathogenicity.

A metagenomics approach to detect *Salmonella* and determine serotypes may be one possible solution for this controversial issue. For example, samples can be lightly enriched in *Salmonella* selective broth and the enrichment broth can be used for further metagenomic analyses. On the other hand, *E. coli* O157:H7 and 6 serotypes of Shiga-toxin producing *E. coli* (STECs) are considered adulterants in ground beef because infective doses are very low and the infections caused by *E. coli* O157:H7 are often severe and life threatening; that is, leading to tragic outcomes such as kidney failure, hemorrhagic colitis, and death. Outbreaks caused by *E. coli* O157:H7 and the "big six" non-O157 Shiga-toxin producing serotypes, following their declaration as adulterants in ground beef, have been reduced by half. The success was possible because of a rapid diagnostic test system and the mandated regulatory requirement and fears of the costs associated with recalls.

Salmonella contamination of meat from food-animals can occur at multiple stages such as transportation to the slaughterhouse and during the slaughter process from hides and feces [152, 157]. Fecal, hide, and lymph-node contamination are believed to be the main sources of carcass contamination [152, 175]. Rinse water or improper handling of the meat are also potential sources of contamination. However, overall contamination levels can be decreased if *Salmonella* can be reduced pre-harvest in cattle and the environment. Moreover, proper use of antibiotics during the finishing period in feedlot may help reduce antibiotic-resistant *Salmonella*. Schmidt *et al.* followed the occurrence of resistant *E. coli* and *Salmonella* in feces and on hides from feedlot to processing plants and until the final carcasses were refrigerated and stored [152]. In that study, *E. coli* and *Salmonella* population existed in feces and hides at the feedlot and during processing.

Salmonella was not detected on the final carcass. However, cephalosporin resistant *E. coli* concentrations on hides during processing were correlated with the contaminant concentration on the final carcass [152]. Interestingly, even though the prevalence of cephalosporin resistant *Salmonella* was only 2.8% in feces at one of the feedlots, prevalence on hides was 55.6%. Samples from an additional two feedlots were only detected with *Salmonella* from 8.1% of hides at the feedlot; however, 100% of hides at processing were detected with *Salmonella*. Moreover, 1.4% were resistant to cephalosporins. These are alarming results, since antibiotic resistant *E. coli* and *Salmonella* derive from feedlots where intervention will be needed to reduce the concentration of resistant bacteria.

As mentioned above, *Salmonella* prevalence in the feedlot varies by region and season. Antibiotics are not labeled nor typically used to control *Salmonella* infections in cattle. Vaccinations are available for chickens, pigs, and cattle for common host-adapted serotypes, such as *S*. Newport and *S*. Dublin; however, vaccination is not a common practice. Some of the obstacles to control *Salmonella* in the feedlot are that *Salmonella* infections often are subclinical and *Salmonella* is shed intermittently [74]. Mechanisms of intermittent shedding are complicated. Stress conditions of the animal can induce shedding, such as transportation or antibiotic treatments. Even though *Salmonella* was not detected before sending an animal to the slaughterhouse, animals may shed during transportation, which may increase the risk of contamination of hides during shipment and the carcass at the slaughterhouse [157].

Ivanek *et al*. modeled that the fecal shedding and immune responses in pigs are strongly dependent on the serotype and dose of infection [176]. They experimentally infected pigs with low ($0.65x10^6$ CFU/pig) or high ($0.65x10^9$ CFU/pig) doses of *Salmonella* using 4 different serotypes, *Salmonella* Typhimurium, Derby, Yoruba, or Cubana. They examined the fecal

shedding of the pigs and classified the pigs into 4 shedding stages; latent, continuous shedding, intermittent non-shedding and intermittent shedding. The pigs infected with high doses of *Salmonella* Typhimurium or *Salmonella* Derby, which are more invasive and pig-adapted serotypes, were likely to enter an intermittent non-shedding state after continuous shedding rather than recovering. Although their shedding time was shorter during continuous shedding and the intermittent shedding state, they were more likely to re-enter into intermittent non-shedding state as well. Therefore, in total, pigs infected with Typhimurium or Derby tend to shed *Salmonella* for a longer duration and at a higher rate than the pigs infected with Yoruba or Cubana. The study suggests that serotypes of *Salmonella* are an important factor in shedding, which ultimately affects the dissemination to other animals within the pen.

Quantification of *Salmonella* has been conducted at multiple stages for risk assessment purposes from pre-harvest to post-harvest stages [177, 178]. The probability of illness from *Salmonella* Enteritidis contamination in eggs was predicted from a dose-response curve by the FAO [177]. Even low doses of contamination in retail meat can multiply to harmful levels due to inappropriate storage conditions; typically, an inadequate cold chain and other ambient factors [178]. The prevalence and quantity of *Salmonella* have been reported in cattle and pigs from hides, lymph nodes, and feces at the feedlot and slaughterhouse to assess the risk of contamination in the food chain and to identify high-shedding cattle [159, 165, 179-182]. Accurate quantification of *Salmonella* is a challenging task, especially from fecal samples with large amount of background microbiota. Kunze *et al*. reported the concentration of *Salmonella* in feces was 1.03 most-probable number (MPN)/g in feedlot cattle with desirable weight for harvest and 0.48 MPN/g in poor productivity cattle. The concentrations of *Salmonella* from hide swabs at the abattoir were 2.32 MPN/g in feedlot cattle and 1.24 MPN/g in dairy cattle. The prevalence

or concentration of *Salmonella* in fecal samples and hides differs by the sampling methods and sampling sites; therefore, caution needs to be exercised when comparing the results.

For example, in fecal samples *E. coli* or *Salmonella* may locate in one part of the voided feces, or in the fecal sample, but not be included in the part used for isolation [182]. Similarly, environmental samples may require multiple samplings from different sites. Agga *et al*. evaluated fecal grab (FG) and recto-anal mucosal swab (RAMS) sampling methods for enumeration of *Salmonella* in feedlot cattle. Fecal samples were collected from eight different commercial feedlots approximately 90 days pre-harvest. Fecal grab samples and RAMS were directly spiral plated for enumeration. Detection of *Salmonella* from FG samples were significantly higher than from RAMS, and overall *Salmonella* prevalence was high in this study (89.6% of RAMS and 98.8% of FG). The mean *Salmonella* concentration was 3.04 log CFU/ml by RAMS and 3.15 log CFU/ml by FG. Kappa agreement was 0.2 and classified as low. This was because the the Kappa calculation depends on the prevalence of the disease. In this experiment, the prevalence of *Salmonella* was 99% by FG, which adversely affected the Kappa calculation. The authors used PABAK (prevalence-adjusted bias-adjusted kappa) and obtained 0.82 agreement between these two sampling methods. The authors classified cattle shedding more than 2.7 log CFU/ml as high-shedders. PABAK agreement between FG and RAMS for identifying high-shedding cattle was 86.3%. The mean concentration of *Salmonella* for the samples that were detected by FG (but not with RAMS) was log 1.07 CFU/ml. This may be due to the heterogeneous distribution of *Salmonella* within the feces. Therefore, the authors suggested RAMS as the best method for enumeration and detection of *Salmonella* and for identifying high-shedding cattle. However, because the prevalence of *Salmonella* in this study was very high, RAMS may not be applicable for the farms with a low prevalence of *Salmonella*

or cattle infected with different serotypes of *Salmonella*. The Agga *et al*. study did not identify the serotypes of the *Salmonella* and these *Salmonella* concentrations may not be applicable to other serotypes. Since many cattle are subclinical and shed intermittently, frequent RAMS sampling may be necessary.

Hide sampling also differs by sampling site and sampling multiple locations is necessary for accurate test results. Kalchayanand *et al*. tested hide samples from the left and right shoulders, left and right ribs, back, and belly for the prevalence of *E. coli* O157:H7 and *Salmonella* [183]. The belly was identified as the site most likely to be naturally contaminated by both bacteria. At one sampling from 64 carcasses, the *Salmonella* prevalence for back samples was 9.4%, while the belly was 54.7%. Therefore, it is recommended to test multiple sites for accurate estimation of the prevalence.

Utilizing different enumeration methods also makes it difficult to compare the *Salmonella* concentrations between studies. There are a number of reported enumeration methods, such as most probable number (MPN), immunomagnetic isolation methods, spiral plating (or serial dilution) methods, and quantitative real-time PCR (qPCR). Each method has advantages and disadvantages in the sensitivity, specificity, labor intensity, accuracy, reproducibility, and repeatability. The advantage and disadvantage of MPN method and qPCR were compared in a mini-review by Malorny *et al*. [178]. The quantitation limit for the MPN method is 1.8 MPN/g and takes 4-5 days, while qPCR can detect as low as 1 cell in 10g of food and can be automated. In addition, studies use different back calculations and units to present the results, and a consensus unit may be required for comparison between studies.

Multiple steps can be taken to control *Salmonella* entering into the food supply. However, to reduce antibiotic resistant *Salmonella*, prudent use of antibiotics at the feedlot is key because this

is the final environment in which the cattle reside and may receive antibiotic treatment. Several strategies were proposed to combat antimicrobial resistance by controlling the use of antibiotics in the food-animal production system. In Europe, the Precautionary Principle, which was justification for banning the growth promotion use of antimicrobials in food animals, was adopted in 2006 based on the assumption that the cause of antimicrobial resistance in humans is due to growth promoter use of antibiotics in food animals [184, 185]. On the other hand, in the United States the use of medically important antibiotics for growth promotion purposes effectively disappeared in 2017 when their labels were withdrawn by the drug sponsors. Also, the judicious use of antimicrobials has been promoted, where judicious use is defined as "drug use practices aimed at maximizing therapeutic efficacy while minimizing the selection of resistant microorganisms" by the FDA. This judicious use policy consists of 1) limiting medically important antimicrobial drugs to uses in food-producing animals that are considered necessary for assuring animal health, and 2) limiting medically important antimicrobial drugs to uses in food-producing animals to those that include veterinary oversight or consultation, which are discussed later.

2.7 Antibiotics and Antibiotic Resistance Mechanisms

2.7.1 Antibiotics and antibiotic resistance

Different classes of antibiotics are available for both human and animal use. Antibiotics are a subclass of antimicrobials, originating from a microbial source, and tend to specifically target bacteria (i.e., are antibacterial). The WHO has categorized medically important antimicrobials into three categories via two criteria [186]. The first criterion (C1) is "The antimicrobial class is the sole, or one of limited available therapies, to treat serious bacterial infections in people". The 2nd criterion (C2) is that "The antimicrobial class is used to treat infections in people caused by

either: (1) bacteria that may be transmitted to humans from non-human sources, or (2) bacteria that may acquire resistance genes from non-human sources." Critically important antimicrobials are those meet both C1 and C2. Highly important classes meet either C1 or C2 and lastly, important classes are those used in humans that meet neither C1 nor C2. Critically important antimicrobials include carbapenems, 3rd and higher generations of cephalosporins, gentamicin, ciprofloxacin, vancomycin, and others. Highly important antimicrobials include chlortetracycline, sulfamethoxazole, trimethoprim, 1st and 2nd generation cephalosporins, and others [186]. There are only 3 classes of antibiotic that the WHO does not classify as medically important for human medicine.

In general, multiple mechanisms for antibiotic resistance are known; 1) modifications of the antimicrobial molecule by production of enzymes that can degrade or inactivate the drug, 2) prevention to reach the antibiotic target by decreasing antibiotic penetration and by efflux pumps, 3) change and/or bypass the target site, 4) and global cell adaptations [187]. Antibiotic resistance can be transmitted both inter- and intrabacterially via transformation (chromosomal), conjugation (plasmid), transduction (phages), and also via transposons and integrons [188]. Along with plasmids, these latter factors are called mobile genetic elements, which often contain antibiotic resistance genes and contribute greatly to the evolution of prokaryotes and the dissemination of antibiotic resistance [189].

2.7.2 Cephalosporins and their resistance mechanisms

Cephalosporins are in the class of β-lactam antibiotics first isolated from a fungus *Cephalosporium acremonium,* now named *Acremonium chrysogenum,* and found in a sewer in 1948. Cephalosporins have a similar structure to penicillin and inhibit bacterial cell wall synthesis by disrupting the synthesis of the peptidoglycan layer by binding to the penicillin-

binding protein (PBP). Penicillin-binding proteins are transpeptidases, which facilitate the transpeptidation step in cell wall synthesis. PBP binds to β-lactam antibiotics because of the chemical structure similarities to D-alanyl-D-alanine, which forms the peptidoglycan layer. This binding process is irreversible and is how cephalosporins act as bactericidal antibiotics.

Cephalosporins became commercially available in 1964 for human use and since then the consumption of cephalosporins has continuously increased [190]. In 2015, cephalosporins were ranked the top 3rd of oral antibiotic classes and agents prescribed for outpatients in the United States [191]. Currently, five generations of cephalosporins have been developed. The spectrum and coverage of Gram-positive and Gram-negative bacteria differ by the generation of cephalosporin used. Third generation and higher cephalosporins are classified as the highest priority within the critically important antimicrobials by the WHO [186]. Critically important cephalosporins have the possibility of "transmission of *Enterobacteriaceae*, including *E. coli* and *Salmonella*, from non-human sources" [186]. Third generation cephalosporins, such as ceftriaxone are one of the few choices for the treatment of invasive salmonellosis in children or immunocompromised people. Fluoroquinolones and azithromycin are other antibiotic options for salmonellosis treatment in adults. Fluoroquinolones are not used for children because of the increased risk of musculoskeletal disorders [24]. Ceftriaxone is effective against Gram-negative and Gram-positive bacteria, and extended-spectrum cephalosporins, more tolerate to β-lactamase produced by the bacteria. Ceftriaxone has a long half-life, which makes it a favorable antibiotic for the treatment of community-acquired infectious diseases, Lyme disease, and sexually-transmitted diseases. Cephalosporins are time-dependent antibiotics, which means they are effective when they are at a concentration higher than the minimum inhibitory concentration (MIC) for a requisite amount of time.

Ceftiofur and cephapirin are the only cephalosporins approved for food-animal use by the FDA. Ceftiofur is in the same class as ceftriaxone and used for the treatment and control of bovine respiratory disease in beef cattle caused by *Mannheimia haemolytica*, *Pasteurella multocida,* and *Histophilus somni* and for foot rot and metritis. Cephalosporins are estimated to encompass only <1% of the mass of antimicrobials sold for food-animal production purposes, around 31,010 kg in 2016 [6]. However, it is estimated that in 2016 80% of cephalosporins marketed for food-animals were intended for use in cattle [6]. Multiple formulas of ceftiofur are available: in powdered form (NAXCEL®, approved 1988), as a sterile suspension (EXCENEL® RTU EZ, approved 1998), and in a long-acting suspension (EXCEDE®, approved 2003) marketed by Zoetis. EXCEDE® or ceftiofur crystalline-free acid (CCFA) is a long-acting formula used in beef cattle, dairy cattle, horses, and pigs. A one-time subcutaneous injection at the base of the ear is required by the label for the treatment or control of BRD in cattle. Ceftiofur metabolizes into desfuroylceftiofur within 10 minutes after injection and this metabolite is equally as effective as ceftiofur for 7 days per label instructions. Withdrawal time for beef cattle is 13 days prior to slaughter following a single injection.

Bacteria most often become resistant to cephalosporins by acquiring β-lactamase producing genes. β-lactamases are enzymes that can break the β-lactam structure through hydrolysis. β-lactamase enzymes are traditionally divided into 4 classes, A, B, C, and D (Ambler classification) based on the protein sequence (Figure 4) [192]. In 2009, Bush & Jacoby updated the Ambler classification into 3 groups based on the enzymatic function to aid clinicians and laboratory microbiologists in clinical decision-making (Figure 4). Relating the Bush and Jacoby clasification to the Ambler classification: group 1 consists of classes A, C, and D which utilize serine to hydrolyze β-lactams, and group 2 consists of class B metalloenzymes that require

divalent zinc ions for the hydrolysis process. An added functional classification separates class C (including AmpC) from classes A and D in that they are more active on cephalosporins than benzylpenicillin [192].

AmpC proteins are resistant to inhibition by clavulanic acid and active on cephamycins (2nd generation cephalosporins), although a few exceptions exist in *Klebsiella pneumoniae* derived AmpC. AmpC expression is inducible by exposure to certain β-lactams in many bacteria. When large quantities of group 1 enzymes are produced, they confer resistance to carbapenems [187]. A *Salmonella* Wien with porin loss and the *bla*$_{CMY-4}$ gene was resistant to imipenem reaching MIC values of 32 μg/ml [193]. As of 2009, 890 β-lactamases have been discovered; including *bla*$_{CMY-2}$ in group 1, and *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$ extended spectrum β-lactamase (ESBL) in group 2. Group 3, or class B, enzymes are functionally and structurally unique because they require a zinc ion at the active site (so-called metallo-beta-lactamases) and are not inhibited by clavulanic acid, but by metal ion chelators, such as EDTA.

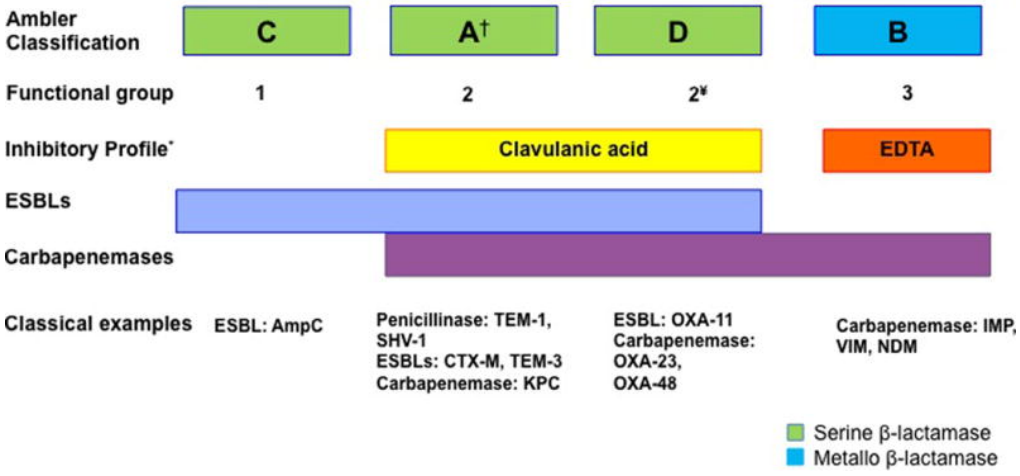


Figure 4. Schematic illustration of β-lactamases according to the Ambler classification scheme (Adapted from Munita and Arias, 2016 [187])

2.7.3 Transmission of cephalosporin resistance via bla$_{CMY-2}$ gene

Until recently, in the United States the *bla*$_{CMY-2}$ gene was the leading ceftiofur/ceftriaxone resistance gene discovered in food-animal production and in humans infected with foodborne *Salmonella*. The *bla*$_{CMY-1}$ gene encoding beta-lactamase enzyme is the oldest cephamycinase discovered. The *bla*$_{CMY-2}$ gene belongs to AmpC enzyme group. These genes can hydrolyze cephamycins including cefoxitin and cefotetan, and oxyiminocephalosporins such as ceftazidime, cefotaxime, and ceftriaxone, and monobactams such as aztreonam. Clavulanic acid does not have much effect on AmpC β-lactamases. The *bla*$_{CMY-2}$ gene originated from the chromosome of *Citrobacter freundii* [194]. Plasmid borne *bla*$_{CMY-1}$ was first reported in 1989, which rapidly spread in hospital and animal-derived *Salmonella* isolates. No chromosomally coded AmpC genes have been reported in *Salmonella* spp., *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis* and multiple other species until that time [193]. Currently, *bla*$_{CMY-2}$ genes are mainly found on large plasmids such as the IncA/C plasmid (>140 kbp), which encodes for multiple resistance genes in *Enterobacteriaceae*.

A study from Canada showed that *bla*$_{CMY-2}$ in *E. coli* and *Salmonella* was found on five types of plasmids (IncA/C, IncI1, IncK, IncFIB, and unidentified plasmid) isolated from poultry, cattle, swine and related food products [195]. All of the IncA/C plasmids isolated from *Salmonella* were multidrug resistant (ACSSuT-Cef). Transmission of ceftiofur and ceftriaxone resistant *Salmonella* carrying *bla*$_{CMY-2}$ genes between humans and food-animals (cattle, chicken) have been reported [28, 196]. In 2001, it was shown that *bla*$_{CMY}$ genes are transferrable between *E. coli* and *Salmonella* by transconjugation, which suggested that the *bla*$_{CMY-2}$ genes were disseminated intra-bacterially with the animal microbiota acting as a reservoir [197]. However, in these studies, the transconjugation results were assessed by phenotypic resistance profile

changes and PCR. Also, the incompatibility types of plasmids were not determined. Later studies showed that IncA/C plasmid carrying MDR genes have a diverse backbone and *E. coli* have a more diverse backbone than *Salmonella* [198]. A similar backbone of a MDR IncA/C plasmid (113,320bp) to that of *Salmonella* Newport (pSN254, 176,473bp) was discovered in *Yersinia pestis* (pIP1202, 182,913bp) and *Yersinia ruckeri* (pYR1, 158,038bp) isolates from fish, which would imply natural transfer between species [198].

Many resistance genes on IncA/C plasmids are encoded between two transposons, Tn21 and Tn10. The pSN254 plasmid carried duplicate copies of the $bla_{\text{CMY-2}}$ genes, while the other two plasmids from *Yersinia* carried none. The pIP1202 and pSN254 plasmid carried the mercury resistance operon, while the pYR1 did not. This mercury resistance operon is a hallmark of the Tn21-transposon family [198]. While Welch *et al*. demonstrated that IncA/C plasmids from 70 *Salmonella* strains (e.g. *S.* Typhimurium, Kentucky, Dublin, Heidelberg) were transferable to *Y. pestis*, none of the IncA/C plasmids derived from *S.* Newport from multiple food-animals were transferable. It is possible that environmental factors or antibiotic pressure may be needed to have a higher success rate of plasmid transfer for this particular serotype.

Heider *et al*. examined the genetic homology of $bla_{\text{CMY-2}}$ genes from *E. coli* and *Salmonella* isolated from humans, cattle, swine, beef, pork, turkeys, and sewage from 7 states [199]. They found that *Salmonella* $bla_{\text{CMY-2}}$ genes have more alleles than *E. coli*. Although the alleles were synonymous mutations, based on the study by Call *et al*. (2006) it was found that plasmids in *Salmonella* are more likely to be transmitted between *Salmonella*, while plasmids in *E. coli* are transferred from *E. coli* and *Salmonella.* They postulated that evolution of the $bla_{\text{CMY-2}}$ gene first occurred in *Salmonella*. They also found the same alleles of $bla_{\text{CMY-2}}$ from different serotypes of *Salmonella* derived from humans and turkey meat, suggesting the occurrence of conjugation,

intergron/transposon, or homologous recombination under some selective pressure [199]. Later, Daniels *et al.* studied the effect of ceftiofur use on the horizontal transfer of *bla*$_{CMY-2}$ plasmids among *E. coli* and *Salmonella* in dairy cattle treated with ceftiofur. Dairy calves were infected with donor bacteria carrying *bla*$_{CMY-2}$ plasmids and recipient bacteria. The transfer of plasmids between the bacteria was detected at a low frequency under ceftiofur selection pressure; however, no difference was observed between calves treated with ceftiofur and those without ceftiofur treatment [30].

Call *et al*. conducted a comparative genomics study of IncA/C plasmids derived from two geographically distinct cattle sources of *E. coli* (Washington and Illinois) and a human-derived *S.* Newport strain (pAM04528, Kansas). Using plasmid pSN254 as a reference, they showed that the IncA/C plasmids were genetically distinct from *Y. pestis* and *Photobacterium damselae* derived IncA/C plasmids [200]. The phylogenetic tree analysis of these IncA/C plasmids showed three distinct subgroups represented by plasmids from *E. coli* and *S*. Newport, and those from *Y. pestis* and *P. damselae*, and lastly *Y. ruckeri* plasmid. Interestingly, genomic comparisons of *bla*$_{CMY}$ genes derived from bacterial isolates of the 1920s, before the antibiotic era, and recent plasmid *bla*$_{CMY}$ genes showed that *bla*$_{CMY}$ from the 1920s were closely related (Figure 5) [194]. Therefore, use of antibiotics may have pressured the dissemination of plasmid borne *bla*$_{CMY}$ genes, but did not contribute to the evolution of the gene. Although single nucleotide polymorphisms are discovered in *bla*$_{CMY}$ genes, it has not evolved for resistance to carbapenems or ESBLs, which may be because the *bla*$_{CMY}$ gene has evolved to its maximum capacity [194].

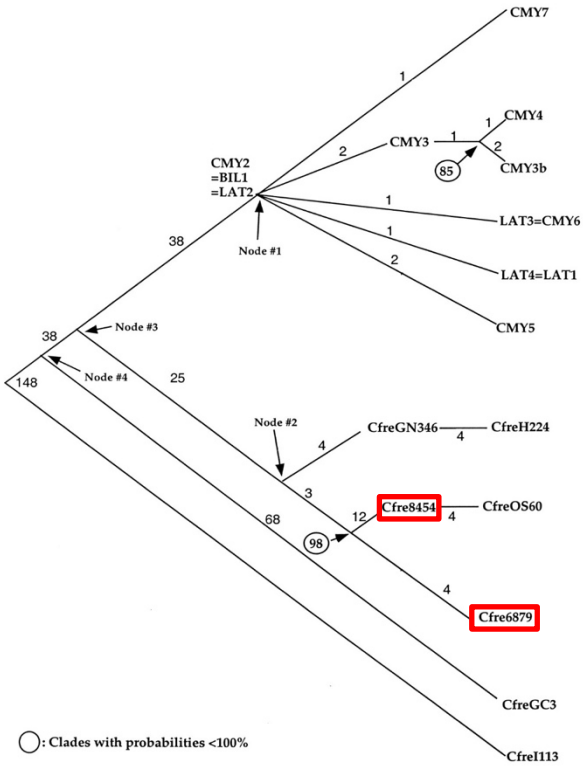


Figure 5. Bayesian consensus phylogenetic tree of *Citrobacter freundii* AmpC allele. Cfre6879 and Cfre8454 are AmpC genes from *Citrobacter* isolates from 1920, before the antibiotic era, locate at the middle and tip of the tree, respectively (Adapted from Barlow *et al.* 2002 [194]).

Other common resistance genes for cephalosporins are *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$. Some of the *bla*$_{TEM}$ and *bla*$_{SHV}$ genes along with the *bla*$_{CTX-M}$ are extended-spectrum beta-lactamases (ESBLs), and show phenotypic resistance to third and fourth generation cephalosporins but not to cefoxitin (cephamycin) and carbapenems. Until recently, *bla*$_{TEM}$ had been the major beta-lactamase gene found in the United States; however, it recently has been replaced by *bla*$_{CTX-M}$. The *bla*$_{CTX-M}$ gene was the most common ESBL found in Europe from humans and animals; however, the gene was not reported in animals in the United States until 2010 when *bla*$_{CTX-M}$ gene was found on a IncN plasmid in *E. coli* from dairy cattle and then feedlot cattle in 2013 [201, 202]. Wittum *et al*. screened 2,034 *Salmonella* isolates from NVSL submitted from

October 2010 and June 2011 for the *bla*$_{\text{CTX-M}}$ gene. They found the *bla*$_{\text{CTX-M}}$ gene in 6.8% of turkey, 0.1% of swine, and 3.5% of equine *Salmonella* isolates originating from Texas [203]. However, the *bla*$_{\text{CTX-M}}$ gene was not detected in the 58 cattle, 83 chicken, and 198 other source derived *Salmonella* isolates. Most recently, NARMS reported an increase in the *bla*$_{\text{CTX-M-65}}$ gene in *S*. Infantis from various sources including humans, and cecal contents of dairy cattle and chickens at slaughter facilities from North Carolina and California collected in 2015 [204].

2.7.4 Tetracycline and resistance mechanism

Tetracycline is a highly important antibiotic per the WHO and one of the oldest antibiotics used in animals and humans [186]. Tetracycline was approved by the FDA for human use in 1948. Tetracyline was discovered by Dr. Benjamin Minge Duggar from *Streptomyces aureofaciens*, which was referred to as "ultra-mold" from the soil of the University of Missouri campus [205]. Originally, it was named aureomycin and is now known as chlortetracycline. Currently, chlortetracycline, oxytetracycline, and tetracycline are approved for use in food-producing animals [205]. Tetracyclines are broad-spectrum antibiotics, with a chemical structure that consists of a four or "tetra" hydrocarbon ring. Tetracycline inhibits bacterial protein synthesis by binding to the bacterial 30S ribosome and work in a bacteriostatic manner by preventing the aminoacyl-tRNA from binding to the 30S ribosome [206]. Tetracyclines have a broad range of activity against Gram-positive, Gram-negative bacteria, and other organisms including Chlamydiae, Mycoplasmas, Rickettsiae, and protozoan parasites such as *Anaplasma marginale*.

Until 2017, it was a common practice to use tetracyclines as in-feed antibiotics for both disease prevention and growth promotion in food-animal production in the United States (subtherapeutic use). In 2016, among all the domestic sales of antimicrobials approved for use in

food-animals, tetracyclines accounted for 70%, with 49% of the tetracycline class intended for use in cattle [6]. Approximately 5.8 million tons of tetracycline were sold in 2016 but sales decreased by 15% from 2015 [6]. Nowadays, tetracycline resistant bacteria have been widely reported in animal and human-derived bacterial populations. In 2015, around 30% of cattle (HACCP) derived *Salmonella* isolates and 10% of human clinical isolates were resistant to tetracycline [32].

The major mechanisms of tetracycline resistance are by traversing membrane systems (efflux proteins) and modifying the ribosomal binding mechanisms. Currently, more than 50 tetracycline resistance genes have been identified. The genes *tet*(A) through *tet*(E), *tet*(H) through *tet*(J), *tet*(Z), *tet*(30), *tet*(31), just to list a few, encode for efflux proteins [206]. The *tet*(M), *tet*(O), *tet*(S), *tet*(W) genes, and others encode for ribosomal protection. In Gram-negative bacteria, *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), and *tet*(G) are most commonly found. The *tet*(B) gene is the most common resistance gene reported in Gram-negative bacteria.

In Gram-negative bacteria, efflux mechanisms are encoded with two genes, one for the efflux protein and another for the repressor protein, each of which is regulated by tetracycline itself [206]. When tetracycline is absent, the repressor protein blocks the transcription of both the repressor and efflux protein. However, when the tetracycline-Mg^{2+} complex enters the cell, it binds to the repressor protein, and the repressor protein no longer controls the transcription of these genes. Then, the efflux protein is produced and tetracycline resistance is expressed. While this is well recognized in Gram-negative bacteria, this mechanism has not been found in Gram-positive *tet*(K) or *tet*(L) genes.

Tetracycline resistance genes are transmitted widely through plasmids. In *Salmonella enterica*, *tet*(A), *tet*(B), *tet*(C), *tet*(D) and *tet*(G) are commonly found [207, 208]. Many

Salmonella isolates that are resistant to tetracycline are co-resistant to other antibiotics such as streptomycin. Commonly, they exhibit a penta-resistance phenotype, which includes resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (ACSSuT). The ACSSuT phenotype is mainly located on a class-1 integron forming a gene cassette on the plasmid. This gene cassette is similar to that of the *Salmonella* Genomic Island 1, which was likely transferred to the IncA/C plasmid. The integron is bounded by insertion sequences (IS) and mobilizable elements. The most common plasmids harboring tetracyline resistance are IncA/C followed by IncI1, IncF, and IncH.

2.7.5 Effect of ceftiofur on Salmonella and E. coli in feedlot cattle

Several experimental and observational studies have been conducted to analyze the effects of ceftiofur on Gram-negative bacteria in feedlot cattle [29, 30, 35, 209-211], dairy cattle [212-214], and swine [215-217]. Many studies have shown that the treatments transiently increase the resistant bacteria but may not have a long-term effect. In this section, each study will be critically reviewed.

A cross-sectional study to examine the association between ceftiofur use and *E. coli* with reduced susceptibility to ceftriaxone was conducted on dairy farms [212]. Fecal samples were collected from 1,266 dairy cows on 18 farms in Ohio. *E. coli* with reduced susceptibility to ceftriaxone were isolated from 12 out of 18 herds. At the individual level, 436 (34.4%) out of 1,266 *E. coli* isolates had reduced susceptibility to ceftriaxone. If more than one cow was isolated with ceftriaxone resistant *E. coli*, the herd was classified as positive. Associations between the use of ceftiofur and reduced susceptibility to ceftriaxone was highly significant at the herd level (odds ratio, 25.0; $p = 0.01$); meanwhile, it was not significant at the individual

level within farm (adjusted odds ratio, 1.01; p = 0.83). The authors suggested that to reduce the ceftiofur resistance on dairy farms, intervention strategies are most effective at the herd level.

This was an observational study and convenience samples were used; therefore, the results could be biased by the age of the cow, feed, lactation stages, housing pens, mixture with treated cows, and other environmental factors that were not taken into consideration. The records for antimicrobial treatment were obtained for a 6-month period prior to sample collection, which is a long period during which microbiota normalization could occur post-treatment. Cows might either have received the treatment 6 months before sampling, or else have received the treatment the day before the sample collection. The resistant bacteria population would be high if the treatment was performed right before the sample collection. This could be adjusted by collapsing the 6-month period into groups at week or months level. Herd level significance may be due to certain herd's use of ceftiofur more frequently, which can increase the resistant *E. coli* population. Ceftiofur use in dairy cattle has a short-term effect and would not require milk to be discarded or withheld from the market. Ceftiofur formulas used in dairy cattle often are different from those used in the feedlot, which typically uses a long-acting formula. Therefore, the results from this study might not be applicable to feedlot cattle.

A longitudinal experimental study design reported by Lowrance *et al*. examined the effects of different concentrations and repeated doses of ceftiofur crystalline-free acid (CCFA) on the antimicrobial susceptibility of *E. coli* populations in feedlot cattle [209]. The study was conducted for 28 days and fecal samples were collected 8 times. The administrated doses and concentrations of CCFA differed as follows: a one-time full-dose regimen (6.6 mg/kg on day 0), a one-time 2/3-dose regimen (4.4 mg/kg on day 0), and a three-time full-dose regimen (6.6 mg/kg on days 0, 6, and 13). Ceftiofur treatment cohorts consisted of 10 steers (5 steers/pen),

and 5 or 6 untreated steers were mixed within each cohort pen at a 1:1 ratio. The MIC values of the isolated *E. coli* were tested by microbroth dilution for 15 antimicrobials. The total amount of *E. coli* and of ceftiofur-resistant *E. coli* were enumerated. In total, 1,441 isolates were recovered and subsets of 953 isolates were subjected to biochemical confirmation of *E. coli*, which 952 were confirmed as *E. coli*.

There were 986 (68.4%) resistant isolates and the most common resistance observed was to sulfisoxazole, tetracycline, and streptomycin. Ceftiofur resistance was found in 289 (20.1%) isolates with perfect agreement to the ACSSuT penta-resistance phenotype. Ceftiofur resistance was more prevalent in the 3-dose regimen followed by the single 2/3-dose regimen (4.4 mg/kg), followed by the single full-dose regimen (6.6 mg/kg). In the 3-dose cohort, the proportion of ceftiofur resistant *E. coli* was significantly higher on days 13, 16, and 20 compared with the control cohort. In the single full-dose cohort and single 2/3-dose cohort, days 2, 6, 9, and 16 were significantly high for ceftiofur resistant *E. coli* compared with control cattle ($p = 0.03$). However, no significant differences in ceftiofur resistance were found between the single full-dose and single 2/3-dose cohorts. The authors argued that the variation between the single 2/3- and full-dose cohorts were likely associated with the with-in pen effects rather than ceftiofur selection pressure. The selection pressure of ceftiofur allowed for the expansion of resistant *E. coli* but the population of resistant *E. coli* returned to baseline levels after 2 weeks. This may indicate that susceptible *E. coli* had a greater fitness then the resistant *E. coli* in the tested feedlot. The concentration of *E. coli* decreased significantly in all treatment groups 2 days after initial treatment. Three-dose cohorts remained low until day 20 and showed recovery on day 28. In all other groups, concentration was not significantly different on day 13. These results show that the

effects of ceftiofur treatment were transient on both the proportion of ceftiofur resistant *E. coli* and the concentration of total *E. coli*.

As a follow-up of Lowrance *et al*., Alali *et al*. [29] studied the quantity of the *bla*$_{\text{CMY-2}}$ gene in the total community DNA extracted from 469 fecal samples from the same feedlot cattle population. Total community DNA represents all the bacterial, viral, archaeal, and eukaryotic community within the feces. Quantification of the *bla*$_{\text{CMY-2}}$ gene using community DNA reflects the entire gene pool and helps in understanding the dynamics of antimicrobial resistance genes in the population. However, it will not provide information regarding from which bacteria the gene is derived. The authors extracted community DNA from the fecal samples derived from cattle treated with 3 different doses of ceftiofur, as mentioned above in the Lowrance study. Quantitative real-time PCR was conducted for the *bla*$_{\text{CMY-2}}$ gene which is the primary gene responsible for ceftiofur and ceftriaxone resistance in the United States. They also quantified the 16s rRNA gene, which allows for the quantification of total bacterial genes in the community DNA and for standardization purposes. While the CCFA treatment regimens increased the quantity of the *bla*$_{\text{CMY-2}}$ gene significantly compared with control groups without standardization, the differences were lowered after the standardization with 16s rRNA. This result showed that the treatment decreased the overall bacterial concentration in addition to the quantity of the *bla*$_{\text{CMY-2}}$ gene. Compared with the Lowrance *et al*. study that measured the number of samples with ceftiofur-resistant *E. coli,* a significant increase of *bla*$_{\text{CMY-2}}$ genes was detected in different sampled days. These two studies show that *E. coli* can be used as an indicator to measure the resistant bacteria but measurement of total bacterial numbers is equally important.

Singer *et al.* and Boyer *et al*. observed the effect of ceftiofur on *E. coli* isolated from 5 dairy cows that were treated for *Leptospira* infection for 32 days [210, 218]. An additional 5 cows from the same cohort were selected randomly as controls. The dairy cows were treated intramuscularly with 2.2mg/kg of ceftiofur once daily for 5 days. Fecal samples were collected prior (days -1, 0) during (day 2, 4) and after (days 5, 11, 14, 18, 25, and 32) treatment. Three *E. coli* isolates from each cow on each day (total 468 isolates) were tested for antibiotic susceptibility. The quantity of *E. coli* was manually counted and the presence of the $bla_{\text{CMY-2}}$ gene in the fecal samples was identified by multiplex PCR. The *E. coli* were genotyped with repetitive element PCR (Rep-PCR).

The total *E. coli* load significantly decreased from days 0 to 2 and day 2 to 4 in the treated cows compared with the controls. The authors calculated an antibiotic resistance index (ARI) by dividing the sum of number of antibiotics that each isolate was resistant to by number of isolates in the population and multiplied by number of antibiotics tested. ARI is a quantitative measurement of the level of antibiotic resistance in a bacterial population, which is used to make comparison among populations within a single study. The ARI of *E. coli* in the treatment group significantly increased on days 4, 5, and 6. Both the *E. coli* load and ARI gradually returned to the base level around day 8. Among 203 *E. coli* isolates, the $bla_{\text{CMY-2}}$ gene was detected by PCR in 12 isolates. Interestingly, the $bla_{\text{CMY-2}}$ gene was detected from all the sampled days from both treatment and control groups from the community DNA. Even though it cannot be determined that these genes were derived from *E. coli*, it implies that resistant bacteria populations reside in the microbiota before the antibiotic treatments.

They also found that the genotype of susceptible *E. coli* was different from that of resistant *E. coli*, which implies little to no horizontal gene transfer occurred between the *E. coli* in the

presence of the antibiotic pressure. Additionally, no resistant strains were isolated from the untreated cows even though they were located in the same cohort and most likely share a common microbiota. These may be due to low sensitivity and a small sample size. As the authors discussed, using agar containing ceftiofur would have helped to detect resistant *E. coli* in untreated cows. The sample size used in the study was small and is not appropriate for the generalization of results between treatment groups. However, they analyzed 10 isolates per animal for genotypic analysis to compare the genomic diversity per animal, which helped to conclude that the horizontal gene transfer between resistant and susceptible isolates likely did not occur as a result of antibiotic pressure.

Benedict *et al.* studied the effect of routine exposure of feedlot cattle to five antibiotics on non-type specific *E. coli* (NTSEC) over 3 years by 2-point sampling; in total, enrolling over 10,000 animals [219]. Cattle were exposed to two in-feed antibiotics (macrolides and tetracycline) for the prevention of live-abscesses. In addition, 44.6% (412/923) of the cattle received macrolides and tetracycline treatments parenterally. Less than 2% of the cattle received beta-lactams, phenicols, quinolones, or sulfonamide treatments parenterally. The first set of samples were collected upon arrival to the feedlot and the second set of samples were collected during the feeding period, which ranged from 33-202 days on feed (DOF), with an average of 95.5 DOF. The majority of pens housed at least one animal that was treated with antibiotics before the second sampling. In the first sampling set, 79.8% of NTSEC were pan-susceptible, while 21.9% were pan-susceptible in the second sampling set.

The most common resistance phenotype was single resistance to tetracycline and additional resistance to streptomycin and sulfisoxazole was also observed. Individual parenteral tetracycline exposure significantly increased the odds of recovering tetracycline and trimethoprim-

sulfamethoxazole resistant isolates. In-feed tetracycline exposure also increased the odds of recovering resistance to tetracycline. Interestingly, parenteral exposure to beta-lactams decreased the streptomycin resistance. Pen-level exposure to these antibiotics was associated with recovery of resistant NTSEC isolates. This study provided abundant information on antibiotic use, which many previous observational studies have lacked. Even though this study included more than 10,000 animals in total, there were few NTSEC isolates that were resistant to medically important antibiotic; therefore, the significance of antibiotic use on such resistance could not be assessed as the authors had anticipated. The fact that beta-lactam exposure was associated with decreased streptomycin resistance shows the complexity of resistance mechanisms in bacteria.

In contrast, Platt *et al.* showed a protective effect of chlortetracycline treatment against ceftiofur resistant *E. coli*, while Kanwar *et al.* showed the opposite results and found that chlortetracycline use increased ceftiofur resistance [34, 35]. As Benedict *et al*. and Kanwar *et al* discussed, indirect selection of NTSEC that were susceptible to streptomycin or tetracycline but resistant to beta-lactams may have replaced the NTSEC that were resistant to streptomycin or tetracycline, respectively. However, since genotypes were not examined in the Benedict study, the mechanisms remain unknown. The temporal relationship between antibiotic use and resistance is not shown in this study since only two-point sampling was conducted and the period ranged from 33-202 DOF. The effects of antibiotics may vary by the days after the treatment, which were not reported in their study.

In contrast with previous studies, Schmidt *et al*. did not find a significant increase of extended-spectrum-cephalosporin-resistant *E. coli* (ESC^r) following therapeutic ceftiofur treatment at the herd level in 763 feedlot cattle over a 10 months study. However, the fecal prevalence of ESC^r *E. coli* in the feces of 50 cattle from 3-8 days post-treatment of ceftiofur,

significantly increased from 8.2% to 92.0% at an individual level. No significance was found in the hide prevalence at slaughter. The study was conducted at the U.S. Meat Animal Research Center (USMARC) feedlot, where the cattle were born and raised on pasture at USMARC. Samples were collected six periods during the study for 10 months. Prevalence of ESC^r *E. coli* upon arrival at the feedlot was 3.9% in feces and 15.0% in hides. The fecal concentration of ESC^r *E. coli* was >2.00 log CFU/swab for 85.2% post-ceftiofur injection, while only 1.6% of the samples remained at this level at subsequent periods. All ESC^r *E. coli* were detected with *bla*$_{\text{CMY-2}}$ gene and ACSSuTCfCtCx phenotype. PFGE genotypes of ESC^r *E. coli* isolates were also studied and 26 unique PFGE genotypes were identified, in which 12 of them were isolated at multiple sampling time points. This suggests a clonal expansion of ESC^r *E. coli* carrying *bla*$_{\text{CMY-2}}$ gene but not a horizontal gene transfer between the isolates with different PFGE genotypes. Most prevalent plasmid replicons were IncA/C plasmid followed by IncY plasmid. Overall results showed that ceftiofur transiently increase ESC^r *E. coli* at an individual level, but not at the herd level in the long-term.

2.7.6 Effects of chlortetracycline and ceftiofur on E. coli *in feedlot cattle*

Previously, the effects of ceftiofur and chlortetracycline were examined in *E. coli* isolated from the same cattle that were studied in this dissertation [35, 220]. In a 2008 study by Platt *et al*., chlortetracycline treatment transiently increased *E. coli* with reduced susceptibility to tetracycline but also reduced phenotypically ceftiofur resistant *E. coli* in cattle [34]. Platt *et al.* administered in-feed chlortetracycline to 10 steers at the concentration of 22 mg/kg for 3 separated 5-day periods with a 1-day interval. Another 10 steers served as controls (without treatment). Fecal samples were collected every 2 to 4 days and three *E. coli* were isolated from each sample and tested for antibiotic susceptibility. Among 525 *E. coli* isolates, 24.4% were

pansusceptible and 75.6% were resistant to one or more antimicrobials. Tetracycline resistant isolates were 294 (56.4%) of the total and 37 of the total (7.1%) were resistant to ceftiofur. Those isolates resistant to ceftiofur always were co-resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (or ACSSuT). In the control cohort, 42.3% were resistant to tetracycline with 68.8% in the treatment cohort (p=0.03). In contrast, the proportion of ceftiofur resistant isolates was significantly lower (p=0.01) in the treatment cohort (2.9%) than control cohort (8.6%).

This observation led to the follow-up study by Kanwar *et al.* in 2009 to utilize chlortetracycline to reduce ceftiofur resistant *E. coli* in feedlot cattle following treatment with CCFA [35]. Unexpectedly, in the Kanwar *et al*. study chlortetracycline contributed to an increased prevalence of ceftiofur resistant *E. coli* isolates. To be more specific, Kanwar *et al.* enrolled 176 steers in the study, randomized into two replicates with 11 steers per pen (16 pens). In half of the pens, ceftiofur crystalline-free acid (CCFA) was administered to one out of the 11 steers and mixed in the pen to observe the effect of mixing with steers not treated with CCFA, to test the hypothesis that mixing with steers that were not treated with antibiotics may restore the microbiota of treated steer. In the other half of the pens, all 11 steers received CCFA treatment. The CCFA treatment was followed by in-feed 5-day chlortetracycline treatments for three times with 1-day in between the treatments (that was the same as described above in the Platt *et al*. study).

Three non-type specific *E. coli* were isolated from the fecal samples on days 0, 4, 12, and 26, and tested for antibiotic susceptibility and genotyped for *tet*(A), *tet*(B), and $bla_{\text{CMY-2}}$ genes. Among the 1,050 *E. coli* isolates collected, 32.4% were pansusceptible and 67.6% were resistant to at least one antibiotic. Most common resistances were to tetracycline (61.1%) followed by

ampicillin and sulfisoxazole. Ceftriaxone-resistant isolates were 25.4%. In the treatment group with the least amount of antibiotic exposure, 55.1% of *E. coli* were pansusceptible and the number of antibiotics to which the isolates were resistant was right-skewed. In the pens with all animals receiving CCFA treatment, the number of antibiotic-resistant isolates were more uniformly distributed to the full range of MDR numbers (note: no pan-resistant phenotypes were observed in any group). Chlortetracycline treatment increased the number of isolates with higher MDR counts but tended to select more of the low MDR isolates than CCFA treatment. Animals in pens treated with both CCFA and CTC exhibited a profound effect of the chlortetracycline, where 3.0% of the isolates were resistant to 12 antibiotics.

Among 1,050 isolates tested, 120 isolates were co-resistant with both *tet*(A) and $bla_{\text{CMY-2}}$. The *tet*(B) gene was detected in 208 isolates and only 14 isolates harbored both *tet*(A) and *tet*(B) together. When phenotype and genotype were compared, the proportion of tetracycline-resistant isolates increased following the chlortetracycline treatment in the pens that received chlortetracycline but with CCFA given only to 1 out of 11 steers. These isolates were carrying tet(B) genes much more so than the *tet*(A) gene. While in the pens that all cattle received CCFA treatment or with additional chlortetracycline treatment, *tet*(A) was favored over *tet*(B).

Unexpected from the Platt *et al*. study, chlortetracycline treatment was not associated with a decrease in ceftiofur resistance. With prior CCFA treatment, chlortetracycline treatment delayed the return of ceftiofur resistant isolates to baseline level. Many phenotypically ceftriaxone resistant isolates were not explained by the presence of the $bla_{\text{CMY-2}}$ gene. In fact, 32.9% of 88 steers in this cohort were detected with $bla_{\text{CTX-M-32}}$ gene located on an IncN plasmid [202]. The genotyping results explain some of the differences between the Platt *et al*. study and Kanwar *et al*. study, where Platt *et al*. study showed a decrease of ceftiofur resistance following CTC and

Kanwar *et al*. showed an increase and delay of return to baseline level. In the Kanwar *et al*. study, the priming of the CCFA treatment may have selected *E. coli* with $bla_{\text{CMY-2}}$, which also carry *tet*(A) gene. While in Platt et al., due to no prior CCFA treatment, *tet*(B) carrying *E. coli* may have been favored over *tet*(A) *E. coli* which led to a decrease of ceftiofur resistant *E. coli* when treated with CTC. In our study, we examined the *Salmonella* population from the same cattle population as Kanwar *et al*.

2.7.7 Therapeutic and subtherapeutic CTC on Salmonella *and* E. coli *in cattle and pigs*

Using similar antibiotics to Kanwar *et al*., but with experimental inoculation of nalidixic acid resistant *S.* Typhimurium in pigs, a study was conducted by Ebner *et al* [221]. Pigs were randomly assigned to four treatment groups after inoculation of *S.* Typhimurium orally and intranasally. Treatment started two days after *Salmonella* inoculation in four treatment groups as follows: 1) intramuscular injection of ceftiofur sodium (Naxcel) for 3 days followed by 100g/ton of oxytetracycline in-feed, 2) apramycin in-feed for 14 days followed by oxytetracycline, 3) carbadox in-feed until pigs reached 35 kg followed by oxytetracycline 4) no antibiotics (control).

Salmonella prevalence reached its peak from 2 to 7 days post-inoculation (DPI); therefore, the authors considered 2 DPI as baseline for each assessment of variation. Apramycin treatment decreased the shedding pig prevalence the most, followed by ceftiofur sodium and carbadox treatments. They found significant interactions between treatment and days. Resistant isolates were the lowest on 4 DPI and peaked at the end of the study (70 dpi) among all treatment groups. However, the percentage of resistant isolates among treatment groups did not differ, except for the carbadox treatment group. This study showed the temporal association of antibiotic treatments and increased resistant isolates. Since pigs were fed with oxytetracycline throughout

the whole experimental period after each antibiotic injection, it is not directly comparable to our study as each injectable antibiotics cannot be assessed in the absence of the tetracycline.

Delsol *et al*. reported the effects of therapeutic (15mg/kg) and sub-therapeutic (1.5mg/kg) doses of chlortetracycline on the *Salmonella* Typhimurium DT104 and *E. coli* populations in pigs [222]. They experimentally inoculated five strains of ACSSuT with nalidixic acid resistant *Salmonella* Typhimurium DT104 to pigs 48-hours before the treatment began and tracked the quantitative changes in the fecal samples up to 41-days post inoculation in three groups: therapeutic, sub-therapeutic, and control groups. *Salmonella* was isolated for 7-weeks after inoculation.

Pigs treated with therapeutic doses shed higher numbers of *Salmonella* compared with the sub-therapeutic dose group up to 7-days post-treatment. However, the significant difference was observed only until 7-days post-treatment, while non-significant differences were found after that. After 41-days post-treatment, sub-therapeutic pigs were shedding higher numbers of *Salmonella* than therapeutic or control groups. The authors discussed that CTC treatment had an effect to establish the *S.* Typhimurium in the gut, which resulted in higher and longer shedding in the fecal samples post-treatment. However, the *Salmonella* in this study were collected from pig feces, which might reflect the shedding more than the establishment. Few studies showed that fecal shedding of *Salmonella* changes with the immune response via inflammation in the gut and may change by stress [74]. Therefore, pigs in the therapeutic treatment groups may have shed higher number of *Salmonella* due to antibiotic treatment stress or due to change in the composition of microbiota; further, it is not clear if the CTC treatment significantly affected the establishment of *Salmonella* population in the gut flora. Experimental inoculation may not be an ideal method to examine the long-term effects of antibiotic treatments on farms. This is because

variations, such as inoculation concentration, inoculation methods, serotypes, time to start and antibiotic treatments, are introduced. More time to comingle the pigs after *Salmonella* inoculation may be needed to uniform the infections and establish the microbiota among pigs may needed to reproduce natural farm environment.

Naturally infected pigs may be infected with multiple serotypes, while experimental inoculation has more control to observe the differences to the response to antibiotic treatments. There was no difference in the MIC of *S.* Typhimurium DT104 after CTC treatment. In *E. coli*, these authors observed a higher tetracycline resistant population in the therapeutic dose treatment group until after 1-2-weeks post-treatment. Since there were 2% of *E. coli* population with MIC of > 50mg / L before treatment, it implies that these populations were selected by CTC administration. Those resistant isolates were carrying *tet*(A), *tet*(B), and *tet*(C) genes, and isolates with *tet*(B) genes had a higher MIC value (256mg / L) than isolates with *tet*(A) or *tet*(C) genes (32 to 128mg / L). Interestingly, some isolates with *tet*(C) genes were susceptible to CTC, which raises a question to the function of *tet*(C) genes. These authors are recommending extending the withdrawal time of CTC so that less resistant bacteria would enter the human population through a food chain, which is an important insight to determine the risk of using antimicrobials in food animals.

Funk *et al*. [223] studied the effects of subtherapeutic doses of chlortetracycline on *Salmonella* prevalence and antimicrobial susceptibility of Gram-negative flora in pigs. Treatment dosage was 50g of CTC per ton of feed for the entire finishing period following the labeled indication. Gram-negative isolates were collected from those that grew on a MacConkey agar plate. The fecal samples derived from three different farms that were willing to alternate their feed and antibiotics. The overall prevalence of *Salmonella* was 0.7% (15/2,112). The

chlortetracycline-fed group had a higher prevalence (1.3%) than the non-fed group (0.09%), but no statistical significance was detected. All the *Salmonella* isolates were derived from one farm. All isolates were ACSSuT-ceftiofur-cefoxitin resistant, but susceptible to ceftriaxone (whose CLSI breakpoint at the time of the research was >= 64 mg / L) and the serotypes were Agona and Ohio. Since the prevalence of *Salmonella* was very low in this study, the effect of subtherapeutic CTC on *Salmonella* prevalence cannot be determined.

However, the authors also tested the antimicrobial resistance of around 76,500 Gram-negative bacteria isolates. In both CTC treatment and control groups, tetracycline resistance was the most common phenotype. In total 97.5% were resistant to tetracycline in the treatment group and 84.3% in control group. Ceftriaxone resistance (that is, with extremely high MIC) was found in 0.7% of samples from the CTC treatment group and 0.3% in control. The odds-ratio of being tetracycline resistant between a CTC fed and a non-fed farm was 7.2, and that of ceftriaxone resistance was 2.4. These results show that subtherapeutic dosages of CTC in feed can select both tetracycline and ceftriaxone resistance in Gram-negative bacteria in swine. In this study, the sampling was conducted only once at the end of the finishing period, which the temporal relation of CTC use and antibiotic resistant bacteria emergence could not be examined. Even though the authors confirmed the presence of *Salmonella* in the tested farm prior to initiating the study, the prevalence was very low, which may be due to the intermittent shedding pattern requiring more sampling days.

Sharma *et al*. reported a study on the effects of the subtherapeutic use of chlortetracycline (T) alone or in combination with sulfamethazine (TS) on the diversity and distribution of *E. coli* in feedlot cattle in Canada [224]. Chlortetracycline was given at 350 mg per head per day (approximately 0.5 mg/lb per day), and so was sulfamethazine: both are common antibiotics used

in Canadian feedlots. Control pen animals were not treated with antibiotics. Each pen consisted of 50 steers. The study was continued until slaughter and fecal samples were collected 9 times based on their diet because the T and TS are both top dressing feed additives; diets were as follows, background silage-based diet (period A to C), diet transition to grain (D), finishing grain-based diet (E to H), and off antimicrobials (I). The *E. coli* was tested by PCR. *E. coli* that grew on plain MacConkey agar, MacConkey agar with ampicillin (32 μg/ml), and MacConkey agar with TE hydrochloride (16 μg/ml) were enumerated. Antimicrobial susceptibility was tested for each isolate and genotyped for the presence of ampicillin, tetracycline, and sulfamethoxazole resistance genes. Genetic diversity was determined by PFGE analysis.

The authors mainly compared the distribution of resistant *E. coli* at period A (day 0) and period H (day 197). Both tetracycline and ampicillin resistant *E. coli* numbers were higher in period H. They also found continuous detection of both resistant *E. coli* in control group at period H, which suggests other factors contributing such as change in animal diet from forage to grain. The diversity of phenotypical resistance decreased as the trial proceeded. It was showing 11 antibiograms at period A, while only 3 antibiograms were found during period H, irrespective of the treatments. Common resistant patterns were resistance to both trimethoprim-sulfamethoxazole (SX) and tetracycline (19.2%), or tetracycline alone (26.7%) in period A and increased to 47.5% and 31.7%, respectively in period H. The authors also showed by PFGE analysis and phylogenetic tree analysis that the genetic diversity of *E. coli* decreased at period H even in control groups. Since the structure of the pens used in this study does not allow direct animal contacts between different treatment groups, the authors concluded that the resistant *E. coli* were spread in the environment at the feedlot and then among cattle. The overall results

indicated that the use of chlortetracycline and sulfamethazine increase tetracycline resistance among *E. coli*.

Agga *et al.* conducted a randomized control study in 300 weaned calves divided into 2 treatment groups: 1) 5 consecutive days of in-feed prophylactic CTC (22mg/kg; 150 calves), and 2) no CTC treatment (150 calves). Calves were divided into 5 pens with 30 calves per pen and each occupied pen was separated by an empty pen from other occupied pens. Fecal swab and pen surface, feed, and water were collected five times: upon arrival, 5 days post-treatment (5-DPT), 27-DPT, 75-DPT, and 117-DPT. The concentrations of generic *E. coli*, cefotaxime-resistant *E. coli* ($3GC^r$), and tetracycline-resistant *E. coli* (TET^r) were enumerated by spiral-plating on plain CHROMagar *E. coli*, CHROMagar *E. coli* supplemented with 2mg / L cefotaxime, and CHROMagar *E. coli* supplemented with 32mg / L tetracycline. Concentrations of generic and TET^r *E. coli* were approximately 4.8 $\log_{10}$ CFU/swab and 3.2 $\log_{10}$ CFU/swab, respectively, upon arrival in both groups. On 5-DPT, generic *E. coli* in CTC group was 4.7 $\log_{10}$ CFU/swab, which was not different from upon arrival. Interestingly, generic *E. coli* concentration decreased in the control group on 5-DPT (3.51 $\log_{10}$ CFU/swab). The concentration of generic *E. coli* was higher in CTC groups on 5-DPT but the differences between the groups diminished after 27-DPT. TET^r *E. coli* increased by CTC treatment to 4.25 $\log_{10}$ CFU/swab on 5-DPT, which was higher than control group (1.86 $\log_{10}$ CFU/swab); however, the differences between the groups diminished after 27-DPT.

The concentrations of generic *E. coli* and TET^r *E. coli* increased in both treatment groups on 75-DPT and 117-DPT compared with that of day upon arrival. However, the concentration did not differ between the two groups on both days. The concentration of cephalosporin-resistant *E. coli* were not enumerable. Prevalence of cephalosporin resistant *E. coli* increased from <10%

upon arrival to >70% after 75-DPT in both groups. Generic and TET^r *E. coli* concentration on the pen surface was around 1.2 $\log_{10}$ CFU / g upon arrival and increased to 5-6 $\log_{10}$ CFU / g on 117-DPT in both pens, except for in the empty pens. These results showed that the effect of CTC treatment was only seen on 5-DPT by increasing tetracycline resistant *E. coli* concentrations. The concentration of generic *E. coli* and tetracycline-resistant *E. coli* increased in spite of the treatment group, which suggests other undetermined environmental factors affected the concentration. The authors discussed that the deposited manure in occupied pens increased the tetracycline and cephalosporin-resistant population, for which further study will be needed. The increase of tetracycline resistance agrees with the Sharma *et al*. study in that resistant isolates increased at the end of the study period regardless of the treatment group; that said, Sharma *et al*. were using subtherapeutic doses of CTC. The consideration of environmental variables is necessary when conducting similar experiments in near future.

2.8 Strategies to tackle antimicrobial resistance derived from animal

2.8.1 Precautionary principle (Ban the growth promoter use)

The Swann report was published in 1969 by U.K. parliament, which was the first formal warning of increased antimicrobial resistant infections in humans due to the use of antibiotics for growth promotion and feed efficiency in food animals in the UK [225]. The Swann report specifically warned of the increase of chloramphenicol resistant *Salmonella*. The first ban on antibiotic use as growth promoters started in the U.K. for tetracycline, penicillin, and streptomycin in 1972 [185]. Sweden was the first country to ban the use of all antibiotics for growth promotion in 1986. Denmark followed and worked with industry to also ban the use of antibiotics for prophylactic purpose in 1995. At the same time, Denmark started the monitoring system called the Danish Integrated Antimicrobial Resistance Monitoring and Research Program

(DANMAP) [226]. The EU banned growth promoter use of all the antimicrobials in 2006. In the UK, vancomycin-resistant enterococci (VRE) were first reported in food animals in 1993, which led to the ban of avoparcin for all purposes in Sweden; later, in the entire European Union (EU) in 1997. Avoparcin has a similar chemical structure as vancomycin (both are glycopeptides), which is a critically important class of antibiotics for use in humans [186]. In the US, avoparcin was never approved for use in animals. The ban in Denmark reduced the overall use of antibiotics but increased the antibiotic use for disease treatment [10]. It is not yet clear whether the ban reduced resistance in human health. In the United States, prudent or judicious use of antimicrobials is promoted at multiple levels. A partial ban on the extra-label use of cephalosporins in animals was announced in 2012, and the voluntary withdrawal of growth promotion labels by product sponsors led to a *de facto* ban on the use of medically important antimicrobials in the U.S starting in 2017.

In 2007, Phillips reviewed the effect of the European ban on growth promoters for human health outcomes using *Enterococcus* and *Campylobacter* as examples. The ban of antibiotics as growth promoters in 2006 in the EU had eliminated the use of antibiotics for that purpose, however, the infection of ciprofloxacin resistance *Campylobacter* infections in the human population increased according to Phillips [227]. However, this was disputed later by Hammerum and colleagues from Denmark, who claimed that one-third of *Campylobacter* infections in Denmark were due to travel [228]. Hammerum and colleagues further noted that 67% of poultry meat imported in Denmark was contaminated with *Campylobacter*, while only 17% was contaminated in domestic poultry meat. Further, 17% of Danish chicken-derived *Campylobacter jejuni* were resistant to ciprofloxacin, compared to 49% in imported chicken [228]. Chickens were imported from Germany, Sweden and United Kingdom to Denmark for

several years. The latest 2015 DANMAP show that ciprofloxacin-resistant *Campylobacter jejuni* from broiler and broiler meat has been increasing since 2006 reaching 27% in 2015. However, this rate is far lower than the EU average of 66% [229]. Additionally, the importation of chicken to Denmark has increased from $10.2M in 2006 to $23M in 2014 [230], which may partially explain the increase of ciprofloxacin resistance even though fluoroquinolone were not used in broiler production since 2009. Among *Enterococcus* spp., Phillips claims that the ban of avoparcin in 1997 in EU and virginiamycin (streptogramin) in 1998 in Denmark had little effect on humans because cases of vancomycin-resistant Enterococcus (VRE) were rare from the beginning. In the United States, avoparcin was never allowed for use in animals due to its purported carcinogenic effects. However, VRE cases are high in the human population in the US, which is probably because of the high vancomycin use in hospitals. Phillips also claimed that 9% of *E. faecium* that were submitted to European Antimicrobial Resistance Surveillance System (EARSS) were VRE in 2004 and increased to 14% in 2005. Hammerum commented that since the EARSS report included both *vanA* (animal origin) and *vanB* (human) gene cases, this cannot be used to explain this paradox. Ironically, the 2015 DANMAP reported that the cases of VRE in human have been increasing, and a 5-times increase was observed from 2012 to 2013. More than 90% of the VRE isolates are *Enterococcus faecium vanA* type, which has a sequence type (ST) that is not of animal origin [229, 231]. Since these *E. faecium vanA* type occurrences have regional differences, Hammerum *et al*. suspect that these may be due to the use of vancomycin for *C. difficile* treatment in hospitalized patients. Interestingly, the authors also mentioned that the use of cephalosporins can help to transmit *vanA* gene to *E. faecium* in mice [231].

Both conclusions from Phillips and Hammerum *et al* are based on DANMAP data but are interpreted in vastly differently ways. The differences between Phillips and Hammerum *et al*.

comes from the different focus on the final purpose of the growth promotion ban. Phillips focused on the effect on human health, which is an ultimate goal, while the current ban policy in EU focuses on reducing the resistant population derived from animal production system [185]. It might be true as Phillips stated that the contributions of animal food production on human resistant bacterial infection were not as large as initially feared in the Swann report, but it remains true that food-borne pathogens are one of the major causes of infection by resistant bacteria. These illustrate the difficulty of controlling the use of antimicrobials and then measuring the effects on the intervention.

2.8.2 Judicious use principles in the United States

In the United States, the FDA proposed the withdrawal of subtherapeutic uses of two important antibiotics in human medicine, penicillin and tetracyclines, in 1977. However, due to a lack of sufficient scientific evidence to approve this proposal, Congress directed FDA to continue the study on the effects of the subtherapeutic use of antimicrobials in animals on human health. Even after this attempt, when the National Academy of Sciences reported the study on the effect of antimicrobial use in animals, the FDA did not make a decision [170]. In 2008, the FDA announced a prohibition of extra-label cephalosporin use in food-animal production. However, critics claimed that this was too broad of a prohibition and cited concerns over negative consequences, and a lack of scientific evidence, which resulted in the FDA delaying the final order.

In April 2012, the FDA Center for Veterinary Medicine (CVM) issued an order of prohibition, which generally prohibits the extra-label use of cephalosporin (excluding cephapirin) in cattle, swine, chickens, and turkeys, with notable exceptions. As long as the use adheres to the labeled regimens, it is approved to be used for treatment and control of labeled and

extra-labeled disease indications. Later that same year, judicious use of antibiotics (or voluntarily withdrawing the use of medically important antibiotics for animal growth promotion use) was announced by the FDA in the Guidance for Industry (GFI) #209 in seeking public comment [170, 232]. The process in reaching this recommendation is summarized in GFI #209 [170]. The GFI #209 is following the two principles. The first principle is "*The use of medically important antimicrobial drugs in food-producing animals should be limited to those uses that are considered necessary for assuring animal health"*, which indicates that as long as the use of antimicrobials for disease prevention is considered necessary by the veterinarian, it is considered judicious use. The second principle is "*The use of medically important antimicrobial drugs in food-producing animals should be limited to those uses that include veterinary oversight or consultation"*, which indicates that use of medically important antimicrobials requires the supervision of veterinarian [232]. In 2017 January, GFI #213 was put into full implementation by FDA. Chlortetracycline in feed changed from over-the-counter to the Veterinary Feed Directive in 2017 as mentioned in GFI #213 (effective October 2015).

2.9 Summary of literature review

Salmonella has been studied extensively, although not to the same degree as commensal *E. coli*, because of its unique pathogenicity towards humans and other animals. *Salmonella* resides in multiple hosts including food-animals, which ultimately can infect humans as a foodborne pathogen. Although most of the *Salmonella* found in food-animals are pan-susceptible, specific serotypes of *Salmonella* are highly MDR. It is crucial to use antibiotics prudently at the feedlot to reduce antibiotic resistant *Salmonella*. There have been no studies conducted to compare the effects of ceftiofur and chlortetracycline on the *Salmonella* populations in feedlot cattle. Studies from pigs and dairy cattle are not directly applicable to the feedlot cattle population. This is

because pigs and dairy cattle have different farm operation styles, metabolisms, and antibiotic use protocols. Moreover, *Salmonella* prevalence and serotypes distribution are different among hosts, geographical locations, and seasons.

Multiple studies on the effects of antibiotics on *E. coli* populations in feedlot cattle have been conducted, largely because *E. coli* are commensal bacteria and readily found in all mammalian enteric samples. *E. coli* tends to acquire plasmids, including plasmids carrying resistance genes, from other bacteria. In multiple studies, antibiotic treatments have increased antibiotic resistant *E. coli* transiently. Some studies have shown that farm management is more important than the use of antibiotics for causing an increase in antibiotic resistant *E. coli*. Even though *E. coli* and *Salmonella* belongs to same *Enterobacteriaceae* family, they have different biology, microbial ecology and genomic backgrounds. *E. coli* has more diverse phenotype and pathogenicity within host species, while *Salmonella* is uniformly more pathogenic, especially to humans. In *Salmonella,* serotype and antibiotic resistance are highly associated. This may be due to serotypes specializing in the response to horizontal gene transfer or recombination. Therefore, the antibiotic response in *E. coli* is rarely directly comparable to that of *Salmonella*.

In the United States, judicious use of antibiotics at the pre-harvest level is adopted to reduce the population of antibiotic resistant bacteria that can contaminate meat at slaughter or escape from the farm via environmental means such as water, dust, or soil. The effects of antibiotic use on the quantity of antibiotic resistant *Salmonella* are not well known. In our study, we have isolated the *Salmonella* in the feedlot cattle population for 26 days following injection of CCFA and administration of in-feed CTC in a randomized control study. The effects of antibiotics were assessed from multiple aspects:1) the prevalence and quantitative dynamics of *Salmonella* by treatment and day, 2) antibiotic resistance by antibiotic phenotype and genotype

by WGS, and 3) genomic dynamics of *Salmonella* by antibiotic treatments. As the advancement of WGS technology proceeds and the opportunity to conduct WGS and bioinformatic analysis within our laboratory expanded, the detailed genomic study of isolates derived from the same population was enabled. Few studies have been conducted to compare the genomics of *Salmonella* collected from different host populations (human versus cattle) or geographically separated populations. However, no study has been reported as of yet comparing isolates from a single feedlot cattle population experimentally treated with antibiotics. Moreover, *Salmonella* isolates collected in our study are wild-type populations, not those experimentally challenging farm animals as orally administered laboratory cultured strains, which induce more variables by inoculation methods, concentration, and serotypes [233]. Our research project will show the effects of CCFA and CTC on feedlot cattle from multiple aspects, which fills the research gaps of current research on antibiotic resistant *Salmonella* in feedlot cattle.

CHAPTER III

POPULATION DYNAMICS OF ENTERIC *SALMONELLA* IN RESPONSE TO ANTIMICROBIAL USE IN BEEF FEEDLOT CATTLE*

3.1 Introduction

Foodborne salmonellosis is estimated to cause more than 1.2 million illnesses annually in the United States, requiring 23,000 hospital admissions and resulting in 450 deaths [19]. Salmonellosis is usually self-limiting, and even severely affected patients generally recover in 5 to 7 days if given rehydration fluids. Antimicrobial treatment options for adults include ceftriaxone, a medically important third-generation cephalosporin, and fluoroquinolones. There are potential side effects of fluoroquinolones for pediatric patients, and hence the first choice of treatment is ceftriaxone [234-236]. Because fewer treatment options are available for antimicrobial-resistant *Salmonella*, infections with these strains are potentially more life threatening [237]. Use of antimicrobials can cause unintentional selection pressure for antimicrobial resistance in the gut microbiota of animals, and therefore can potentially lead to more severe cases of salmonellosis [238-240].

The National Antimicrobial Resistance Monitoring System (NARMS) has previously reported an increase in ceftriaxone resistant *Salmonella* carrying the *bla*$_{\text{CMY-2}}$ gene, a class C beta-lactamase and the chromosome encoded *amp*C gene, in human cases of salmonellosis [193, 241, 242]. The *bla*$_{\text{CMY-2}}$ gene also confers resistance to ceftiofur, a third-generation cephalosporin approved for veterinary use, as well as ampicillin, amoxicillin-clavulanic acid,

cephalothin, and cefoxitin [193, 241]. Observed increases in ceftriaxone resistant *Salmonella* in humans may be due, at least in part, to the increased use of third-generation cephalosporins in food animals [28, 169]. This is considered a high public health risk since ceftriaxone and ceftiofur belong to the same class of 3rd generation cephalosporins, which the World Health Organization (WHO) has classified as critically important for human medicine. A variety of risk management strategies have been employed to help maintain antimicrobial efficacy for human medicine and to reduce the spread of antimicrobial-resistant bacteria derived from food animals [243]. In 2008, the U.S. Food and Drug Administration (FDA) announced a plan to prohibit the extra-label use of all cephalosporins in food animals (with no exceptions); later, this was revoked due to concerns about overly broad restrictions and the potential for unintended negative consequences [244]. Following re-examination by the FDA, extra-label use of cephapirin, some extra-label uses for indications involving the same route of administration, dose, and duration, and the use in minor food-producing species were excluded from the 2012 prohibition on extra-label use of cephalosporins in food-producing animals [244]. The FDA also promoted judicious use of antimicrobials of importance to human medicine, by working to remove growth promotion labels as of January 1, 2017 [31, 245]. The effects of such strategies on reducing human infections with resistant bacteria have yet to be determined [10, 185, 227]. In the current study, we investigated treatment strategies involving a 3rd generation cephalosporin and chlortetracycline in fed beef cattle and their effects on intestinal *Salmonella enterica* populations.

In the recent past, several studies have been conducted to investigate the selection of resistant *E. coli* and *Salmonella* with the use of ceftiofur in cattle, both in experimental and observational settings [30, 35, 209-213]. In one study, ceftiofur administration in beef cattle transiently increased ceftiofur-resistant *E. coli*; however, the bacterial population returned to the before-

treatment level after 2 weeks [209]. Daniels and others showed that ceftiofur use in a dairy herd was not associated with the occurrence of ceftiofur-resistant *Salmonella* and *E. coli* [30]. Singer and others reported that the therapeutic use of ceftiofur in dairy cattle opened the "window" to detect resistant *E. coli*, but it was not concluded that such use resulted in the emergence or expansion of resistant *E. coli* [210]. Another dairy farm study reported that ceftiofur use and *E. coli* with reduced susceptibility to ceftriaxone are associated at the herd level, but not at the individual cow level [212]. A 10-month long study by Schmidt *et al*. showed that ceftiofur use in feedlot cattle did not increase the extended-spectrum cephalosporin resistant *E. coli* [211]. These studies suggest that the therapeutic use of ceftiofur can transiently increase the detection of cephalosporin resistant *E. coli*; however, the susceptible bacterial population returns after a suitable washout period. Since *E. coli* and *Salmonella* both belong to the Enterobacteriaceae family, it would seem likely that *Salmonella* would exhibit a similar response to ceftiofur as *E. coli*; however, it is not yet known since studies involving *Salmonella* require consistent presence at the high prevalence of pathogen like commensal bacteria. An observational study investigating varying levels of ceftiofur use and an association with resistant *Salmonella* isolated on swine farms, showed that the barns with rare and common ceftiofur use had 4.1% and 6.0% recovery of *Salmonella* carrying the bla_{CMY} gene, respectively, while only 0.15% recovery occurred in the barns with moderate uses of ceftiofur [215]. The results suggest that other factors, such as farm management or environmental factors may have a greater impact than ceftiofur use on resistant *Salmonella*.

Chlortetracycline (CTC) is a common feed additive used to treat and control bacterial pneumonia (bovine respiratory disease complex) in feedlot cattle; this is in addition to vaccination used to prevent respiratory disease and other injectable antimicrobials used for

disease control purposes [45]. In previous work reported from our group by Platt *et al*., CTC treatment paradoxically reduced the prevalence of ceftiofur resistant *E*. *coli* [34]. However, contradictory results were found in a subsequent study by Kanwar *et al*. in which CTC treatment increased ceftiofur resistance, most likely due to co-selection [35]. In a longer-term study, the effects of prophylactic use of CTC on antimicrobial-resistant *E*. *coli* in beef cattle were studied for 117 days by Agga *et al*.; their findings showed an increased level of tetracycline resistant *E*. *coli* on Day 5 post-treatment, but not on Days 27, 75, and 117 [63]. Additionally, prevalence of cephalosporin-resistant *E*. *coli* remained the same among CTC and control groups throughout the study period [63]. We have further investigated the antimicrobial resistance profiles of the *Salmonella* population dynamics in response to both ceftiofur and chlortetracycline administration in the very same cattle studied by Kanwar *et al*. [35].

A randomized controlled study concerning the use of CTC and ceftiofur use and antibiotic resistant *Salmonella enterica* in feedlot cattle, has not previously been reported.

The current study is focused on the effects of ceftiofur crystalline-free acid (CCFA) and CTC on the prevalence of *Salmonella* in feedlot cattle, and on changes in the profile of antimicrobial susceptibilities of *Salmonella* resulting from the selective pressures of CTC and CCFA. Furthermore, we investigate and report on the temporal dynamics of the *Salmonella* population in response to the use of antimicrobials.

3.2 Materials and Methods

3.2.1 Experimental design

A randomized, controlled, longitudinal field trial was conducted involving two sequential 26-day replicates in an experimental feedlot at West Texas A&M University in Canyon, Texas,

USA. The first replicate began in early August 2009 and a second replicate began in the middle of September. The cattle were owned by a third party and been purchased from a single operation in the far western United States. The cattle were shipped directly to the experimental feed yard one month before the trial started. The cattle were yearling steers that were predominantly of the Angus breed. The cattle were fed diets typical of regional feedlots; that is, a flaked-corned based diet with added roughage, protein, vitamins and minerals. Any cattle that became sick and required antibiotic treatments were excluded from the study. In each replicate, 88 steers were assigned into 8 pens (n = 11 cattle) to distribute the body weights among the pens evenly, in a two-by-two factorial design with four treatment regimens (Figure 6), as described in a previous paper by our group [35].

Across both replicates, in 8 pens all 11 steers received 6.6 mg/kg of CCFA (Excede®, Zoetis Animal Health, Florham Park, NJ) subcutaneously at the base of the ear ("All-CCFA & CTC" and "All-CCFA / no CTC" in Figure 6; group metaphylaxis model), and in the remaining 8 pens, a single steer treated with CCFA on day 0 was co-housed (mixed) with 10 non-treated steers. Repeated across both replicates, four of the pens receiving "All-CCFA" and four of the pens receiving "1-CCFA" treatment later received three 5-day pulses of 22 mg/kg CTC (Aureomycin®, chlortetracycline complex equivalent to 220.5 g/kg of chlortetracycline, Alpharma, Bridgewater, NJ). The CTC was top-dressed in feed with a one-day break between each 5-day pulse. The CTC feeding occurred during the period from day 4 until day 20 ("All-CCFA & CTC", "1-CCFA & CTC"). The remaining 8 pens across both replicates did not receive CTC i.e., (four each of "All-CCFA / no CTC" and "1-CCFA / no CTC"). Fecal samples were collected every other day *per rectum* as described previously [35]. These samples were mixed with glycerol at a 1:1 ratio and preserved at −80 °C.

The animal experiments were approved by the Amarillo-Area Cooperative Research, Education, and Extension Triangle Animal Care and Use Committee (Protocol No. 2008-07), and by the Clinical Research Review Committee at Texas A&M University (CRRC # 09–35). All experiments were performed in accordance with institutional and the United States Department of Agriculture (USDA) guidelines and regulations governing the oversight and conduct of experiments involving food producing animals in the U.S. The Texas A&M University Institutional Biosafety Committee approval # IBC 2014-043 permitted the microbiological laboratory experiments involving *Salmonella enterica* isolates.

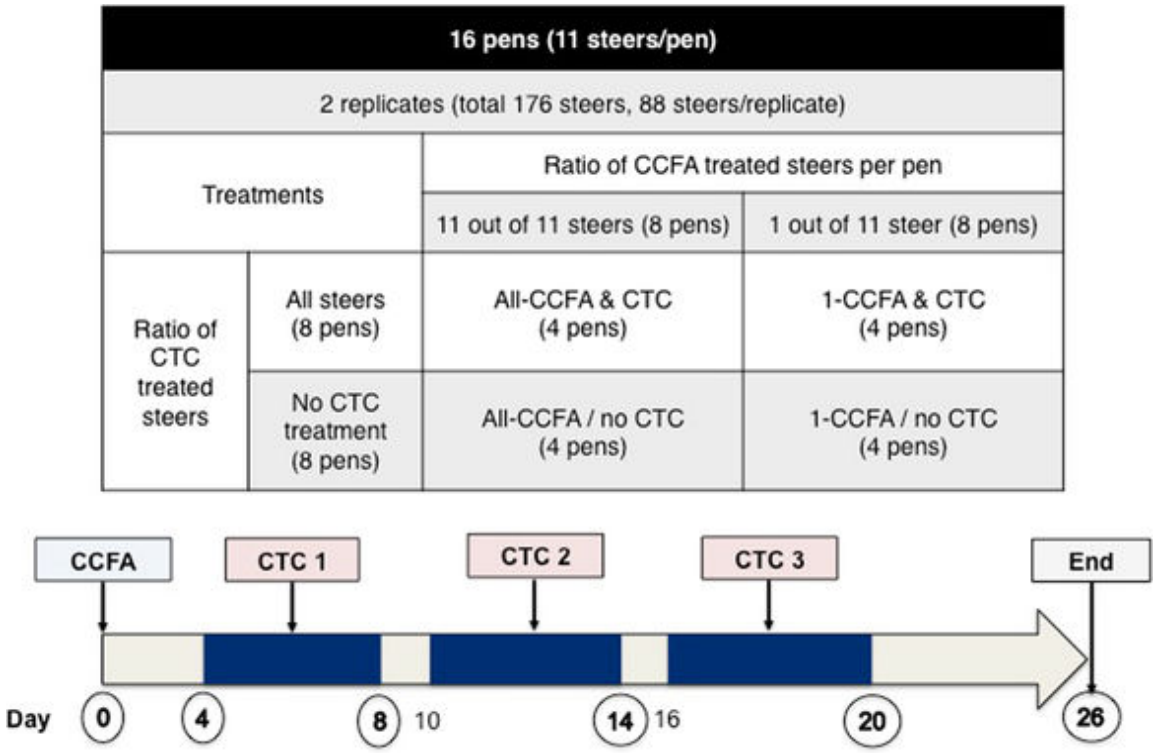


Figure 6. Study design. Four pens were allocated to each treatment over two replicates. Samples were tested from the circled days on the arrow (0, 4, 8, 14, 20, and 26). Un-circled numbers represent the days when 'pulsed' CTC was added back into the feed bunks for five days.

3.2.2 Salmonella *isolation from fecal samples*

A total of 1,040 fecal samples obtained across days 0, 4, 8, 14, 20, and 26 were cultured for *Salmonella*. The maximum effect of CCFA treatment on multidrug resistant *E. coli* isolates was seen on day 4 in the previous study [35], and we expected to observe a similar trend for *Salmonella*. Days 8, 14, and 20 were chosen because these were the last days of each of the 5-day CTC treatment pulses, and we predicted they would reflect the maximum effect on *Salmonella* prevalence. The study was completed on day 26.

We isolated *Salmonella* by following a modified enrichment process as described previously [246] (see Tables 1-4 for media preparation instructions). Samples were thawed on ice and mixed thoroughly with a transfer pipette. In total, 500 mg of feces were pre-enriched in 5 ml of tryptic soy broth (TSB) (Difco, Becton Dickinson, Franklin Lakes, NJ) for 2 hours at room temperature, then incubated for 6 hours at 37 °C, and then kept at 4 °C for 14 hours. A 1 ml aliquot of the enriched feces in TSB was transferred into 9 ml of tetrathionate broth (Difco, Becton Dickinson) and incubated at 37 °C for 24 hours. After incubation, 100 µl of the tetrathionate broth culture was transferred into 10 ml of Rappaport-Vassiliadis R10 (RV) broth (Difco, Becton Dickinson) and incubated at 42 °C for 18 hours. The next day, 50 µl of RV broth was spiral-plated onto Brilliant Green agar (BGA) (Difco, Becton Dickinson) using an Eddy Jet 2 spiral plater (Neutec Group Inc., Farmingdale, NY). A single presumptive *Salmonella* isolate was plated on tryptic soy agar (TSA) with 5% sheep blood agar (Remel^{TM}, Lenexa, KS) for isolation and incubated at 37°C for 18 hours. Isolates were verified with *Salmonella* O-antiserum Poly A-I & Vi Factors 1–16, 19, 22–25, 34, Vi (Becton, Dickinson and Company, Franklin Lakes, NJ). Confirmed *Salmonella* isolates (n = 566) were preserved in cryobeads at −80 °C for further characterization.

Table 1.Tetrathionate broth formulas per liter purified water (modified from http://www.bd.com/europe/regulatory/Assets/IFU/HB/CE/BA/BA-257103.pdf Becton Dickinson)

BD Tetrathionate Broth Base

Bacto™ Proteose Peptone 5.0 g	Provides nitrogen, carbon, vitamins and acids.
Bacto™ Bile Salts 1.0 g	Suppresses coliform bacteria and inhibits Gram-positive organisms.
Sodium Thiosulfate 30.0 g	Suppresses commensal intestinal organisms. Forms tetrathionate by the addition of iodine solution. *Salmonella* carries the enzyme tetrathionate reductase and proliferates in the medium.
Calcium Carbonate 10.0 g	Neutralizes and absorbs toxic metabolites and provides a stable pH value.
pH 8.4 ± 0.2	

Table 2 Iodine solution formula

Iodine Solution
Iodine 300.0 g
Potassium Iodide 250.0 g

Table 3 Rappaport-Vassiliadis broth (modified from http://www.bd.com/europe/regulatory/Assets/IFU/HB/CE/BA/BA-257257.pdf)

BD Rappaport Vassiliadis Broth	
Bacto™ Tryptone 4.54 g	Source of carbon and nitrogen for general growth requirements.
Sodium Chloride 7.2 g	
Potassium Dihydrogen Phosphate 1.45 g	
Magnesium Chloride, Anhydrous 13.4 g	Raises the osmotic pressure in the medium. *Salmonella* can grow at higher osmotic pressure, because they can survive dehydration. Inhibits the growth of *Proteus* spp. and *Escherichia coli*.
Malachite Green Oxalate 0.036 g	Inhibitory to organisms other than *Salmonella*e. *Salmonella* Typhi cannot grow under this concentration of Malachite Green.
pH 5.1 ± 0.2	Inhibits the growth of other microorganisms.

Table 4. Brilliant Green Agar

BD Brilliant Green Agar	
Yeast Extract 3.0 g	Nutrient
Bacto Proteose Peptone 10.0 g	Nutrient
Lactose 10.0 g	Differentiation from lactose fermenters
Sucrose 10.0 g	Differentiation from sucrose fermenters
Sodium Chloride 5.0 g	
Phenol Red 0.08 g	Differentiation by color from lactose/sucrose fermenters; *Salmonella* do not produce acid.
Agar 20.0 g	Agar base
Brilliant Green 12.5 mg	Inhibit the growth of other coliform bacteria.
pH 6.9 ± 0.2	

3.2.3 Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MIC) of 14 antimicrobials (9 antimicrobial classes) for *Salmonella* isolates were determined by the broth microdilution method using the Sensititre® system (TREK, Thermo Scientific Microbiology, Oakwood Village, OH). Tested antimicrobials were ampicillin (AMP), amoxicillin/clavulanic acid (AUG2), azithromycin (AZI), cefoxitin (FOX), ceftiofur (XNL), ceftriaxone (AXO), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (GEN), nalidixic acid (NAL), streptomycin (STR), sulfisoxazole (FIS), tetracycline (TET), and trimethoprim/sulfamethoxazole (SXT) (Table 5; Figure 7). Briefly, isolates were plated onto TSA with 5% sheep blood agar and incubated at 37 °C for 18 hours. Then, 1 or 2 colonies were suspended in 4 ml of sterilized water adjusted to 0.5 McFarland standard and 50 µl of the culture suspension was transferred into 11 ml of Mueller-Hinton broth; thereafter, 50 µl of suspension was inoculated onto Gram-negative National Antimicrobial Resistance Monitoring System (NARMS) CMV3AGNF plates using the Sensititre® automated inoculation delivery system (TREK). Plates were incubated at 37 °C for 18 hours.

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 strains for susceptibility testing. Plates were read on a Sensititre OptiRead™ (TREK, Thermo Scientific Microbiology). The results were interpreted as susceptible, intermediate, or resistant according to Clinical and Laboratory Standards Institute (CLSI) guidelines using SWIN software (TREK, Thermo Scientific Microbiology) [247]. When breakpoints were undetermined, we followed the consensus breakpoints established by NARMS for *Salmonella*.

Intermediate isolates were reclassified as susceptible for further statistical analysis. Isolates resistant to three or more classes of antimicrobials were considered multidrug resistant (MDR) as defined by NARMS.

Table 5. MIC range and breakpoints for *Salmonella spp.* antimicrobial susceptibility testing by NARMS (2014) [26]

Antibiotic	**Range**	**Breakpoint**
Ampicillin	1 - 32	≥ 32
Amoxicillin/Clavulanic Acid	1/0.5 - 32/16	≥ 32 / 16
Azithromycin	0.12 - 16	≥ 32
Cefoxitin	0.5 - 32	≥ 32
Ceftiofur	0.12 - 8	≥ 8
Ceftriaxone	0.25 - 64	≥ 4
Chloramphenicol	2 - 32	≥ 32
Ciprofloxacin	0.015 - 4	≥ 1
Gentamicin	0.25 - 16	≥ 16
Nalidixic Acid	0.5 - 32	≥ 32
Streptomycin	2 - 64	≥ 64
Sulfisoxazole	16 - 256	≥ 512
Tetracycline	4 - 32	≥ 16
Trimethoprim/Sulphamethoxazole	0.12/2.4 - 4/76	≥ 4 / 76

SENSITITRE™ GRAM NEGATIVE NARMS PLATE FORMAT

Plate Code: **CMV3AGNF**

	1	2	3	4	5	6	7	8	9	10	11	12
A	FOX 32	AZI 8	CHL 16	AXO 64	AXO 0.25	CIP 2	GEN 16	NAL 16	XNL 2	FIS 32	AMP 32	STR 16
B	FOX 16	AZI 4	CHL 8	AXO 32	AUG2 32/16	CIP 1	GEN 8	NAL 8	XNL 1	FIS 16	AMP 16	STR 8
C	FOX 8	AZI 2	CHL 4	AXO 16	AUG2 16/8	CIP 0.5	GEN 4	NAL 4	XNL 0.5	SXT 4/76	AMP 8	STR 4
D	FOX 4	AZI 1	CHL 2	AXO 8	AUG2 8/4	CIP 0.25	GEN 2	NAL 2	XNL 0.25	SXT 2/38	AMP 4	STR 2
E	FOX 2	AZI 0.5	TET 32	AXO 4	AUG2 4/2	CIP 0.12	GEN 1	NAL 1	XNL 0.12	SXT 1/19	AMP 2	NEG
F	FOX 1	AZI 0.25	TET 16	AXO 2	AUG2 2/1	CIP 0.06	GEN 0.5	NAL 0.5	FIS 256	SXT 0.5/9.5	AMP 1	POS
G	FOX 0.5	AZI 0.12	TET 8	AXO 1	AUG2 1/0.5	CIP 0.03	GEN 0.25	XNL 8	FIS 128	SXT 0.25/4.75	STR 64	POS
H	AZI 16	CHL 32	TET 4	AXO 0.5	CIP 4	CIP 0.015	NAL 32	XNL 4	FIS 64	SXT 0.12/2.38	STR 32	POS

ANTIMICROBICS

FOX	Cefoxitin
AZI	Azithromycin
CHL	Chloramphenicol
TET	Tetracycline
AXO	Ceftriaxone
AUG2	Amoxicillin / clavulanic acid 2:1 ratio
CIP	Ciprofloxacin
GEN	Gentamicin
NAL	Nalidixic Acid
XNL	Ceftiofur
FIS	Sulfisoxazole
SXT	Trimethoprim / sulfamethoxazole
AMP	Ampicillin
STR	Streptomycin
NEG	Negative Control
POS	Positive Control

Figure 7. Trek Sensititre® CMV3AGNF plate design

3.2.4 Salmonella *DNA extraction for whole-genome sequencing*

Salmonella DNA was isolated in a QIAcube HT robot using the QIAamp 96 DNA QIAcube HT Kit (Qiagen, Valencia, CA). A single *Salmonella* colony was suspended into 5 ml of TSB and incubated overnight at 37 °C. From the suspension culture, 1 ml was transferred into a 1.2 ml micro-collection tube and centrifuged at 4,000 rpm for 15 minutes at room temperature. After the supernatant was removed, the pellet was re-suspended in ATL buffer (Qiagen) and mixed with reagent DX (Qiagen). One tube of small pathogen lysis beads (Qiagen) was mixed with the suspension and disrupted with the Qiagen TissueLyser system (Qiagen) 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 a ThermoMixer (Eppendorf, Hauppauge, NY) followed by a heat shock for 10 minutes at 95 °C. The suspension was cooled to room temperature and 4 µl of RNAse A was added. The prepared samples were set in the QIAcube HT for DNA extraction using a modified protocol

provided by Qiagen. The quality of the DNA was determined by the 260/280 ratios on the FLUOstar Omega Microplate Reader (BMG LABTECH, Cary, NC). The DNA quantity was measured by fluorescence with the Quant-iT™ Pico Green® ds DNA Assay kit (Thermo Fisher Scientific) on the FLUOstar Omega Microplate Reader and the DNA was stored at −20 °C until future use.

3.2.5 Whole-genome sequencing by Illumina MiSeq

To determine the serotypes and genotypes of the *Salmonella* isolates, we conducted whole-genome sequencing (WGS) using the Illumina MiSeq platform (Illumina, San Diego, CA, USA) on 566 isolates as shown in Figure 8. Libraries for 32 *Salmonella* DNA samples were multiplexed and pooled per sequence run using the Illumina Nextera XT library preparation kit as per the manufacturer's instructions and were run with the Illumina MiSeq 600 cycle v3 Reagent Kit with paired-end 2 reads (Illumina).

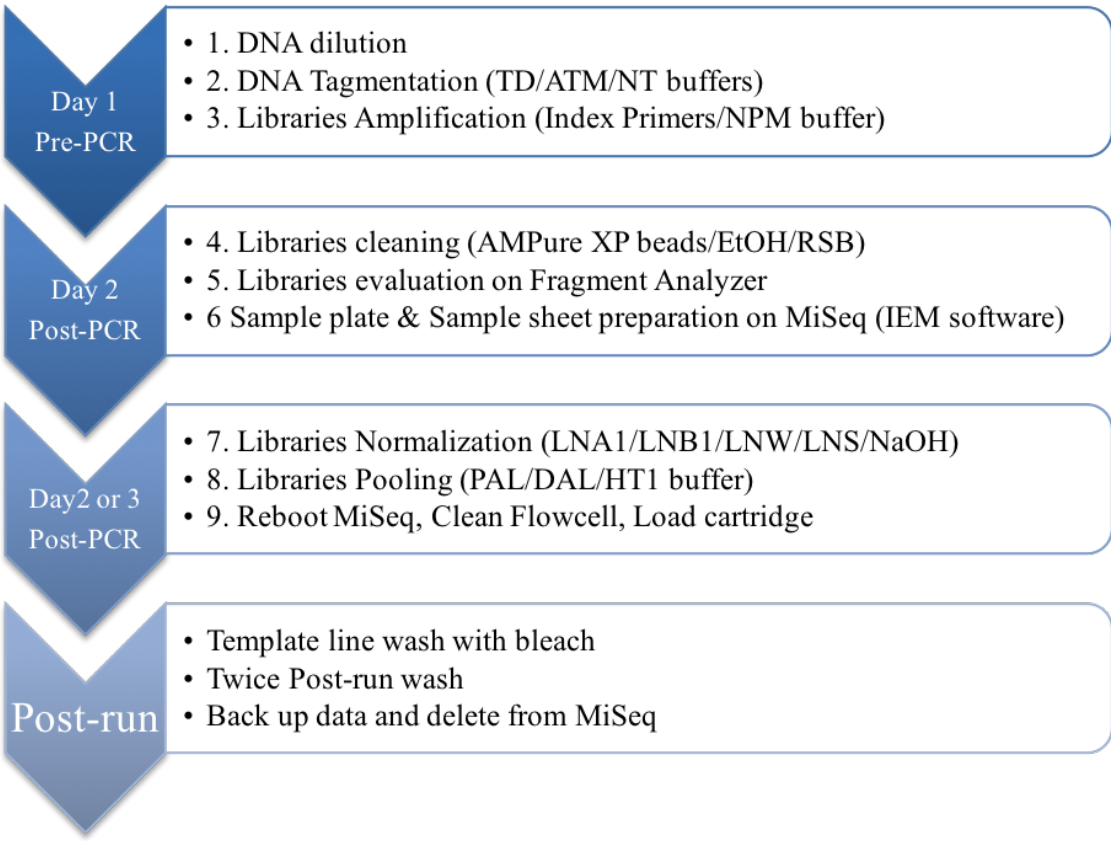


Figure 8. Library preparation workflow with Nextera XT library preparation kits

3.2.6 Illumina Sequencing data analysis

3.2.6.1 *de novo* assemblies and gene annotations

Obtained raw reads in fastq files were trimmed with Trimmomatic ver. 0.36 [248]. The quality of the reads was confirmed by FastQC software and *de novo* assembled by Spades ver. 3.10 [249]. The assembly quality was assessed on the QUAST web server, which provided the number of contigs, total length of genome by bp, GC (%), N50, and L50 statistics [250]. When the number of contigs was over 1,000 in SPAdes 3.10, the contigs were reassembled on the

PATRIC web resource using SMART mode, which assembles with SPAdes, Velvet, and IDBA assembler. The best-assembled contigs were chosen for downstream analysis.

Raw fastq files obtained from the forward and reverse reads were uploaded to the web-based database SeqSero 1.0 to determine the serotypes from WGS data (http://www.denglab.info/SeqSero) [96]. Multi-Locus Sequence Type (MLST) of each of the *Salmonella* isolates was determined by the combination of 7 gene alleles (*aro*C, *dna*N, *hem*D, *his*D, *pur*E, *suc*A and *thr*A) using the PubMLST database (30-Nov-2014) in the SRST2 pipeline in the Illumina® BaseSpace® Sequence Hub [251, 252]. The PlasmidFinder database (20-Feb-2017) [253] was used for plasmid determination on the Center for Genomics Epidemiology (CGE) web server.

3.2.7 Statistical analysis

3.2.7.1 Descriptive Statistics

The proportion of *Salmonella* positive (binary outcome) fecal samples and the proportion of isolates resistant to greater than or equal to 3 classes of antimicrobials, or else pan-susceptible by sampling day and by treatment group were cross-tabulated in Stata® version 12.1 (StataCorp LLC, College Station, TX). Crude associations between the serotypes, sampling days, and treatment groups were initially tested via the Likelihood-ratio based Chi-Square test or the Fisher's exact test for rare combinations. Graphics for descriptive statistics were created on Tableau Desktop Professional Edition 10.3.2.

3.2.7.2 Multivariable analysis

Prevalence of *Salmonella* (a binary variable) was modelled using a multilevel mixed-effects logistic regression model (-melogit- in Stata® ver. 15.1) considering replicate (2 replicates), pen (16 pens) and animal identifier (176 animals) as potential clustering variables with repeated observations. Following initial assessment of these three potential sources of over-dispersion, all three variables were included as significant ($P < 0.05$) random effects in the final statistical model. A 3-way full factorial statistical model incorporating fixed effects for CCFA (mixing) and CTC antibiotic regimens and sampling day was built.

The final model for the prevalence of MDR *Salmonella* (a binary variable) was similarly modelled using multilevel mixed-effects logistic regression (-melogit- in Stata® ver. 15.1). Replicate, pen, and animal identifier were considered as potential clustering variables. Following the initial assessment, replicate was instead included as a fixed effect in the final model since repeated observations within animal identifier was not significant in the presence of pen level effects. In addition to replicate, a statistical model incorporating the fixed effects for each of the CCFA and CTC antibiotic regimens, sampling day, and 2-way interactions between day and antibiotic regimens was built. Marginal mean estimates from the final models were produced and graphical representations of the temporal dynamics were plotted.

3.2.8 Data Availability

The datasets generated during and/or analyzed during the current study are not publicly available due to on-going sequencing analyses at the time of publication; however, they will

become available from the corresponding author upon request. The sequencing data will be publicly available on NCBI as a Bioproject upon completion of all publications.

3.3 Results

3.3.1 Descriptive statistics of Salmonella *among fecal samples of feedlot cattle*

In total, *Salmonella* were recovered from 566 out of 1,040 fecal samples. The mean *Salmonella* prevalence before the antibiotic treatments began (day 0) was 75.0% (95% CI: 67.9–81.2%), with 132 out of 176 samples testing positive. Among the two groups in which all cattle were treated with CCFA (All-CCFA / no CTC, All-CCFA & CTC), the prevalence of *Salmonella* was 34.1% (15 positive out of 44 samples) and 27.3% (12 positive out of 44 samples) on day 4, respectively. By day 14, the *Salmonella* prevalence in cattle receiving no subsequent CTC treatment (All-CCFA / no CTC; 31 positives out of 43 samples, 72.1%) was similar to that of those steers with the least antibiotic exposure (1-CCFA / no CTC; 35 positives out of 44 samples, 79.5%) as shown in Figure 9 (second row). When CTC treatment followed CCFA treatment, the prevalence dropped even further to 16.3% by day 14 (All-CCFA & CTC; 7 out of 43 positive). By day 26, the prevalence in both CTC treatment groups (1-CCFA & CTC, All-CCFA & CTC) returned to 47.1% (40 out of 85 positive). By comparison, the overall mean prevalence among steers in those pens that received the least antibiotic treatment (1-CCFA / no CTC) was estimated at 72.7% (95% CI: 66.8–78.0%) throughout the study period.

3.3.2 Descriptive statistics of Salmonella *isolates resistant to antibiotics*

The total number of *Salmonella* isolates and the percentage of MDR *Salmonella* (defined as resistant to ≥ 3 antimicrobial classes) for each sampling day by antibiotic treatment groups

are shown in an inner circle of Figure 9. Among the steers with the least exposure to antibiotic treatment, the vast majority of isolates remained pan-susceptible throughout the study period, although 3.2%, 5.7%, and 3.8% of multidrug resistant *Salmonella* isolates were detected on days 4, 14 and 20, respectively (Figure 9, first row). Among the group in which all cattle were treated with CCFA on day 0, 26.7% of *Salmonella* isolates were MDR on day 4 and 22.7% were MDR on day 8; however, MDR prevalence thereafter declined by day 14 (Figure 9, second row). In the group treated with CTC (1-CCFA & CTC) starting from day 4, pan-susceptible isolates decreased and MDR isolates increased (73.3%) on day 8 and further increased to 80% and 100% on days 14 and 20, respectively (Figure 9, third row). By the end of the study (day 26), 50% of the isolates in this latter group remained MDR. Among animals sequentially receiving both CCFA and CTC treatment, the pattern of MDR dynamics resembled that of the treatment groups receiving CTC (Figure 9, fourth row).

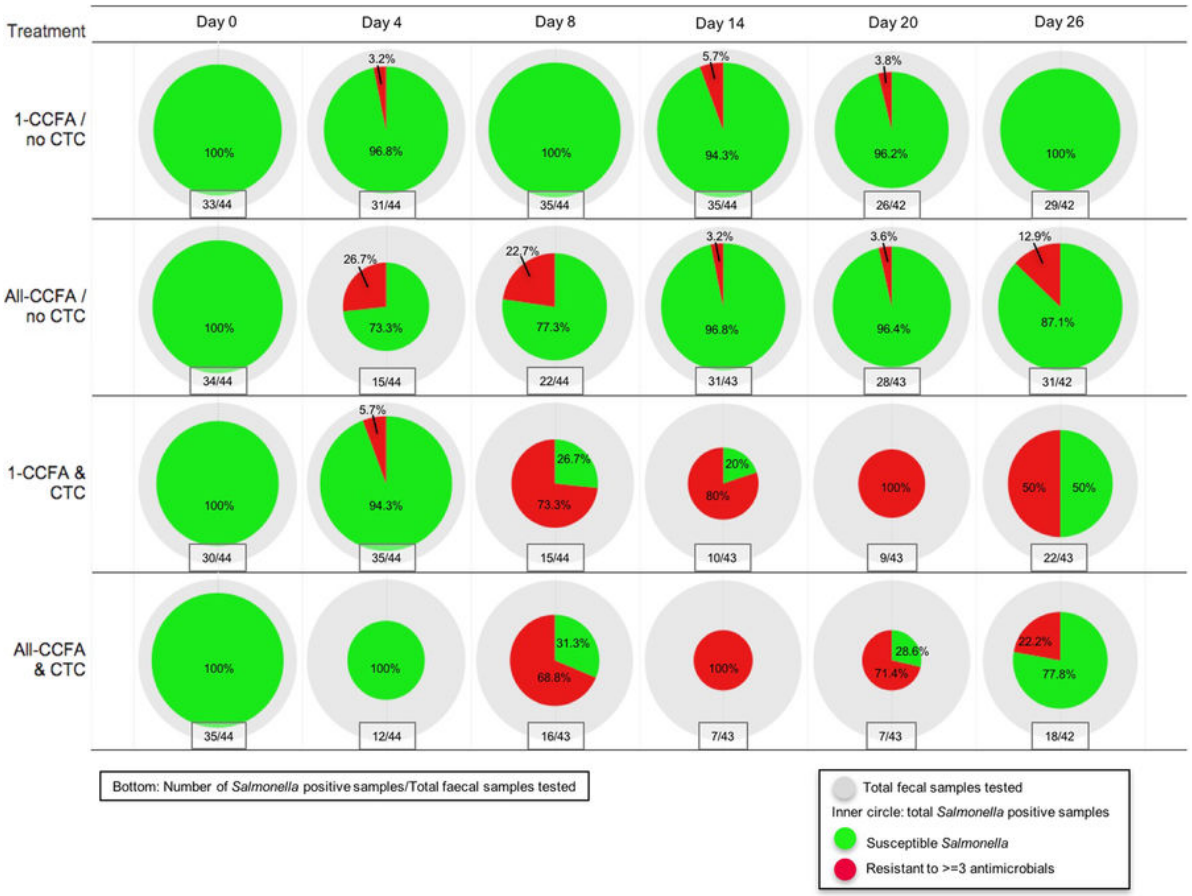


Figure 9. Total number of isolates and proportion of MDR isolates per day and treatment group The outer circle (grey) reflects the number of fecal samples tested per pen each sampling day (n = 42 to 44). The size of the inner circle corresponds to the number of *Salmonella* isolates obtained each sampling day. The green piece of the inner circle represents pan-susceptible *Salmonella* and the red piece represents resistance to 3 or more classes of antimicrobials (MDR). Total number of the *Salmonella* positive samples and total fecal samples tested are shown below each pie in the text box. Percentages of pan-susceptible and MDR isolates are shown inside the circle.

3.3.3 Mixed effects logistic regression model of Salmonella *prevalence and MDR*

Salmonella *prevalence*

CCFA and CTC treatments were coded as binary variables, with 0 (no treatment) used as the referent category. Days were 0, 4, 8, 14, 20, and 26, with day 0 used as the referent. CCFA

treatment effects were highly significant on days 4 ($p < 0.004$) and 8 ($p < 0.003$). CTC treatment significantly decreased the prevalence of *Salmonella* on days 8 ($p < 0.004$), 14 ($p < 0.001$), and 20 ($p < 0.024$) when compared to day 0 (Figure 10).

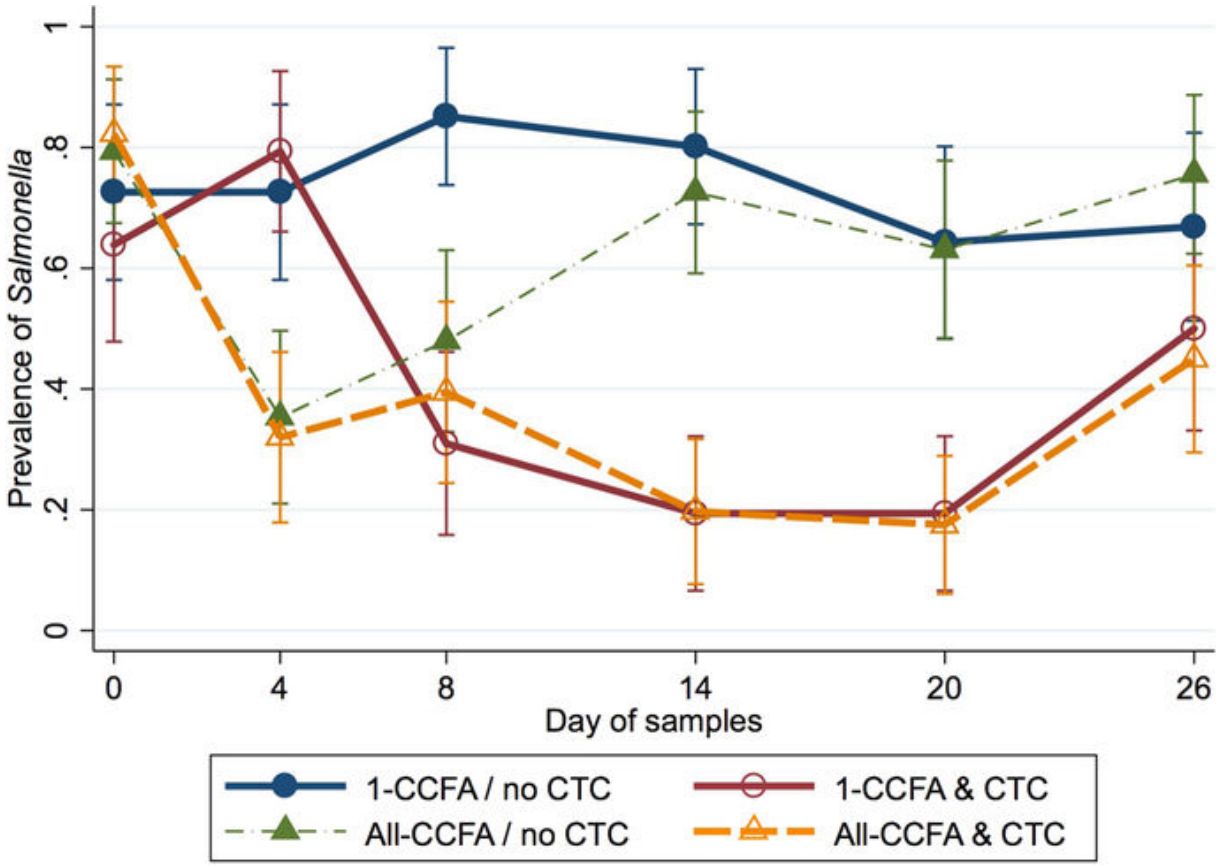


Figure 10. Modelled marginal mean prevalence of *Salmonella* by day and treatment group.
Modelled marginal mean prevalence of *Salmonella* and 95% confidence intervals in cattle fecal samples by sample day and treatment group.
Solid lines represent treatment groups in which a single CCFA treated steer was mixed within an otherwise untreated group while dashed lines represent groups in which all steers received CCFA treatment.

The prevalence of MDR *Salmonella* was modelled similar to that of overall *Salmonella* prevalence, as above, except that day 4 was used as a referent category. This is because MDR isolates first appeared on day 4, yielding unstable model parameters for day 0. Interaction between CTC treatment and day was associated with an increase of MDR *Salmonella* from day 8 ($p < 0.008$) to day 26 ($p < 0.041$) (Figure 11). CCFA treatment on day 0 further increased MDR *Salmonella* probability on day 8 in the All-CCFA & CTC treated groups, compared with the group treated only with CTC. A slight increase of MDR *Salmonella* was seen on day 4 in the CCFA-only treated group, compared to the referent group (1-CCFA / no CTC); however, this was not significant ($P = 0.159$).

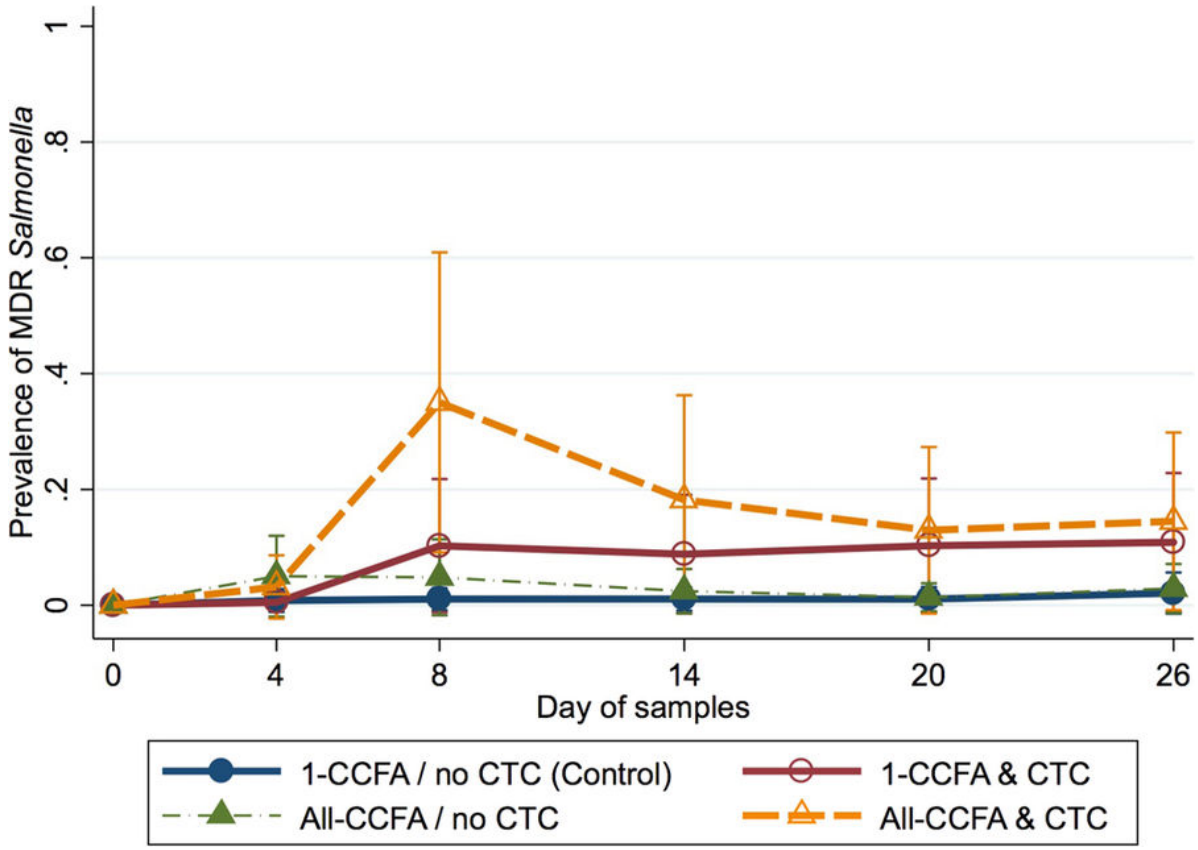


Figure 11. Modelled marginal mean prevalence of MDR *Salmonella* by day and treatment group. Modelled marginal mean prevalence of *Salmonella* and 95% confidence intervals in cattle fecal samples by sample day and treatment group. Solid lines represent treatment groups in which a single CCFA treated steer was mixed within an otherwise untreated group while dashed lines represent groups in which all steers received CCFA treatment.

3.3.4 Identification of serotypes and MLST

Salmonella serotypes were identified by whole-genome sequencing using the SeqSero pipeline [96], which determines the serotype based on the sequence of the O-antigen gene cluster and H1 and H2 antigens. Six serotypes were detected; the most common serotype was *Salmonella* Mbandaka (38.0%), followed by *S.* Give (19.1%), *S.* Kentucky (13.6%), *S.* Reading (15.2%), *S.* Montevideo (13.4%), and *S.* Anatum (0.7%) as shown in Table 6.

Table 6. Multi-Locus Sequence Type (MLST) and serotypes of the *Salmonella* isolates

Serotype	Number of isolates	Antigenic profile from Seqsero				MLST	Gene Alleles						
		O	H1 (*fli*C)	H2 (*flj*B)	Predicted profile		*aro*C	*dna*N	*hem*D	*his*D	*pur*E	*suc*A	*thr*A
Anatum	4 (0.7%)	O-3,10	e,h	1,6	3,10:e,h:1,6	64	10	14	15	31	25	20	33
Kentucky	77 (13.6%)	O-8	i	z6	8:i:z6	198	76	14	3	77	64	64	67
Mbandaka	215 (38.0%)	O-7	z10	e,n,z15	7:z10:e,n,z15	413	15	70	93	78	113	6	68
Montevideo	76 (13.4%)	O-7	g,m,s	-	7:g,m,s:-	138	11	41	55	42	34	58	4
Give	108 (19.0%)	O-3,10	l,v	1,7	3,10:l,v:1,7	654	111	47	49	42	12	58	3
Reading	86 (15.2%)	O-4	e,h	1,5	4:e,h:1,5	1628	46	60	10	9	6	12	17

All serotypes and MLST data matched and no divergent serotypes were detected from any single MLST (Table 6). *Salmonella* Reading was detected starting on day 4, and the prevalence increased greatly by day 8 in the All-CCFA / no-CTC groups, and especially in the 1-CCFA & CTC and All-CCFA & CTC treatment groups (Figure 12), where its presence extended well past day 8. Five serotypes, except *S*. Reading, were identified on day 0. In the 1-CCFA / no CTC group, the diverse composition of serotypes remained similar throughout the study. The *S*. Reading isolate detected on day 4 in the 1-CCFA / no CTC group was derived from the single steer that received CCFA and was mixed in the group comprised of 10 other non-CCFA-treated cattle; however, the *S*. Reading isolates from day 14 and 20 were from steers that were not treated with antibiotics. In the All-CCFA/no CTC group, the susceptible serotypes (*S*. Anatum, Give, Kentucky, Mbandaka, Montevideo) decreased on day 4 while *S*. Reading was increasingly

present. In the 1-CCFA & CTC group, all isolates were *S*. Reading on day 20; however, susceptible serotypes were once again detected on day 26. The All-CCFA & CTC group exhibited less serotype diversity and *S*. Montevideo was detected only on day 0 in this group. More *S*. Reading were isolated from the treatment groups that received CTC. Serotype distribution was similar between replicates 1 and 2, except that *S*. Anatum was identified only in replicate 1 (Figure 13). The most common serotype detected in the 1-CCFA / no CTC group was *S*. Mbandaka and *S*. Reading was the least detected serotype (Table 7). The most common serotype detected in All-CCFA & CTC group was *S*. Give followed by *S*. Reading. In the 1-CCFA & CTC group, overall 33.1% were *S*. Reading.

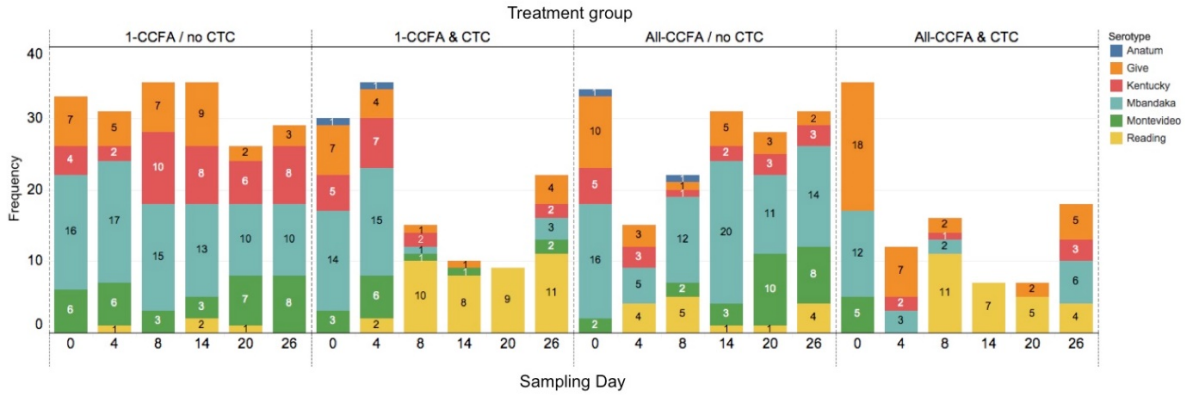


Figure 12. Number of *Salmonella enterica* isolates within serotypes by treatment group and sample day. Six serotypes (Anatum, Give, Kentucky, Mbandaka, Montevideo, and Reading) were found among tested fecal samples. Numbers shown in the bars are the number of isolates for each serotype by treatment group across both trial replicates.

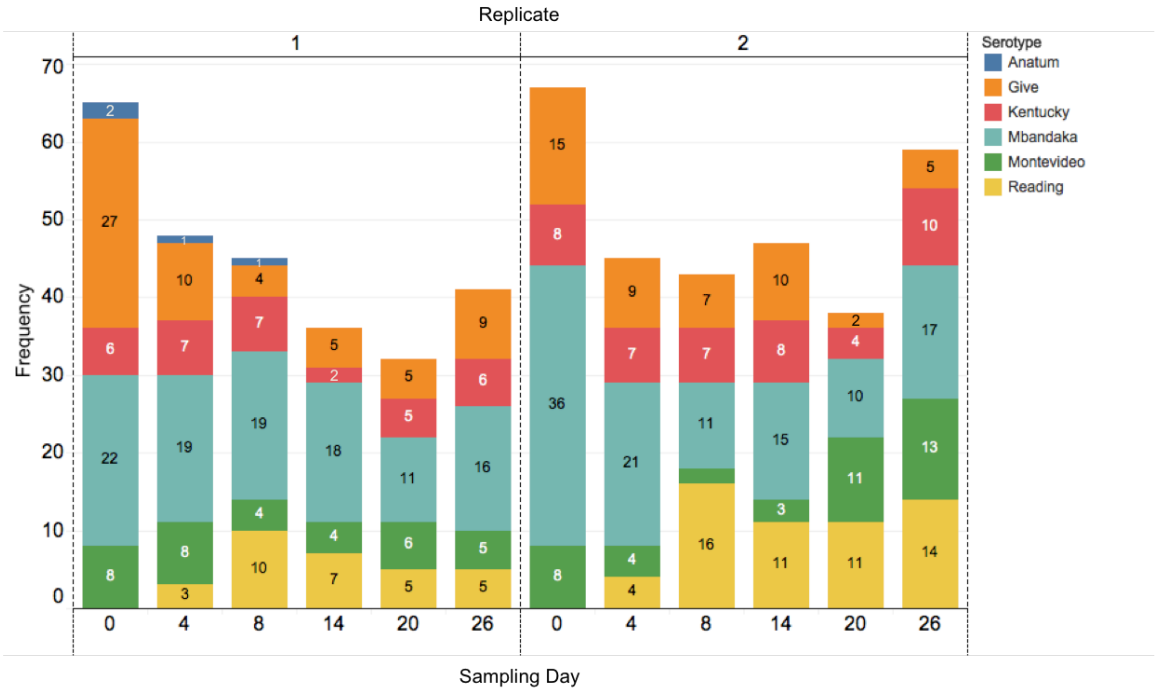


Figure 13 Distribution of *Salmonella enterica* isolates within serotypes by replicate and day of sampling across all treatment groups.

For quality control purposes, 2 isolates randomly chosen from each of the 5 serotypes identified by whole-genome sequencing (WGS) were sent to the National Veterinary Services Laboratories for traditional serotyping. All traditional serotyping results matched exactly with sequence based serotyping results.

3.3.5 Associations of phenotypic antimicrobial resistance profile and serotypes of Salmonella

All isolates (n = 566) were tested against a standard NARMS panel that included 14 antimicrobials arising from 9 antibiotic classes. Serotype and resistant phenotypes were

significantly associated ($p < 0.05$). All the *S.* Anatum, *S.* Give, *S.* Mbandaka and *S.* Montevideo were phenotypically pan-susceptible. Nearly all (96.5%) of the *S.* Reading isolates had at least the penta-resistant profile, ACSSuT (resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline), with additional resistance to the 3rd generation cephalosporins, ceftiofur and ceftriaxone (Table 7). The other 3.5% of Reading isolates detected from the 1-CCFA & CTC group were not resistant to sulfonamides. The *S.* Reading resistance profiles were AMP-AUG2-AXO-FOX-TIO-STR-CHL-TET for 8 antimicrobials and AMP-AUG2-AXO-FOX-TIO-FIS-STR-CHL-TET for 9 antimicrobials. One *S.* Kentucky isolate from the 1-CCFA & CTC group was resistant to 3 antimicrobials: STR, FIS, and TET (Table 7). No resistance to azithromycin, ciprofloxacin, gentamicin, or nalidixic acid was detected.

Table 7. Proportion of *Salmonella* serotype and AMR phenotype by treatment group

Serotype	Serotype per Treatment Group				AMR Phenotype
	1-CCFA / no CTC	**1-CCFA & CTC**	**All-CCFA / no CTC**	**All-CCFA & CTC**	
Anatum (n=4)	0.0% (0)	1.7% (2)	1.2% (2)	0.0% (0)	Pansusceptible: 100.0% (4)
Give (n=108)	17.5% (33)	14.0% (17)	14.9% (24)	35.8% (34)	Pansusceptible: 100.0% (108)
Kentucky (n=77)	20.1% (38)	13.2% (16)	10.6% (17)	6.3% (6)	Pansusceptible: 98.7% (76) **STR-SUL-TET: 1.3% (1)**
Mbandaka (n=215)	42.9% (81)	27.3% (33)	48.4% (78)	24.2% (23)	Pansusceptible: 100.0% (215)
Montevideo (n=76)	17.5% (33)	10.7% (13)	15.5% (25)	5.3% (5)	Pansusceptible: 100.0% (76)
Reading (n=86)	2.1% (4)	33.1% (40)	9.3% (15)	28.4% (27)	**AMP-AUG2-AXO-FOX-TIO-SOX-STR-CHL-TET: 96.5% (83)** **AMP-AUG2-AXO-FOX-TIO-STR-CHL-TET: 3.5% (3)**
Total (n=566)	100.0% (189)	100.0% (121)	100.0% (161)	100.0% (95)	

3.3.6 Serotype pattern per individual animal

Salmonella serotypes that were detected in the same animal from different sampling days were aggregated to identify the serotype patterns (Figure 14). In one steer, 5 serotypes (*S.* Give, Kentucky, Mbandaka, Montevideo, and Reading) were detected across the different sampling days. In 4.5% (8 steers) of animals, 4 serotypes with different combinations were

detected. Three serotypes were detected from 20.5% (36 steers) of animals and 2 serotypes were detected from 38.6% (68 steers) of animals. A single serotype was detected from 34.7% (61 steers) of animals. No steers were detected with *S*. Reading alone; that is, it was always detected with other serotypes across different sampling days (Figure 15). Four steers were classified as not harboring any *Salmonella* throughout the study period.

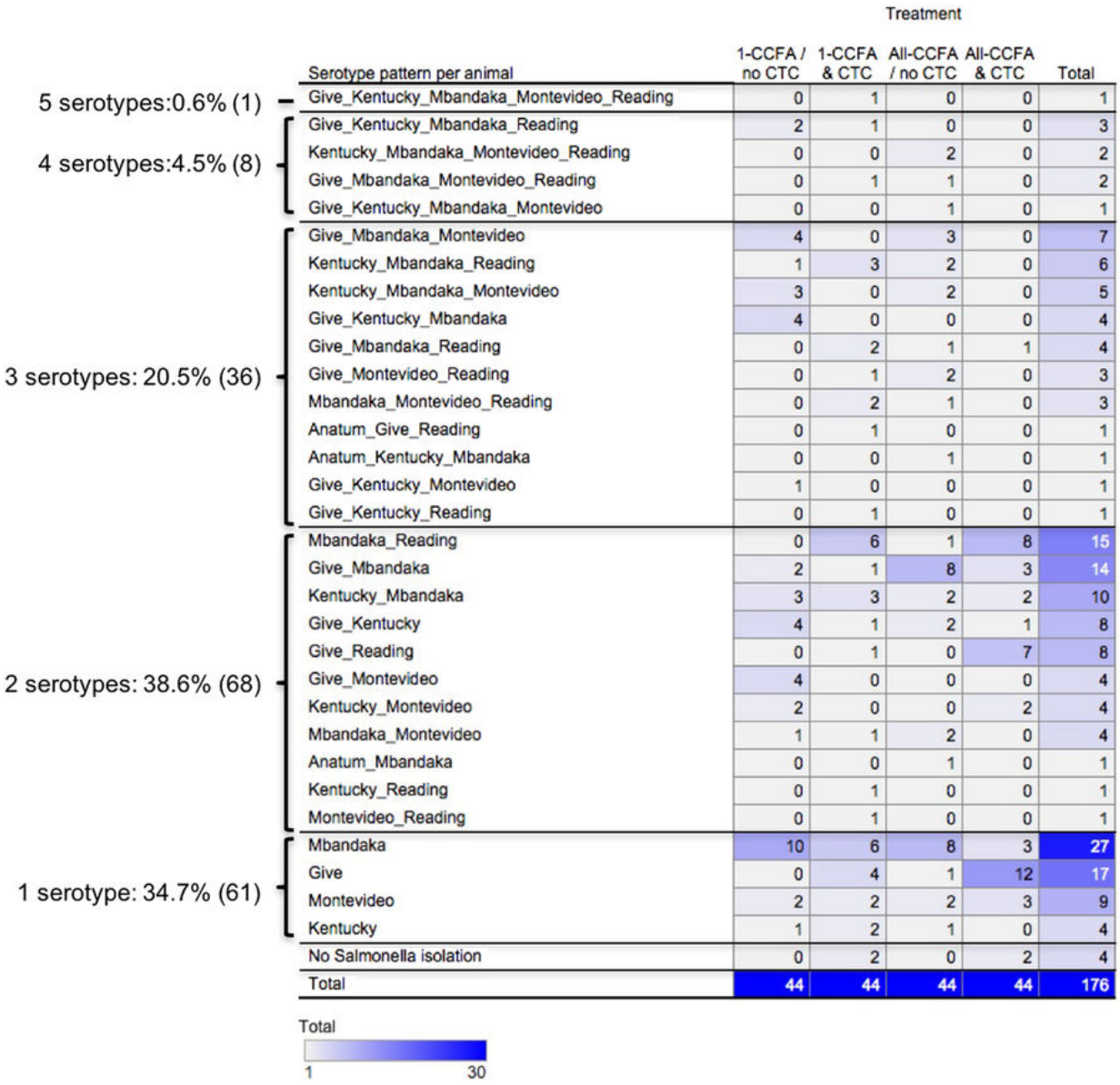


Figure 14. Total number of animals harboring each serotype pattern across all sampling days. Serotype patterns were clustered per treatment group across different sampling days. Darker blue indicates more animals with specific serotype patterns; conversely, lighter blue shaded serotype combinations had fewer animals.

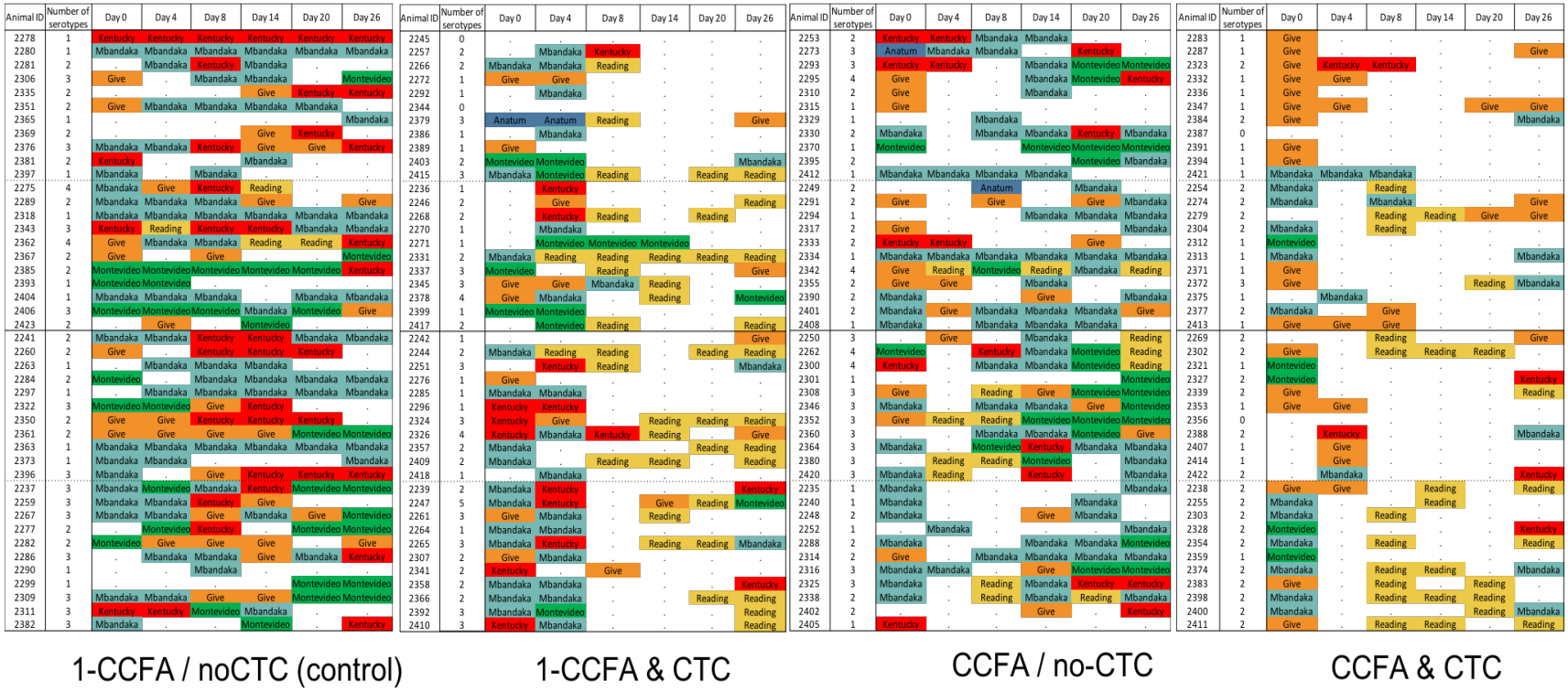


Figure 15. *Salmonella* serotypes detected per individual animal per day and treatment group
Red: Kentucky; orange: Give; green: Montevideo; teal blue: Mbandaka; yellow: Reading; dark blue: Anatum

3.4 Discussion

This randomized, controlled, longitudinal field trial has clearly demonstrated that the use of antibiotics shifts the antibiotic resistance status of the *Salmonella* population by selecting for MDR *Salmonella* and against the pan-susceptible *Salmonella* serotypes that are highly prevalent in beef cattle in this region of the USA. Since resistance phenotype and *Salmonella* serotype are so strongly associated, this effectively means that antibiotic use selects for particular serotypes. Chlortetracycline treatment alone, or with prior treatment with a 3rd generation cephalosporin, decreased the overall prevalence of *Salmonella*; further, each of these treatments increased the proportion of MDR *Salmonella* that remained. Ceftiofur treatment alone did reduce the prevalence of *Salmonella*; however, the reduction was relatively transient. The MDR phenotypic pattern was virtually identical among the *S.* Reading isolates (Table 7). Six *Salmonella* serotypes, which have commonly been reported in feedlot cattle and the feedlot environment, were detected in the fecal flora of these cattle [153, 254-256]. As a point of reference, *Salmonella* Anatum, Montevideo, and Kentucky accounted for 50.4% of the serotypes detected in the National Animal Health Monitoring Systems (NAHMS) Feedlot 2011 study [256].

We found a high prevalence of *Salmonella* in the feces of feedlot cattle prior to antibiotic treatment. Previous attempts to determine the dynamics of *Salmonella* in experimental studies were likely not successful due to a low prevalence overall, and of resistant *Salmonella*, in the study population; thus, requiring a vast number of animals on trial to have enough power for the analyses [209, 213]. Even in large-scale observational studies, the prevalence can vary a great deal, illustrating that in many U.S. locations the prevalence is very low. The NAHMS

Feedlot 2011 study reported that the overall pen-level prevalence of *Salmonella* in feedlot cattle across the United States was 35.6%, with 9.1% sample (cattle)-level prevalence [154].

Our study successfully showed that the *Salmonella* population changes in response to antibiotic treatments, in part due to the high prevalence at the beginning of the study (i.e., 70 + %). Some of the factors explaining the high initial prevalence in our study include geography (southern United States) and season (August-October). Several studies have shown a seasonal variation in *Salmonella* prevalence, such that sampling during the summer months yields the highest percentage of *Salmonella* positive samples [155, 156, 159, 257]. One study from North Dakota found 62.2% prevalence of *Salmonella* in the tested herd during spring months, but only one serotype *S.* Typhimurium var. Copenhagen was isolated from the entire herd [258]. It is possible that if our study had been conducted during the colder months of the year, such an obvious change in the *Salmonella* population might not have been observed.

The *Salmonella* prevalence and serotype populations differ by region, country, and ambient environment which implies that the resistance phenotype will vary at the same time [154]. Therefore, our results may not necessarily be generalized to feedlots at other geographic locations. Sampling only from fecal grabs might not be an ideal method to determine the overall prevalence in feedlot cattle. One study showed that a combination of samples from perineum swab and fecal grab samples increased the prevalence of *Salmonella* to 88% from 50% versus when only fecal grab was tested for the prevalence [259]. We collected the fecal samples only by fecal grabs, which may return false negative results if *Salmonella* are distributed unequally within the feces.

Several observational and experimental studies have explored the association between antimicrobial use (including ceftiofur) and the temporal dynamics of resistant *E. coli* and

Salmonella in cattle and pigs [30, 209, 211, 214-217, 260, 261]. One-time CCFA treatment (as is typically used to control respiratory disease in beef feeder cattle) on day 0 reduced the overall prevalence of *Salmonella* in feces. CCFA is not labeled for the control of *Salmonella* in cattle; however, its broad-spectrum nature, presence in multiple tissues, and effectiveness against Gram-negative bacteria appears to result in a temporal decrease of *Salmonella* prevalence immediately following treatment. The dose of CCFA (6.6 mg/kg) used in the current study was for the treatment and control of bovine respiratory diseases, such that the serum concentration is maintained over the minimum inhibitory concentration (0.2 μg/ml) for up 10 days following a single-dose administration. A previous study illustrated that an extra-label regimen of ceftiofur (5 mg/kg, intramuscularly) decreased the detection and quantity of *Salmonella* and effectively treated salmonellosis in neonatal calves [262].

In our study, CCFA treatment also increased the proportion of MDR *Salmonella* on days 4 through 8. Despite only receiving a single dose of CCFA, by the end of the study (day 26) 12.5% of isolated *Salmonella* remained multidrug resistant (Figure 9). With the follow-up of CTC treatment to earlier CCFA treatment, MDR isolates increased further to 75% of total *Salmonella* by day 14. We chose to explore day 14 to examine the status of antibiotic resistant *Salmonella* in cattle feces on the first day post-treatment that the animals were eligible to be sent to slaughter based on residue avoidance; that is, because the labelled slaughter withholding time of CCFA is 13 days. Our findings indicate that at the point at which this compliance requirement is met, MDR *Salmonella* prevalence persists far above the baseline starting values, a finding that has not been previously reported. However, we are well aware that such a scenario is highly unlikely in real feedlot settings; that is, CCFA treatment at the whole pen level is extremely unlikely to occur anytime close to when cattle are sent to

slaughter. Typically, fed cattle would instead be sent to slaughter at least 6 months post-arrival. To understand longer-term dynamics, additional studies are needed to further investigate the effects of CCFA and CTC treatments from treatment in the early feeding period through to slaughter.

While CTC has been used for many decades in animal agriculture for prevention and growth promotion purposes, the effects of therapeutic doses of CTC on the prevalence and resistance of *Salmonella* in cattle have not been studied extensively. CTC was added to the feed (as a top-dress) for 3 pulses of 5 continuous days, with a one-day interval in between. This regimen was designed to observe the maximum effect of CTC, to follow the product label in the U.S., and to be consistent with a previous study published by Platt *et al*. [34]. CTC treatment alone reduced *Salmonella* prevalence to the same levels as cattle first injected with CCFA and then subsequently treated with CTC. This further suggests that the initial CCFA treatment did not have a significant long-term effect on *Salmonella* prevalence. It is possible that the initial CCFA may have selected for MDR *Salmonella*, which will be discussed later.

The effects of CTC given at therapeutic or sub-therapeutic doses on pathogens including *Salmonella* have been reported in pigs [15, 263]. Although not statistically significant, pulsed CTC feeding during the finishing period lowered *Salmonella* prevalence in pigs [263]. Wells *et al*. have shown that CTC supplemented in the diet reduced the prevalence of both *Campylobacter* and Shiga-toxin producing *E. coli* [15]. Prevalence of *Salmonella* was at a negligible level in their study. A study by Agga *et al*. found that one-set of 5-day CTC in-feed treatments transiently increased tetracycline resistant *E. coli* concentration in the fecal swabs of feedlot cattle. Importantly, generic *E. coli* concentrations in the CTC treatment group

remained the same, thus indicating that tetracycline resistant *E. coli* effectively replaced the susceptible *E. coli* population [63].

Previous work by our group illustrated an inexplicable and paradoxical reduction of ceftiofur resistant *E. coli* via CTC treatment (despite mechanistic potential for co-location via *tet*(A) and $bla_{\text{cmy-2}}$ genes housed on a single IncA/C plasmid in many *E. coli*). We explored the potential that this might serve as a potential intervention to reduce ceftiofur resistance by instead favoring singly resistant *E. coli* strains harboring the *tet*(B) gene on the bacterial chromosome [34, 35]. In our current study, three pulsed (intermittent) CTC treatments displayed a stronger selection pressure on MDR *Salmonella* than CCFA treatment alone, which was completely the opposite of the previous published work exploring the effects of these same regimens in *E. coli* populations [35]. In that study, fecal *E. coli* from CCFA administrated pens showed more resistance to a full range of antibiotics than in cattle from the pens administered only CTC (the latter effect was negligible) [35]. As mentioned, while Agga *et al*. showed that a 5-day single-pulsed CTC treatment increased tetracycline resistant *E. coli* in fecal swabs of beef cattle at 5 days-post-treatment, no differences in cephalosporin resistant *E. coli* were found [63]. It is possible that the resistant *E. coli* population were different between these two studies. Comparatively, phenotypic resistance patterns and sequence type (or PFGE patterns) of *E. coli* in earlier studies of food animals are often diverse, while *Salmonella* are often either pan-susceptible or else MDR (assuming they are present at all) [35, 211, 255, 264].

Importantly, in *Salmonella*, drug resistance phenotypes and serotypes are much more highly associated than is seen across the more prevalent and diverse populations of *E. coli* [255, 265, 266]. These differences in resistance patterns may indicate that *Salmonella* and *E.*

coli are not regularly sharing their resistance elements in the cattle gut microbiome [264]; conversely, they also might not be sharing the same ecological niches in the varying cross-sections of intestinal regions [81]. One previous experimental study suggested that the use of ceftiofur in dairy cattle did not promote the transfer of $bla_{\text{CMY-2}}$ coding plasmids among *Salmonella* and commensal *E. coli* in calves inoculated with plasmid-bearing bacteria and in dairy herds using ceftiofur [30]. However, another study found that the presence of an inflammatory condition in the gut could boost horizontal gene transfer between *Salmonella* and *E. coli* [267], something that is often lacking in studies involving healthy animals. Although limited to phenotypic resistance, our study reveals that *Salmonella* and *E. coli* isolated from the same gut microbiome did not share similar resistance patterns, either at baseline or during specified treatment periods and across treatment regimens [35].

We detected 6 serotypes in our study population; in decreasing overall prevalence, these were *Salmonella* Mbandaka, Give, Kentucky, Reading, Montevideo, and Anatum. The serotypes detected in this study were consistent with those published in previous studies and isolated from the lymph nodes, hides, and feces of feedlot and dairy cattle from the same region of Texas [150, 151, 159, 160, 167, 255]. *Salmonella* serotypes often appear to adapt to specific animal hosts. Among human clinical isolates reported to the CDC, *S.* Montevideo was 10th among frequently reported serotypes in 2013 [21]. In the current study, *S.* Mbandaka (serogroup C1) was the dominant serotype followed by Give (serogroup E1). The dominance of certain serotypes has been observed in other studies in feedlot cattle as well [151, 160]. However, it is possible that *Salmonella* enrichment via RV media may bias the detection towards serogroups C1, C2, and E [21]. We detected *S.* Reading, which belongs to serogroup B, but none of these were pan-susceptible. It remains a possibility that pan-susceptible *S.*

Reading isolates were not detected in our study due to enrichment bias or else the resistant *S.* Reading population was stable in the gut microbiota but was not able to outcompete the susceptible strains prior to antibiotic pressure. Our study has illustrated that serotype and MDR are often strongly associated. Most (96.5%) *S.* Reading isolates had the ACSSuT resistance profile, while the other serotypes did not; this agrees with previous work that suggests that *S.* Reading is more likely to be of the ACSSuT resistance phenotype than other serotypes [159, 167]. It remains unclear why certain serotypes are more highly resistant to antimicrobials, while others are not. However, all these cited studies suggest that the antimicrobial susceptibility of the various *Salmonella* serotypes might be determined not only by their genetic predispositions, but also by coexisting serotypes as well as environmental factors, such as antimicrobial selection pressure.

The first and second replicates of these cattle trials were conducted at the same feedlot with a one-month interval between the studies. Cattle were housed in porous-fenced pens through which contact could be made with cattle in adjacent pens. Treatments were randomly assigned to balance the contact potential across pens; however, sterility could not be maintained between pens in such a field setting as is standard in the beef feeding industry. The serotype distribution of the first and second replicates was similar throughout the study and no *S.* Reading was found on day 0, even in the second replicate (Figure 13). This latter point strongly suggests that MDR *Salmonella* were transiently detected solely due to the antibiotic pressure applied during this trial. All those steers that were detected with *S.* Reading on at least one sampling day also had other serotypes isolated during at least one other sampling day (Figure 14, 15). Since these serotypes were not detected at the same time point (i.e., since we limited the number of colonies we assayed from each plate), it does not

necessarily mean that the steers were infected with 2 or more serotypes at once; however, this point does support the idea that *S.* Reading likely co-resided with other serotypes under "normal" non-antibiotic selection pressure conditions, and was more readily detected after the antibiotic treatments.

The steer from which 5 different serotypes were isolated on 5 different days illustrates the complexity of *Salmonella* colonization in cattle; further, this is consistent with another study in dairy cattle that showed multiple serotype colonization per animal [255, 268, 269]. In agreement with our study, confirmation of *Salmonella* infection or colonization sometimes requires multiple testing methods, enrichment, and sequential sampling time-points. Four steers in our study were negative for *Salmonella* throughout the whole study period (26-days); even after this, these could be interpreted as being below the detection limit or else intermittent shedding of *Salmonella* or as being truly negative for *Salmonella* colonization.

One limitation of this approach was that we terminated the study on day 26 and thus did not track the cattle to the age at which they would be sent to slaughter; that is, when fecal, hide, and lymph node *Salmonella* populations would represent a greater potential threat to public health. The feedlot is the final production stage for beef cattle, and thus represents a more proximate source of MDR *Salmonella* contamination at the slaughterhouse than occurs earlier in the beef production cycle [165]. In the future, we plan to conduct a similarly designed randomized controlled study with follow-up extending to slaughter approximately 150 days post-treatment. We currently hypothesize that any temporal antibiotic treatment effects will wane over such an extended period of time, yielding few, if any, differences among the treatment groups by the time animals are ready for slaughter as shown in previous studies in *E. coli* populations [63, 211].

In conclusion, we found that CTC and CCFA treatments dramatically decreased overall *Salmonella* prevalence in feedlot cattle; however, multi-drug resistant *Salmonella* strains proliferated after antibiotic susceptible *Salmonella* population were killed. Even after the mandatory slaughter withholding period for ceftiofur crystalline-free acid was complete at 13 days post treatment, the prevalence of MDR *Salmonella* remained elevated when compared to baseline levels, suggesting it may be important to consider the prevalence of MDR isolates when exploring adaptive withholding periods for antibiotics in the future. When new classes of antibiotics are introduced to the market, there may be no known resistance elements circulating in bacteria. However, after decades of use the situation will change which may warrant consideration of a microbial safety endpoint in addition to residue avoidance parameters. *Salmonella* serotypes and antibiotic resistance phenotypes displayed strong associations and suggest that specific serotypes may be more likely to carry MDR genes. Although it was transient, this study indicates that the use of CCFA and CTC exerts a strong selection pressure on the *Salmonella* populations in the gut of feedlot cattle; therefore, judicious use of antimicrobials is necessary for beef cattle feeding operations to aid in preventing increased levels of MDR *Salmonella* being present in cattle destined for slaughter.

CHAPTER IV

QUANTITATIVE DYNAMICS OF SALMONELLA IN FECES OF FEEDLOT CATTLE TREATED WITH CEFTIOFUR AND CHLORTETRACYCLINE

4.1 Introduction

The objective of this chapter is to explore the quantitative dynamics of *Salmonella* impacted by antibiotic treatments in feedlot cattle. The first part describes a comparison of three detection and quantification methods including hydrolysis probe real-time qPCR (qPCR), direct spiral plating, and broth enrichment followed by spiral plating. We also compared quantitative results arising from qPCR versus direct spiral plating. In the second part, utilizing the quantitative data from qPCR and direct culture, we analyzed the temporal change of *Salmonella* and antibiotic resistant *Salmonella* quantity impacted by antibiotic treatments from Day 0 to Day 26. Other variables affecting *Salmonella* quantitative dynamics, in addition to antibiotic treatments, were investigated.

Salmonellosis is one of the most common foodborne diseases in the United States. Detection of *Salmonella* in retail beef has been low (0.4-2%) in reports from the National Antimicrobial Resistance Monitoring System (NARMS) from 2002 to 2015. However, retail beef is one of the primary sources of *Salmonella* infection for humans *via* food in the United States and multiple outbreaks have been reported due to consumption of under-cooked ground beef [32, 146]. The finishing period for cattle occurs in the feedlot and this is where *Salmonella* can spread *via* fecal-oral route or the environment (e.g., feed and flies) between infected and uninfected cattle residing in the same pen [270, 271]. Additionally, a few calves may be vertically infected from their dams [272]. Most often, cattle are asymptomatic carriers, and no clinical signs are shown. Some cattle are super-shedders (i.e., those that shed

$>10^4$ CFU per gram of feces) and may spread *Salmonella* to other cattle in the pen and beyond. An intermittent shedding pattern makes it more difficult to control the dissemination of *Salmonella* among cattle. Currently, vaccines for *Salmonella* Newport (*S.* Newport) and *S.* Dublin are available; however, only 6.5% of feedlots vaccinate for *Salmonella* because of the expense [45]. Additionally, no antibiotics are approved for the treatment and control of *Salmonella* because of the risk that antibiotic use might increase colonization with antibiotic-resistant *Salmonella*.

Salmonella contamination of retail meat mainly occurs at the slaughterhouse through fecal contamination of hides and from infected lymph nodes that are incorporated into batches of ground beef [159, 180, 181, 273-275]. Several quantitative studies have shown higher prevalence of *Salmonella* on hides than in feces at slaughter; therefore, hides and lymph-nodes are likely sources of carcass contamination [13, 159, 276]. Since *Salmonella* is not considered an adulterant in raw meat by the USDA, it is crucial to decrease the overall *Salmonella* quantity that enters the slaughterhouse rather than rely on punitive product recalls to effect changes at the plant. Additionally, contamination of meat with antibiotic resistant *Salmonella* is a serious public health concern due to there being fewer treatment options for infected individuals, especially infants, children, pregnant women, and immunocompromised persons.

In lieu of fluoroquinolones, which may cause musculoskeletal disorders, cephalosporins are the choice of treatment for these high-risk populations. Approximately 80% of cephalosporins that are sold for food animals are used in cattle production [8]. Since the feedlot is the final stage where cattle receive antibiotics for disease treatment, control, and prevention, it is crucial to control the antibiotic resistant *Salmonella* pre-harvest to reduce

Salmonella entering slaughter. Proper intervention strategies to reduce antibiotic resistant *Salmonella* are needed. Controlling *Salmonella* infection and antibiotic use at the feedlot will ultimately mitigate hide and carcass contamination of *Salmonella* and antibiotic resistant *Salmonella* at slaughter; ideally, such reductions will extend to lymph nodes as well.

The effects of antibiotic use on the quantity of *E. coli* in cattle has been studied previously [30, 210, 211]. However, despite the pathogenicity of *Salmonella* to humans, the effects of antibiotics on the concentration of *Salmonella* (including susceptible and resistant *Salmonella*) have not been studied extensively in cattle fecal samples but have been studied in pigs and mice [222, 277]. Most studies of *E. coli* have demonstrated that antibiotic treatment decreases *E. coli* concentrations transiently [210]. On the other hand, some studies have shown that antibiotic treatments help establish *Salmonella* populations and induce super shedding in mice and pigs [80, 278]. One study that included experimental inoculation of *Salmonella* in pigs showed that pigs that received antibiotic treatment had higher fecal shedding. In mice, *Salmonella* populations were established after antibiotic treatment, presumably by the disruption of the healthy microbiota.

We conducted our study in the Texas Panhandle, where a high prevalence of *Salmonella* in cattle is commonly observed. In this studied population, a third-generation cephalosporin, ceftiofur crystalline-free acid (CCFA), and chlortetracycline (CTC) were experimentally given to investigate their effects on the quantity of *Salmonella* present in bovine feces. CCFA was given on Day 0 followed by CTC treatment from Day 4 to Day 20 for five consecutive days over three time periods with a one-day interval in between. For the CCFA treatment, 8 pens were mixed with one steer treated with CCFA on Day 0 among 10 steers without CCFA to investigate the effect of mixing CCFA treated steer among non-treated cattle. These

antibiotics are not approved for the treatment of *Salmonella*, and their direct impact on amount of susceptible and resistant *Salmonella* populations have not been investigated. Their use in feedlot cattle would typically be for the treatment and control of bovine respiratory disease.

Resistance to chlortetracycline is common among *E. coli* and *Salmonella*. Tetracycline resistant isolates are often co-resistant to cephalosporins and conferred by plasmids carrying resistance gene cassettes. In our previous study, we observed that the prevalence of *Salmonella* decreased after antibiotic treatment; however, the proportion of multidrug resistant (MDR) *Salmonella* increased, as determined by broth enrichment methods [88]. While it has been shown that the proportion of MDR *Salmonella* increased with antibiotic treatment, it is not clear if the susceptible *Salmonella* populations were replaced by resistant *Salmonella* within the animals.

Quantification of *Salmonella* from cattle, pig, poultry, and horse feces has been reported previously using various methods, including most probable number (MPN), spiral plating, automated immunomagnetic separation, and quantitative real-time PCR (qPCR) [159, 277, 279-281]. For accurate and rapid detection and quantification of *Salmonella* in fecal samples, molecular methods like qPCR also can be utilized [282-285]; however, qPCR quantification of *Salmonella* in fecal samples is challenging because of the presence of a complex microbiota and PCR inhibitors. A few studies have successfully used hydrolysis probe (Taqman®) quantitative real-time PCR and TaqMan® quantitative reverse-transcript real-time PCR to detect *Salmonella* in food and environmental samples [285, 286]. The studies that have compared culture methods and qPCR for the detection of *Salmonella* in food, mice, and biosolid samples, demonstrated lower detection limits with qPCR [277, 284, 287].

Comparison of culture and qPCR methods have also been studied in Shiga-toxin producing *E. coli* [288-290]. Semi-quantification by enrichment of fecal samples and plating can recover weak *Salmonella* and increase the counts but at the cost of reduced accuracy. Direct plating of diluted fecal samples on selective agar is easy and fast, and we used this approach for comparison with qPCR.

In the current study, quantification of *Salmonella* and antibiotic resistant *Salmonella* was performed by direct spiral plating on plain Brilliant Green Agar (BGA) and BGA containing tetracycline (16 mg/L) and ceftriaxone (4 mg/L). Additionally, hydrolysis-probe based real-time quantitative PCR (qPCR) was conducted targeting the *invA* gene, which is a specific invasion gene necessary for *Salmonella* to infect epithelial cells [291, 292]. We examined the Kappa statistics to compare the results obtained from qPCR, spiral plating, and broth enrichment. Additionally, we analyzed the effects of CCFA and CTC treatments by zero-inflated Poisson models. Lastly, we conducted qPCR performance and analysis following the MIQE guidelines for qPCR [293].

4.2 Materials and Methods

4.2.1 Experiment design and sample collection

The experimental design was the same as described in Chapter III. Briefly, 2 replicates of cattle with 4 treatment groups were treated with either CCFA, CTC or both and were sampled every other day for 26 days. Glycerol fecal samples from Days 0, 4, 8, 14, 20, and 26 were used for the quantification by spiral plating. Fecal samples stored at -80 °C without glycerol from the identical sampling days were used for the extraction of total community DNA. At the end of the trial, 16 samples were not collected because either cattle required additional antimicrobial treatments or else were culled or died. A total of 1,040 samples were included in the study.

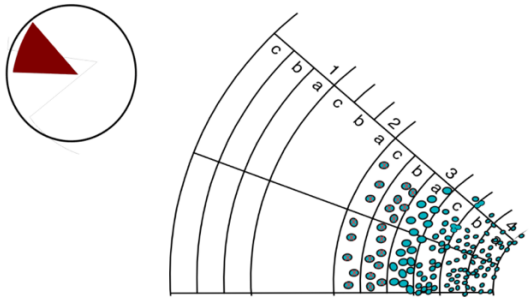
4.2.2 *Broth enrichment method for* Salmonella *detection*

Salmonella was isolated following a modified enrichment process, as described in the Chapter III [88]. Fecal samples were thawed on ice and mixed thoroughly with a transfer pipette. In total, 500 mg of feces were pre-enriched in 5 ml of tryptic soy broth (TSB) (Difco, Becton Dickinson, Franklin Lakes, NJ) for 2 hours at room temperature and then incubated 6 hours at 37 °C and then kept at 4 °C for 14 hours. A 1 ml aliquot of the enriched fecal sample in TSB was transferred into 9 ml of tetrathionate broth (Difco, Becton Dickinson) and incubated at 37 °C for 24 hours. After incubation, 100 µl of the tetrathionate broth culture was transferred into 10 ml of Rappaport-Vassiliadis R10 (RV) broth (Difco, Becton Dickinson) and incubated at 42 °C for 18 hours. The following day, 50 µl of RV broth was spiral-plated onto Brilliant Green agar (BGA) (Difco, Becton Dickinson) using an Eddy Jet 2 spiral plater (Neutec Group Inc., Farmingdale, NY). A single presumptive *Salmonella* isolate was plated on tryptic soy agar (TSA) with 5% sheep blood agar (Remel™, Lenexa, KS) for isolation and incubated at 37°C for 18 hours.

4.2.3 *Quantification of* Salmonella *by colony counting*

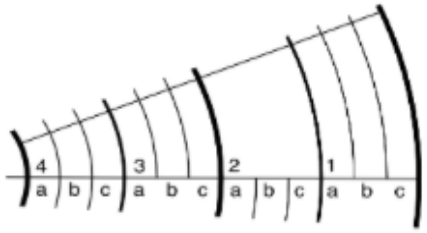
Fecal samples (500 mg) were diluted in 4.5ml of phosphate buffered saline (PBS) in a 1:10 ratio. Diluted fecal samples were plated on BGA, BGA with 16 mg/L of tetracycline (BGA-tet), and BGA with 4 mg/L of ceftriaxone (BGA-cef) by an Eddy Jet 2 spiral plater with the E-Mode 50 µl setting and incubated at 37 °C for 18 hours. The concentrations of antibiotics were determined following CLSI breakpoints for tetracycline and ceftriaxone, respectively. Presumptive *Salmonella* colonies were counted by an automated Flash & Go® colony counter the next day following the manufacturer's instructions (Figure 16, 17) (Neutec Group Inc, Farmingdale, NY).

Note: an E-Mode 50µl distribution was supposedly used to inoculate the plate being counted in the following example.



N' = N_{3c+3b} = 22 CFU

The first count reaches 20 CFU in the 3b ring sector and it therefore stops here. Thus all colonies in the 3c and 3b ring sectors are counted. Then, the opposite end of the grid is counted in the same fashion:



Counting Grid

	3c	3c+3b	3c+3b+3a	3+4c	3+4c+4b	3+4c+4b+4a
E-Mode 50 µl	**2,56**	**6,44**	**12,08**	**20,36**	**32,32**	**50**

Figure 16. Illustration of E-Mode 50 µl spiral plating and methods for colony counting (Adapted from Eddy Jet 2 user's guide, IUL. S.A.) [294]. The area where colonies are growing at the correct density and the dilution factor were incorporated into the final calculation of colony forming unit (CFU) per gram of feces.

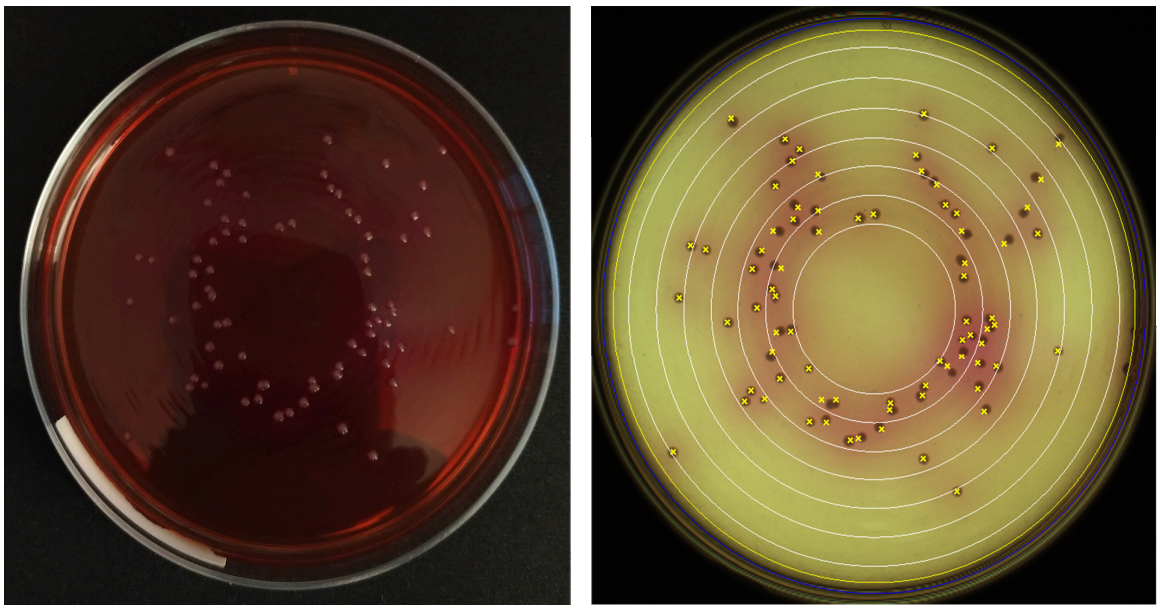


Figure 17. Figure on the left is the presumptive *Salmonella* colonies grown on Brilliant Green Agar (BGA). Figure on the right is the same plate shown on the Flash & Go® colony counter.

4.2.4 Hydrolysis probe quantitative real-time PCR (qPCR)

Gene-based quantification of *Salmonella* in the community DNA was performed using the *invA* gene primers and probes on the AriaMx Real-Time PCR system (Agilent Technologies, Santa Clara, California) [281, 285, 286]. Total community DNA extracted from fecal samples from days 0, 4, 8, 14, 20, and 26 of replicate 1 and 2 were quantified. The community DNA was used directly as a template in the reaction. The reaction plates were set up manually and samples were run in duplicate. Quantities were standardized via the 16s rRNA quantity in the community DNA as described previously [220].

4.2.4.1 Total community DNA extraction for qPCR

Total community DNA was extracted from 200mg feces by the QIAamp DNA Stool Mini Kit ™ (Qiagen, Valencia, CA) in the QIAcube robot ™ (Qiagen, Valencia, CA) following the manufacturer's instructions as described previously [220]. The quality and quantity of the community DNA was determined *via* the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at wavelengths of 260 and 280 nm. DNA samples were stored at -20 °C for further genotypic analysis.

4.2.4.2 Standard curve generation

The genomic DNA extracted from *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC® 700720™ (ATCC®, Manassas, Virginia) by the QIAcube ™ robot (Qiagen, Valencia, CA) was used as a template for standard curve generation [286]. The standard curve was serially diluted and each standard curve reaction contained 3 µl of diluted genomic DNA, in which the final copy numbers were $1x10^6$, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$, and $1x10^1$. Gene copy numbers were calculated following the Thermo Fisher Scientific web tool (https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html). The total size of the *Salmonella* Typhimurium ATCC 700720 strain is 4,857,450bp (GenBank: AE006468.2). The molar mass per base pair was set as 650 (g/mol)/bp. Efficiency was calculated following the equation: efficiency = $10^{(-1/\text{slope of standard curve})} - 1$

4.2.4.3 Primers/probes and reactions setup

The primers and probes targeting the *invA* gene and standard curve generation were adapted and modified from Gonzalez-Escalona *et al.* (Table 8) [285]. FAM was used for the 5'-Reporter

dye (Table 8). The 16s rRNA data were obtained from a previous study [220, 295]. Primer specificity was confirmed by BLAST, which returned only *Salmonella enterica* subsp. *enterica*. The number of *invA* gene copies in *Salmonella* isolates from the study population was confirmed by whole genome sequencing data to be 1-gene copy per genome. Each reaction was composed of 10 µl of Brilliant III Ultra-Fast QPCR Master Mix with Low ROX (Agilent Technologies, Santa Clara, California), 4.6 µl of nuclease-free water, 0.4 µl of probe (0.2 µM), 1 µl each of forward and reverse primers (0.5 µM), and 3 µl of total community DNA (Table 9). Thermocycler conditions for the reaction were 95°C for 3 min (activation of master mix), and 40 cycles of 95°C for 5 sec (denaturing), 60°C for 10 sec (annealing/extension) (Table 10). Data collection from fluorescence was conducted at the annealing/extension step of each cycle. Each sample was run in duplicate.

Table 8. Primers and probes used for qPCR

Gene	Primer	Sequence	Tm (°C)	Product size (bp)	Gene bank accession number
invA	*invA*_176_F	5'-CAACGTTTCCTGCGGTACTGT-3'	60	116	M90846
	*invA*_291_R	5'-CCCGAACGTGGCGATAATT -3'			M90846
	*invA*_FAM_208 Probe	FAM-CTCTTTCGTCTGGCATTATCGATC AGTACCA-Iowa Black RQ-Sp			M90846

Table 9. qPCR reaction protocol

Brilliant III Probe Master Mix Reaction

Component	Volume/Rxn (µl)	Primer/Probe Final con. (µM)
NF water	4.6	
2xBrilliant III qPCR MM Low ROX	10	
invA Probe	0.4	0.2
invA Primer F	1	0.5
invA Primer R	1	0.5
DNA	3	
Total	20	

Table 10. qPCR thermocycler protocol

Segment	Number of cycles	Temp. (°C)	Duration	Comment
Activation	1	95	3 min.	
Denaturation	40	95	5 sec.	
Annealing		60	10 sec.	Data collection

4.2.5 Statistical Analysis

After each run, qPCR data were analyzed using the AriaMx ver. 1.0 software (Agilent). Gene copy numbers were back-calculated to gene copies per gram of wet feces by taking feces loss and dilutions into account at each step of community DNA extraction. Cq derived gene copy numbers were multiplied by 583.33 for the complete back calculation. Both non-standardized *invA* gene copies per gram wet feces and the quantities obtained by standardizing with the 16S rRNA gene were calculated and analyzed. All standardized, non-standardized, and colony count derived quantities were logarithmically transformed to log base 10 for use as a dependent variable in both multiple imputation and zero-inflated Poisson models using Stata/IC 14.2 (StataCorp LLC, College Station, TX). Detection based on qPCR and direct spiral plating were used as binary data for detection analyses. Kappa statistics were calculated for the agreement among detection methods. *Salmonella* positive samples by broth enrichment, qPCR and direct spiral plating methods were visualized by a Venn-diagram using BioVenn [296].

4.2.5.1 Analysis of observed qPCR and direct plating data with zero-inflated Poisson model and linear regression

Direct plating and qPCR derived $\log_{10}$ transformed outcomes were rounded and converted from continuous numeric to integer data for analysis purposes since many of the qPCR reactions and colony counts were below the limit-of-quantification (LOQ) and marked as zero. Counts were set as dependent variables, and CCFA (binomial), CTC (binomial), and days (ordinal) were included as independent variables specified in a 3-way full factorial model. No-CCFA treatment (0), No-CTC treatment (0), and day 0 were set as the baseline.

The same variables were used as inflation variables. The $\log_{10}$ transformed counts were used for linear regression analyses of qPCR and direct plating.

4.2.5.2 Imputation of missing values

When the quantity of the *invA* gene and colony numbers on BGA were under the LOQ they were recorded as missing values. Originally, missing values were given a value of 1 and $\log_{10}$ transformed to 0 for visualization purposes. The missing values were estimated by imputation procedures in Stata/IC 14.2 with linear regression, truncated regression, and interval regression to provide a censored continuous variable [297, 298]. Each imputation was conducted with the full factorial model including trial replicate as a fixed effect, and a 3-way full factorial model using CCFA, CTC, and days as independent variables. The upper limit of missing values was set as 3.6 $\log_{10}$ for those with missing observations in truncated regression and interval censored regression. The lower limit was set to -∞. The imputed values were diagnosed for fit with the observed data [298]. Multiple imputed values from interval censored regression estimation were estimated for further analyses utilizing mixed-level linear regression with the command "mimrgns" available through "ssc install mimrgns" in Stata version 14.2 [299].

4.3 Results

4.3.1 Descriptive statistics of broth enrichment, qPCR, and spiral plating methods

4.3.1.1 Description of qPCR runs

Each community DNA sample was tested in duplicate by qPCR and each run included a standard curve. A total of 30 qPCR runs were performed on 96 well plates. The efficiency of

qPCR was calculated from the standard curve, in which the mean for 30 runs was 97.8% (range: 90.5%-106.9%) (Figure 18). The mean coefficient of correlation (R^2) was 98.1% (range: 93.8%-99.9%) and the mean slope was -3.38 (ranged -3.57 to -3.17). The Cq value ranged from 18.49 to 39.98 with a mean of 29.95. The negative control wells, which contained master mix, probe, primer pairs, and water in the reaction, were negative in all of the runs.

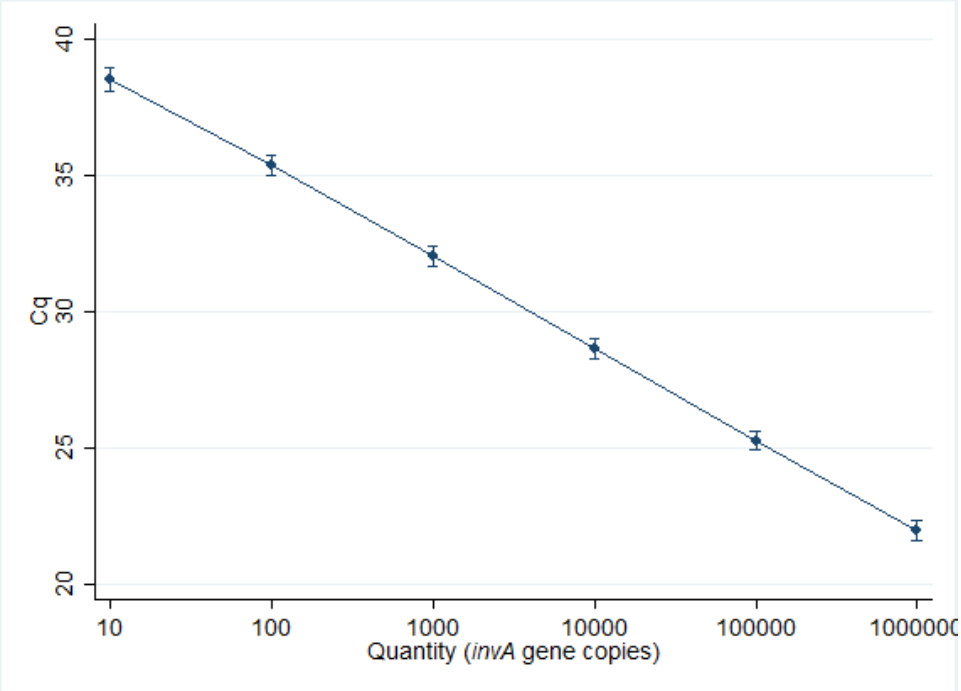


Figure 18. Example of the standard curve regression plot amplified from template genomic DNA of *Salmonella enterica* Typhimurium targeting the *invA* gene.

4.3.1.2 Comparison of detection and quantitation methods

From 1,040 fecal samples tested, *Salmonella* was detected in 566 samples (54.4%) after the broth enrichment method. *Salmonella* was detected by qPCR and direct spiral plating in 324/1040 (31.2%) and 252/1040 (24.2%) samples, respectively (Table 11). Further, 474 (45.6%), 716 (68.8%), and 788 (75.7%) samples were below the LOQ by enrichment broth, qPCR, and direct spiral plating, respectively, and therefore were initially recorded as zero. The LOQ was $\log_{10}$ 2.78 per gram feces for qPCR and $\log_{10}$ 2.60 per gram feces for direct plating. The 16s rRNA gene was detected in all samples. The mean $\log_{10}$ gene copies per gram of feces for qPCR was 4.33 (95% CI: 4.24-4.42) and the $\log_{10}$ CFU per gram feces for direct plating was 3.54 (95% CI:3.44-3.63).

Table 11. Number of samples detected with *Salmonella* in broth enrichment, probe qPCR, and direct spiral plating.

Detection method	Detected	Below LOQ	Total	Min-Max ($\log_{10}$ / g feces)	Mean ($\log_{10}$ / g feces)
Broth enrichment	566 (54.4%)	474 (45.6%)	1040	-	-
Probe qPCR	324 (31.2%)	716 (68.8%)	1040	2.78-7.99	4.33 (95% CI: 4.24-4.42)
Direct spiral plating	252 (24.2%)	788 (75.7%)	1040	2.60-6.18	3.54 (95% CI: 3.44-3.63)

4.3.1.3 Comparison of three detection methods

A total of 623/1040 (59.9%) samples were detected with *Salmonella* in at least one of the detection methods among all of the tested days. A total of 187/1040 (18.0%) samples were positive for all 3 methods and 417/1040 (40.1%) samples were negative for all three detection methods. *Salmonella* was detected only by broth enrichment method in 237/1040 (22.8%) samples, only by qPCR in 48/1040 (4.6%), and only by direct spiral plating in 6/1040 (0.6%) samples (Figure 19). The sample numbers that were detected by the three methods are shown in a Venn diagram (Figure 19). At the individual animal level, the *Salmonella* detection by each method and the detected pattern by day is shown in Figure 20. Among the 4 animals in which *Salmonella* was not detected by broth enrichment from all 6 tested days (ID 2245, 2344, 2387, and 2356), 3 animals had detection with qPCR on day 0 (ID 2245, 2344) or on both day 0 and 4 (ID 2387). Therefore, only 1 animal (ID 2356) out of 176 did not have *Salmonella* detected with any of the methods on any of the tested days.

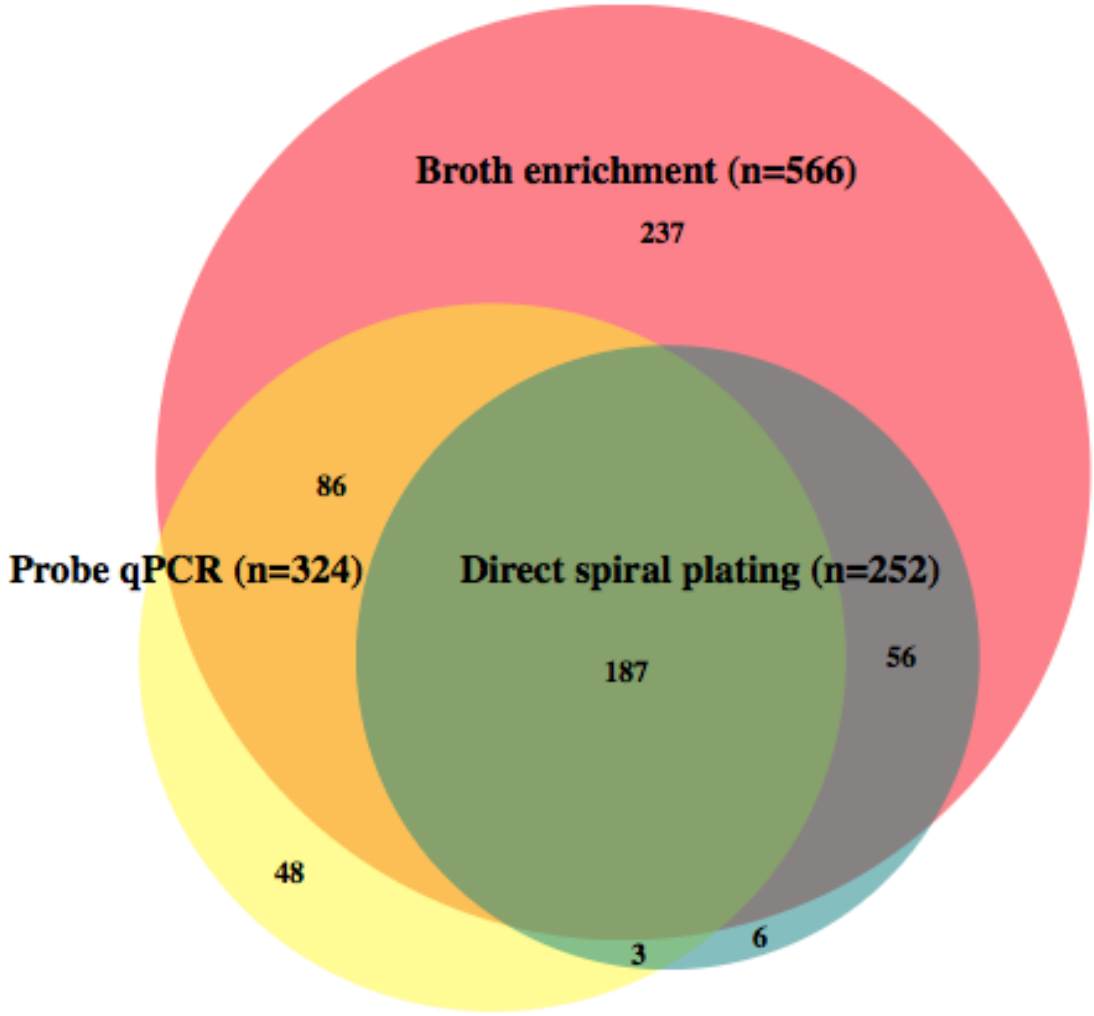
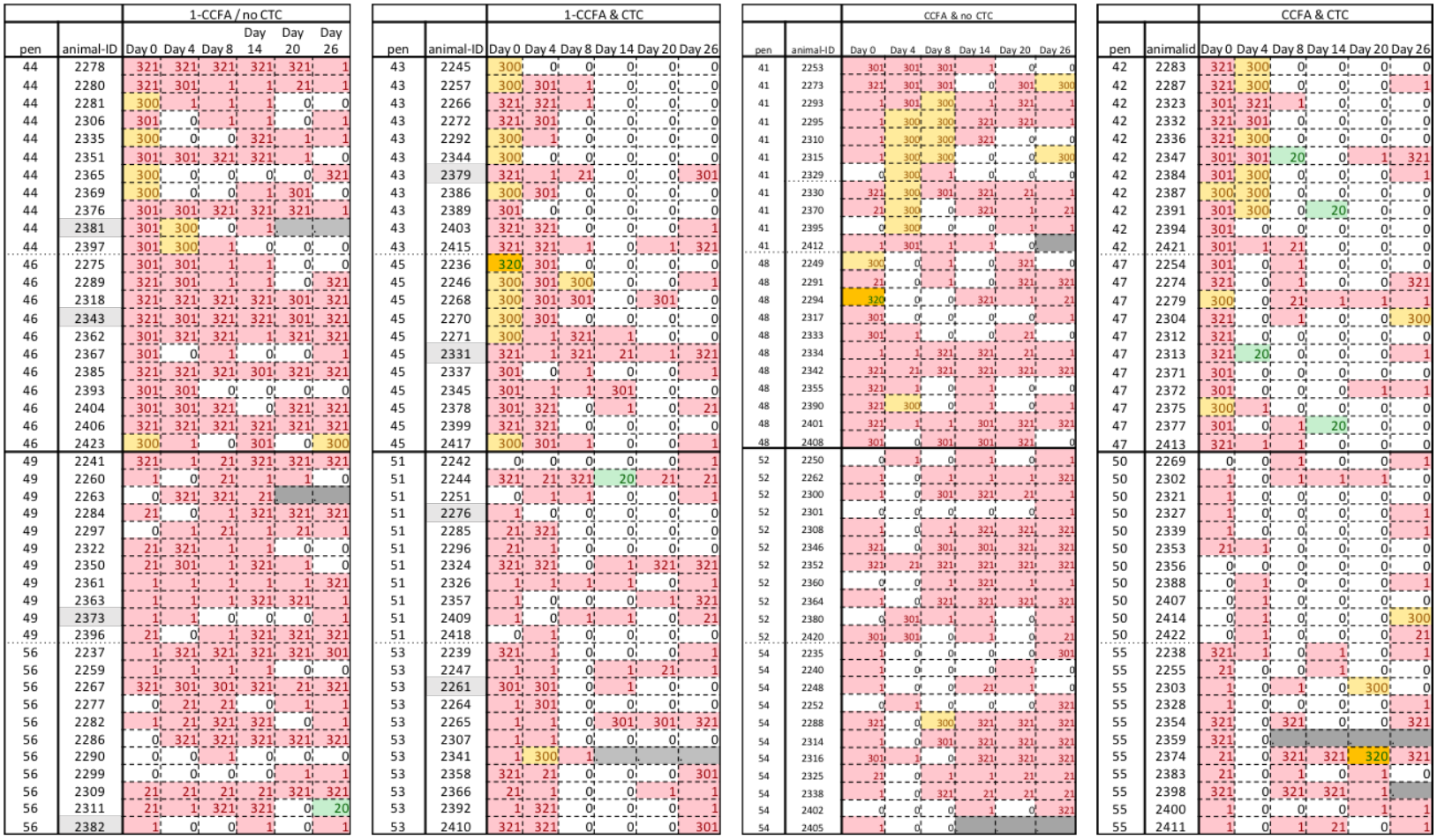


Figure 19 Number of samples detected by broth enrichment, probe qPCR, and direct spiral plating methods shown in a Venn diagram out of 1,040 samples tested in total by each of the three methods. Red: Broth enrichment, Yellow: Probe invA qPCR, Green: Direct spiral plating. Numbers corresponds to the number of samples detected with Salmonella in each portion of the circle.



		1-CCFA / no CTC					
pen	animal-ID	Day 0	Day 4	Day 8	Day 14	Day 20	Day 26
44	2278	321	321	321	321	321	1
44	2280	321	301	1	1	21	1
44	2281	300	1	1	1	0	0
44	2306	301	0	1	1	0	1
44	2335	300	0	0	321	1	1
44	2351	301	301	321	321	1	0
44	2365	300	0	0	0	0	321
44	2369	300	0	0	1	301	0
44	2376	301	301	321	321	321	1
44	2381	301	300	0	1		
44	2397	301	300	1	0	0	0
46	2275	301	301	1	1	0	0
46	2289	321	301	1	1	0	321
46	2318	321	321	321	321	301	321
46	2343	321	301	321	321	301	321
46	2362	301	321	321	1	321	321
46	2367	301	0	1	0	0	1
46	2385	321	321	321	301	321	321
46	2393	301	301	0	0	0	0
46	2404	301	301	321	0	321	321
46	2406	321	321	321	321	321	321
46	2423	300	1	0	301	0	300
49	2241	321	1	21	321	321	321
49	2260	1	0	21	1	1	0
49	2263	0	321	321	21		
49	2284	21	0	1	321	321	321
49	2297	0	1	21	1	21	1
49	2322	21	321	1	1	0	0
49	2350	21	301	1	321	1	0
49	2361	1	1	1	1	1	321
49	2363	1	1	1	321	321	1
49	2373	1	1	0	0	0	1
49	2396	21	0	1	321	321	321
56	2237	1	321	321	321	321	301
56	2259	1	1	1	1	0	0
56	2267	321	301	301	321	21	321
56	2277	0	21	21	0	1	1
56	2282	1	21	321	321	0	1
56	2286	0	321	321	321	321	321
56	2290	0	0	1	0	0	0
56	2299	0	0	0	0	1	1
56	2309	21	21	21	21	321	321
56	2311	21	1	321	321	0	20
56	2382	1	0	0	1	0	1

		1-CCFA & CTC					
pen	animal-ID	Day 0	Day 4	Day 8	Day 14	Day 20	Day 26
43	2245	300	0	0	0	0	0
43	2257	300	301	1	0	0	0
43	2266	321	321	1	0	0	0
43	2272	321	301	0	0	0	0
43	2292	300	1	0	0	0	0
43	2344	300	0	0	0	0	0
43	2379	321	1	21	0	0	301
43	2386	300	301	0	0	0	0
43	2389	301	0	0	0	0	0
43	2403	321	321	0	0	0	1
43	2415	321	321	1	0	1	321
45	2236	320	301	0	0	0	0
45	2246	300	301	300	0	0	1
45	2268	300	301	301	0	301	0
45	2270	300	301	0	0	0	0
45	2271	300	1	321	1	0	0
45	2331	321	1	321	21	1	321
45	2337	301	0	1	0	0	1
45	2345	301	1	1	301	0	0
45	2378	301	321	0	1	0	21
45	2399	321	321	0	0	0	0
45	2417	300	301	1	0	0	1
51	2242	0	0	0	0	0	1
51	2244	321	21	321	20	21	21
51	2251	0	1	1	0	0	1
51	2276	1	0	0	0	0	0
51	2285	21	321	0	0	0	0
51	2296	21	1	0	0	0	0
51	2324	321	321	0	1	321	321
51	2326	1	1	1	1	0	1
51	2357	1	0	0	0	1	321
51	2409	1	0	1	1	0	21
51	2418	0	1	0	0	0	0
53	2239	321	1	0	0	0	1
53	2247	1	1	0	1	21	1
53	2261	301	301	0	1	0	0
53	2264	1	301	0	0	0	0
53	2265	1	1	0	301	301	321
53	2307	1	1	0	0	0	0
53	2341	1	300	1			
53	2358	321	21	0	0	0	301
53	2366	21	1	0	0	1	1
53	2392	1	321	0	0	0	1
53	2410	321	321	0	0	0	301

		CCFA & no CTC					
pen	animal-ID	Day 0	Day 4	Day 8	Day 14	Day 20	Day 26
41	2253	301	301	301	1	0	0
41	2273	321	301	301	0	301	300
41	2293	1	301	300	1	321	1
41	2295	1	300	300	321	321	1
41	2310	1	300	300	321	0	0
41	2315	1	300	300	0	0	300
41	2329	0	300	1	0	0	0
41	2330	321	300	301	321	21	1
41	2370	21	300	0	321	1	21
41	2395	0	300	0	0	1	1
41	2412	1	301	1	1	0	
48	2249	300	0	1	0	321	0
48	2291	21	0	1	0	321	321
48	2294	320	0	0	321	1	21
48	2317	301	0	0	0	0	1
48	2333	301	1	0	0	21	0
48	2334	1	1	321	321	21	1
48	2342	321	21	321	321	321	321
48	2355	321	1	0	1	0	0
48	2390	321	300	0	1	0	1
48	2401	321	1	1	301	321	321
48	2408	301	0	301	301	321	0
52	2250	0	1	0	1	0	1
52	2262	1	0	1	1	1	321
52	2300	1	0	301	321	21	1
52	2301	0	0	0	0	0	1
52	2308	1	0	1	321	321	321
52	2346	321	0	301	301	321	321
52	2352	321	21	321	321	321	321
52	2360	0	0	1	321	1	1
52	2364	1	0	321	321	321	321
52	2380	0	301	1	1	0	1
52	2420	301	301	0	1	0	21
54	2235	1	0	0	0	0	301
54	2240	1	0	0	0	1	0
54	2248	1	0	0	21	1	0
54	2252	0	1	0	0	0	321
54	2288	321	0	300	321	321	321
54	2314	1	0	301	321	321	321
54	2316	301	1	0	321	321	321
54	2325	21	0	1	1	21	21
54	2338	1	0	321	21	21	21
54	2402	0	0	0	1	0	321
54	2405	1	0	0			

		CCFA & CTC					
pen	animalid	Day 0	Day 4	Day 8	Day 14	Day 20	Day 26
42	2283	321	300	0	0	0	0
42	2287	321	300	0	0	0	1
42	2323	301	321	1	0	0	0
42	2332	321	301	0	0	0	0
42	2336	321	300	0	0	0	0
42	2347	301	301	20	0	1	321
42	2384	301	300	0	0	0	1
42	2387	300	300	0	0	0	0
42	2391	301	300	0	20	0	0
42	2394	301	0	0	0	0	0
42	2421	301	1	21	0	0	0
47	2254	301	0	1	0	0	0
47	2274	321	0	1	0	0	321
47	2279	300	0	21	1	1	1
47	2304	321	0	1	0	0	300
47	2312	321	0	0	0	0	0
47	2313	321	20	0	0	0	1
47	2371	301	0	0	0	0	0
47	2372	301	0	0	0	1	1
47	2375	300	1	0	0	0	0
47	2377	301	0	1	20	0	0
47	2413	321	1	1	0	0	0
50	2269	0	0	1	0	0	1
50	2302	1	0	1	1	1	0
50	2321	1	0	0	0	0	0
50	2327	1	0	0	0	0	1
50	2339	1	0	0	0	0	1
50	2353	21	1	0	0	0	0
50	2356	0	0	0	0	0	0
50	2388	0	1	0	0	0	1
50	2407	0	1	0	0	0	0
50	2414	0	1	0	0	0	300
50	2422	0	1	0	0	0	21
55	2238	321	1	0	1	0	1
55	2255	21	0	0	1	0	0
55	2303	1	0	1	0	300	0
55	2328	1	0	0	0	0	1
55	2354	321	0	321	0	0	321
55	2359	321	0				
55	2374	21	0	321	321	320	321
55	2383	21	0	1	0	1	0
55	2398	321	0	321	321	1	
55	2400	1	0	0	0	1	1
55	2411	1	0	1	21	0	1

Figure 20 Detection pattern of individual cattle by treatment group and detection methods. Each row represents an individual steer with pen and animal identifier. The colors and numbers correspond to the detection methods. Pink: detected with enrichment (all containing number 1), containing number 2: detected additionally with direct plating, containing number 3: detected additionally with *invA* qPCR, Yellow: only detected with qPCR (number 300), Green: only detected with direct plating (number 20), Orange: detected with direct plating and qPCR, but not with enrichment (number 320), 0: no detection in all methods, Grey: no samples were collected.

4.3.1.4 Agreement between direct spiral plating and qPCR

Measures of association between two tests are shown in 2x2 tables for broth enrichment vs. direct spiral plating (Table 12), broth enrichment vs. qPCR (Table 13), and direct spiral plating vs. qPCR (Table 14). Kappa (κ) , which measure the agreement of two tests beyond chance and prevalence and bias-adjusted kappa (PABAK) statistics, adjusting for prevalence and bias, were calculated (Table 15) [300] [301]. Kappa and PABAK between broth enrichment vs. direct spiral plating was 0.3894 and 0.3615, respectively, and broth enrichment vs. qPCR was 0.3581 and 0.3385, respectively, both of which represent fair agreement. Kappa and PABAK between direct spiral plating vs. qPCR was 0.5292 and 0.6230, respectively, which indicates moderate agreement (Table 15) [302].

Table 12. 2x2 table comparing the detection of *Salmonella* by broth enrichment and direct spiral plating methods.

		Salmonella detection after broth enrichment		
		+	-	Total
Direct spiral plating	+	243	9	252
	-	323	465	788
	Total	566	474	1,040

Table 13. 2x2 table comparing the detection of Salmonella by broth enrichment and probe qPCR methods.

		Salmonella detection after broth enrichment		
		+	-	Total
qPCR	+	273	51	323
	-	293	423	717
	Total	566	474	1,040

Table 14. 2x2 table comparing the detection of *Salmonella* by direct spiral plating and probe qPCR methods.

		qPCR		
		+	-	Total
Direct plating	+	190	62	252
	-	134	654	788
	Total	324	716	1040

Table 15. Kappa (κ) and PABAK and agreement between direct spiral plating, broth enrichment, and probe qPCR.

	Broth enrichment vs.		qPCR vs. direct plating
	Direct plating	qPCR	
Agreement	68.08%	66.92%	81.15%
Expected Agreement	47.72%	48.33%	59.71%
Kappa (κ)	0.3894	0.3598	0.5322
PABAK	0.3615	0.3385	0.6230
Level of agreement	Fair	Fair	Moderate
Significance	P=0.0000	P=0.0000	P=0.0000
Prevalence (95% CI)	54% (51-57.5)		31% (28-34.1)

Agreement: observed agreement between two tests, Expected Agreement: agreement by chance, Level of agreement was adapted from Viera *et al*. [302]. PABAK: prevalence-adjusted bias-adjusted kappa

4.3.2 Comparison of the quantity by qPCR and direct plating on BGA-plain, BGA-tet, and BGA-cef

4.3.2.1 Quantity difference between qPCR and direct plating on BGA-plain

The mean quantities determined by qPCR and direct plating are summarized in Table 16. The quantity of $\log_{10}$ *invA* gene copy numbers by qPCR and $\log_{10}$ CFU counts by direct plating on plain BGA were significantly correlated by linear regression analysis ($p < 0.0000$). When the samples detected by either qPCR or direct plating on BGA were included (n=386) the R^2 was 0.3345, the coefficient was 0.86 and the intercept was 2.68 (Figure 21a). When only samples that were detected in both methods (n=190) were included, the R^2 was 0.4261,

the coefficient was 0.54 and the intercept was 0.61 (Figure 21b). The mean quantity difference between qPCR and BGA-plain was $\log_{10}$ 0.96 and ranged from -1.13 to 3.46 (Figure 22a). Modelled marginal prediction of $\log_{10}$ *invA* gene copies were linearly regressed by the $\log_{10}$ CFU counts, and were significantly associated (p = 0.000) (Figure 22b).

Table 16. Numbers of samples and mean quantities by qPCR and direct plating on BGA by tested days.

		invA qPCR		BGA	
	Total # of Samples	Number positive (%)	Mean Log_{10} gene copies (Min-Max)	Number positive (%)	Mean Log_{10} CFU (Min-Max)
Day 0	176	95 (54.0%)	4.40 (2.81-8.00)	61 (34.6%)	3.6 (2.60-6.19)
Day 4	176	65 (36.9%)	3.99 (2.78-6.53)	27 (15.3%)	3.13 (2.60-4.60)
Day 8	175	40 (22.9%)	4.29 (3.21-6.83)	34 (19.4%)	3.62 (2.60-5.08)
Day 14	173	41 (23.7%)	4.59 (3.26-6.59)	43 (24.9%)	3.60 (2.60-5.9)
Day 20	171	36 (21.1%)	4.32 (3.09-6.66)	40 (23.4%)	3.57 (2.60-5.33)
Day 26	169	47 (27.8%)	4.48 (2.85-6.48)	47 (27.8%)	3.55 (2.60-5.83)
Total	1040	324 (31.2%)	4.33 (2.78-8.00)	252 (24.2%)	3.54 (2.60-6.19)

BGA: Brilliant Green Agar, $\log_{10}$ gene copies or CFU per gram feces

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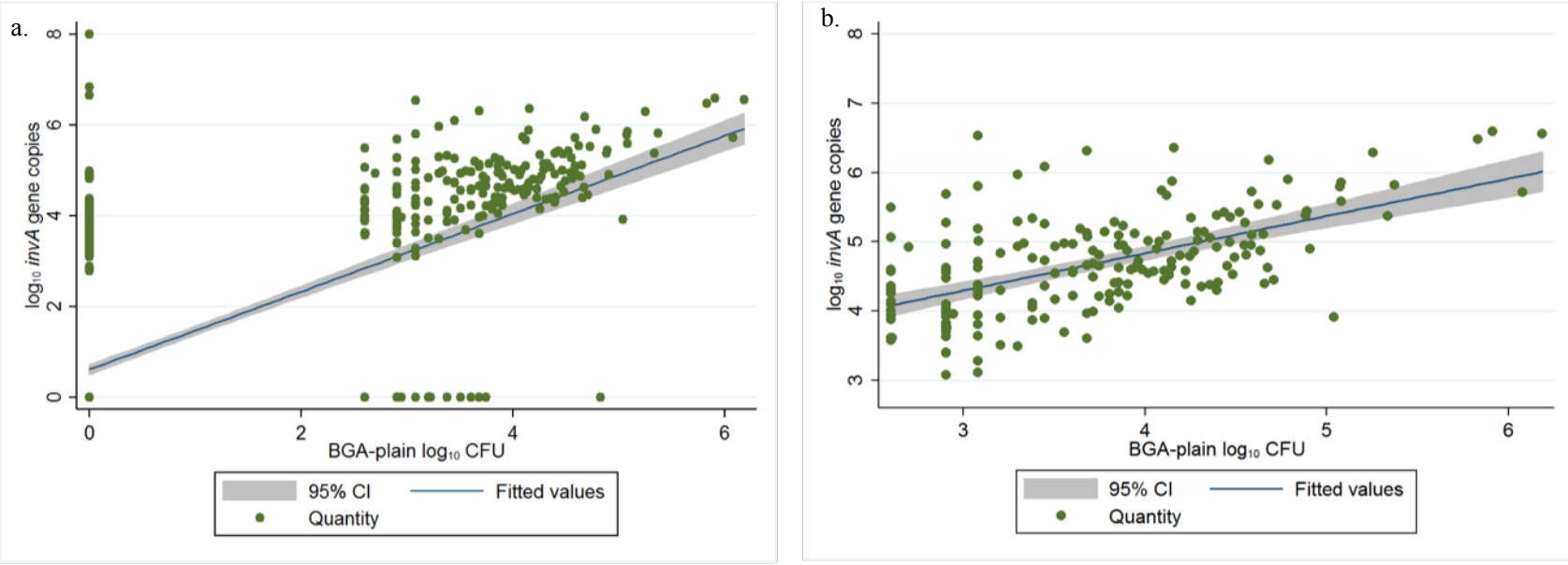


Figure 21. Linear prediction line and scatter plot of quantities of log10 *invA* genes via qPCR and $\log_{10}$ CFU on BGA-plain agar. (a) Including samples detected with either method (n=386), (b) Only samples detected with both methods (n=190).

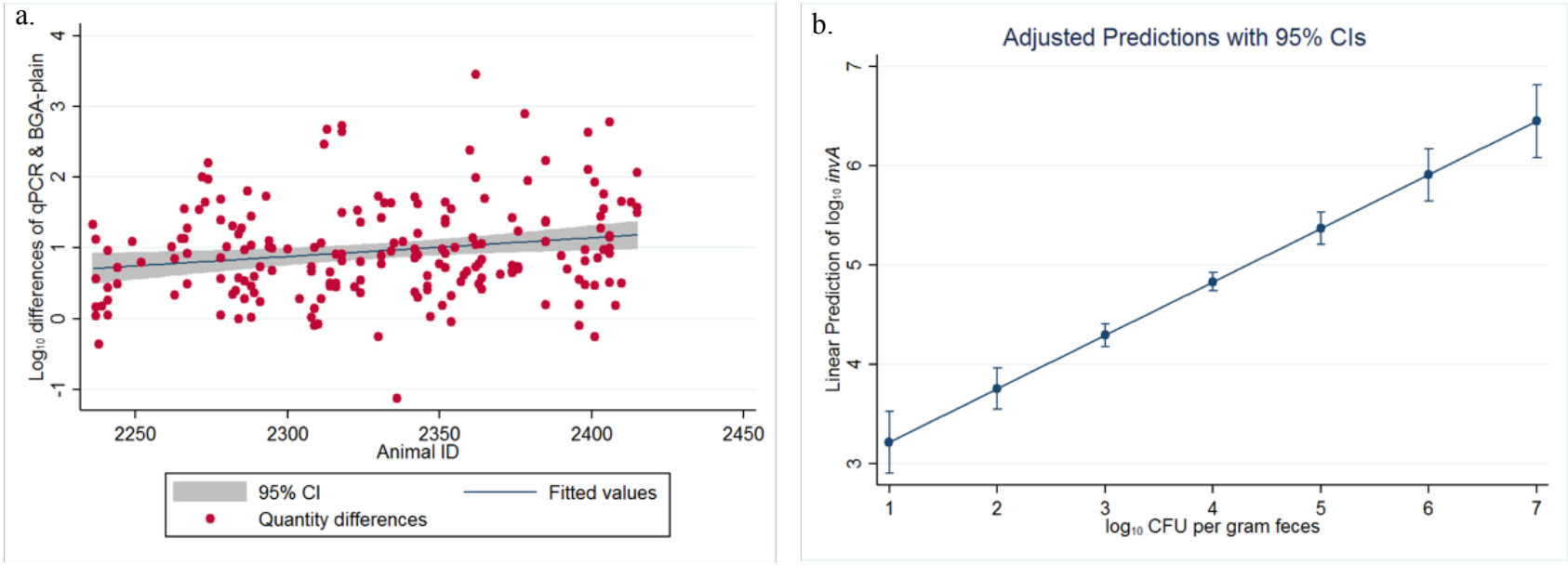


Figure 22. Quantity differences of qPCR and BGA-plain agar counts. (a) Quantity differences of qPCR and BGA-plain agar. BGA-plain agar was subtracted from qPCR. (b) Linear prediction of $\log_{10}$ *invA* qPCR by rounded $\log_{10}$ CFU of direct plating

4.3.2.2 Quantity distribution of samples detected with qPCR classified in 4 groups

The overall mean $\log_{10}$ *invA* gene copies distribution of the 324 samples that were detected by qPCR is shown in Figure 23a. The distribution was right skewed. The distribution was divided into 4 groups based on the overlap between detection methods: 1) samples detected only by qPCR (Figure 23b,c: yellow), 2) samples detected with qPCR and broth enrichment (Figure 23c: orange), 3) samples detected with all three methods (Figure 23c: dark green), and 4) samples detected with qPCR and direct plating (Figure 23c: light green). The quantity distribution of samples was different between groups as shown in Figure 23c. Mean quantity of *Salmonella* in the 1st group, from samples that were detected only by qPCR (48 samples, yellow part in Figure 23b, c), was $\log_{10}$ 3.6 gene copies per gram feces ranging from a minimum of 1.62 to a maximum of 4.22, which were on the lower end of the quantity compared to samples detected by both qPCR and broth enrichment (Figure 23c). This compartment may have consisted of animals with very low infection rates or else those that had carried viable *Salmonella* in the past but from which only dead bacteria may be detected in the present.

Among 48 positive samples, 19 samples were from day 0 (40%) and 17 samples from day 4 (35%). A significant association ($P < 0.05$) was found by Fisher's exact test (Table 17). The sample numbers in this fraction increased on day 4 in the CCFA treatment group, which may include dead cells killed by CCFA treatment on day 0. Samples shown in the orange bar (group 2) had a mean and median of 3.8, and ranged from 2.78 to 7.99. Group 2 had 3 samples that exhibited a very high quantity. Samples in group 3 (pink) had median 4.6 and mean 4.7, and ranged from 3.1 to 6.6, which were normally distributed. These samples are

certain to be infected with *Salmonella*. Samples shown in blue (group 4) were 3 samples that ranged from 3.9 to 4.7. These samples may be the samples carrying undetected *Salmonella* serotypes by broth enrichment.

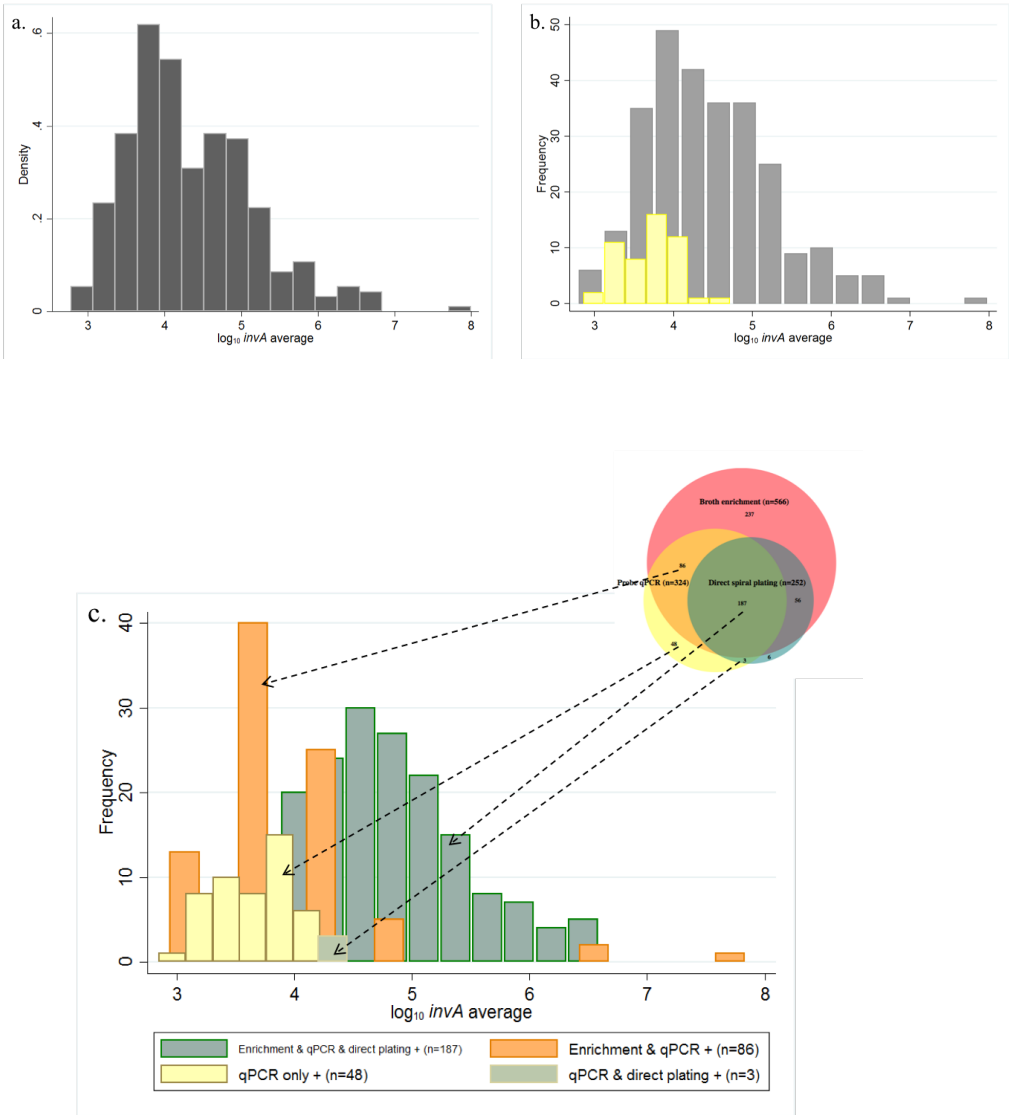


Figure 23. Distribution of mean quantity of *invA* gene copies that was detected with qPCR. (a) Over all distribution. (a) was further breakdown into those detected only with qPCR (yellow) and broth enrichment (gray) in (b). (b) was further broken down in (c).

Table 17. Sample numbers tabulated by day and treatment groups among 48 detected with qPCR

Treatment | Days	0	4	8	14	20	26	Total
1-CCFA / no CTC	5	2	0	0	0	1	8
1-CCFA & CTC	10	1	1	0	0	0	12
CCFA / no CTC	1	8	5	0	0	2	16
CCFA & CTC	3	6	0	0	1	2	12
Total	19	17	6	0	1	5	48

4.3.2.3 Quantification of total tetracycline, and ceftriaxone resistant *Salmonella*

For the quantification of total *Salmonella*, tetracycline-resistant *Salmonella*, and ceftriaxone-resistant *Salmonella* by direct spiral plating, 1,040 fecal samples were plated on BGA, BGA-tet (16 mg/L), and BGA-cef (4 mg/L) agar. On BGA, BGA-tet, and BGA-cef, 252/1040 (24.2%), 32/1040 (3.0%), and 43/1040 (4.1%) samples had colony growth, respectively. The sample numbers by day are summarized in Table 18 with mean $\log_{10}$ CFU and minimum and maximum quantity values. The growth comparison between the samples and different agars are shown in a Venn diagram (Figure 24). Although most of the samples that had growth on BGA-tet and BGA-cef had growth on BGA, 7 over these samples did not grow on BGA. Twelve samples grew on BGA-cef and BGA, but not on BGA-tet. Two samples grew on BGA-tet and BGA, but not on BGA-cef.

Table 18. Prevalence and mean quantity of *Salmonella* on BGA, BGA-tet, and BGA-cef.

		BGA		BGA-tet		BGA-cef	
Day	Total	Number (%)	Mean Log_{10} CFU	Number (%)	Mean Log_{10} CFU	Number (%)	Mean Log_{10} CFU
Day 0	176	61 (34.6%)	3.6 (2.60-6.19)	2 (1.1%)	3.73 (3.08- 4.38)	3 (1.7%)	4.01 (3.30-4.38)
Day 4	176	27 (15.3%)	3.13 (2.60-4.60)	1 (0.6%)	2.90 (-)	1 (0.6%)	3.30 (-)
Day 8	175	34 (19.4%)	3.62 (2.60-5.08)	6 (3.4%)	3.79 (2.90-5.13)	10 (5.7%)	3.78 (2.90-5.06)
Day 14	173	43 (24.9%)	3.60 (2.60-5.9)	6 (3.5%)	3.66 (2.90-4.13)	8 (4.6%)	3.39 (2.60-3.98)
Day 20	171	40 (23.4%)	3.57 (2.60-5.33)	7 (4.0%)	3.74 (2.60-4.29)	9 (5.3%)	3.73 (2.90-4.58)
Day 26	169	47 (27.8%)	3.55 (2.60-5.83)	10 (5.9%)	3.45 (2.6-4.59)	12 (7.1%)	3.69 (2.60-4.74)
Total	1040	252 (24.2%)	3.54 (2.60-6.19)	32 (3.1%)	3.62 (2.60-5.13)	43 (4.1%)	3.68 (2.60-5.06)

BGA: Brilliant Green Agar, BGA-tet: supplement with 16 mg/L of tetracycline, BGA-cef: supplement with 4 mg/L of ceftriaxone

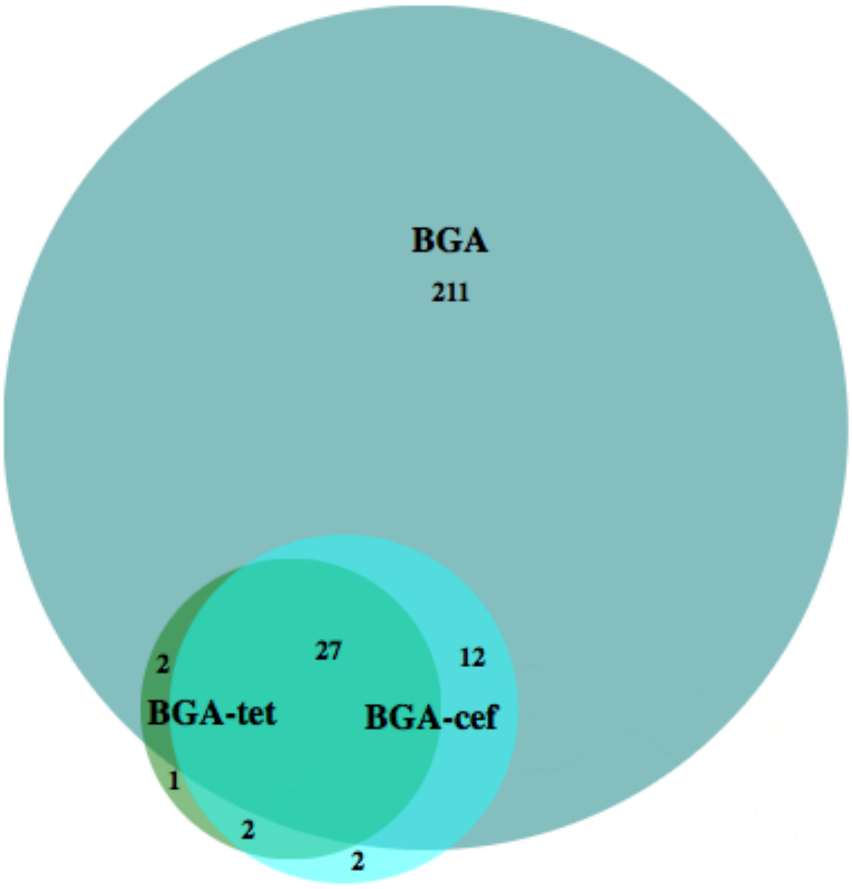


Figure 24. Growth of *Salmonella* on brilliant green agar (BGA), BGA-tetracycline (BGA-tet), and BGA-ceftriaxone (BGA-cef) from PBS diluted fecal samples.

4.3.3 Effects of antibiotic treatments on quantifiable levels of Salmonella

4.3.3.1 Change on the numbers of animals shedding *Salmonella* over or below LOQ by antibiotic treatments

In total, 1,040 community DNA (in duplicate for qPCR) and fecal samples were tested from 176 steers from days 0, 4, 8, 14, 20, and 26. During the study, 16 fecal samples from 7

animals were not collected because either the animal became sick and required retreatment or else died during the study. To analyze the change of the proportions of animals shedding more than the limit-of-quantification (LOQ), the change of the proportions of animals that started to shed more than the LOQ after Day 0 was plotted in Figure 25.

Overall, the proportion of animals shedding more than the LOQ remained similar throughout the study period in the control group (1-CCFA / no CTC; Figure 25). In this group, more animals shed ceftriaxone and tetracycline resistant *Salmonella* on Day 14. With CTC treatment alone (1-CCFA & CTC group; Figure 25), most of the animals that were shedding quantifiable *Salmonella* on Day 0 were shedding below the LOQ on Day 8 until 20 and the amount of *Salmonella* was once again quantifiable on Day 26. In this group, more animals were shedding ceftriaxone and tetracycline resistant *Salmonella* on Day 8 and on Day 26 in comparison to other days, which implies an expansion of the resistant population from below LOQ to enumerable levels after CTC treatment. In the All-CCFA treatment group, animals shedding ceftriaxone resistant *Salmonella* over the LOQ increased and remained shedding from Day 8 until Day 26. Lastly, All-CCFA & CTC treatment decreased the animals shedding *Salmonella* until Day 20. After the CTC treatment started on Day 4, animals shedding tetracycline and ceftriaxone resistant *Salmonella* increased on Day 8 and gradually decreased to Day 26.

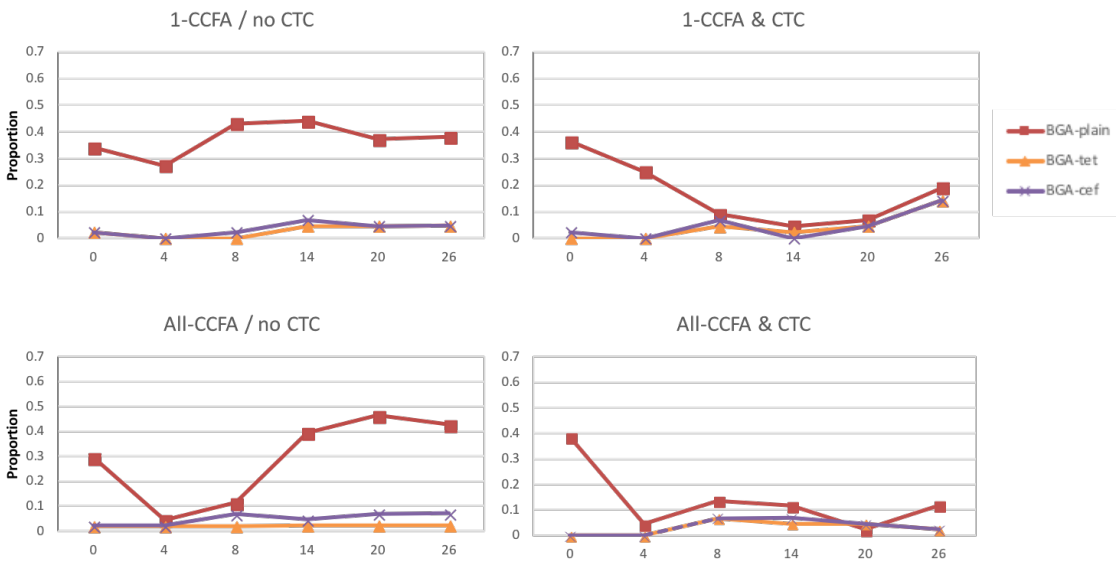


Figure 25. Proportion of animals with *Salmonella* shedding at levels over the limit-of-quantification (LOQ) by direct plating.

4.3.3.2 Observed quantity change of Salmonella by antibiotic treatments

The observed $\log_{10}$ gene copies or CFU /gram feces per treatments and days are plotted for *invA* qPCR, BGA-plain, BGA-tet, and BGA-cef in Figure 26 The number of animals and mean quantity per treatment group and by day are summarized in Tables 19 and 20. The significant differences ($P < 0.05$) in mean $\log_{10}$ *invA* gene copies were observed in the CCFA treatment group (All-CCFA / no CTC) between days 0 & 4 and days 4 & 8, 14, 20, and 26. Also, in the All-CCFA & CTC group, following CCFA treatment, the quantity significantly decreased from Day 0 to 4 ($p < 0.05$). The mean $\log_{10}$ CFU counts were significantly different between days 0 & 8 and 0 & 14 in the 1-CCFA / no CTC group.

Considering the animals shedding $>10^4$ of *Salmonella* as super shedders, in the control group (1-CCFA / no CTC), super shedders increased from 1/15 (7%) to 6/16 from Day 0 to

Day 26, respectively based on BGA growth (Table 21). Additionally, among those shedding tetracycline and ceftriaxone resistant *Salmonella*, 2/2 (100%) of the cattle were shedding more than 10^4 CFU on Day 20 in control group (Figure 26). In the CTC treatment group, the number of super shedders increased from 2/16 (12.5%) to 3/8 (37.5%) from Day 0 to Day 26, respectively, based on BGA growth. Half of the cattle that were shedding tetracycline and ceftriaxone resistant *Salmonella* were shedding more than 10^4 CFU on Day 20 and 26. In the CCFA treatment group (All-CCFA / no CTC), the number of super shedders dropped to 0% on Day 4 from 46% (6/13) on Day 0 and returned to 40% (8/20) on Day 20. In the All-CCFA & CTC treatment group, 7/17 (41%) were super shedders on Day 0 and dropped to 0% during the study; however, returned to 2/5 (40%) on Day 26. In this group, most of the animals were shedding less than 10^4 CFU.

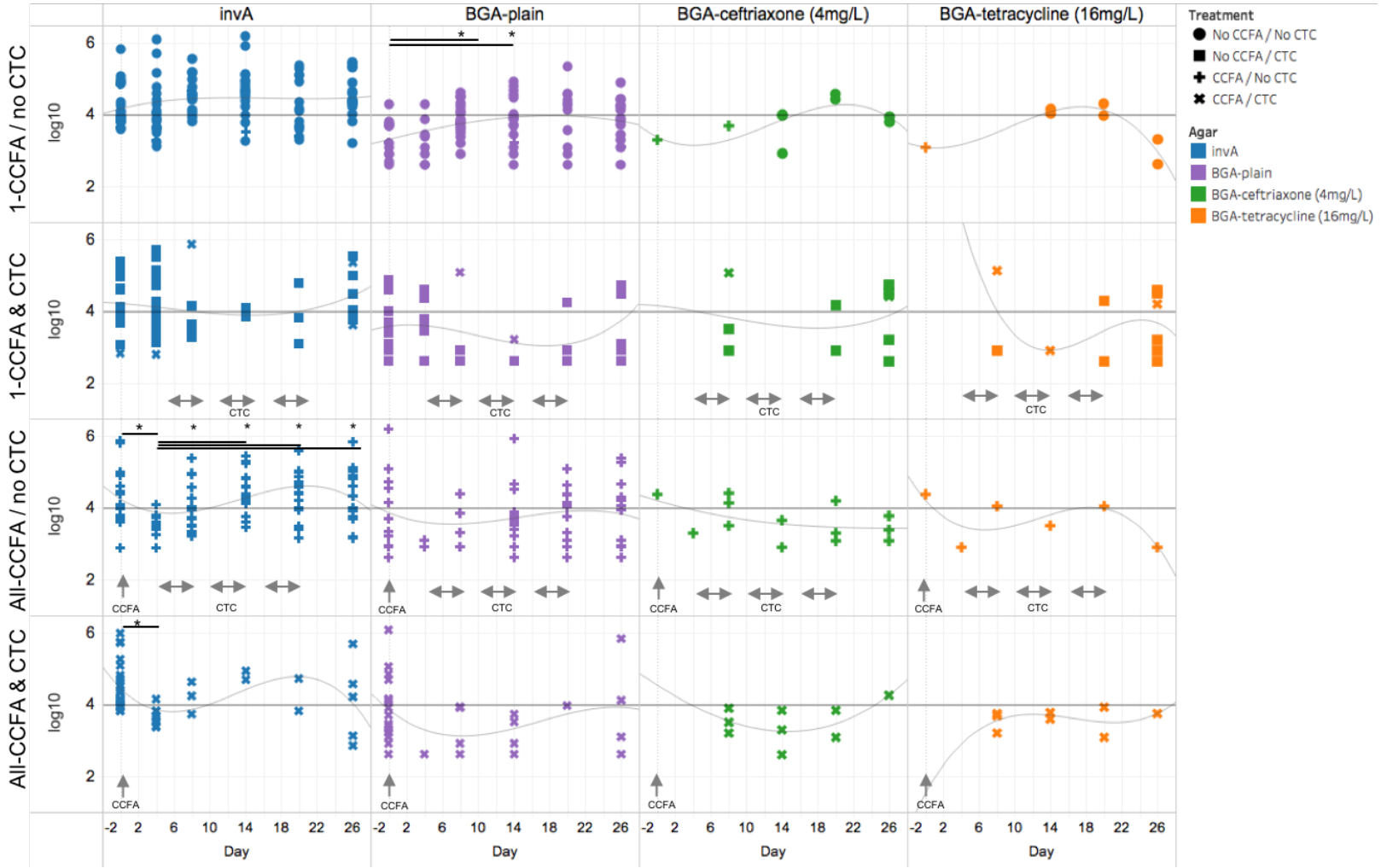


Figure 26. Observed quantity of susceptible, tetracycline, and ceftriaxone resistant *Salmonella* by antibiotic treatments by day. Blue: *invA* gene copies, Purple: Brilliant green agar (BGA) without antibiotics, Green: BGA with 4 mg/L of ceftriaxone, Orange: BGA with 16 mg/L of tetracycline. *indicates significance between days where the line is starting and where the * mark is. Quantity above the reference line at $\log_{10}$ 4 are supershedders

Table 19 Prevalance and *Salmonella* quantity by day and treatment group by qPCR and direct plating on BGA

invA qPCR	Sample numbers / Log_{10} gene copies (95% CI)	Day 0	Day 4	Day 8	Day 14	Day 20	Day 26
1-CCFA / no CTC (p=0.36)	113/260 (43.4%)	24/44 (54.5%)	22/44 (47.7%)	15/44 (34.1%)	19/44 (43.2%)	16/42 (38.1%)	17/42 (40.5%)
	4.4 (4.2-4.5)	4.2 (4.0-4.5)	4.2 (3.8-4.6)	4.6 (4.3-4.8)	4.6 (4.3-5.0)	4.4 (3.9-4.9)	4.5 (4.2-4.8)
1-CCFA / CTC (p=0.92)	66/261 (25.3%)	28/44 (63.6%)	20/44 (45.5%)	5/44 (11.4%)	2/43 (4.7%)	3/43 (7.0%)	8/43 (18.6%)
	4.2 (4.0-4.4)	4.2 (4.0-4.5)	4.2 (3.7-4.6)	4.1 (2.8-5.4)	4.0 (2.4-5.5)	3.9 (1.8-6.0)	4.44 (3.8-5.1)
CCFA & no CTC (p=0.008)	97/260 (37.3%)	17/44 (38.6%)	14/44 (31.8%)	17/44 (38.6%)	18/43 (41.9%)	15/43 (34.9%)	16/42 (38.1%)
	4.3 (4.1-4.4)	4.49 (4.0-5.0)[a]	3.57 (3.4-3.7)[b]	4.13 (3.6-4.6)[a]	4.6 (4.2-5.0)[a]	4.36 (4.0-4.7)[a]	4.46 (4.0-4.9)[a]
CCFA & CTC (p=0.12)	48/259 (18.5%)	26/44 (59.1%)	9/44 (20.5%)	3/43 (7.0%)	2/43 ((4.7%)	2/43 (4.7%)	6/42 (14.3%)
	4.3 (4.0-4.7)	4.7 (4.3-5.1)[c]	3.6 (3.5-3.9)[d]	4.2 (3.1-5.3)	4.8 (3.2-6.4)	4.3 (-1.4-9.9)	4.5 (3.0-6.0)

BGA-plain	Sample numbers / Log_{10} CFU (95% CI)	Day 0	Day 4	Day 8	Day 14	Day 20	Day 26
1-CCFA / no CTC (p=0.03)	97 /260 (37.3%)	15/44 (34.1%)	12/44 (27.3%)	19/44 (43.2%)	19/44 (43.2%)	16/42 (38.1%)	16/42 (38.1%)
	3.6 (3.4-3.7)	3.2 (3.0-3.5)[e]	3.1 (2.8-3.5)	3.8 (3.6-4.0)[f]	3.7 (3.4-4.1)[ef]	3.6 (3.2-4.1)	3.7 (3.3-4.0)
1-CCFA / CTC (p=0.94)	44/261 (16.9%)	16/44 (36.4%)	11/44 (25%)	4/44 (9.0%)	2/43 (4.7%)	3/43 (7.0%)	8/43 (18.6%)
	3.3 (3.1-3.6)	3.4 (3.1-3.8)	3.2 (2.7-3.7)	3.3 (1.4-5.2)	2.9 (-0.9-6.7)	3.3 (1.1-5.4)	3.5 (2.7-4.2)
CCFA & no CTC (p=0.65)	75/260 (28.8%)	13/44 (29.5%)	2/44 (4.5%)	5/44 (11.4%)	17/43 (39.5%)	20/43 (46.5%)	18/42 (42.9%)
	3.6 (3.4-3.8)	3.9 (3.3-4.5)	3.0 (3.0-4.4)	3.7 (2.9-4.4)	3.6 (3.3-4.1)	3.5 (3.2-3.9)	3.5 (3.0-3.9)
CCFA & CTC (p=0.23)	36/259 (13.9%)	17/44 (38.6%)	2/44 (4.5%)	6/43 (14.0%)	5/43 (11.6%)	1/43 (2.3%)	5/42 (11.9%)
	3.5 (3.2-3.9)	3.9 (3.4-4.3)	2.6 (2.6-2.6)	3.1 (2.5-3.8)	3.1 (2.5-3.7)	4.0 (-)	3.6 (1.9-5.3)

[abcdef]: specified with different alphabet indicates significant difference between days ($p<0.05$) by regression analysis. In one row, if same alphabet is shared between different days, they are not significantly different ($P > 0.05$).

Table 20 Prevalence and *Salmonella* quantity per treatment group and days by direct plating on BGA-tet and BGA-cef

BGA-tet	Sample numbers / Log_{10} CFU (95% CI)	Day 0	Day 4	Day 8	Day 14	Day 20	Day 26
1-CCFA / no CTC (p=0.07)	7/260 (2.7%)	1/44 (2.3%)	0	0	2/44 (4.5%)	2/42 (4.8%)	2/42 (4.8%)
	3.6 (3.0-4.2)	3.1	-	-	4.1 (3.3-4.8)	4.1 (2.1-6.2)	3.0 (-1.5-7.4)
1-CCFA / CTC (p=0.84)	11/261 (4.2%)	0	0	2/44 (4.5%)	1/43 (2.3%)	2/43 (4.7%)	6/43 (14.0%)
	3.6 (3.0-4.2)	-	-	4.0 (-10.1-18.1)	2.9	3.4 (-7.2-14.2)	3.7 (2.8-4.6)
CCFA & no CTC (p=0.37)	6/260 (2.3%)	1/44 (2.3%)	1/44 (2.3%)	1/44 (2.3%)	1/43 (2.3%)	1/43 (2.3%)	1/42 (2.4%)
	3.6 (3.0-4.3)	4.4	2.9	4.0	3.5	4.0	2.9
CCFA & CTC (p=0.45)	8/259 (3.1%)	0	0	3/43 (7.0%)	2/43 (4.7%)	2/43 (4.7%)	1/42 (2.4%)
	3.6 (3.3-3.8)	-	-	3.5 (2.8-4.2)	3.7 (2.6-4.8)	3.5 (-1.9-8.8)	3.7

BGA-cef	Sample numbers / Log_{10} CFU (95% CI)	Day 0	Day 4	Day 8	Day 14	Day 20	Day 26
1-CCFA / no CTC (p=0.30)	9/260 (3.5%)	1/44 (2.3%)	0	1/44 (2.3%)	3/44 (6.8%)	2/42 (4.8%)	2/42 (4.8%)
	3.8 (3.4-4.2)	3.1	-	3.7	3.6 (2.1-5.1)	4.5 (3.5-5.5)	3.8 (2.8-4.9)
1-CCFA / CTC (p=0.90)	12/261 (4.6%)	1/44 (2.3%)	0	0	0	2/43 (4.7%)	6/43 (14.0%)
	3.7 (3.2-4.3)	4.4	-	-	-	3.5 (-4.5-11.5)	3.7 (2.6-4.7)
CCFA & no CTC (p=0.37)	13/260 (5%)	1/44 (2.3%)	1/44 (2.3%)	3/44 (6.8%)	2/43 (4.7%)	3/43 (7.0%)	3/42 (7.1%)
	3.6 (3.3-3.9)	4.4	3.3	4.0 (2.9-5.2)	3.3 (-1.4-8.0)	3.5 (2.0-5.0)	3.4 (2.5-4.3)
CCFA & CTC (p=0.45)	9/259 (3.5%)	0	0	3/43 (7.0%)	3/43 (7.0%)	2/43 (4.7%)	1/42 (2.4%)
	3.5 (3.1-3.9)	-	-	3.5 (2.8-4.3)	3.7 (2.6-4.8)	3.5 (-1.3-8.2)	3.7

Table 21 Counts of super shedder (shedding >10^4 per gram feces)

Number super shedder/detected (%)	Detection method	Day 0	Day 4	Day 8	Day 14	Day 20	Day 26
1-CCFA / no CTC	BGA-plain	1/15 (7%)	1/12 (8%)	7/19 (37%)	6/19 (32%)	7/16 (44%)	6/42 (38%)
	invA qPCR	11/24 (46%)	11/21 (52%)	13/15 (87%)	15/19 (79%)	9/16 (56%)	14/17 (82%)
1-CCFA & CTC	BGA-plain	2/16 (12.5%)	2/11 (18.2%)	1/4 (25%)	0/2 (0%)	1/3 (33.3%)	3/8 (37.5%)
	invA qPCR	11/28 (39.3%)	9/20 (45%)	2/5 (40%)	1/2 (50%)	1/3 (33.3%)	5/8 (62.5%)
All-CCFA / no CTC	BGA-plain	6/13 (46%)	0/2 (0%)	1/5 (20%)	3/17 (18%)	8/20 (40%)	6/18 (33.3%)
	invA qPCR	11/17 (65%)	1/14 (7%)	7/17 (41%)	15/18 (83%)	10/15 (67%)	10/16 (63%)
All-CCFA & CTC	BGA-plain	7/17 (41%)	0/2 (0%)	0/6 (0%)	0/5 (0%)	0/1 (0%)	2/5 (40%)
	invA qPCR	20/26 (77%)	1/9 (11%)	2/3 (67%)	2/2 (100%)	1/2 (50%)	4/6 (67%)

4.3.3.3 Serotype and quantity of *Salmonella*

No significant correlation was detected between the numbers of serotypes detected in an individual animal and treatment with CCFA (p=0.54), treatment with CTC (p=0.06), pens (p=0.26), or replicate (p=0.1), using Poisson regression analysis (Figure 27). No association was detected between serotypes and the quantity of *Salmonella* (Figure 28a). No association was detected between the number of serotypes that an animal carried and the quantity of *Salmonella* quantified by qPCR or direct plating (Figure 28b). Most of the animals from which *S.* Reading was isolated by enrichment methods, MDR serotype had growth on BGA-tet and BGA-cef (odds ratio: 16.1 (CI:3.6-70.9) and 23.0 (CI:5.3-100.2), respectively) by direct plating (Figure 28c, d). However, 2 samples (animals 2318 and 2396) from 1-CCFA / no CTC group (Figure 29a) and 1 sample (animal 2364) from All-CCFA / no CTC (Figure

29c) had growth on BGA-tet and BGA-cef, even though *S.* Reading was not isolated from all 6 days. Among them, animal 2318 was only detected with *S.* Mbandaka throughout the study period with high quantity ranged $\log_{10}$ 4.36-6.66 gene copies, which can be classified as high-shedder where the *Salmonella* population may have been dominated by *S.* Mbandaka.

4.3.3.4 Two animals had growth on BGA-cef on Day 0

On BGA-tet and BGA-cef agar, 2 samples (from animal 2342 and 2343 placed in separate pens) had growth on day 0, which was approximately 4.4 $\log_{10}$ CFU/gram feces in the samples from animal 2342 and 3.1 $\log_{10}$ CFU/gram feces in sample from animal 2343 on BGA-tet and BGA-cef. The colony growth on the plain-BGA of each sample was 6.2 and 3.2 log10 CFU/gram feces, respectively. The animal 2342 was detected with *S.* Give on day 0 and the animal 2343 was detected with *S.* Kentucky on day 0.

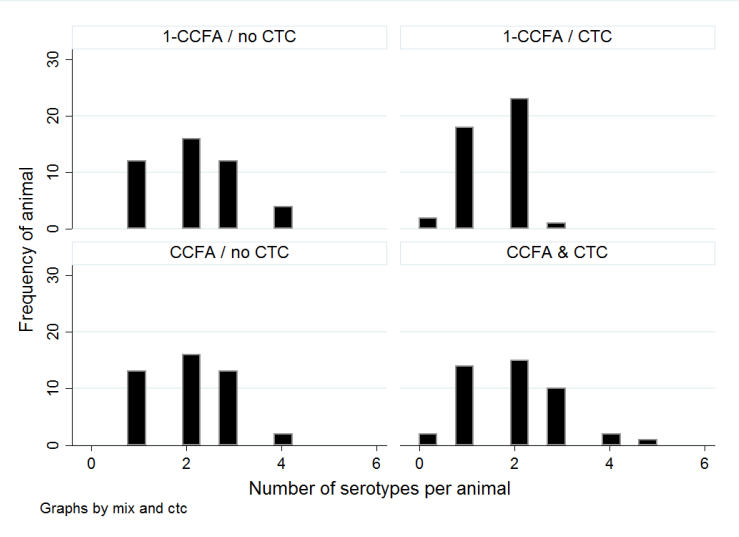


Figure 27 Frequency of animals per number of serotypes detected in one animal by treatment groups.

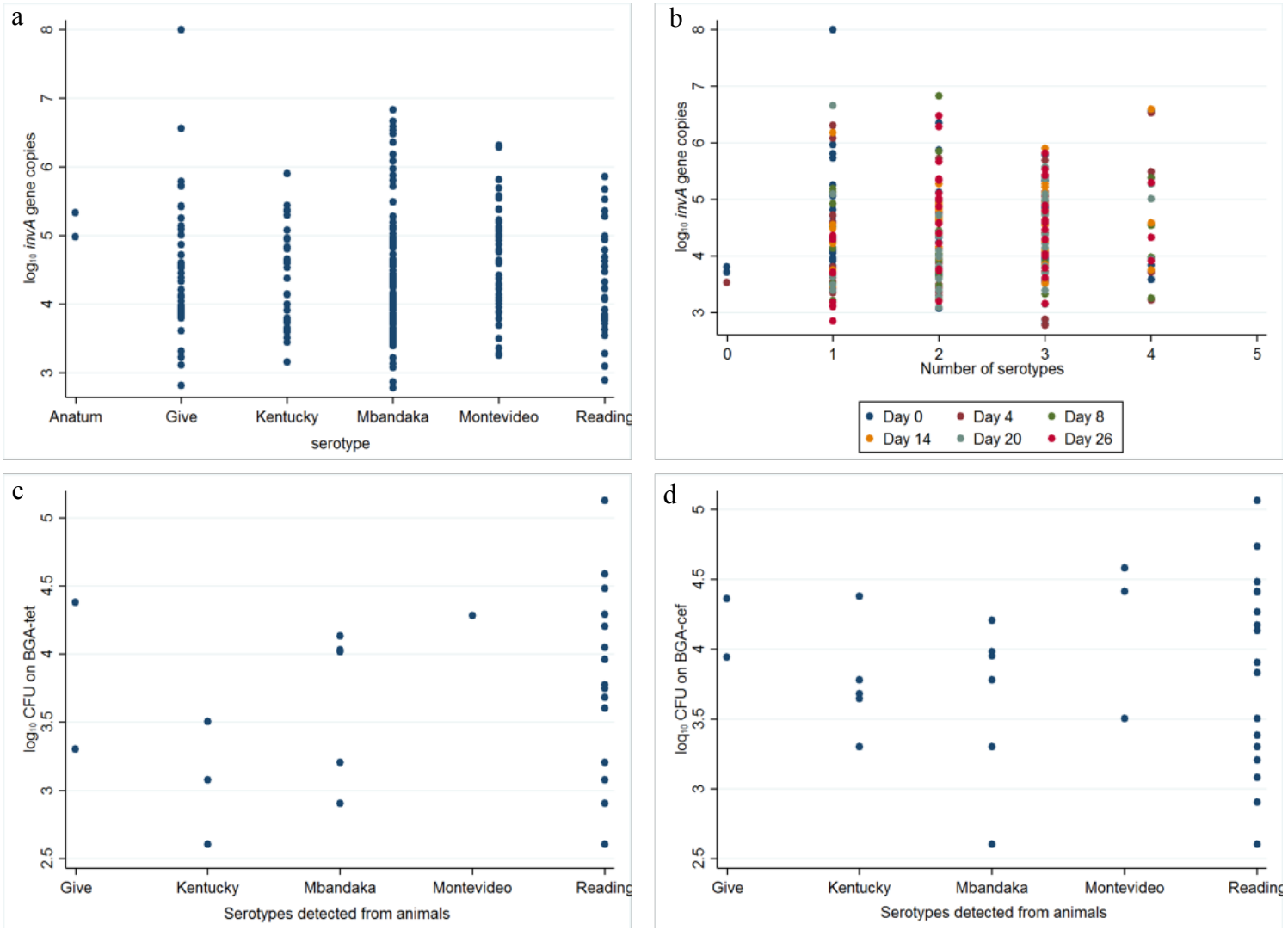


Figure 28 (a) Serotype and *invA* gene quantity, (b) number of serotypes detected per animal and *invA* gene quantity, (c) and (d) Serotypes detected from animals and growth on BGA-tet and BGA-cef.

a.

			serotypes						plain_logcolony						tet_logcolony						cef_logcolony						log_invA_average					
pen	animalid	Number of serotypes	Day 0	Day 4	Day 8	Day 14	Day 20	Day 26	0	4	8	14	20	26	0	4	8	14	20	26	0	4	8	14	20	26	0	4	8	14	20	26
44	2278	1	Kentucky	Kentucky	Kentucky	Kentucky	Kentucky	Kentucky	3.68	2.9	4.48	2.9	3.08	0	0	0	0	0	0	Pen 44	0	0	0	0	0	0	5.08	4.59	4.53	3.76	3.64	0
44	2280	1	Mbandaka	Mbandaka	Mbandaka	Mbandaka	Mbandaka	Mbandaka	2.6	0	0	0	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	3.61	4.27	0	0	0	0
44	2281	2	.	Mbandaka	Kentucky	Mbandaka	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.81	0	0	0	0	0
44	2306	3	Give	.	Mbandaka	Mbandaka	.	Montevideo	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.91	0	0	0	0	0
44	2335	2	.	.	.	Give	Kentucky	Kentucky	0	0	0	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.9	0	0	3.98	0	0
44	2351	2	Give	Mbandaka	Mbandaka	Mbandaka	Mbandaka	.	0	0	3.86	3.68	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.99	4.11	4.04	4.66	0	0
44	2365	1	.	.	.	.	.	Mbandaka	0	0	0	0	0	2.6	0	0	0	0	0	0	0	0	0	0	0	0	4.07	0	0	0	0	4.3
44	2369	2	.	.	.	Give	Kentucky	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.81	0	0	0	3.6	0
44	2376	3	Mbandaka	Mbandaka	Kentucky	Give	Give	Kentucky	0	0	3.2	3.86	3.08	0	0	0	0	0	0	0	0	0	0	0	0	0	4.98	1.9	3.91	5.09	3.81	0
44	2381	2	Kentucky	.	.	Mbandaka			0	0	0	0			0	0	0	0			0	0	0	0			3.63	3.27	0	0		
44	2397	1	Mbandaka	.	Mbandaka	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.12	3.12	0	0	0	0
46	2275	4	Mbandaka	Give	Kentucky	Reading	.	.	0	0	0	0	0	0	0	0	0	0	0	Pen 46	0	0	0	0	0	0	3.58	3.23	0	0	0	0
46	2289	2	Mbandaka	Mbandaka	Mbandaka	Give	.	Give	4.27	0	0	0	0	4.23	0	0	0	0	0	0	0	0	0	0	0	0	4.86	3.62	0	0	0	4.59
46	2318	1	Mbandaka	Mbandaka	Mbandaka	Mbandaka	Mbandaka	Mbandaka	3.08	3.45	4.33	4.68	0	3.45	0	0	0	4.13	0	0	0	0	0	3.95	0	0	5.81	6.09	5.14	6.18	6.66	4.36
46	2343	3	Kentucky	Reading	Kentucky	Kentucky	Mbandaka	Mbandaka	3.2	0	3.75	3.2	0	3.08	3.08	0	0	0	0	0	3.3	0	3.68	0	0	0	4.83	4.07	4.65	3.51	3.39	4.29
46	2362	4	Give	Mbandaka	Mbandaka	Reading	Reading	Kentucky	0	3.08	3.51	0	4.55	3.3	0	0	0	0	3.96	0	0	0	0	2.9	4.41	0	3.84	6.53	4.55	0	5.28	5.3
46	2367	2	Give	.	Give	.	.	Montevideo	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.94	0	0	0	0	0
46	2385	2	Montevideo	Montevideo	Montevideo	Montevideo	Montevideo	Kentucky	2.7	3.38	3.6	0	3.08	4.26	0	0	0	0	0	0	0	0	0	0	0	0	4.93	4.77	4.97	4.22	3.28	5.35
46	2393	1	Montevideo	Montevideo	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.78	1.75	0	0	0	0
46	2404	1	Mbandaka	Mbandaka	Mbandaka	.	Mbandaka	Mbandaka	0	0	3.64	0	4.12	2.6	0	0	0	0	0	0	0	0	0	0	0	0	4.34	3.65	5.19	0	5.1	4.36
46	2406	3	Montevideo	Montevideo	Montevideo	Mbandaka	Montevideo	Give	3.72	2.9	4.62	3.94	4.59	4.43	0	0	0	4.02	4.28	3.3	0	0	0	3.98	4.58	3.94	4.87	5.69	5.54	5.12	5.1	5.42
46	2423	2	.	Give	.	Montevideo	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.91	0	0	3.26	0	3.2
49	2241	2	Mbandaka	Mbandaka	Kentucky	Kentucky	Mbandaka	Mbandaka	3.81	0	3.75	4.56	4.31	3.9	0	0	0	0	0	Pen 49	0	0	0	0	0	0	4.24	0	0	4.81	4.36	4.87
49	2260	2	Give	.	Kentucky	Kentucky	Kentucky	.	0	0	3.38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
49	2263	1	.	Mbandaka	Mbandaka	Mbandaka			0	4.29	3.81	2.6			0	0	0	0			0	0	0	0			0	5.14	4.14	0		
49	2284	2	Montevideo	.	Mbandaka	Mbandaka	Mbandaka	Mbandaka	3.68	0	0	4.91	2.9	3.83	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.9	4.1	4.41
49	2297	1	.	Mbandaka	Mbandaka	Mbandaka	Mbandaka	Mbandaka	0	0	3.38	0	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
49	2322	3	Montevideo	Montevideo	Give	Kentucky	.	.	2.6	3.45	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.89	0	0	0	0
49	2350	2	Give	Give	Kentucky	Kentucky	Kentucky	.	2.9	0	0	3.86	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.88	0	4.63	0	0
49	2361	2	Give	Give	Give	Give	Montevideo	Montevideo	0	0	0	0	0	3.08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.22
49	2363	1	Mbandaka	Mbandaka	Mbandaka	Mbandaka	Mbandaka	Mbandaka	0	0	0	3.72	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.49	3.39	0
49	2373	1	Mbandaka	Mbandaka	.	.	.	Mbandaka	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
49	2396	3	Mbandaka	.	Give	Kentucky	Kentucky	Kentucky	2.6	0	0	4.45	4.26	4.89	0	0	0	0	0	2.6	0	0	0	0	3.78	0	0	0	0	4.65	4.15	5.44
56	2237	3	Mbandaka	Montevideo	Mbandaka	Kentucky	Montevideo	Montevideo	0	3.86	4.23	4.79	5.34	0	0	0	0	0	0	Pen 56	0	0	0	0	0	0	0	4.42	4.39	5.9	5.38	3.79
56	2259	3	Mbandaka	Mbandaka	Kentucky	Give	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
56	2267	3	Mbandaka	Mbandaka	Give	Mbandaka	Give	Montevideo	2.9	0	0	3.45	2.6	4.15	0	0	0	0	0	0	0	0	0	0	0	0	3.83	4.02	3.81	4.73	0	4.64
56	2277	2	.	Montevideo	Kentucky	.	Montevideo	Montevideo	0	2.6	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
56	2282	2	Montevideo	Give	Give	Give	.	Give	0	2.6	4.11	3.08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.45	4.38	0	0
56	2286	3	.	Mbandaka	Mbandaka	Give	Mbandaka	Kentucky	0	2.6	4.05	4.02	4.39	3.72	0	0	0	0	0	0	0	0	0	0	0	0	0	3.58	4.58	4.55	4.92	4
56	2290	1	.	.	Mbandaka	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
56	2299	1	.	.	.	.	Montevideo	Montevideo	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
56	2309	3	Mbandaka	Mbandaka	Give	Give	Montevideo	Montevideo	3.75	2.61	3.51	2.6	3.56	4.39	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.69	4.3
56	2311	3	Kentucky	Kentucky	Montevideo	Mbandaka	.	.	3.2	0	4.49	3.88	0	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.77	4.95	0	0
56	2382	3	Mbandaka	.	.	Montevideo	.	Kentucky	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 29. Description of overall population per treatment pens by detected serotypes per sampled days (0, 4, 8, 14, 20, and 26) and quantity of *Salmonella* per gram feces by direct plating and *invA* qPCR for 176 steers tested in this study.
Each serotype is color coded; Anatum: dark blue, Give: orange, Kentucky: red, Mbandaka: teal blue, Montevideo: Green, Reading: yellow. Serotypes were determined from the isolates derived after broth enrichment. Quantity of direct plating is $\log_{10}$ transformed and each number indicates the quantity per gram feces. Zero indicates no detection (LOQ). Gray squares are samples not collected. Number of serotype is the total serotypes detected from the animal from 6 sampled days. In each figure, the upper half is replicate 1 and the lower half is replicate 2. Each replicate is divided into 2 pens with dotted line. In 1-CCFA group, the one steer that received CCFA is colored in light gray. (a) 1-CCFA / no CTC group (b) 1-CCFA & CTC group (c) All-CCFA / no CTC (d) All-CCFA & CTC

b.

			serotypes						plain_logcolony						tet_logcolony						cef_logcolony						log_invA_average					
pen	animalid	Number of serotypes	Day 0	Day 4	Day 8	Day 14	Day 20	Day 26	0	4	8	14	20	26	0	4	8	14	20	26	0	4	8	14	20	26	0	4	8	14	20	26
43	2245	0	.	.	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	Pen 43	0	0	0	0	0	0	3.72	0	0	0	0	0
43	2257	2	.	Mbandaka	Kentucky	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.67	3.5	0	0	0	0
43	2266	2	Mbandaka	Mbandaka	Reading	.	.	.	3.08	4.59	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.63	5.72	0	0	0	0
43	2272	1	Give	Give	.	.	.	.	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.6	1.9	0	0	0	0
43	2292	1	.	Mbandaka	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.97	0	0	0	0	0
43	2344	0	.	.	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.72	0	0	0	0	0
43	2379	3	Anatum	Anatum	Reading	.	.	Give	3.38	0	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.33	0	0	0	0	3.6
43	2386	1	.	Mbandaka	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.92	3.56	0	0	0	0
43	2389	1	Give	.	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.83	0	0	0	0	0
43	2403	2	Montevideo	Montevideo	.	.	.	Mbandaka	3.68	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.13	3.88	0	0	0	0
43	2415	3	Mbandaka	Montevideo	Reading	.	Reading	Reading	2.9	2.6	0	0	0	2.9	0	0	0	0	0	2.9	0	0	0	0	0	3.2	4.97	4.1	0	0	0	4.5
45	2236	1	.	Kentucky	.	.	.	.	2.6	0	0	0	0	0	0	0	0	0	0	Pen 45	0	0	0	0	0	0	3.94	3.44	0	0	0	0
45	2246	2	.	Give	.	.	.	Reading	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.98	3.31	3.48	0	0	0
45	2268	2	.	Kentucky	Reading	.	Reading	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.97	4.97	3.28	0	3.09	0
45	2270	1	.	Mbandaka	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.12	3.44	0	0	0	0
45	2271	1	.	Montevideo	Montevideo	Montevideo	.	.	0	0	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.77	0	4.14	0	0	0
45	2331	2	Mbandaka	Reading	Reading	Reading	Reading	Reading	3.56	0	5.08	3.2	0	4.47	0	0	5.13	2.9	0	4.2	0	0	5.06	0	0	4.41	4.98	0	5.86	0	0	5.4
45	2337	3	Montevideo	.	Reading	.	.	Give	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.96	0	0	0	0	0
45	2345	3	Give	Give	Mbandaka	Reading	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.98	0	0	3.85	0	0
45	2378	4	Give	Mbandaka	.	Reading	.	Montevideo	0	2.6	0	0	0	3.08	0	0	0	0	0	0	0	0	0	0	0	0	3.98	5.49	0	0	0	0
45	2399	1	Montevideo	Montevideo	.	.	.	.	3.08	3.68	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.19	6.31	0	0	0	0
45	2417	2	.	Montevideo	Reading	.	.	Reading	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.98	3.36	0	0	0	0
51	2242	1	.	.	.	.	.	Give	0	0	0	0	0	0	0	0	0	0	0	Pen 51	0	0	0	0	0	0	0	0	0	0	0	0
51	2244	2	Mbandaka	Reading	Reading	.	Reading	Reading	3.38	2.6	2.9	2.6	2.6	2.6	0	0	0	0	0	0	0	0	3.51	0	0	2.6	3.87	0	3.63	0	0	0
51	2251	3	.	Kentucky	Reading	.	.	Mbandaka	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.9	0	0	0	0	0	0	0	0	0
51	2276	1	Give	.	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
51	2285	1	Mbandaka	Mbandaka	.	.	.	.	2.9	3.45	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.73	0	0	0	0
51	2296	1	Kentucky	Kentucky	.	.	.	.	3.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
51	2324	3	Kentucky	Give	.	Reading	Reading	Reading	4.58	3.78	0	0	4.25	4.73	0	0	0	0	4.29	4.59	4.38	0	0	0	4.17	4.74	4.95	5.14	0	0	4.79	5.5
51	2326	4	Kentucky	Mbandaka	Kentucky	Reading	.	Give	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
51	2357	2	Mbandaka	.	.	.	Reading	Reading	0	0	0	0	0	4.47	0	0	0	0	0	4.48	0	0	0	0	0	4.48	0	0	0	0	0	5
51	2409	2	Mbandaka	.	Reading	Reading	.	Reading	0	0	0	0	0	2.6	0	0	2.9	0	0	2.6	0	0	0	0	0	0	0	0	0	0	0	0
51	2418	1	.	Mbandaka	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
53	2239	2	Mbandaka	Kentucky	.	.	.	Kentucky	2.9	0	0	0	0	0	0	0	0	0	0	Pen 53	0	0	0	0	0	0	3.08	0	0	0	0	0
53	2247	5	Mbandaka	Kentucky	.	Give	Reading	Montevideo	0	0	0	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
53	2261	3	Give	Mbandaka	.	Reading	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.81	2.78	0	0	0	0
53	2264	1	Mbandaka	Mbandaka	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.13	0	0	0	0
53	2265	3	Mbandaka	Kentucky	.	Reading	Reading	Mbandaka	0	0	0	0	0	2.9	0	0	0	0	2.6	3.2	0	0	0	0	2.9	2.6	0	0	0	4.09	3.82	4
53	2307	2	Give	Mbandaka	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
53	2341	2	Kentucky	.	Give				0	0	0				0	0	0				0	0	0				0	3.75	0			
53	2358	2	Mbandaka	Mbandaka	.	.	.	Kentucky	3.98	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.6	0	0	0	0	3.7
53	2366	2	Mbandaka	Mbandaka	.	.	Reading	Reading	3.51	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
53	2392	3	Mbandaka	Montevideo	.	.	.	Reading	0	4.36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.06	0	0	0	0
53	2410	3	Kentucky	Mbandaka	.	.	.	Reading	4.88	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.38	4.26	0	0	0	3.8

Figure 29, Continued.

c.

			serotypes						plain_logcolony						tet_logcolony						cef_logcolony						log_invA_average					
pen	animalid	Number of serotypes	Day 0	Day 4	Day 8	Day 14	Day 20	Day 26	0	4	8	14	20	26	0	4	8	14	20	26	0	4	8	14	20	26	0	4	8	14	20	26
41	2253	2	Kentucky	Kentucky	Mbandaka	Mbandaka	.	.	0	0	0	0	0	0	0	0	0	0	0	Pen 41	0	0	0	0	0	0	4.38	3.79	3.48	0	0	0
41	2273	3	Anatum	Mbandaka	Mbandaka	.	Kentucky	.	3.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.98	4.08	3.7	0	3.15	3.2
41	2293	3	Kentucky	Kentucky	.	Mbandaka	Montevideo	Montevideo	0	0	0	0	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.73	3.33	0	4.63	0
41	2295	4	Give	.	.	Mbandaka	Montevideo	Kentucky	0	0	0	5.91	4.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.71	1.63	6.59	5.02	0
41	2310	2	Give	.	.	Mbandaka	.	.	0	0	0	3.68	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.79	3.22	3.6	0	0
41	2315	1	Give	.	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.62	3.51	0	0	3.2
41	2329	1	.	.	Mbandaka	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.48	0	0	0	0
41	2330	2	Mbandaka	.	Mbandaka	Mbandaka	Kentucky	Mbandaka	4.15	0	0	4.66	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	5.88	3.45	6.83	4.4	0	0
41	2370	1	Montevideo	.	.	Montevideo	Montevideo	Montevideo	3.68	0	0	3.6	0	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	3.8	0	4.22	0	0
41	2395	2	.	.	.	.	Montevideo	Mbandaka	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.39	0	0	0	0
41	2412	1	Mbandaka	Mbandaka	Mbandaka	Mbandaka	.		0	0	0	0	0		0	0	0	0	0		0	0	0	0	0		0	3.54	0	0	0	
48	2249	2	.	.	Anatum	.	Mbandaka	.	0	0	0	0	2.9	0	0	0	0	0	0	Pen 48	0	0	0	0	0	0	4.1	0	0	0	3.99	0
48	2291	2	Give	.	Give	.	Give	Mbandaka	3.23	0	0	0	4.64	4.29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.87	5
48	2294	1	.	.	.	Mbandaka	Mbandaka	Mbandaka	2.95	0	0	3.2	0	2.6	0	0	0	0	0	0	0	0	0	0	0	0	3.96	0	0	4.3	0	0
48	2317	2	Give	.	.	.	.	Mbandaka	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.04	0	0	0	0	0
48	2333	2	Kentucky	Kentucky	.	.	Give	.	0	0	0	0	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	3.66	0	0	0	0	0
48	2334	1	Mbandaka	Mbandaka	Mbandaka	Mbandaka	Mbandaka	Mbandaka	0	0	3.3	3.6	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.93	4.55	0	0
48	2342	4	Give	Reading	Montevideo	Reading	Mbandaka	Reading	6.19	3.08	4.39	2.9	3.08	2.6	4.38	2.9	0	0	0	0	4.36	3.3	4.41	2.9	3.3	3.08	6.56	0	5.38	3.76	3.94	4.3
48	2355	2	Give	Give	.	Mbandaka	.	.	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.61	0	0	0	0	0
48	2390	2	Mbandaka	.	.	Give	.	Mbandaka	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.79	3.23	0	0	0	0
48	2401	2	Mbandaka	Give	Mbandaka	Mbandaka	Mbandaka	Give	4.71	0	0	0	4.11	3.08	0	0	0	0	0	0	0	0	0	0	0	0	4.45	0	0	4.83	4.58	5
48	2408	1	Mbandaka	.	Mbandaka	Mbandaka	Mbandaka	.	0	0	0	0	3.3	0	0	0	0	0	0	0	0	0	0	0	0	0	3.76	0	3.21	3.46	3.49	0
52	2250	3	.	Give	.	Mbandaka	.	Reading	0	0	0	0	0	0	0	0	0	0	0	Pen 52	0	0	0	0	0	0	0	0	0	0	0	0
52	2262	4	Montevideo	.	Kentucky	Mbandaka	Montevideo	Reading	0	0	0	0	0	2.9	0	0	0	0	0	0	0	0	0	0	0	3.38	0	0	0	0	0	3.9
52	2300	4	Kentucky	.	Mbandaka	Mbandaka	Montevideo	Reading	0	0	0	3.6	3.08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.98	4.58	0	0
52	2301	1	.	.	.	.	.	Montevideo	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
52	2308	3	Give	.	Reading	Give	Montevideo	Montevideo	0	0	0	3.38	4.36	3.92	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.11	4.38	4.6
52	2346	3	Mbandaka	.	Mbandaka	Mbandaka	Give	Montevideo	4.55	0	0	0	3.75	4.19	0	0	0	0	0	0	0	0	0	0	0	0	4.95	0	4.03	4.25	4.21	4.8
52	2352	3	Give	Reading	Reading	Montevideo	Montevideo	Montevideo	5.07	2.9	2.9	3.88	4.08	2.6	0	0	0	0	0	0	0	0	0	0	0	0	5.79	0	4.55	5.23	5	4
52	2360	3	.	.	Mbandaka	Mbandaka	Montevideo	Give	0	0	0	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.28	0	0
52	2364	3	Mbandaka	.	Montevideo	Kentucky	Mbandaka	Mbandaka	0	0	3.86	3.75	4.15	4.06	0	0	0	3.51	4.03	2.9	0	0	3.51	3.64	4.2	3.78	0	0	4.27	4.81	4.72	4.9
52	2380	3	.	Reading	Reading	Montevideo	.	Mbandaka	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.54	0	0	0	0
52	2420	3	Mbandaka	Reading	.	Kentucky	.	Mbandaka	0	0	0	0	0	2.95	0	0	0	0	0	0	0	0	0	0	0	0	2.87	2.89	0	0	0	0
54	2235	1	Mbandaka	.	.	.	.	Mbandaka	0	0	0	0	0	0	0	0	0	0	0	Pen 54	0	0	0	0	0	0	0	0	0	0	0	3.7
54	2240	1	Mbandaka	.	.	.	Mbandaka	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
54	2248	2	Mbandaka	.	.	Give	Mbandaka	.	0	0	0	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
54	2252	1	.	Mbandaka	.	.	.	Mbandaka	0	0	0	0	0	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.7
54	2288	2	Mbandaka	.	.	Mbandaka	Mbandaka	Montevideo	4.13	0	0	3.83	4.4	5.25	0	0	0	0	0	0	0	0	0	0	0	0	0	4.59	0	3.92	5.28	4.42	6.3
54	2314	2	Give	.	Mbandaka	Mbandaka	Mbandaka	Mbandaka	0	0	0	3.51	2.9	4.65	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.73	4.17	3.41	5.1
54	2316	3	Mbandaka	Mbandaka	.	Give	Montevideo	Montevideo	0	0	0	4.52	5.08	5.37	0	0	0	0	0	0	0	0	0	0	0	0	4.9	0	0	5.43	5.58	5.8
54	2325	3	Mbandaka	.	Reading	Mbandaka	Kentucky	Kentucky	3.22	0	0	0	2.9	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
54	2338	2	Mbandaka	.	Reading	Mbandaka	Reading	Mbandaka	0	0	3.86	2.6	2.92	2.6	0	0	4.05	0	0	0	0	0	4.13	0	3.08	0	0	0	4.95	0	0	0
54	2402	2	.	.	.	Give	.	Kentucky	0	0	0	0	0	2.91	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.8
54	2405	1	Kentucky	.	.				0	0	0				0	0	0				0	0	0				0	0	0			

Figure 29, Continued.

d.

			serotypes						plain_logcolony						tet_logcolony						cef_logcolony						log_invA_average					
pen	animalid	Number of serotypes	Day 0	Day 4	Day 8	Day 14	Day 20	Day 26	0	4	8	14	20	26	0	4	8	14	20	26	0	4	8	14	20	26	0	4	8	14	20	26
42	2283	1	Give	.	.	.	.	.	4.13	0	0	0	0	0	0	0	0	0	0	Pen 42	0	0	0	0	0	0	4.52	3.62	0	0	0	0
42	2287	1	Give	.	.	.	.	Give	3.45	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.26	3.35	0	0	0	0
42	2323	2	Give	Kentucky	Kentucky	.	.	.	0	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.33	4.13	0	0	0	0
42	2332	1	Give	Give	.	.	.	.	3.08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.71	1.9	0	0	0	0
42	2336	1	Give	.	.	.	.	.	5.04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.91	3.5	0	0	0	0
42	2347	1	Give	Give	.	.	Give	Give	0	0	2.9	0	0	3.08	0	0	0	0	0	0	0	0	0	0	0	0	8	3.81	0	0	0	3.1
42	2384	2	Give	.	.	.	.	Mbandaka	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.97	3.38	0	0	0	0
42	2387	0	.	.	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.8	1.77	0	0	0	0
42	2391	1	Give	.	.	.	.	.	0	0	0	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.1	3.67	0	0	0	0
42	2394	1	Give	.	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.82	0	0	0	0	0
42	2421	1	Mbandaka	Mbandaka	Mbandaka	.	.	.	0	0	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.82	0	0	0	0	0
47	2254	2	Mbandaka	.	Reading	.	.	.	0	0	0	0	0	0	0	0	0	0	0	Pen 47	0	0	0	0	0	0	3.91	0	0	0	0	0
47	2274	2	Mbandaka	.	Mbandaka	.	.	Give	4.16	0	0	0	0	2.6	0	0	0	0	0	0	0	0	0	0	0	0	6.36	0	0	0	0	4.6
47	2279	2	.	.	Reading	Reading	Give	Give	0	0	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.03	0	0	0	0	0
47	2304	2	Mbandaka	.	Reading	.	.	.	3.68	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.97	0	0	0	0	4.2
47	2312	1	Montevideo	.	.	.	.	.	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.06	0	0	0	0	0
47	2313	1	Mbandaka	.	.	.	.	Mbandaka	3.3	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.97	0	0	0	0	0
47	2371	1	Give	.	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.33	0	0	0	0	0
47	2372	3	Give	.	.	.	Reading	Mbandaka	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.13	0	0	0	0	0
47	2375	1	.	Mbandaka	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.15	0	0	0	0	0
47	2377	2	Mbandaka	.	Give	.	.	.	0	0	0	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.16	0	0	0	0	0
47	2413	1	Give	Give	Give	.	.	.	4.09	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.74	0	0	0	0	0
50	2269	2	.	.	Reading	.	.	Give	0	0	0	0	0	0	0	0	0	0	0	Pen 50	0	0	0	0	0	0	0	0	0	0	0	0
50	2302	2	Give	.	Reading	Reading	Reading	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	2321	1	Montevideo	.	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	2327	2	Montevideo	.	.	.	.	Kentucky	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	2339	2	Give	.	.	.	.	Reading	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	2353	1	Give	Give	.	.	.	.	4.83	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	2356	0	.	.	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	2388	2	.	Kentucky	.	.	.	Mbandaka	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	2407	1	.	Give	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	2414	1	.	Give	.	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.9
50	2422	2	.	Mbandaka	.	.	.	Kentucky	0	0	0	0	0	2.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
55	2238	2	Give	Give	.	Reading	.	Reading	6.08	0	0	0	0	0	0	0	0	0	0	Pen 55	0	0	0	0	0	0	5.72	0	0	0	0	0
55	2255	2	Mbandaka	.	.	Reading	.	.	3.21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
55	2303	2	Mbandaka	.	Reading	.	.	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.82	0
55	2328	2	Montevideo	.	.	.	.	Kentucky	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
55	2354	2	Mbandaka	.	Reading	.	.	Reading	4.67	0	3.9	0	0	4.12	0	0	3.75	0	0	3.75	0	0	3.51	0	0	4.26	4.62	0	4.22	0	0	5.7
55	2359	1	Montevideo	.					3.38	0					0	0					0	0					4.05	0				
55	2374	2	Mbandaka	.	Reading	Reading	.	Mbandaka	2.9	0	3.94	3.51	3.96	5.83	0	0	3.68	3.6	3.92	0	0	0	3.9	3.3	3.83	0	0	0	4.63	4.9	4.72	6.5
55	2383	2	Give	.	Reading	.	Reading	.	3.21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
55	2398	2	Mbandaka	.	Reading	Reading	Reading		3.91	0	2.9	3.72	0		0	0	3.2	3.78	3.08		0	0	3.2	3.83	3.08		4.39	0	3.72	4.7	0	
55	2400	2	Mbandaka	.	.	.	Reading	Mbandaka	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
55	2411	2	Give	.	Reading	Reading	.	Reading	0	0	0	2.6	0	0	0	0	0	0	0	0	0	0	0	2.6	0	0	0	0	0	0	0	0

Figure 29, Continued.

4.3.4 Modelling the effects of antibiotics on quantity dynamics

4.3.4.1 Distribution of $\log_{10}$ invA gene copies per gram feces per antibiotic treatment before and after imputation

Among 1,040 samples tested in duplicate (2,080 reactions), 571 reactions (27.5%) were detected with the *invA* gene. The lowest and highest quantified gene copies ranged from $\log_{10}$ 2.78 to 8.00 *invA* gene copies per gram feces with a median of $\log_{10}$ 4.32 and mean of $\log_{10}$ 4.43 (Table 22, Figure 30). To increase the resolution of observed values in Figure 30a, missing values (0) were removed in Figure 30b and Figure 30c. From our annotated WGS data, only 1 gene copy of *invA* gene was detected in the representative genomic sequence per isolate. The quantities of *invA* gene copies were standardized with 16s rRNA quantities by taking the ratio and these were $\log_{10}$ transformed (Table 22, Figure 31).

Data for missing 1,509 (72.5%) wells (i.e., those with Cq values > 40) from qPCR runs were imputed using interval censored regression on Stata. The assumption was that samples containing *Salmonella* at concentrations less than the LOQ were likely to not harbor exactly zero bacteria. Multiple imputation methods impute the missing data as censored data by a parametric method given other predictors and assuming a normal distribution from observed data. The upper limit value of the left censoring data was set to $\log_{10}$ 3.6, which is the mean and median of the *invA* gene copy numbers in the samples with one-missing qPCR value (Table 22). The lower limit was set to $-\infty$ on the $\log_{10}$ scale. Predictors were the full factorial model of CCFA and CTC treatments by day of sampling. The $\log_{10}$ *invA* gene copies per gram feces were imputed as shown in Figure 32. The average of smallest quantity after 8 imputation runs was as low as $\log_{10}$ -2.81 and the largest was $\log_{10}$ 3.59 (Figure 32). Figure 33 is the distribution of observed (Figure 33a) and imputed (Figure 33b) $\log_{10}$ *invA* gene copies per gram feces, grouped by

missing values per qPCR reaction. Figure 34 is observed (Figure 34a) and imputed (Figure 34b) $\log_{10}$ *invA* gene copies per gram feces by CCFA and CTC treatments. Figure 35 is observed and imputed by CCFA and CTC treatments by day.

Table 22. Observed and 16s rRNA standardized $\log_{10}$ *invA* gene copies per gram feces per qPCR reaction.

invA gene detection	N	Mean ($\log_{10}$)	Median ($\log_{10}$)	Min ($\log_{10}$)	Max ($\log_{10}$)
Overall runs summary	571	4.43	4.32	2.78	8.00
Both duplicate detected	494	4.55	4.47	3.06	8.00
Missing in 1 reaction	77	3.65	3.65	2.78	4.58
16s standardized *invA*	N	Mean ($\log_{10}$)	Median ($\log_{10}$)	Min ($\log_{10}$)	Max ($\log_{10}$)
Overall runs summary	571	-5.13	-5.24	-7.20	-1.29
Both duplicate detected	494	-5.00	-5.08	-7.20	-1.29
Missing in 1 reaction	77	-5.95	-5.98	-7.03	-4.59

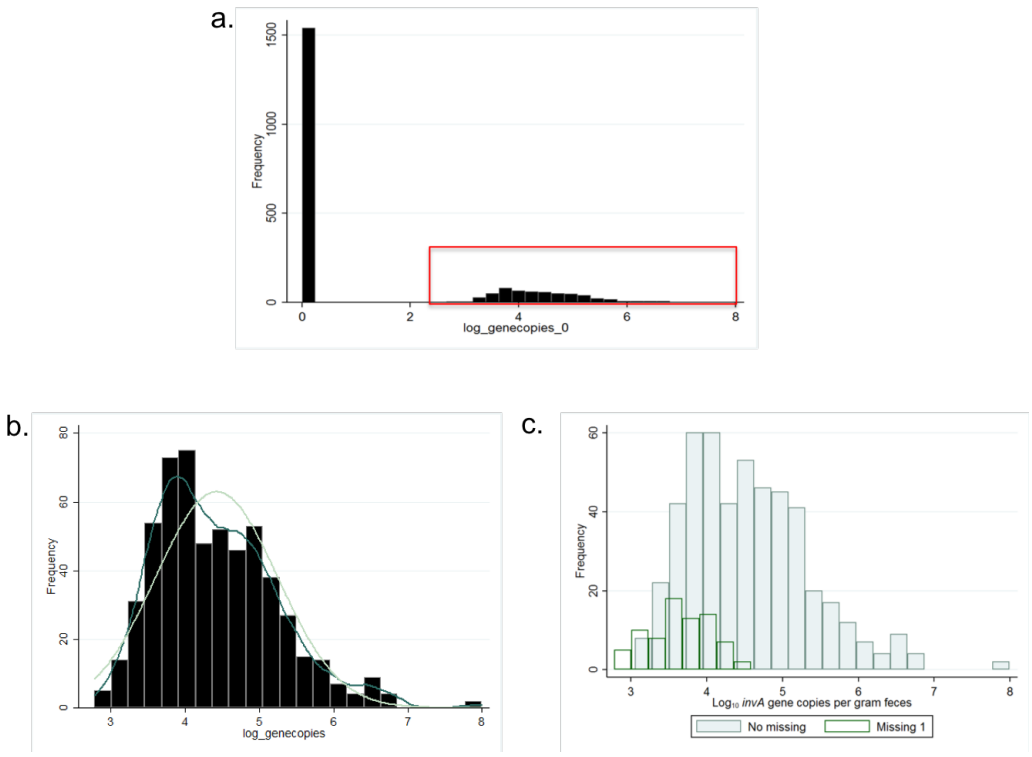


Figure 30 Distribution of $\log_{10}$ *invA* gene copies from observed data. (a) Overall distribution of $\log_{10}$ *invA* gene copies per gram feces.
Missing observations (below LOQ) were given a value 1 and $\log_{10}$ transformed to 0 in (a). (b) Distribution of red squared area from (a). (c) Distribution of $\log_{10}$ *invA* gene copies of samples that had no missing values (light blue) and missing 1 (green).

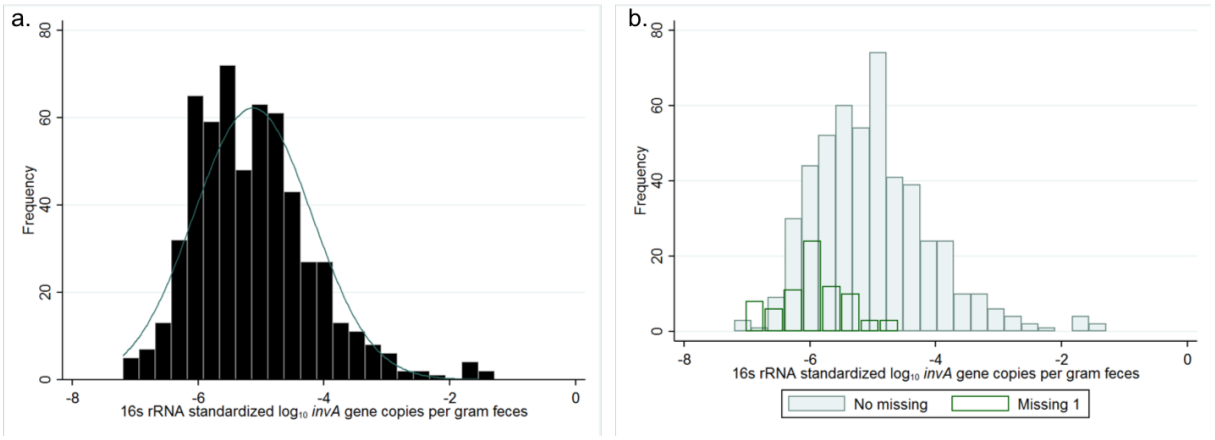


Figure 31. Distribution of 16s rRNA standardized $\log_{10}$ *invA* gene copies from observed data. (a). Distribution of $\log_{10}$ *invA* gene copies per gram feces after 16s rRNA standardization. (b.) Distribution of $\log_{10}$ *invA* gene copies per gram feces after 16s rRNA standardization that had no missing values (light blue) and missing 1 (green).

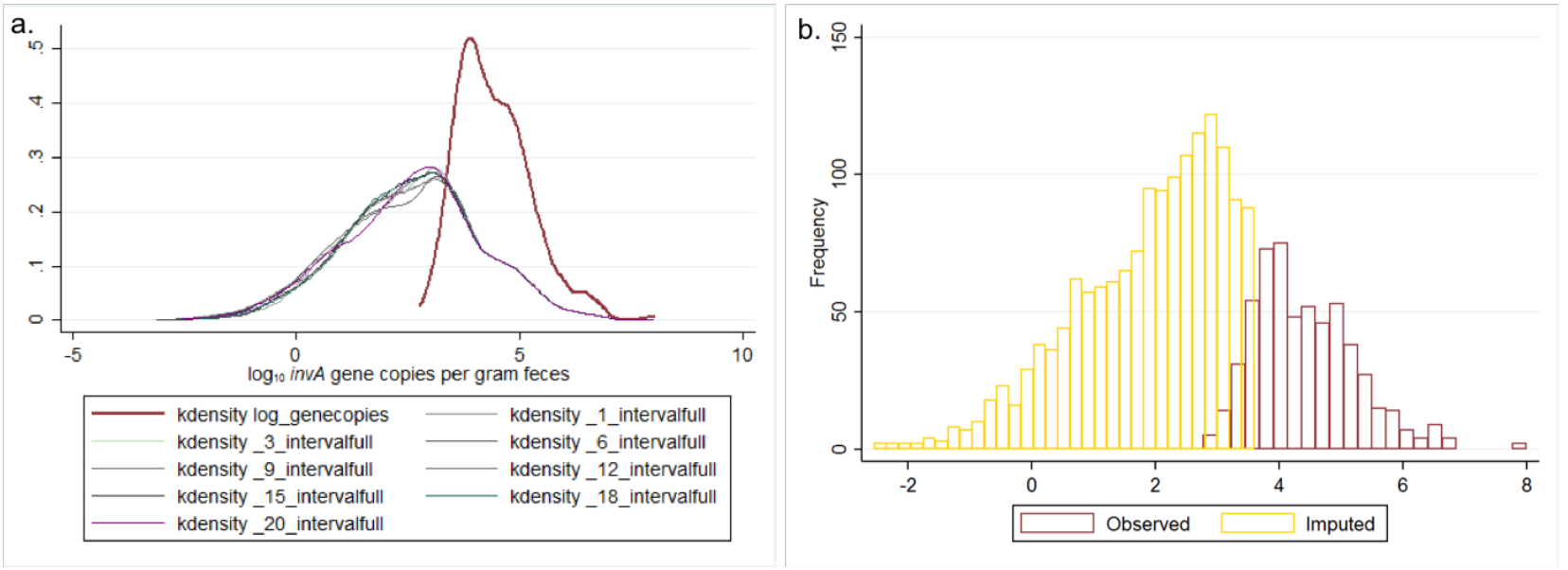


Figure 32. Histogram and Kernel density of observed and imputed data. (a) Observed data (red) and imputed data from 8 imputations (other lines), X-axis is log10 invA gene copies and Y-axis is density. (b) Distribution of observed (red) and represented imputed data (yellow)

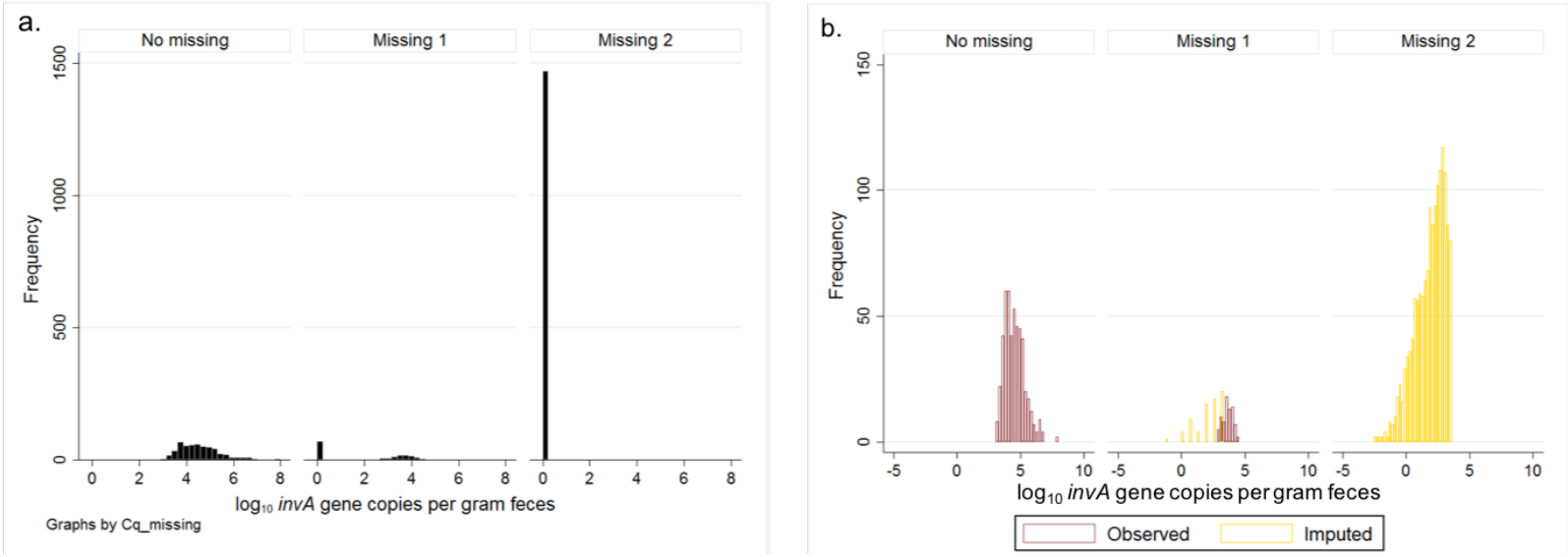


Figure 33. Distribution of $\log_{10}$ *invA* gene copies before (a) and after (b) interval censored regression per missing value groups. (a) is with missing value 0 and (b) is after imputation.
The upper limit was set to $\log_{10}$ 3.6 for imputation. No missing: all duplicates have values after qPCR; Missing 1: one of the duplicate reaction had an observed Cq value; Missing 2: neither of the reaction had observed Cq values.

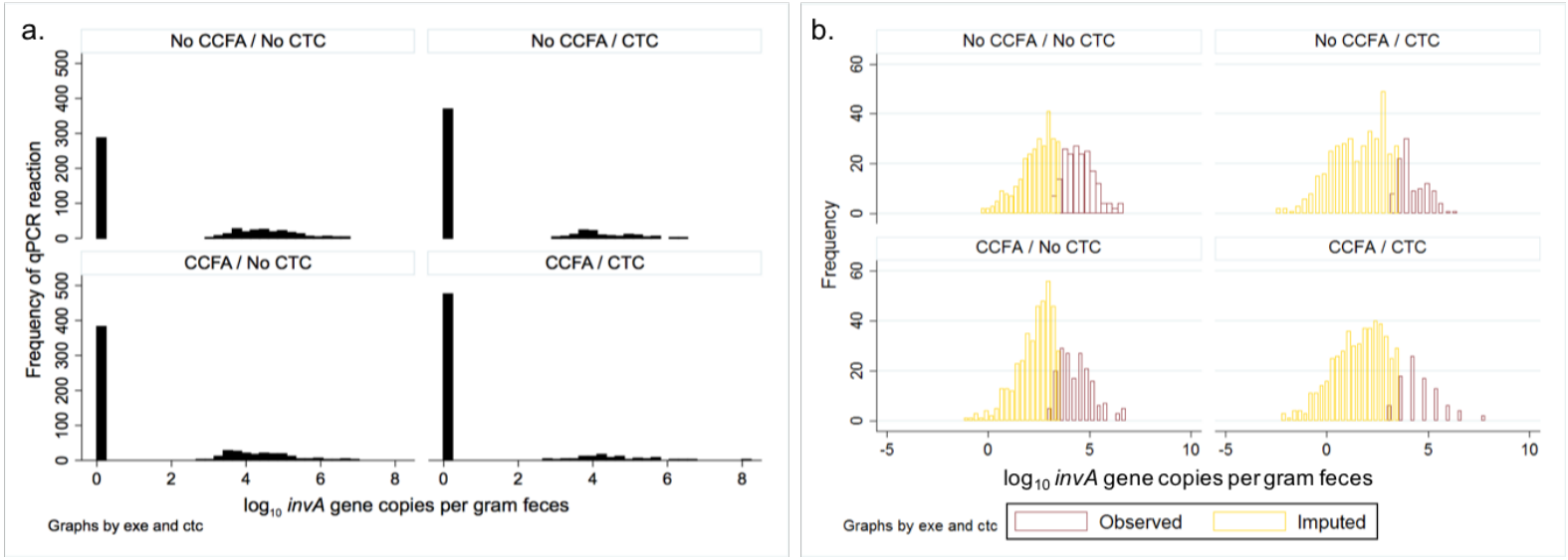


Figure 34. Distribution of $\log_{10}$ *invA* gene copies per gram feces per treatments before (a) and after imputation (b). Missing value was given a value 1, which are shown as 0 after $\log_{10}$ transformation. CCFA: ceftiofur crystalline-free acid, CTC: chlortetracycline

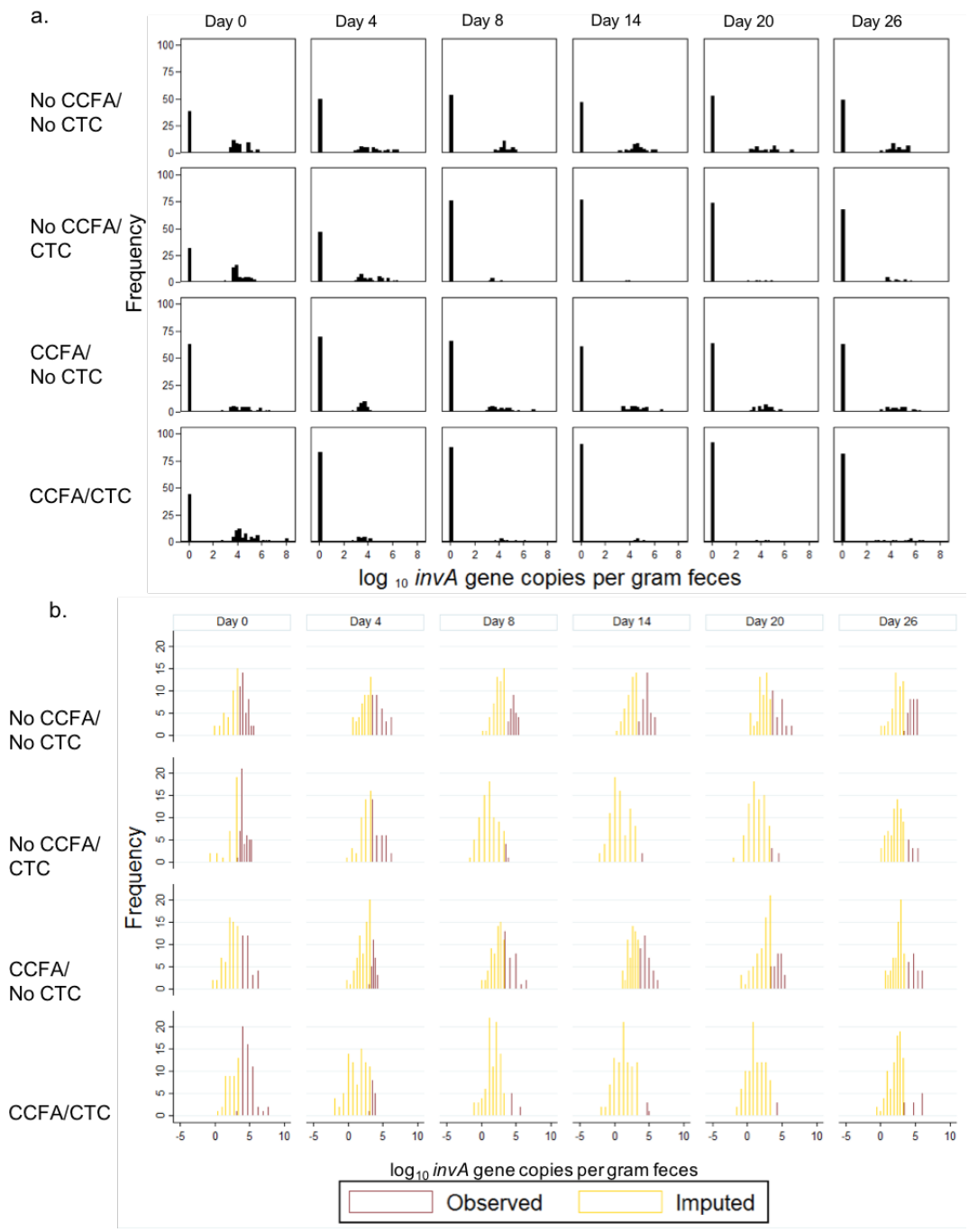


Figure 35. Distribution of $\log_{10}$ *invA* gene copies per gram feces by treatments and day from (a) observed and (b) imputed data.
CCFA: ceftiofur crystalline-free acid, CTC: chlortetracycline

4.3.4.2 Effects of antibiotic treatments on quantity of invA gene copies by zero-inflated Poisson regression model with observed data and 16s standardized observed data

The effects of antibiotic treatment on the $\log_{10}$ *invA* gene copies first were modelled by a zero-inflated Poisson regression model with the observed data (before imputation). The $\log_{10}$ *invA* values were rounded and converted to numerical values. The distribution of the numerical counts are shown in Figure 36a, which are zero-inflated. The median rounded $\log_{10}$ was 4. The zero-inflated Poisson Regression model predicted that treatment by CTC significantly decreased the rounded $\log_{10}$ count of *Salmonella* on Day 8, and it remained at low levels until the end of the study period (Figure 36b). CCFA treatment on Day 0 drastically decreased the count on Day 4 in the All-CCFA & CTC group. The zero-inflated model predicted that CTC treatment increased the zero inflation significantly ($P<0.05$); interestingly, CCFA treatment was not significant in the same way. Figure 36c and 36d illustrates the distribution of 16s standardized $\log_{10}$ *invA* gene copies, which show a similar pattern to that of the non-standardized data.

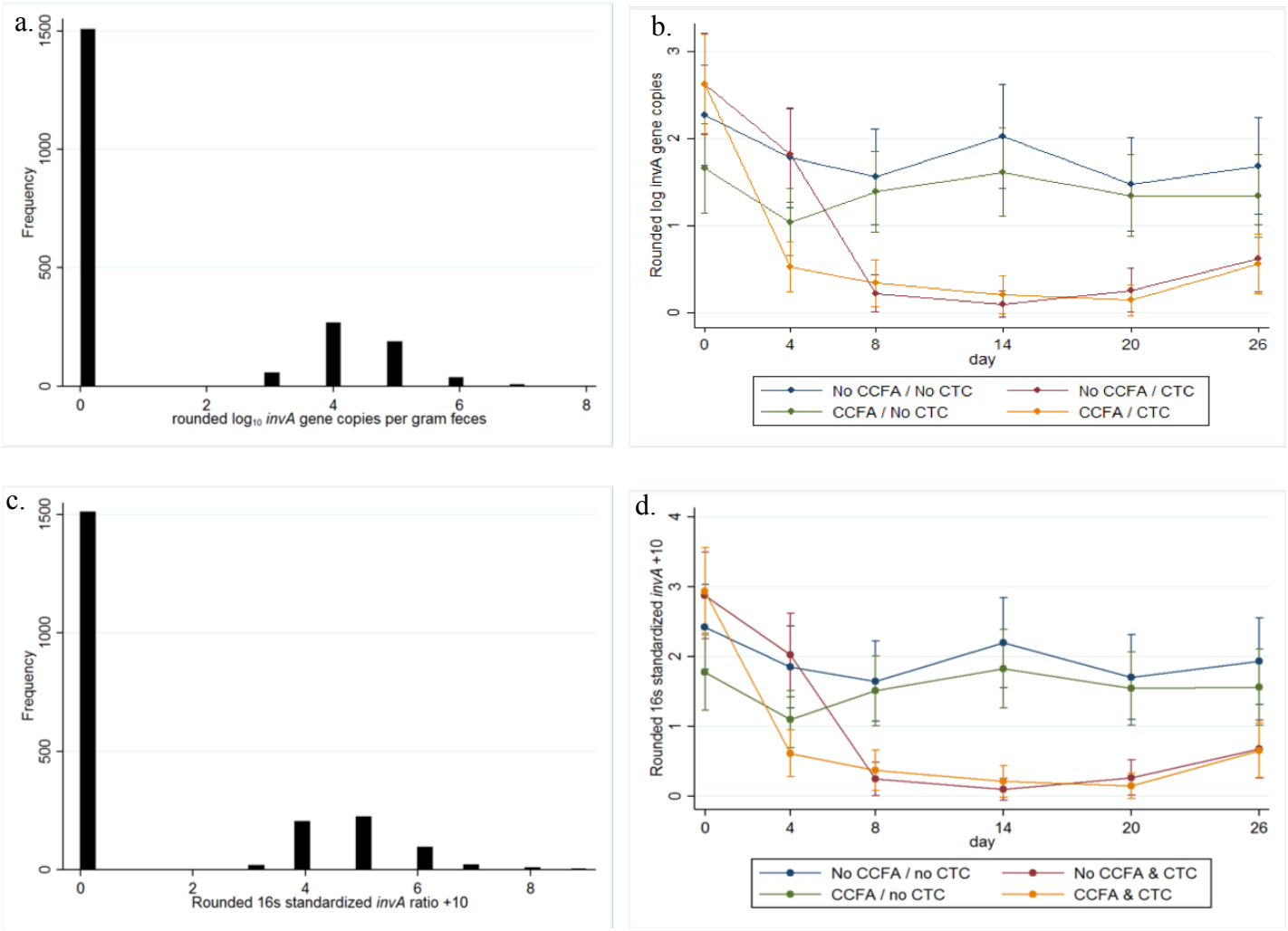


Figure 36. (a) Distribution of rounded $\log_{10}$ *invA* gene copies per gram feces and (b) modelled margins prediction of *invA* quantity by zero-inflated Poisson regression with 95% CI. (c). Distribution of 16s standardized rounded $\log_{10}$ *invA* gene copies per gram feces and (d) modelled prediction of *invA* by zero-inflated Poisson regression 95% CI.
Missing observations were given a value 1 and $\log_{10}$ transformed to 0. CCFA: ceftiofur crystalline-free acid, CTC: chlortetracycline

4.3.4.3 Multilevel mixed-linear regression model with representative imputed values

A representative imputed value set was chosen for multilevel mixed-linear regression to assess the effects of antibiotic treatments by day on the quantity of *Salmonella* represented by *invA* gene copies per gram of feces. In Stata, estimation following multiple imputation can be performed; however, no programmed methods are provided to predict margins from the estimation model. As shown in Figure 32, all the imputed values distributed similarly; therefore, only one out of the 20 imputed data models was chosen for further estimation and margins prediction. Margins of treatment effects on $\log_{10}$ *invA* gene copies per gram feces were predicted by multilevel mixed linear regression (Figure 37a). Random effects were replicate, pen, animal, and duplicate repeats of the qPCR reaction. A significant decrease in imputed and observed gene copies can be seen with CTC treatment, especially on Day 8, where the animals treated with only CTC (red) show a dramatic decrease. With CCFA treatment on Day 0, gene copies also significantly decreased (orange). A slight increase in gene copies is observed following CTC treatment on Day 8 (orange). In the animals treated with CCFA on Day 0 (Green), only a slight decrease of gene copies was observed.

4.3.4.4 Multilevel mixed-linear regression model with multiple imputed values

Following the multiple imputations, the multiple imputed values were further used to estimate the effects of antibiotics by multilevel mixed-effects linear regression model. Stata does not officially provide a method to compute margins following model estimation with multiple imputed values; however, a command "mimrgns" provided by UCLA is available for installation for margins prediction. To set as panel data, pen was set as the clustering (panel) variable and trial replicate was included as a fixed effect. Treatments by CCFA and CTC by

day were included for a 3-way full factorial model. Animal ID was included as a random effect. Accurate confidence intervals cannot be calculated by this method and therefore are not included in Figure 37b. The overall results were very similar to that of Figure 37a. Similarly, 16s standardized *invA* values were imputed and modelled by multilevel mixed-effects linear regression (Figure 38).

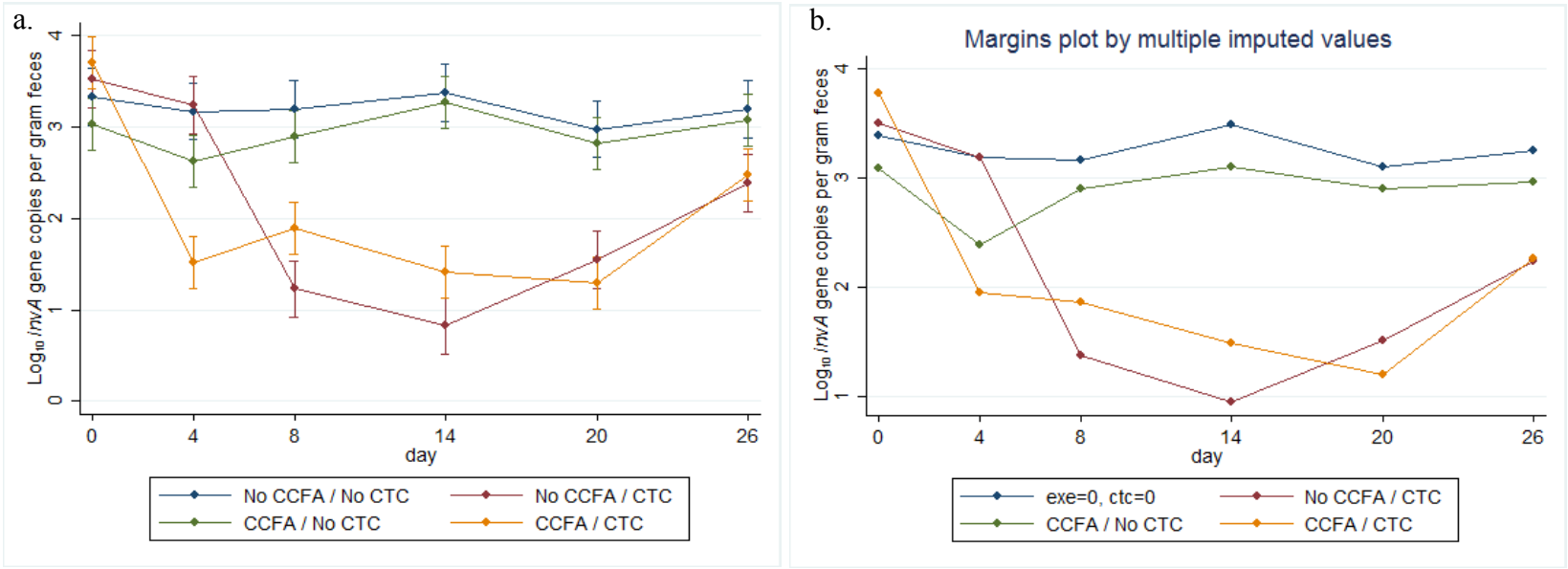


Figure 37. Modelled margins prediction with single imputed data (a) and 20 multiple imputed data (b) for $\log_{10}$ *invA* gene copies per gram feces by treatments and days with 95% CI. 95% CI is not shown in multiple imputed data because of the limitation of the mimrgns program to predict accurate values. CCFA: ceftiofur crystalline-free acid, CTC: chlortetracycline

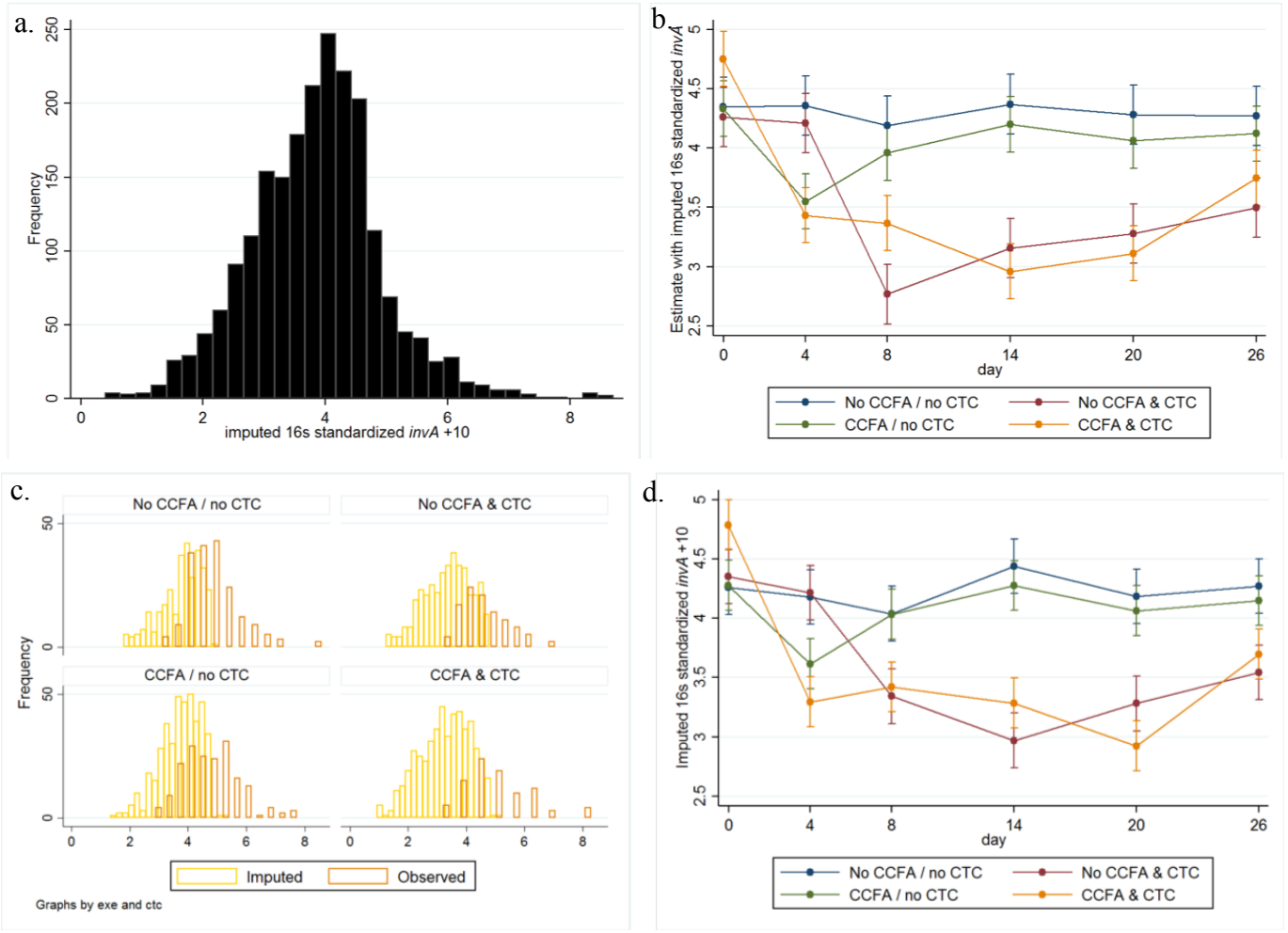


Figure 38. Distribution of 16s standardized *invA* +10. (b) Mixed linear regression model with observed value. (c) imputed 16s standardized *invA* (+10) $\log_{10}$ gene copies per gram feces. (d) Mixed linear regression model with imputed value

4.3.4.5 Distribution of $\log_{10}$ CFU per gram feces per antibiotic treatment before and after imputation

Direct spiral plating was conducted in singlicate. The lowest and highest CFU per gram of feces ranged from $\log_{10}$ 2.60 to 6.19 in a total of 252 samples (24.2%) for which growth was quantifiable on BGA (Figure 39a). Mean count was $\log_{10}$ 3.54 and median was $\log_{10}$ 3.45 CFU per gram feces. Missing values were imputed as shown in Figure 39b. Figure 40 and 41 show per treatment and per treatment and day, respectively. 1-CCFA / no CTC animals had the highest detection rate (8.9%, 93/1040) among all treatment groups. In the 1-CCFA / no CTC animals, *Salmonella* were detected constantly throughout the study period (Table 19 bottom). With All-CCFA / no CTC treatment, less animals were detected with *Salmonella* on Day 4 (Figure 19 bottom).

Multiple imputation by interval censoring regression was conducted for the missing values. After trying a range of different upper limit values (mean 3.6 and higher 4.0), the final upper limit of left censored value was set to $\log_{10}$ 2.6, which was the lowest observed value. Imputed values are visualized for all distributions as per treatments, and per treatments and days as green bars in the following figures 40, 41, and 42, respectively. The red bars are the observed data.

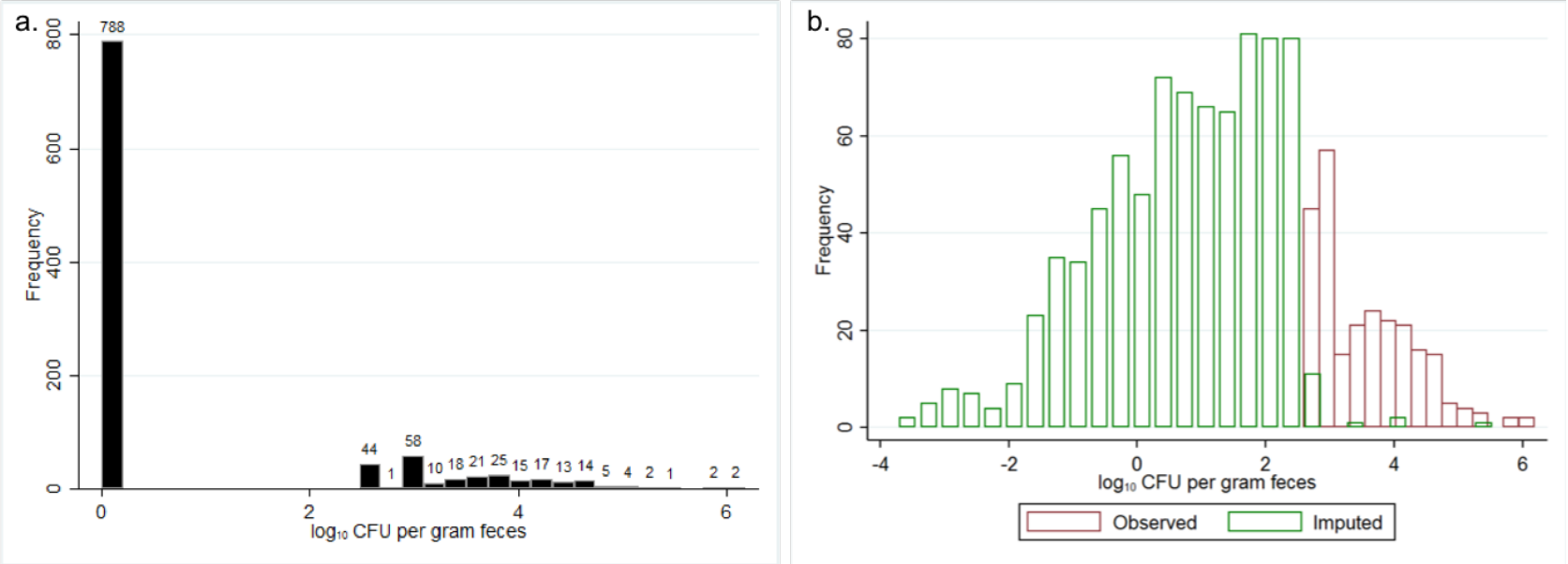


Figure 39. Histogram of $\log_{10}$ CFU per gram feces counted on BGA (a) before and (b) after imputation.
Red: observed values, Green: imputed values. Numbers are the $\log_{10}$ bacterial counts per sample.

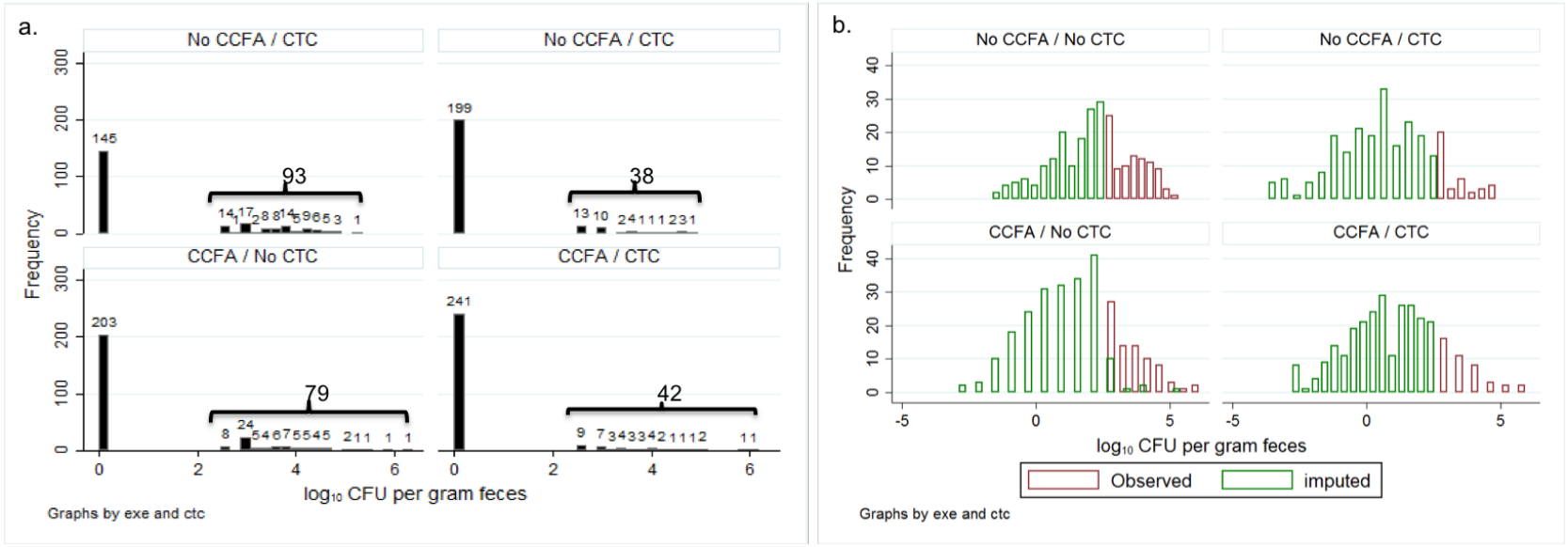


Figure 40. Distribution of $\log_{10}$ CFU per gram feces per individual antibiotic treatment from (a) observed and (b) imputed data by treatments. Missing value was given a value 1, which are shown as 0 after log10 transformation. CCFA: ceftiofur crystalline-free acid, CTC: chlortetracycline

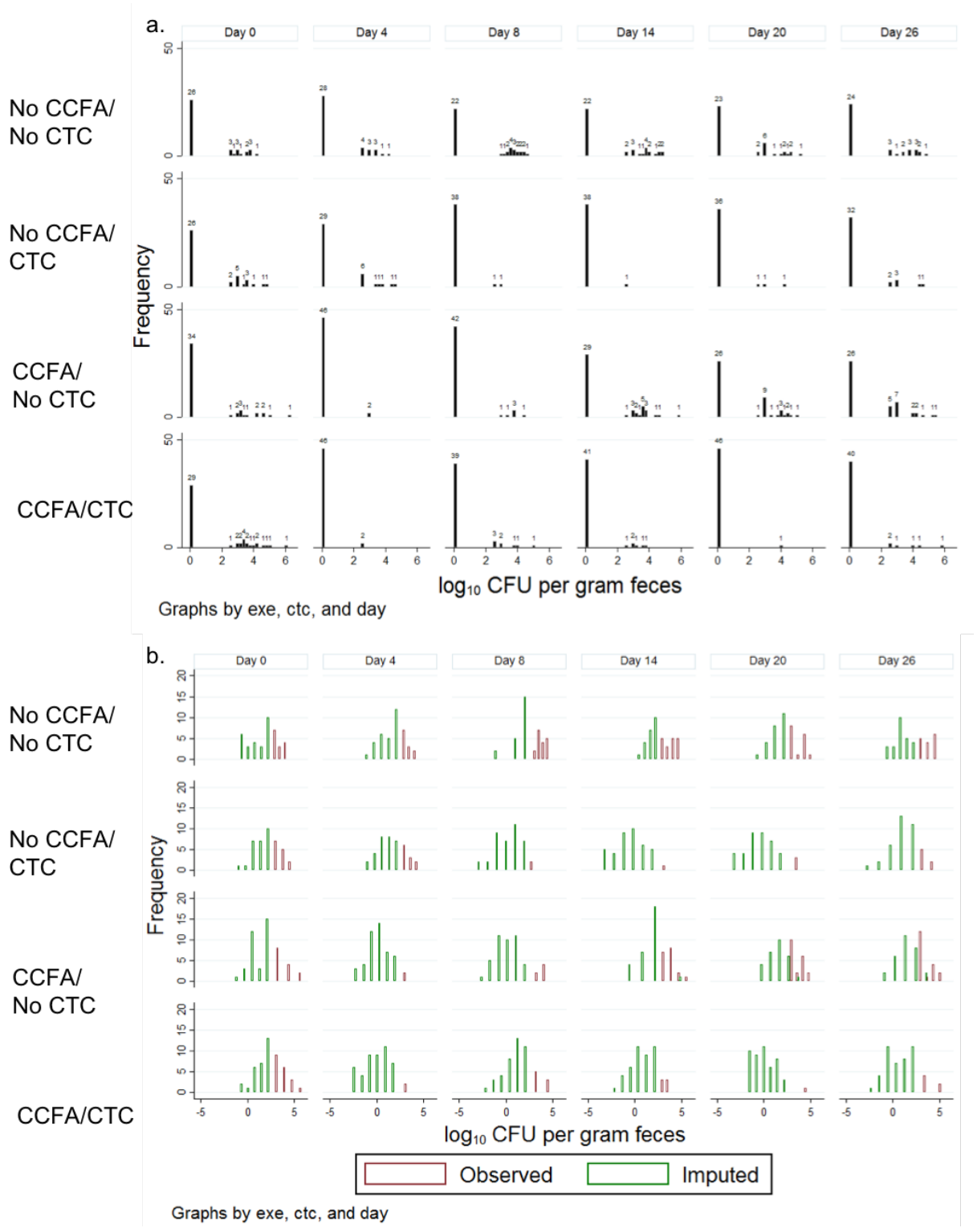


Figure 41. Distribution of $\log_{10}$ CFU per gram feces by treatment and day from (a) observed and (b) imputed data. CCFA: ceftiofur crystalline-free acid, CTC: chlortetracycline

4.3.4.6 Model prediction with observed and imputed data

With the observed spiral plating data, much the same as with $\log_{10}$ *invA* gene copies analysis, a zero-inflated Poisson regression was performed because the majority of the values were below the LOQ (replaced to 0). The $\log_{10}$ CFU per gram of feces were rounded and converted to integer values (Figure 42a). CCFA treatment on Day 0 decreased the $\log_{10}$ CFU per gram of feces significantly on Day 4 ($p=0.032$) (Figure 42b). CTC treatment starting on Day 4 decreased *Salmonella* quantities on Day 8. The animals in pens without any treatment showed an increase in *Salmonella* on Day 8 but remained at a similar quantity until the end of the study.

Following multiple imputation, the imputed values were included in the model prediction using multilevel mixed linear regression (Figure 43 a, b). The changes in the quantity of *Salmonella* was similar to that of the zero-inflated model. However, a larger increase on day 8 in the All-CCFA & CTC treatment group was predicted. Since the growth of *Salmonella* on BGA includes both susceptible and resistant *Salmonella*, the increase may reflect the resistant *Salmonella*.

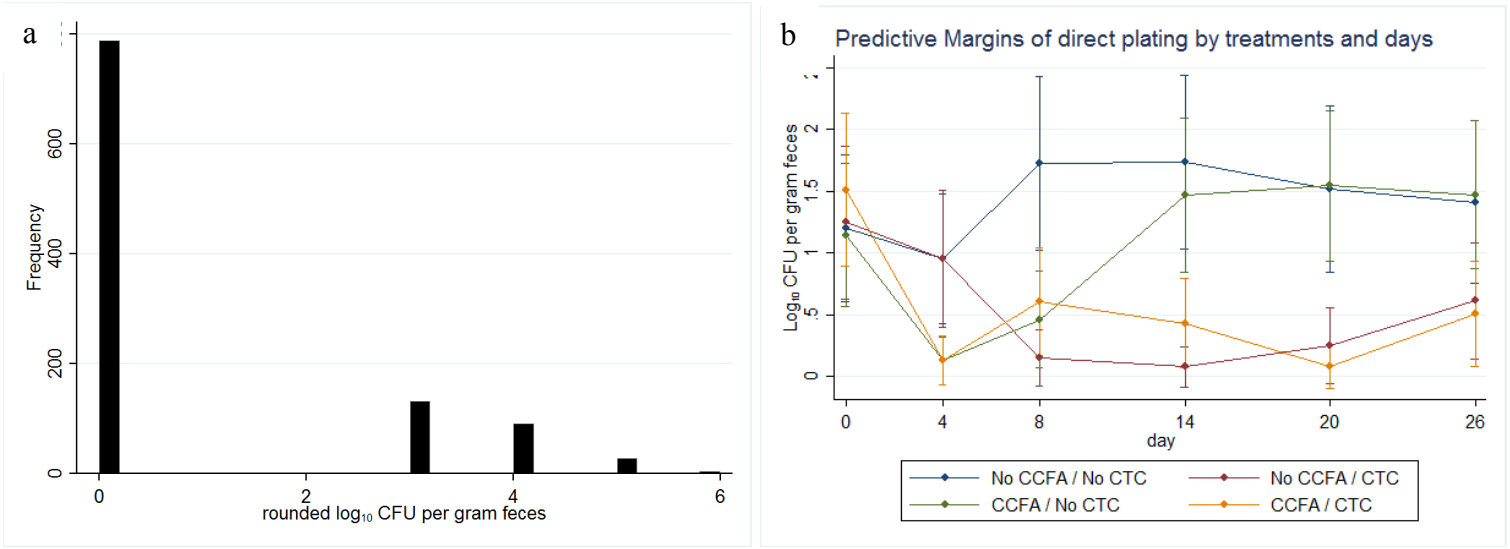


Figure 42. (a) Distribution of rounded integer $\log_{10}$ CFU per gram of feces and (b) zero-inflated Poisson regression on observed $\log_{10}$ CFU per gram feces from direct plating on BGA.

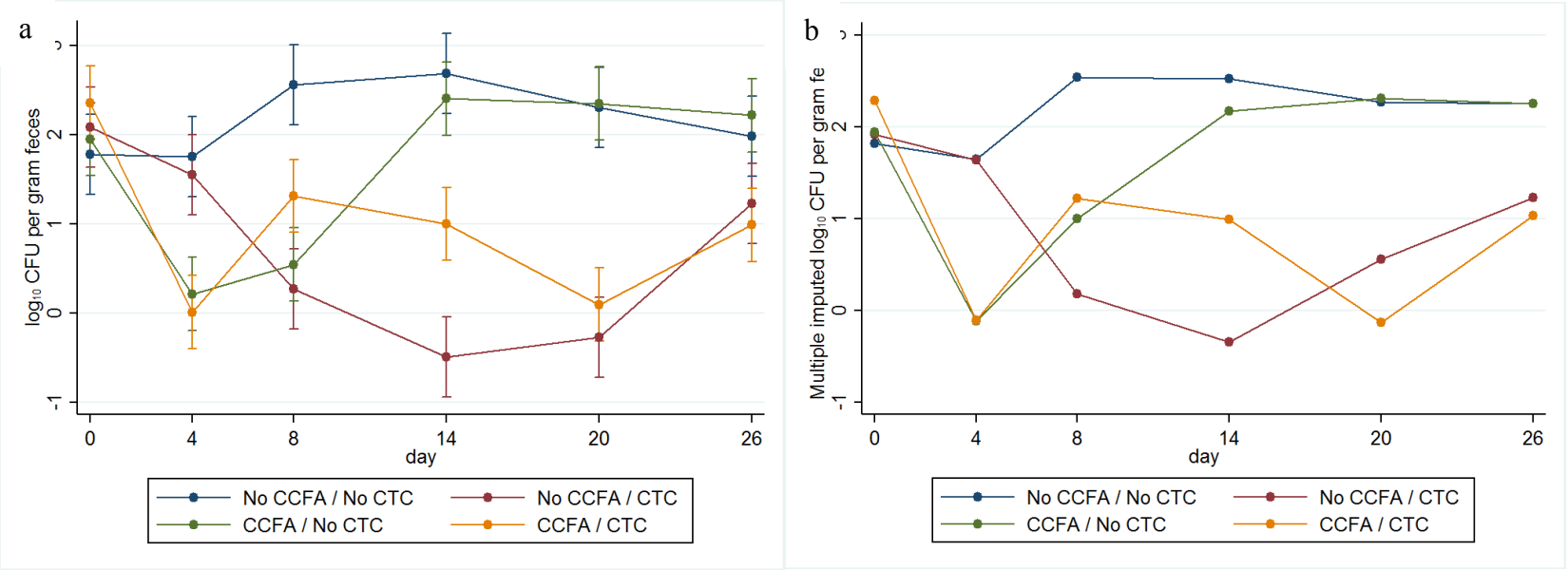


Figure 43. (a) Multilevel mixed linear regression model with representative imputed log10 CFU per gram of feces from direct plating on BGA and (b) multiple imputation multilevel mixed linear regression model with data from all 20 imputations.
Upper $\log_{10}$ CFU limit for unobserved data was set to 2.6.

4.4 Discussion

4.4.1 Detection methods

We conducted broth enrichment methods for the accurate prevalence estimation of *Salmonella* from fecal samples, using standard methods [151, 246]. Additionally, we conducted qPCR and direct plating of fecal samples for the quantification purposes. Since the ultimate purpose was to evaluate the effect of antibiotics on *Salmonella* quantities so as to aid in assessing of risk, we did not conduct any pre-enrichment steps for qPCR or for direct plating on BGA. Pre-enrichment will bias the results in that growth rate of *Salmonella* and background bacteria differ, and when the concentration of *Salmonella* is low, they may be less likely to be detected [178]. Here, since qPCR and direct plating were also used for *Salmonella* detection, the three detection methods were compared. Among these, the broth enrichment method had the highest detection rate as expected. For accurate detection of *Salmonella*, broth enrichment steps are necessary for diagnostics. However, broth enrichment takes 2-5 days to complete, which is time-consuming when the results are required in a short time, such as in clinical settings. Also, enrichment steps may bias the serotypes detected. Even without the selection pressure of antibiotics in the broth, enrichment broths may provide a fitness advantage for particular serotypes.

In our study, we used two direct *Salmonella* detection methods, real-time hydrolysis probe-based qPCR and direct spiral plating; in addition, we used a broth enrichment method as a comparator. For the qPCR, community DNA was extracted directly from fresh fecal samples and qPCR was conducted targeting the *invA* gene. This gene is encoded in the *Salmonella* Pathogenic Island 1, and is a necessary gene for the invasion of epithelial cells using the Type III secretion system [303]. The *invA* gene is present in all known *Salmonella*

genus but not in *E. coli* [304]. It is specific to *Salmonella*, and only one gene copy exists per *Salmonella enterica* isolate [292]. Previous studies on the comparison of detection methods between real-time PCR and enrichment from feces of cattle, horses, and pigs have shown mixed results [277, 283, 305-307]. Target genes in previous publications ranged among *invA*, *spaQ*, and *ttrRSBCA* genes. Most of these studies enriched the feces for 20-24 hours before DNA extraction for the detection of the genes.

We did not conduct an enrichment step prior to total community DNA extraction because our purpose was to quantify antibiotic resistant genes from metagenomic samples and not for clinical diagnosis [220]. For the direct spiral plating method, we diluted the fecal samples with PBS and spiral plated on BGA, BGA-tet, and BGA-cef and then incubated overnight. Studies by Pusterla *et al*. [306]and Kurowski *et al*. [283] had better detection with real-time PCR (RT-PCR) from enriched samples in horse feces, cattle, and dogs compared with culture, which is consistent with our study. In the study of Pusterla *et al*. *Salmonella* was not detected by direct plating the feces on BGA and XLT-4 agars [306]; however, the detection rate was 100% in RT-PCR using the DNA template from an 18-24 hour enrichment of feces in selenite broth. Plating on the enriched broth detected *Salmonella* in only 32% of those samples.

Overall, the broth enrichment method had the best detection our study. Agreement between qPCR and direct plating was moderate. In our assays, all the negative control reactions had no detection at all. In a hypothetical situation when broth enrichment is not conducted, and only qPCR or direct plating are conducted, vast number of samples will result as false-negative. Between qPCR and direct plating, qPCR detected 72 more samples as positive than direct plating. Further, only 190 samples were detected by both qPCR and direct plating. For the detection/diagnostic purpose and *Salmonella* prevalence estimation, even

though time-consuming, broth enrichment remains the best method for the cattle fecal sample, which is in agreement with studies by Eriksson *et al*. and Jensen *et al*. [305, 307].

4.4.2 Quantification by qPCR vs. direct spiral plating

The LOQ of qPCR and direct plating to BGA was similar, around 2.6 $\log_{10}$ gene copies and CFU per gram of feces, respectively (Figure 21). However, among the samples detected by both qPCR and direct plating, the quantity of *Salmonella* was detected at 10^1 times higher in qPCR compared with direct plating. Because qPCR detects both viable and non-viable *Salmonella*, it is possible that the quantity differences between qPCR and direct plating were due to the DNA arising from non-viable *Salmonella*. To detect *Salmonella* contamination in food, RNA has been used for the detection and distinguishing of viable *Salmonella* by qRT-PCR (Quantitative Reverse-Transcriptase PCR), which may result in a similar quantity with direct plating, although we have not tested this hypothesis in the current study [285]. The sensitivity and specificity of the primers and probes used in this study have been tested against other common Enterobacteriaceae. Therefore, it is not likely that other bacteria were detected [285].

In qPCR, multiple steps affect the quantity calculation, including efficiency of DNA extraction, type of template used for creating the standard curve, original fecal sample condition, and back calculation of gene copies to per gram of feces. We generated the standard curves from the whole-genome of *Salmonella* Typhimurium isolates, which can have better amplification than actual total community DNA samples mixed with various other bacterial and non-bacterial DNA. Although using the community DNA for standard curve generation is ideal, reproducibility may be sacrificed; especially when setting a qPCR reaction for 30 runs. The total community DNA used in this study was extracted after the fecal

samples were collected, having been stored at -80 °C for up to two years, and the extracted DNA subsequently stored in the freezer at -20 °C. On the other hand, the fecal samples that we used for the direct plating quantification had been stored at -80 °C with 50% glycerol for eight years, which could weaken or degrade the bacteria and therefore decrease the counts. However, our study showed that even after 8 years of appropriate storage, a quantifiable *Salmonella* can be recovered and quantified.

We divided the samples that were detected by qPCR into 4 groups as shown in Figure 23c: 1) detected with qPCR, direct plating, and enrichment (187 samples), 2) detected with qPCR and enrichment but not with direct plating (86 samples), 3) detected only with qPCR (48 samples), and 4) detected with qPCR and direct plating, but not with enrichment (3 samples). The first group represent the samples that are highly likely to be *Salmonella* positive. The distribution of the quantity of *Salmonella* within these samples was normally distributed. Samples from the second group were not detected with direct plating but with qPCR and enrichment. These samples may have a relatively low quantity of *Salmonella*, and therefore were detected by qPCR and enrichment which have a higher sensitivity. As the histogram shows in Figure 23c, the orange bars are on the lower end of the distribution. However, very few samples that had the highest quantities were also in this population. The reason why they were not detected by spiral plating is unknown; however, the explanation for these could be due to a biased localization of *Salmonella* in the fecal sample, where the portion of sample containing *Salmonella* was not plated.

The third group that was only detected with qPCR (48 samples) may contain non-viable *Salmonella* or very low quantities of *Salmonella* that were still below the LOQ by enrichment. In fact, 14 out of 48 of these samples were from Day 4 and in animals that were treated with

CCFA on Day 0. It is possible that the non-viable *Salmonella* quantified by qPCR were earlier killed by CCFA treatment. Additionally, this idea supports the finding that 3 out of 4 animals were not detected with any *Salmonella* by enrichment on six tested days. Since these three animals were detected with the *invA* gene on day 0 and day 4, but not on the later days, it may be due to the non-viable *Salmonella* shedding at the LOQ or intermittently shedding. CTC is a bacteriostatic agent and therefore static bacteria may have been maintained in the gut. The quantity differences between direct plating and qPCR may explain this phenomenon. Lastly, in the fourth group, 3 samples were detected with direct plating and qPCR but not with enrichment. Although we have not confirmed, these samples may have carried *Salmonella* serotypes other than 6 serotypes that were detected in this population since the enrichment process can favor certain serotypes [86].

Again, the portion of samples used for enrichment may not have contained *Salmonella*. Even though broth enrichment was the best method for detection of *Salmonella*, conducting direct detection by qPCR and direct plating in parallel may increase the diversity and numbers of samples detected with *Salmonella*. However, the fact that qPCR detects DNA derived from both viable and non-viable *Salmonella* can bias the quantitative results. Even though qPCR is known to have a high sensitivity, applying the technique to community DNA samples with substantial background microbiota, such as fecal samples, will require more adjustments. For risk assessment purposes, it seems desirable to run qPCR and broth enrichment in parallel if resources are available to do so.

4.4.3 Effects of antibiotics on the quantity of Salmonella

No antibiotic (MDR) resistant isolates (*S*. Reading in this population) were detected by broth enrichment on day 0 [88]. However, by directly plating the diluted fecal samples on

BGA-cef, three samples exhibited growth, of which two were *S.* Reading. These samples were confirmed to be carrying resistant genes by WGS analysis and the ACSSuT-cef phenotype was confirmed by microbroth dilution. Therefore, we were able to determine that antibiotic resistant *Salmonella* were present in the population before antibiotic treatment started. We were able to determine this by direct plating onto antibiotic selective media rather than by the broth enrichment method. We only picked one colony per sample in the broth enrichment procedure. Since it is likely that the dominant strains on Day 0 were susceptible serotypes and those were equally enriched by broth enrichment procedure, resistant strains were less likely to be detected.

By directly plating the fecal sample onto agar containing antibiotics, it is more likely that resistant strains can grow under preferred or favorable conditions. In two animals, an additional 2 or 3 serotypes were detected in addition to *S.* Reading. Therefore, it is highly probable that susceptible serotypes were dominant before antibiotic treatment began. In fact, in one of the animals that was detected with *S.* Reading on Day 0 (animal 2342), the quantity of *Salmonella* on BGA on Day 0 was very high (6.2 $\log_{10}$ CFU/gram feces), while colony growth on BGA-cef and BGA-tet was around 4.4 $\log_{10}$ CFU/gram feces. These differences are likely due to a high number of susceptible *Salmonella.* After the treatment with CCFA on Day 0, the quantity on BGA and BGA-cef were similar, which implies that the susceptible *Salmonella* were killed or supressed and only the *S.* Reading survived the CCFA treatment. On the other hand, even though we expected similar growth between BGA-cef and BGA-tet because of the ACSSuT-Cef phenotype of *S.* Reading, no colonies grew on BGA-tet from Days 8 to 26.

There are two possible explanations for this. The first possibility is the failure of BGA-tet, which was initially suspected. Therefore, the initial growth on BGA-cef plates was replica plated onto a new batch of BGA, BGA-tet, and BGA-cef agars. Still, no growth was seen on new BGA-tet, which suggests that the BGA-tet agar was correctly functioning. Additionally, plating of the fecal-sample was conducted per day of the samples and not per animal. Fecal samples from same animal (but on different days) were not processed together. Therefore, it is not likely that this was a failure of the batch of BGA-tet agar. The second possibility is the presence of *Salmonella* that is explicity resistant to ceftriaxone but behaves differently in the presence of tetracycline, which was not detected by our broth enrichment detection method. Since fecal samples were directly plated without enrichment on BGA-cef agar, it is possible that this population of *Salmonella* was more dominantly detected than *S.* Reading.

We have not confirmed the serotypes nor genotypes of those isolates that grew on BGA-cef, but not on BGA-tet; therefore, the musings of the previous paragraph are purely speculative. Since growth on BGA-tet was seen on days 0 and 4, this *Salmonella* might have been selected for by the CCFA treatment. In other animals, such cases were found but sporadically and not consistently within animals. A study by Martin *et al*. showed that *S.* Heidelberg and *S.* Kentucky from poultry sources carried the $bla_{\text{CMY-2}}$ gene on an IncI1 plasmid that only had phenotypic resistance to cephalosporins and not tetracyclines [195]. Although not very common in cattle, such *Salmonella* are commonly detected in food-animal derived sources such as poultry.

The quantity of total quantity of *Salmonella* significantly decreased on Day 4 in CCFA treated animals by approximately 2 to 3 $\log_{10}$ CFUs and also in gene copies. In the animals treated only with CCFA, the quantity recovered to that of Day 0 by Day 8, which shows that

the decrease was transient. Treatment with CTC also decreased the quantity of *Salmonella* more than the LOQ and the quantity of *Salmonella* recovered by Day 26. Although previous mouse studies have shown that antibiotic treatments cause microbiota disruptions and increase *Salmonella* colonization, the quantity of *Salmonella* before (Day 0) and after (Day 26) were similar in antibiotic treated to untreated cattle. A study from Barman *et al.* showed that *Salmonella* Typhimurium infection diminished 95% of the indigenous microbiota in mice but the microbiota recovered after 30 days [79]. Stetcher *et al*. studied the relation of Microbiota-Host-Pathogen response in *Salmonella*.

While healthy microbiota protects the host from pathogenic infections, *Salmonella* can overcome the colonization resistance by invading cells and causing inflammation in the host [308]. It has been shown that *Salmonella* can utilize tetrathionate and sialic acids produced as a result of inflammation for their growth, hence providing a selective advantage. It is known that antibiotic treatment itself can cause disruption of host microbiota. Pathogens like *Salmonella* can take advantage of such conditions to grow to where the host becomes a supershedder [278, 309]. A mouse study showed that the presence of one supershedder can increase transmission of *Salmonella* to its cage mates [278]. In our study, around 15 cattle increased their *Salmonella* quantity after the antibiotic treatments. Especially with CCFA treatment, around 5 cattle increased by 10^1 by Day 26 though the sample size remains too small to derive conclusions. Additional cattle were detected with *Salmonella* after CCFA treatment. These cattle are not grown in in a controlled laboratory setting like mice but in the feedlot, where not only antibiotic treatments but other factors such as feed contamination, temperature, the pen environment, and insect bites may affect *Salmonella* infection and

quantities. Even so, cattle treated with antibiotics may be more susceptible to *Salmonella* infection because of a disruption in the gut microbiota.

Salmonella infection is primarily subclinical in cattle. *S*. Dublin and *S*. Newport are known to be highly pathogenic to cattle (and humans); however, we did not find these serotypes in our study. The serotypes that are not host-adapted and do not cause systematic infection may act similar to that of commensal bacteria. Among the *Salmonella* that were detected in our study, resistant *Salmonella* have an advantage during antibiotic exposure; as proof, tetracycline and ceftriaxone resistant *Salmonella* were detected more frequently from those cattle and pens treated with CCFA and CTC. Many mouse *Salmonella* studies are conducted with *S*. Typhimurium infection following streptomycin treatment to artificially induce an enteric colitis model. If our studied cattle had instead been infected with *S*. Dublin and *S*. Newport, we might have seen a significant increase in their quantities after antibiotic treatment, assuming they harbored such resistance. Although we do not know how long our studied cattle were infected with *Salmonella*, they were likely persistently residing in these cattle as we have shown the high prevalence of *Salmonella* on Day 0 and steady prevalence in the control group. Additionally, we do not have data on the condition of the host cattle such as diarrhea, temperature, and immune response, which may require further investigation.

It was expected that the antibiotic resistant *Salmonella* population expands after antibiotic treatment. However, no significant increases in quantity were observed after the antibiotic treatment at the population level. The quantity of tetracycline and ceftriaxone resistant *Salmonella* were constant around 4 $\log_{10}$ CFU/gram feces. However, as the days increased more animals with growth on BGA-tet and BGA-cef were observed, which implies that much

of the resistant *Salmonella* population resided below the LOQ before treatment and increased to more than the LOQ, especially following CTC treatment.

Even though some cattle were detected with 4 to 5 different serotypes, the quantity of *Salmonella* in those cattle was similar to those of cattle detected with only 1 or 2 serotypes. However, the detection of serotypes was not conducted at the same time, so cattle were not necessarily infected with multiple serotypes at the same time. Even so, there might be a biological control such as quorum sensing within *Salmonella* concerning the maximum cell density that can reside in gut microbiota [310]. As expected, the animals detected with *S.* Reading were highly likely to exhibit growth on both BGA-tet and BGA-cef.

4.4.4 Imputation of missing values

The qPCR results from our study contained many missing values (72.9% of the total wells reacted using qPCR). This is problematic for data analysis due to bias produced because incomplete data will not be included in the analysis. To overcome this, we used zero-inflated Poisson regression models for the analysis of treatment effects on the count of *invA* gene copies. The log-transformed values were integer-rounded and considered as counts. The zero-inflated model runs in two separate processes: one part is to analyze the effects on the actual counts, and the other is to determine the effects associated with the zero values (binary outcome).

Multiple imputation is a useful estimation procedure to fill in missing values when there is evidence that the values are not truly zero. It has been shown that an appropriate imputation leads to less bias and better prediction of the model. In our qPCR results, an interval censored regression model was chosen between linear regression and truncated regression because we expected that most of the values were missing due to being below the LOQ. Other imputation

methods impute values within the range of observed values which is clearly inappropriate for the situation in this study. Several possibilities for values below the LOQ can be considered; 1) cattle were not infected with *Salmonella* (true negative), 2) susceptible *Salmonella* were killed by antibiotic treatments, therefore Ct values are higher than 40 (censored data), 3) failure of qPCR runs due to PCR inhibitors, or 4) other reasons. Except for the first reason, the negative results can be interpreted as a false negative. It is highly probable that most samples belong to 2), because, in the prevalence study, only 4 animals were not detected with any *Salmonella* across the investigated 6 days (24 samples/1040 samples) [88].

Imputation is useful when the missing values are "missing at random" or "missing not at random". McCall *et al.* have shown that missing values in qPCR are classified as "missing not at random" [311]. In fact, the number of samples with a missing value increased on day 8 after CTC treatment shown in the second and fourth rows of Figure 26, while it remained constant in the animals without antibiotic treatment (first row). Therefore, it is reasonable to assume the samples without values in qPCR were below the LOQ in our study. We imputed with linear regression and truncated regression models before deciding on the interval censored regression model. Both linear regression and truncated regression imputed the values only within the observed data, which was not suitable for this study. The missing values in the qPCR runs were assumed to be left censored (only upper limit data is known). The upper limit value was set for $\log_{10}$ 3.6, which was the mean value in the samples that had only one observation. While few studies utilizing imputation methods to estimate the missing values in qPCR data have been reported, more established methods are clearly needed [220, 311-313].

To examine the effects of antibiotics from only raw (observed) qPCR data, zero-inflated Poisson regression was used. The log-transformed gene copy data were integer-rounded;

therefore, gene copy data were transformed to counts. Although the data were over-dispersed and the negative binomial is therefore more suitable than Poisson regression, the zero-inflated negative binomial model would not converge in Stata for our sudy. In addition, compared with the multilevel mixed linear regression model using imputed values, the zero-inflated Poisson regression model had a wider standard error suggesting less stable estimates of effects.

4.5 Summary and conclusion of Chapter IV

In this chapter, we explored the temporal change in quantity of *Salmonella caused* by antibiotic treatments in a longitudinal study in feedlot cattle. Such explorations have not been previously conducted. We also compared the three detection methods: broth enrichment, probe-based qPCR, and direct plating, as well as two quantitative methods, probe qPCR and direct spiral plating. We modelled the effects of antibiotic treatments by day on the quantity of *Salmonella* using a zero-inflated Poisson regression model with integer-rounded data and a mixed-level linear regression model using multiple imputed data.

Overall, antibiotic treatments decreased the quantity of *Salmonella* by approximately 2-3 $\log_{10}$ per gram of feces. More specifically, CCFA treatment decreased the total *Salmonella* on Day 4 and the quantity recovered by Day 8 absent additional antibiotic treatment (specially, CTC). Also, the number of cattle shedding over the LOQ was diminished by CCFA treatment while additional cattle were shedding *Salmonella* on Day 26 after the antibiotic effects washed out. Similarly, CTC treatment decreased the quantity of *Salmonella* being shed by cattle; however, by Day 26 the quantity was recovering. In CTC treated cattle, cattle shedding ceftriaxone and tetracycline resistant *Salmonella* over the LOQ increased by Day 26 which is potentially troubling as it represents a change from the baseline day 0 values.

Broth enrichment had the highest detection rate compared with qPCR and direct plating. Between qPCR and direct plating, qPCR had higher rate of detection. The qPCR and direct plating methods detected samples differently, which may have occurred because qPCR can detect both viable and non-viable cells.

CHAPTER V

GENOMIC COMPARISON AND CHARACTERIZATION OF *SALMONELLA ENTERICA* ISOLATED FROM FEEDLOT CATTLE TREATED WITH CEFTIOFUR AND CHLORTETRACYCLINE

5.1 Introduction

In this chapter, genomic analyses were performed on *Salmonella* isolates that were obtained from the study reported in Chapter III. The isolates were derived from feedlot cattle treated with CCFA and/or CTC. In total, 566 isolates derived from cattle naturally infected with *Salmonella* were whole-genome sequenced and the effects of antibiotics on the genomic population of *Salmonella* were explored via phylogenetics and other methods.

The spread of antibiotic resistant bacteria has been a public health concern for several decades. Misuse and overuse of antibiotics in food-animals have been blamed for the dissemination of antibiotic resistant bacteria into the human population; typically, via contaminated food or the environment [314]. It has been shown that antibiotic use transiently increases phenotypically resistant *Salmonella* in previous work from our group and in other studies conducted in feedlot cattle and pigs [88]. In our current study, a third-generation cephalosporin, ceftiofur crystalline-free acid (CCFA) and a tetracycline, chlortetracycline (CTC) transiently increased phenotypically multidrug resistant (MDR) *Salmonella* following treatment. However, we have not investigated the resistance genes carried by these isolates, nor the relatedness of serotypes across animals, pens, and over time. The presence of resistance genes was highly correlated with phenotypic resistance in previous studies [136, 315, 316]. The presence of plasmids was also investigated in our study and associations with the resistant genes. All the MDR isolates investigated in this study were of the ACSSuT-Cef

phenotype (ampicillin, chloramphenicol, streptomycin, sulfonamide, tetracycline, ceftiofur, and ceftriaxone).

Previously, epidemiologic and genomic comparisons of *Salmonella* have been explored in the isolates derived from different locations and sources (i.e., human vs animal, often in different countries across many years), derived from different studies, or outbreaks [317]. However, genomic comparisons of *Salmonella* isolates that were longitudinally collected in a single study population that had been treated with antibiotics has not been previously reported. The genomic analysis herein was conducted by core-genome single nucleotide polymorphism (SNP) analysis, which is a common method applied in outbreak analyses. Since our isolates were derived from a single host population over the course of 67 days (replicate 1 Day 0 to replicate 2 Day 26), they were treated similarly in an analytical sense to an outbreak population. We also phylogenetically explored the similarity between our cattle derived isolates with other publicly available *Salmonella* serotypes. Temporal comparisons of genomes and SNP analysis were performed within each serotype to explore the effects of antibiotic treatment on the genome evolution of *Salmonella*.

5.2 Materials and Methods

5.2.1 Study design and Salmonella *isolation*

Salmonella isolates used in this study were cultured from cattle feces from a 26-day longitudinal, experimental study described previously. Briefly, 176 steers were allocated into 2 replicates, and 4 treatment groups. In each replicate, 88 steers were assigned into 8 pens (n = 11 cattle) to distribute the body weights among the pens evenly in a two-by-two factorial design with four treatment regimens (Chapter III Fig. 6), as described in previous papers by our research group [35, 88]. In 8 pens (4 pens per replicate), all 11 steers received 6.6 mg/kg

of CCFA (Excede®, Zoetis Animal Health, Florham Park, NJ) subcutaneously at the base of the ear ("All-CCFA & CTC" and "All-CCFA / no CTC" in Figure 6; metaphylaxis treatment model), and in the remaining 8 pens (4 pens per replicate), a single steer treated with CCFA on day 0 was co-housed (mixed) with 10 non-treated steers. Half of the pens in the CCFA treatment group and mixed group were treated with three 5-day pulses of 22 mg/kg CTC (Aureomycin®, chlortetracycline complex equivalent to 220.5 g/kg of chlortetracycline, Alpharma, Bridgewater, NJ). The CTC was top-dressed in feed with a one-day break between each 5-day pulse. The CTC treatment occurred from day 4 until day 20 ("All-CCFA & CTC", "1-CCFA & CTC"). The remaining 8 pens in each replicate did not receive CTC ("All-CCFA / no CTC" and "1-CCFA / no CTC"). Fecal samples were collected every other day *per rectum* as described previously [35]. Fecal samples were mixed with glycerol at a 1:1 ratio and preserved at −80 °C. Suspected *Salmonella* were isolated by standard enrichment broths including Tryptic Soy Broth, Tetrathionate Broth, and Rappaport-Vassiliadis broth enrichment. One *Salmonella* isolate was collected from each steer fecal sample that grew on brilliant green agar (BGA) as described previously [88]. In total, 566 isolates were isolated.

For the isolation of potential cephalosporin resistant *Salmonella* isolates from Day 0 fecal samples, 88 fecal samples were plated onto BGA with 4 μg/ml of ceftriaxone (BGA-cef). Only 2 samples grew colonies on BGA-cef. Two isolates from each BGA-cef plate were streaked on blood agar and the species were confirmed by MALDI-TOF. All 568 *Salmonella* isolates were preserved in cryobeads at -80 °C for further characterization and analysis.

5.2.2 Salmonella *DNA extraction for whole-genome sequencing*

Salmonella DNA was isolated in a QIAcube HT robot using the QIAamp 96 DNA QIAcube HT Kit (Qiagen, Valencia, CA). A single *Salmonella* colony was suspended into

5 ml of TSB and incubated overnight at 37 °C. From the suspension culture, 1 ml was transferred into a 1.2 ml micro-collection tube and centrifuged at 4,000 rpm for 15 minutes at room temperature. After the supernatant was removed, the pellet was re-suspended in ATL buffer (Qiagen) and mixed with reagent DX (Qiagen). One tube of small pathogen lysis beads (Qiagen) was mixed with the suspension and mechanically disrupted using the Qiagen TissueLyser system (Qiagen) 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 a ThermoMixer (Eppendorf, Hauppauge, NY) and followed by a heat shock for 10 minutes at 95 °C. The suspension was cooled to room temperature and 4 µl of RNAse A was added. The prepared samples were set in the QIAcube HT for DNA extraction using a modified protocol provided by Qiagen. The quality of the DNA was determined by the 260/280 absorbance ratios on the FLUOstar Omega Microplate Reader (BMG LABTECH, Cary, NC). Absorbance ratio between 1.8-2.0 were required. The DNA quantity was measured by fluorescence with the Quant-iT™ Pico Green® ds DNA Assay kit (Thermo Fisher Scientific). A minimum 10 ng/µl for accurate dilution was set as threshold concentration. The DNA was stored at −20 °C until future use.

5.2.3 Whole-genome sequencing by Illumina MiSeq and de novo assembly

To determine the serotypes and genotypes of the *Salmonella* isolates, we performed whole-genome sequencing (WGS) using the Illumina MiSeq platform (Illumina, San Diego, CA, USA) on the 568 isolates. Libraries for DNA from 32 *Salmonella* isolates were multiplexed and pooled per sequencing run using the Illumina Nextera XT library preparation kit and were run with the MiSeq Reagent Kit v3 600 cycle kit with paired-end reads (Illumina). Obtained raw reads in fastq format were trimmed with Trimmomatic ver. 0.36 [248].

The quality of the reads was confirmed with FastQC (Babraham Bioinformatics). Reads were *de novo* assembled by Spades ver. 3.10 [249]. The assembly quality was assessed on the QUAST web server, which analyzes the number of contigs, total length of genome by bp, GC (%), N50, and L50 [250]. Assembled genomes were annotated by Prokka and RAST [318, 319]. The results of FastQC and Prokka were aggregated by MultiQC [320]. Minimum requirement for contig number was set to 250 base pairs. Contamination was speculated and sequencing repeated if total length was more than 5.2Mb. The assembly process was conducted on the Ada cluster provided by the High-Performance Research Computing Center at Texas A&M University.

5.2.4 Whole-genome sequencing by Oxford Nanopore MinION

Two isolate DNA samples, *S.* Mbandaka (Day 0, no-CCFA/no-CTC treatment group) and *S.* Reading (Day 4, CCFA/no-CTC treatment), extracted as described above and sequenced on the Illumina MiSeq, were also sequenced on the Oxford Nanopore MinION R9 flowcell. The library preparation and sequence runs were performed following the manufacturer's protocol at the Genomics and Bioinformatics Service of Texas A&M University AgriLife Research.

5.2.5 Serotype, MLST, resistant genes, and plasmids determination

Raw fastq files obtained from the forward and reverse reads were uploaded to the web-based database SeqSero to determine the serotypes from the WGS data (http://www.denglab.info/SeqSero) [96]. The multi-locus sequence type (MLST) of each *Salmonella* isolate was determined by the combination of 7 gene alleles (*aro*C, *dna*N, *hem*D, *his*D, *pur*E, *suc*A and *thr*A) using the PubMLST database in the SRST2 application at the Illumina® BaseSpace® Sequence Hub [251, 252]. The PlasmidFinder (20-Feb-2017) and

ResFinder (03-July-2017) databases on the Center for Genomic Epidemiology (CGE) web server (Danish Technical University) were used to determine plasmid replicons and resistance genes [253, 321]. We also *de novo* assembled the plasmids by the plasmidspades commands available on SPAdes ver. 3.10 for those isolates that carried plasmids. The *de novo* assembled plasmids were confirmed with CGE PlasmidFinder. Plasmids were visualized with DNAPlotter and BRIG [322, 323]. Genomic islands on the plasmids were analyzed with IslandViewer 4 [324].

5.2.6 Assembly of MinION data and Illumina short reads

The reads from the MinION in fastq format were first assembled using Canu assembling software [325]. Second, for two isolates, the trimmed paired-end short-reads from the Illumina MiSeq and the MinION reads were aligned with Bowtie 2 [326]. After converting the obtained Sequence Alignment/Map (SAM) format to Binary Alignment/Map (BAM) format by Samtools [327], Pilon software was used to polish the draft assemblies [328]. The assembled reads were annotated with RAST, utilizing the PATRIC web resource. Synteny of pairs of plasmids was visualized with Gepard [329].

5.2.7 Core-genome alignment, single nucleotide polymorphism (SNP) analysis and phylogenetic tree construction

The *de novo* assembled genomes were run through Parsnp software (Harvest Suite) to analyze the SNP differences in the core genome [330]. For the reference genome, we used the isolates within our study from day 0, when present, and if not, from day 4 in addition to the complete genome of *S.* Typhimurium strain LT2 (NCBI accession # PRJNA241). Multiple other complete genome sequences and contigs from NCBI were included as well. The tree was

visualized with FigTree v1.4.3 and Gingr in the HarvestTools suite [330]. Temporal associations between sequences were explored using TemEst software [331]. STRUCTURE software was used to analyze the population structure from the SNP data [332]. The SNP output from Gingr was exported as a VCF and converted to STRUCTURE format with PGDSpider [333].

5.2.8 Validation of WGS based serotyping with traditional serotyping

Ten isolates (2 isolates per serotype: *S*. Give, *S*. Kentucky, *S*. Mbandaka, *S*. Montevideo, *S*. Reading) also were sent to the National Veterinary Services Laboratories (NVSL), United States Department of Agriculture (USDA) in Ames, Iowa for traditional serotyping to compare and validate the results from the serotyping based on WGS data.

5.2.9 Statistical analysis

All statistical analyses were conducted in Stata ver. 14 (Stata Corp, College Station, TX). Assembly statistics of total length, serotypes, and plasmids were analyzed using a linear regression model. Total length was set as a dependent variable (continuous) and serotypes (categorical) and plasmids (binary) were independent variables. Figures were created with Tableau Desktop 10.3. Agreement between resistance phenotype and the presence of corresponding resistance genes were calculated by Kappa agreement for *Salmonella* isolates. Assembly statistics were calculated on QUAST. Depth of coverage was calculated from data obtained from FastQC and aggregated with MultiQC. The following calculation was used: (average sequence length x total sequence) / assembly size. We performed exact logistic regression analysis to analyze the association between independent variables (i.e., treatment, pen, replicate, days) and tree clusters for *S*. Reading isolates.

5.3 Results

5.3.1 Description of studied population from prevalence study

Salmonella was isolated from all four different treatment groups (i.e., 1-CCFA / no CTC, All-CCFA / no CTC, 1-CCFA & CTC, and All-CCFA & CTC) and from all days analyzed in this study (i.e., 0, 4, 8, 14, 20, and 26). In total, 566 *Salmonella* isolates were collected by standard enrichment broth method. Two additional isolates were collected from Day 0 by direct plating of fecal samples on BGA-cef and BGA-tet. As shown in Chapter III Table 6 and Figure 12, 6 serotypes were detected from four treatment groups. In Figure 44, the numbers of isolates per serotype and treatment group is illustrated in a circle. Numbers in each circle represent the number of the isolates from each serotype/treatment combination. Red colored isolates represent MDR isolates and are comprised of 86 *S.* Reading isolates and one *S.* Kentucky MDR isolate.

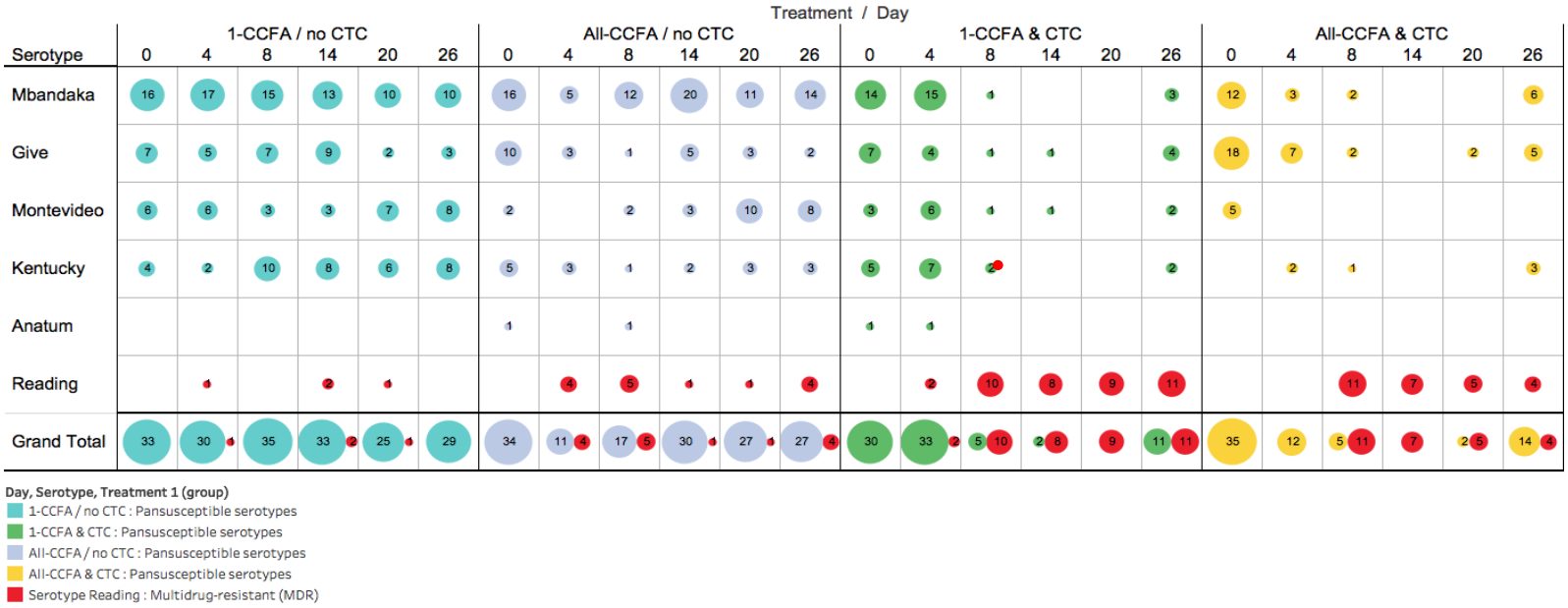


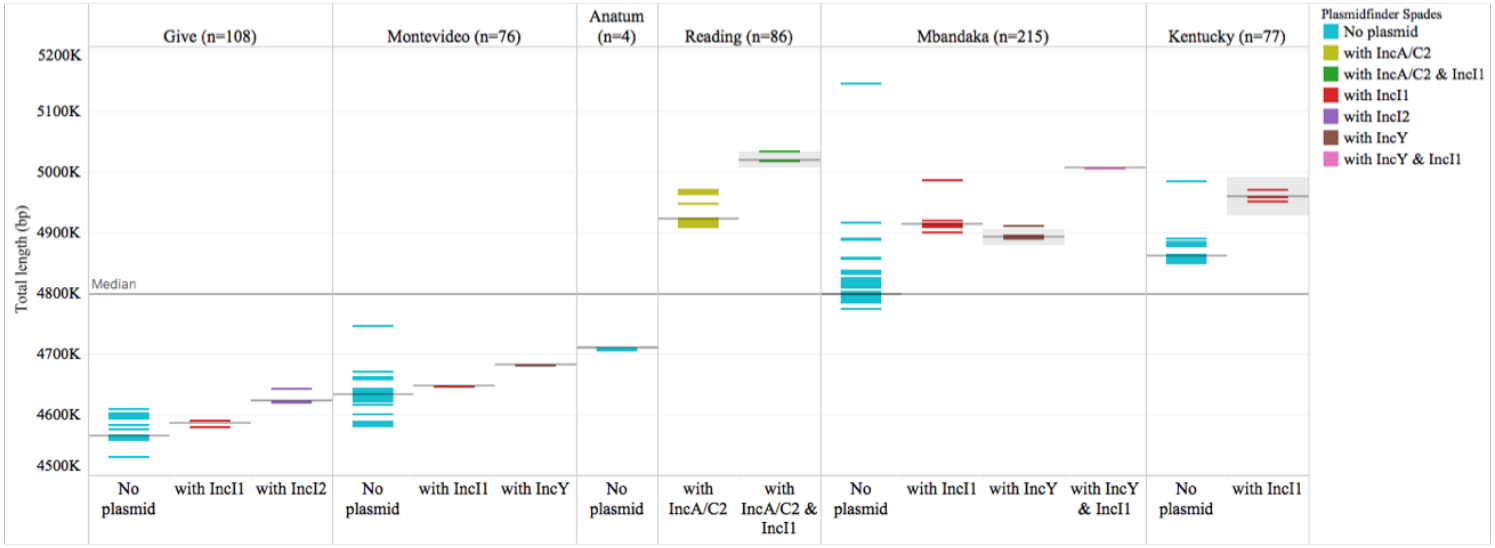
Figure 44. Numbers of *Salmonella* isolates detected per treatment group, serotype, and day. Teal blue: isolates from 1-CCFA / noCTC group, grey blue: All-CCFA / no CTC, green: 1-CCFA & CTC, yellow: All-CCFA & CTC, red: MDR isolates. Column: treatment and day, Rows: serotype. Size of the circle corresponds to the numbers of isolates cultured per day and serotype.

5.3.2 Descriptive statistics of WGS assembly results

We trimmed and *de novo* assembled the paired-end reads by SPAdes 3.10 for all 566 isolates. The assembly quality was assessed by QUAST, which included the following parameters: number of contigs, total length of genome, GC (%), and N50. Serotypes included *S.* Anatum (4 isolates), Give (108 isolates), Kentucky (77 isolates), Mbandaka (215 isolates), Montevideo (76 isolates), and Reading (86 isolates). None of the assemblies contained an uncalled bases (N) in the sequence. The median number of contigs (≥500bp) was 45 (Min. 21-Max. 236) and the median N50 was 279,322 bp (Min. 281,811-Max. 435,129). The median GC content was 52.19% (Min. 52.12-Max. 52.27). The median total length was 4,798,144 bp (Min. 4,531,234-Max. 5,148,251) (Table 23). The total length of the assembly was assessed per serotype since the total size varied by serotype and the presence of plasmids (Figure 45). The total length of assembly was significantly different between serotypes and by the presence of plasmids within serotypes (Figure 45). *Salmonella* Give had smallest total length of 4.5Mb followed by *S.* Montevideo (4.6Mb), *S.* Anatum (4.7Mb), *S.* Reading (approximately 4.7Mb: 4.9Mb minus the length of an IncA/C plasmid 152,290bp), *S.* Mbandaka (4.79Mb), and *S.* Kentucky (4.8Mb) (Figure 45). The average sequence coverage depth was 33.8. The number of coding sequences (CDS) per serotype correlated to the total length, which was around 4,244 in *S.* Give and 4,560 in *S.* Kentucky.

Table 23. Descriptive statistics of *de novo* assemblies by serotype. The numbers in parentheses are the minimum and maximum. CDS: coding sequence

Serotype	Median Total length (bp)	Median # Contigs (>= 500 bp)	Median N50 (bp)	Median Largest contig size (bp)	Median GC (%)	Average number of CDS
Give (n=108)	4,564,362 (4,531,234-4,642,779)	28 (21-173)	417,287 (62,292-522,819)	1,221,249 (222,784-1,509,885)	52.27 (52.14-52.29)	4244
Montevideo (n=76)	4,632,614 (4,581,068-4,746,725)	32 (27-210)	390,744 (72,128-646,889)	1,056,719 (266,322-1,172,373)	52.26 (52.18-52.28)	4330
Anatum (n=4)	4,709,217 (4,707,216-4,710,190)	25 (24-42)	435,129 (241,646-444,567)	892,990 (420,722-1,055,510)	52.21 (52.21-52.21)	4397
Reading (n=86)	4,923,461 (4,910,560-5,034,752)	44 (37-198)	281,811 (52,385-389,669)	624,283 (173,929-624,393)	52.26 (52.14-52.29)	4645
Mbandaka (n=215)	4,798,308 (4,774,684-5,148,251)	47 (29-268)	235,381 (34,731-406,590)	680,539 (164,067-1,020,015)	52.18 (51.05-52.28)	4548
Kentucky (n=77)	4,861,093 (4,851,243-4,985380)	56 (49-236)	306,522 (41,269-409,502)	560,453 (228,542-653,444)	52.12 (52.03-52.15)	4560
All serotypes	4,7981,44 (4,531,234-5,148,251)	45 (21-236)	279,322 (281,811-435,128.5)	653,022 (560,453-1,221,249)	52.19 (52.12-52.27)	4476



Serotype	No plasmid	with IncA/C2	with IncA/C2 & IncI1	with IncI1	with IncI2	with IncY	with IncY & IncI1
Give (n=108)	4,564,307 (n=97: 4,531,234-4,610,606)	-	-	4,584,897 (n=2: 4,579,601-4,590,192)	4,622,138 (n=9: 4,621,184-4,642,779)	-	-
Montevideo (n=76)	4,632,561 (n=74: 4,581,068-4,746,725)	-	-	4,647,254 (n=1)	-	4,682,342 (n=1)	-
Anatum (n=4)	4,709,217 (n=4: 4,707,216-4,710,190)	-	-	-	-	-	-
Reading (n=86)	-	4,923,450 (n=82: ,910,560-4,972,289)	5,019,891 (n=4: 5,018,809-5,034,752)	-	-	-	-
Mbandaka (n=215)	4,798,165 (n=194: 4,774,684-5,148,251)	-	-	4,913,204 (n=15: 4,902,124-4,988,447)	-	4,892,766 (n=5: 4,891,317-4,912,609)	5,006,476 (n=1)
Kentucky (n=77)	4,861,081 (n=72: 4,851,243-4,985,380)	-	-	4,959,551 (n=3: 4,861,936-4,959,551)	-	-	-

Figure 45. Descriptive statistics of *de novo* assemblies by serotype and presence of plasmids. Total length of *de novo* assembled *Salmonella* genome by serotypes and plasmids. The numbers in parentheses are the number of isolates per serotype with corresponding plasmid followed by minimum and maximum total length. When there was only one isolate with a corresponding plasmid, no confidence interval is shown. CI: 95% confidence interval of median total length.

5.3.3 Oxford Nanopore MinION and Illumina short reads assembly

The Oxford Nanopore MinION reads and trimmed Illumina short-reads were assembled for a single *S.* Mbandaka and *S.* Reading isolate each. The final numbers of contigs from this hybrid assembly were 9 and 10, respectively. The numbers of contigs were fewer than the contigs assembled only with Illumina reads using SPAdes, which were 51 and 47, respectively (Table 24).

Table 24. Comparison of the assembly results between Illumina reads and the hybrid assembly of Oxford Nanopore MinION and Illumina reads

Serotype	Reads	Assembly	Total length (bp)	# of contigs	N50 (bp)	Largest contig size	CDS
Mbandaka	Illumina paired end reads	Spades 3.10	4,820,156	51	235,381	912,030	4551
	Illumina and Nanopore MinION	Canu, Samtools, Pilon	4,866,881	9	766,303	1,495,141	4662
Reading	Illumina paired end reads	Spades 3.10	4,927,006	47	261,317	624,393	4630
	Illumina and Nanopore MinION	Canu, Samtools, Pilon	4,975,355	10	737,941	1,339,520	4808

5.3.4 Antimicrobial Resistant genes and plasmids

Following assembly with SPAdes, antimicrobial resistant genotypes were assessed for all of the isolates using the ResFinder database. Resistance phenotype and genotype were completely matching (100%) for *S.* Anatum, Give, Kentucky, and Montevideo isolates. In *S.* Mbandaka, 1 isolate carried *aadA24*, a gene responsible for streptomycin resistance, but the isolate was phenotypically pan-susceptible (Table 25). In *S.* Reading, all 86 isolates displayed a multidrug resistance (ACSSuT-Cef) phenotype and carried the resistance genes, *aadA7, strA, strB, sul1, sul2, floR,* bla_{CMY-2}, and *tet(A)*. Three *S.* Reading isolates were not sulfisoxazole resistant

phenotypically; however, *sul1* and *sul2* genes were detected (Table 25). In one *S.* Reading isolate, *aph(3')-IIa* and *aph(6)-Ic* genes, which correspond to kanamycin and streptomycin resistance, respectively, were detected in addition to the resistance genes listed above. Kappa agreement was calculated for each resistance phenotype-genotype, and agreement beyond chance was found to be between 0.97-1.0 (Table 26).

Three resistance genes *aadA7*-truncated, *sul1*, and *tet*(A) and an IncI1 plasmid were detected from the single *S.* Kentucky isolate exhibiting antimicrobial resistance. The paired-reads from the *S.* Kentucky isolate were assembled with plasmidSPAdes, which produced 55 contigs and a total length of 240,563bp. The annotation by RAST identified all resistance genes from plasmidSPAdes derived contigs, which suggests that the genes were located on the IncI1 plasmid. The results were confirmed with Island Viewer 4, in which the resistance genes were shown within the IncI1 plasmid.

IncI1 plasmids were detected in all the other serotypes, except *S.* Anatum; however, genes encoding for antibiotic resistance were not detected in these isolates. *S.* Give carried IncI1 and IncI2 plasmids. In *S.* Mbandaka, 21 isolates carried IncI1, IncY, or both plasmids. Interestingly, IncY plasmid-replicons were not detected from those fastq files that were assembled with Velvet software (instead of SPAdes) and run with PlasmidFinder. Two *S.* Montevideo carried IncI1 and IncY plasmids, respectively and 3 *S.* Kentucky carried the IncI1 plasmid. All *S.* Reading were detected with the IncA/C2 plasmid and 4 isolates also had an additional IncI1 plasmid (Table 25).

Table 25. Antimicrobial resistance phenotype and genotype patterns per serotype and plasmid replicon type

Serotype	Phenotype	Genotype	Agreement of Phenotype-Genotype	Plasmids
Anatum (n=4)	Pansusceptible (100%)	No resistance genes (100%)	Yes	N/D
Give (n=108)	Pansusceptible (100%)	No resistance genes (100%)	Yes	IncI1 (2, 1.8%) IncI2 (9, 8.3%)
Mbandaka (n=215)	Pansusceptible (100%)	No resistance genes (214, 99.5%)	Yes	IncI1 (15, 6.9%) IncY (5, 2.3%) IncI1&IncY (1, 0.5%)
		aadA24 (1, 0.5%)	No (Pansusceptible phenotype)	N/D
Montevideo (n=76)	Pansusceptible (100%)	No resistance genes (100%)	Yes	IncI1 (1, 1.3%) IncY (1, 1.3%)
Kentucky (n=77)	Pansusceptible (76, 98.7%)	No resistance genes (76, 98.7%)	Yes	IncI1 (2, 2.6%)
	STR-FIS-TET (1, 1.3%)	*aadA7-truncated_sul1_tet*(A) (1, 1.3%)	Yes	IncI1 (1, 1.3%)
Reading (n=86)	STR-FIS-CHL-AMP-FOX-AXO-XNL-TET (83, 96.5%)	*aadA7_strA_strB_sul1_sul2_floR_bla*$_{CMY\_2}$*_tet*(A) (82, 95.3%)	Yes	IncA/C2 (82, 95.3%) IncA/C2 & IncI1 (4, 4.6%)
		aadA7_strA_strB_aph(3')-Iia_aph(6)-Ic_sul1_sul2_floR_bla$_{CMY\_2}$*_tet*(A) (1, 1.1%)	Yes	
	STR-CHL-AMP-FOX-AXO-XNL-TET (3, 3.4%)	*aadA7_strA_strB_sul1_sul2_floR_bla*$_{CMY\_2}$*_tet*(A) (3, 3.4%)	No (no Sulfisoxazole resistant phenotype)	

N/D: Not detected

Table 26. Kappa agreement between antimicrobial resistance phenotype and detected antimicrobial resistance genes

Antibiotic class	Phenotypic resistance	Resistance gene	Agreement	Kappa
Aminoglycoside	Streptomycin	*aadA7, aadA7-truncated*	99.65%	0.9864
	Streptomycin	*strA*		
	Streptomycin	*strB*		
	Streptomycin	*aadA24*		
	Streptomycin	*aph(6)-Ic*		
Sulfonamides	Sulfisoxazole	*sul1*	99.47%	0.9793
	Sulfisoxazole	*sul2*		
Chloramphenicol	Florfenicol	*floR*	100%	1.0
Beta-lactamase	Cefoxitin	bla_{CMY-2}	100%	1.0
	Ceftriaxone	bla_{CMY-2}	100%	1.0
	Ceftiofur	bla_{CMY-2}	100%	1.0
	Ampicillin	bla_{CMY-2}	100%	1.0
	Amoxicillin/clavulanic acid 2:1 ratio	bla_{CMY-2}	100%	1.0
Tetracycline	Tetracycline	*tetA*	100%	1.0

5.3.5 IncA/C2 Plasmids from S. *Reading*

5.3.5.1 Description of IncA/C2 plasmid

All *S*. Reading isolates carried resistance genes and were determined to harbor IncA/C2 plasmids. IncA/C2 plasmids are known to be multidrug-resistance plasmids [334]. To localize the resistance genes in an *S*. Reading isolate *in silico*, the plasmid replicon gene *rep*A for IncA/C2 was located on one of the contigs of the *S.* Reading isolate hybrid-assembled with Illumina and Nanopore MinION reads. The *rep*A gene was located on a single contig among 10 contigs, identified as contig_04 (152,294bp). Resistance genes *aadA7, strA, strB, sul1, sul2, floR,* $bla_{CMY\text{-}2}$, and *tet*(A) were all also located on contig_04. The plasmid contig_04 carried *flo*R, *tet*(A)-*tet*R, *str*B, *str*A, and *sul*2 in one region, a $bla_{CMY\text{-}2}$ with insertion sequence ISEc9 in a second region, and *aadA7*, *sul1* and a mercury resistance operon on transposon Tn21 in a third region (Figure 46). One *S*. Reading isolate was detected with the additional genes *aph(3')-IIa* and *aph(6)-Ic* genes and this isolate was carrying both IncA/C2 and IncI1α plasmids. The *aph(3')-IIa* and *aph(6)-Ic* genes were located on a contig (1,714bp) surrounded by repeated regions based on PlasmidSpades assembly; however, it could not be determined whether the contig was located on the chromosome, the IncA/C2 plasmid, or the IncI1α plasmid. These genes are encode aminoglycoside-modifying enzymes commonly found in Tn5 transposons and they specify resistance to kanamycin, neomycin, butirosin, paromomycin, and ribostamycin [335].

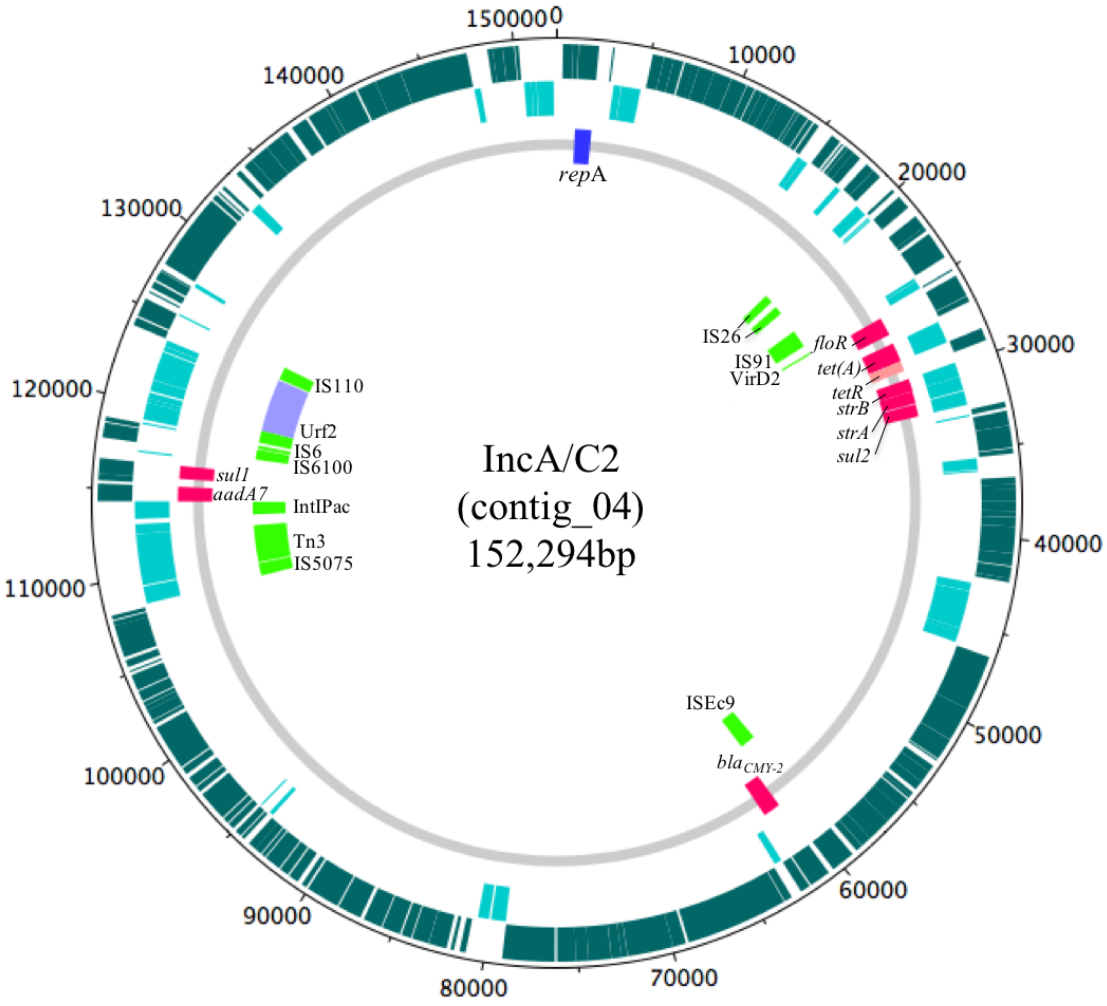


Figure 46. Circular map representation of the IncA/C2 plasmid derived from *S.* Reading hybrid-assembled by MinION and Illumina sequencing and visualized with DNA Plotter.
The dark green region on the outer circle represents genes in the forward CDS region and the inner light teal green are genes in the reverse CDS genes. Red genes are antimicrobial resistance genes. Bright green genes represent mobile elements including transposons and insertion sequences. Purple genes represent a mercury resistance operon. The blue gene is the repliconA (*repA*) gene of the IncA/C2 plasmid.

5.3.5.2 Comparison of contig04 plasmid with closed public IncA/C plasmids

Contig_04 was run through BLASTn at the NCBI website and matched with several complete, closed IncA/C plasmid sequences (GenBank: KJ90290.1 (pSN254b, 152,216bp), CP012682.1 (p33676, 161,461bp), FJ621587.1 (pAM04528, 158,213bp), CP014658.1 (pSAN1-1736, 160,227bp), and CP011429.1 (pYU39, 156,323bp)). The closest matching plasmid sequence found by a BLASTn search was plasmid pSN254b from *Aeromonas salmonicida*, a bacterium which often causes disease in fish [336]. The query coverage was 100% and the identity match was 99%. The pSN254b plasmid has a similar plasmid structure to the pSN254 plasmid which was derived from *S.* Newport strain SL254. Plasmid pSN254 has an IncA/C plasmid backbone [198]. The differences between pSN254 and pSN254b are that pSN254 carries 2 copies of the $bla_{\text{CMY-2}}$ gene, while pSN254b has only one copy; additionally, pSN254b has two insertion sequences (IS) and genes are absent from the Tn21 transposon [336]. Dot plot analysis was conducted between our contig04_IncA/C2 plasmid and pSN254b, pSAN1-1736 (*S.* Anatum derived), and pAM04528 (*S.* Newport derived) (Figure 47). All sequences shared high similarity. Contig04 and pSN254b shared the highest similarity. In both pSAN1-1736 and pAM04528, inversion sequences can be seen as a diagonal line. pAM04528 also has frame shifts caused either by deletion, insertion, or mutation of nucleotides.

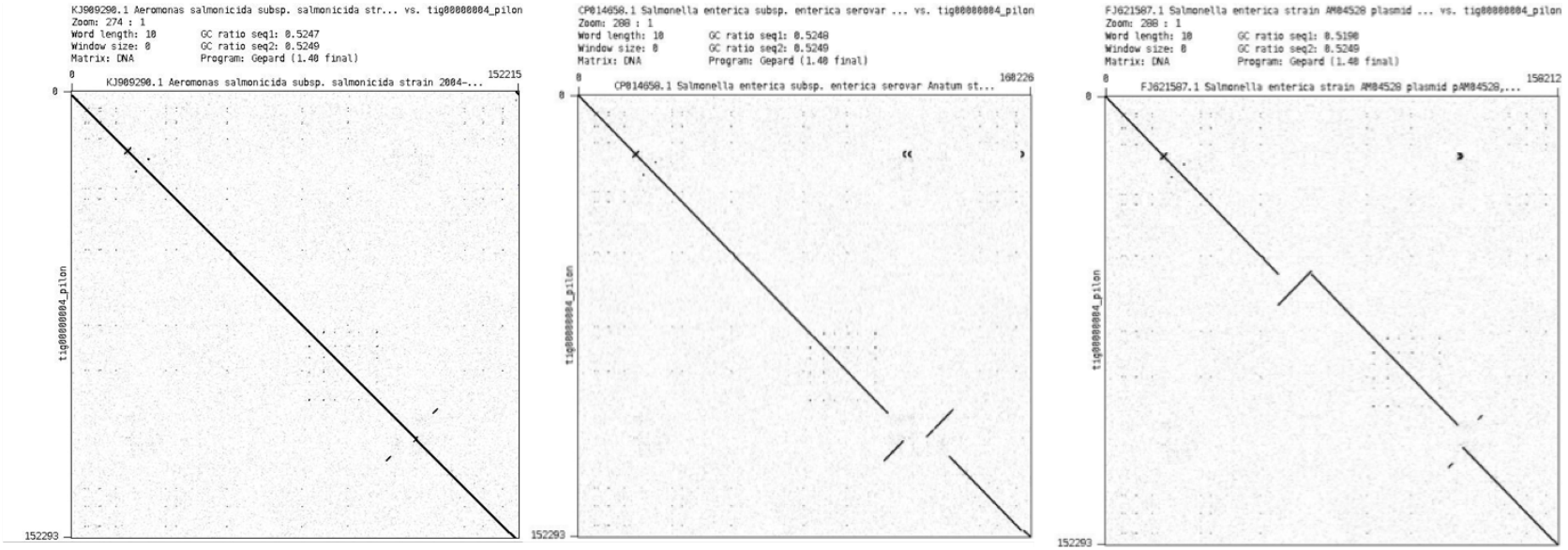


Figure 47. Dotplot analysis of *S.* Reading derived IncA/C2 and pSN254b (left), pSAN1-1736 (CP014658.1) (middle), and pAM04258 (FJ621587.1) (right).
Vertical sequence is Contig 04 and horizontal sequences are pSN254b, pSAN1-1736, and pAM04258 from left to right, respectively.

5.3.5.3 Plasmid assembly with plasmidSPAdes

The plasmidSPAdes command on SPAdes (ver. 3.10) was used to assemble only the plasmid sequences on those isolates that were detected with plasmid replicons from the PlasmidFinder results. In total, 85 *S.* Reading and 30 isolates from among the other five serotypes were run on plasmidSPAdes. The fasta files generated from the assembly were run on PlasmidFinder to confirm the results of plasmidSPAdes. Of the 85 *S.* Reading isolates originally detected with an IncA/C2 plasmid, 71/85 isolates (83.5%) were detected with IncA/C2 plasmid and 4/4 isolates (100%) were detected with additional IncI1_alpha plasmids; however, 10 isolates in which plasmids were initially detected (11.8%) were not detected with plasmid replicons by PlasmidFinder following assembly with plasmidSPAdes. Among the 30 isolates from *S.* Mbandaka, *S.* Give, and *S.* Kentucky, 26 matched with the original PlasmidFinder results, two isolates were not detected with a plasmid replicon, one isolate was detected with an extra IncY plasmid, and one isolate was detected with only one plasmid but originally was expected to harbor two plasmids. PlasmidSPAdes is known to work with those sequences that have more than 40x depth coverage, which can result in failed assemblies of plasmid [337]. Two IncI2_delta plasmids detected in *S.* Give were assembled into 1 contig and BLAST search matched with the complete sequence of *E. coli* plasmid tig00013784_pilon (CP024858.1). The sizes of both contigs were 58,287 bp and GC content (%) were 42.16 and 42.13, respectively. Assembly of plasmids from WGS sequencing is challenging [337]; however, this study illustrated the potential to close the plasmids when the sequence depth coverage is high enough.

5.3.5.4 Comparison of Velvet and SPAdes assembler on plasmid detection by PlasmidFinder

Initially, all of our raw paired-end reads were uploaded onto the CGE webserver for plasmid detection by PlasmidFinder. PlasmidFinder uses Velvet for assembly [100]. Since we later assembled with SPAdes, the assembled genomes were reanalyzed with PlasmidFinder. Interestingly, IncY plasmids were identified from 8 isolates after SPAdes assembly, which were not initially detected when the reads were assembled with Velvet. The same PlasmidFinder database from February 2017 was used for both analyses. The detection of the IncY plasmids may be explained by differences in the assembly alogrithms between Velvet and SPAdes. However, the reason why only IncY plasmids could not be detected by Velvet assembled genomes but from SPAdes assembled genomes remains unclear.

5.3.5.5 Comparison of plasmid assembly results of plasmidSpades and IncA/C2_contig04 plasmid

The plasmid assembled with plasmidSPAdes was compared with the Canu/Pilon assembled contig_04 (D4-50 *S.* Reading isolate) plasmid. plasmidSPAdes assembly resulted in 3 contigs with a total length of 142,840bp. Since the total length of the IncA/C2_contig04 was 152,294bp, around 9,454bp were missing from the plasmidSPAdes assembled plasmid. The missing region from the plasmidSPAdes assembled plasmid coded for an insertion sequence (IS) (Figure 48). One possibility is that IS are repeated regions, which may not have been properly assembled by plasmidSPAdes using only short read sequences. Two other *S.* Reading isolates were successfully assembled into one contig using plasmidSPAdes assembly. Similarly, these assembled plasmid genomes were also missing this insertion sequence region. However, the true sequences of these two isolates are not known, and therefore it cannot be

concluded that this IS area was not assembled properly. It is possible that this region is not present in these isolates; however, it is likely that these isolates were carrying similar plasmids. In comparison to differences in the detection of plasmids by plasmidSPAdes, all resistance genes identified on the Canu/Pilon assembled contig_04 were also detected on the plasmidSPAdes assembled plasmid.

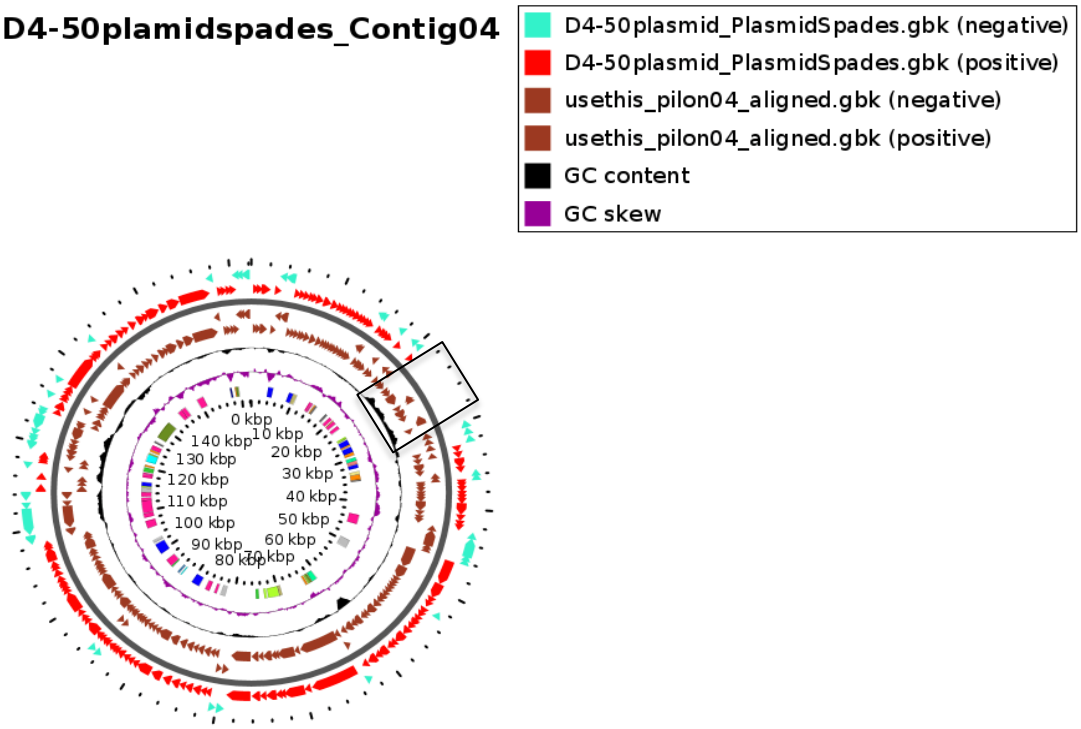


Figure 48. Comparison of plasmids assembled from Illumina reads with plasmidSPAdes and Illumina&MinION. The inner arrowed circle in brown is the forward and reverse reads of the Illumina&MinION assembled contig_04 (reference genome). The outer arrowed circle with red (forward) and green (reverse) colors are the plasmid assembled with plasmidSPAdes aligned with contig_04. The rectangle area represents the area missing from the plasmidSPAdes assembly.

5.3.6 Phylogenetic relation of all isolates by core-genome SNPs with publicly obtained sequences

The core genomes of the *Salmonella* were aligned across and within serotypes using Parsnp software. Maximal unique matches (MUMs) are used in Parsnp to recruit similar genomes and are used by many researchers for the rapid alignment of multiple genomes [330, 338, 339]. Because only the core genomes are aligned, any reference genome can be used in this process. It is ideal to use a reference genome that is closely related to the analyzed strain so that the maximum number of core genomes are selected [330]. Temporal associations between isolates and genetic divergence through sampling dates were assessed with the TempEst program, using a regression model.

We aligned the core-genome of 600 *Salmonella* isolates including 568 isolates from our study, 23 other publicly available sequences of the 6 representative serotypes from our study (2 of *S.* Anatum, 4 of *S.* Kentucky, *S.* Reading, *S.* Mbandaka, and *S.* Give, respectively, and 5 of *S.* Montevideo, respectively), 7 other common zoonosis serotypes (*S.* Enteritidis, *S.* Typhimurium, *S.* Newport, *S.* Heidelberg, *S.* Dublin, *S.* Typhi), and 2 *Salmonella enterica* subspecies (*S. houtenae* and *S. arizonae*). Alignments and phylogenetic analyses were used to determine where our feedlot cattle-derived isolates belong within a previously generated phylogenetic analysis of *Salmonella* (Figure 49). One *S.* Mbandaka that was acquired from the public database (ASM183359v1) created an isolated branch; therefore, a BLAST search was conducted. The sequence turned out to be from a *Salmonella* subspecies IIIb (*Salmonella enterica subsp. diarizonae*) and was labeled as such in our analysis. In total of 600 isolates, core-genome coverage was 43.7%. All isolates within a serotype belonged to the same cluster and had the same ST (Figure 49). Therefore, to increase the resolution of the tree, 2

representative isolates were chosen to be aligned with the other publicly available sequences named above (Figure 50).

Previous studies have shown that *Salmonella enterica* subsp. *enterica* can be divided into at least 2 clades (Clade A and B). From our study, *S.* Kentucky, *S.* Anatum, *S.* Mbandaka, and *S.* Reading belonged to Clade A, while *S.* Montevideo and *S.* Give belonged to Clade B. Within Clade A, *S.* Anatum and *S.* Reading formed a separate sub-cluster (Clade A2) from *S.* Kentucky and *S.* Mbandaka (Clade A1). Since sequences from these 6 serotypes were also included from the NCBI database, we found that, more broadly, *S.* Montevideo and *S.* Reading had isolates that belonged to both Clade A and B. *S.* Montevideo from our study was strictly of ST138 and belonged to Clade B, while CFSAN004346 belonged to Clade A2. *S.* Reading from our study was ST1628 and belonged to Clade A2, while ST412 isolated from turkey in previous work belonged to Clade B, and demonstrates that *S.* Reading is a polyphyletic serotype. *S.* Mbandaka from our study was ST413 and belonged to Clade A1 while ATCC51958 (ST3016) belonged to Clade A2. This corresponds with data from previous studies. As mentioned above, one *S.* Mbandaka turned out to be *S.* diarizonae (IIIb). Only ST64 from *S.* Anatum was included in this tree; however, *S.* Anatum also has multiple STs. *S.* Give from our study was ST654 and the other 3 different STs included from NCBI belong to a different cluster. The same is true for *S.* Kentucky.

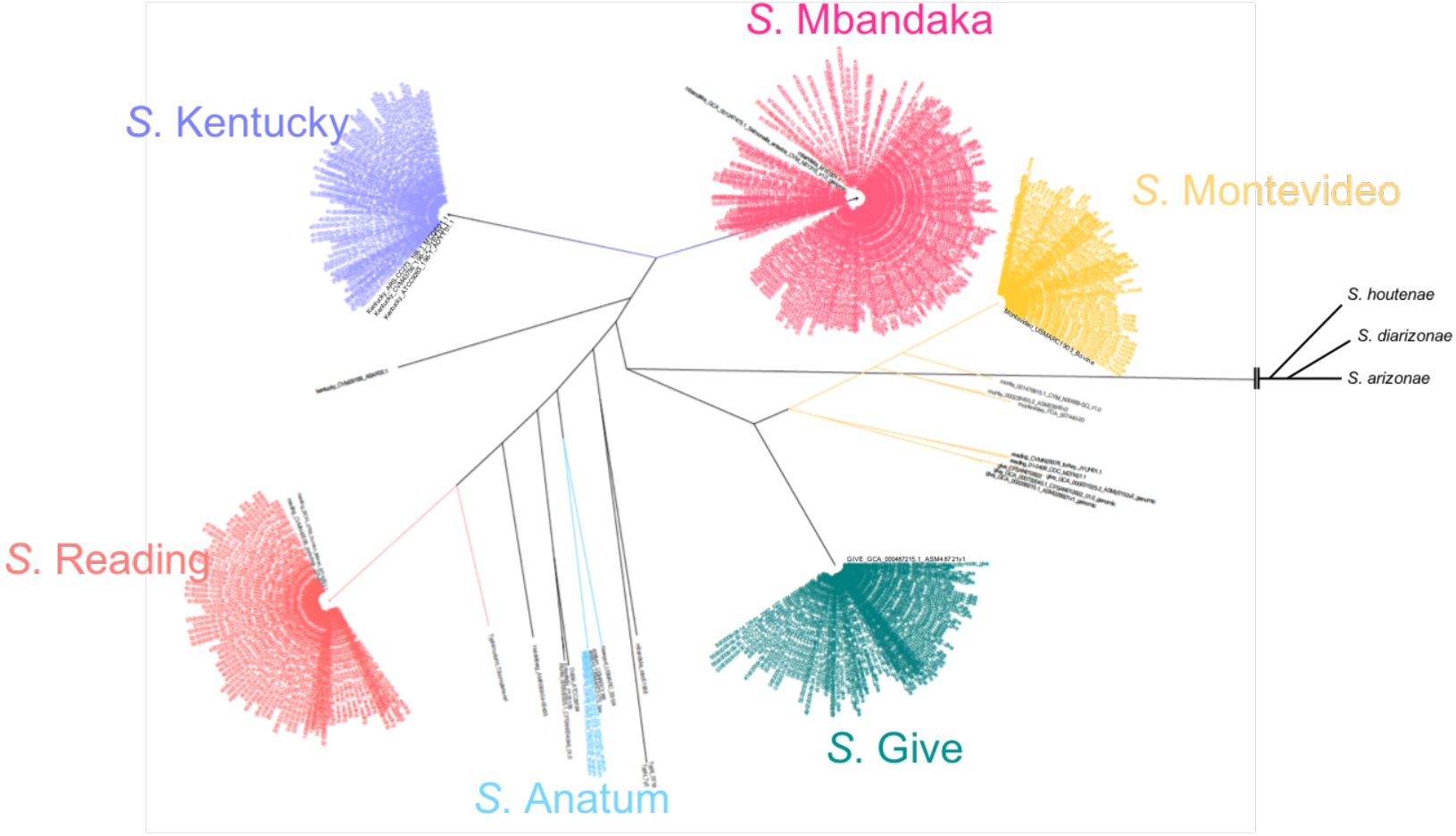


Figure 49. Spider tree of 568 isolates (6 serotypes) from this study, and 32 NCBI sequences from the same 6 serotypes, and other common serotypes analyzed by core-genome SNP analysis. The S. Typhimurium LT2 complete genome was used as a reference genome. The length of S. houtenae, S. arizonae, and S. dizrizonae are modified to fit in the figure.

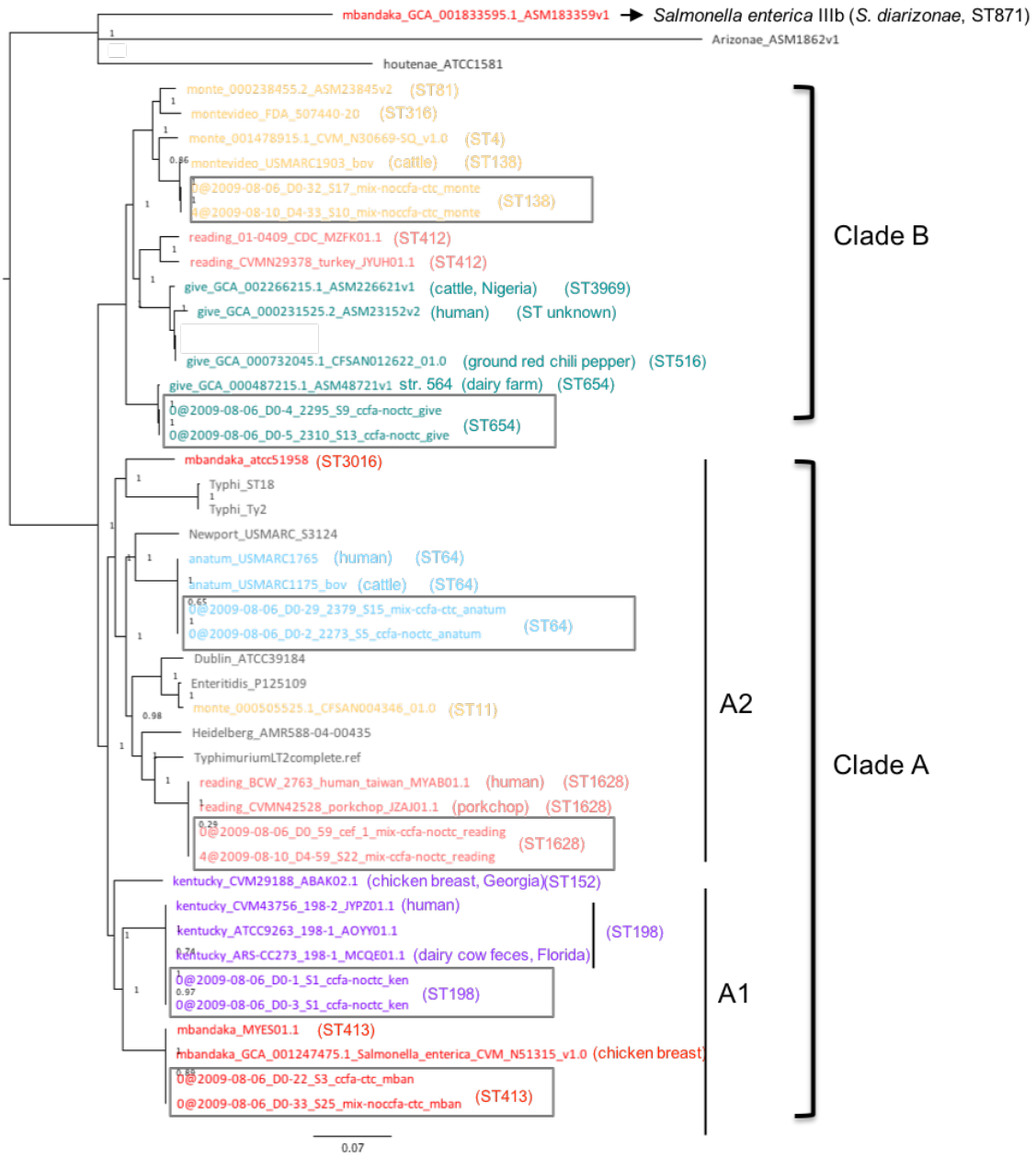


Figure 50. Maximum-likelihood tree of *Salmonella enterica* including two representative isolates from each of the 6 serotypes identified in this study, publicly available sequences from the 6 representative serotypes, and other common serotypes analyzed by core-genome SNP alignment with Parsnp. Isolates in the rectangles are isolates from this study. ST: sequence type from MLST data. Yellow: S. Montevideo, green: S. Give, blue: S. Anatum, salmon: S. Reading, purple: S. Kentucky, red: S. Mbandaka, gray: other common serotypes. An S. Typhimurium LT2 complete genome was used as the reference genome. The host of each of the isolates from publicly acquired databases is shown in parenthesis, when provided. Clades A and B: based on den Bakker et al., and Timme et al.[107, 108]

5.3.7 Salmonella *Reading core-genome analysis*

5.3.7.1 Description of *S.* Reading isolates

The temporal association of MDR *S.* Reading isolates was analyzed by TempEst software. *S.* Reading were isolated from replicate 1 Day 0 until replicate 2 Day 26 (day 67) (Figure 51). The numbers of animals that were newly isolated with *S.* Reading on any given corresponding day were plotted in Figure 51. TempEst analyze the correlation between the sample date (day 0 to day 67) and branch length of the core-genome SNPs phylogenetic tree by regression analysis. There were no temporal associations among the isolates ($P > 0.05$).

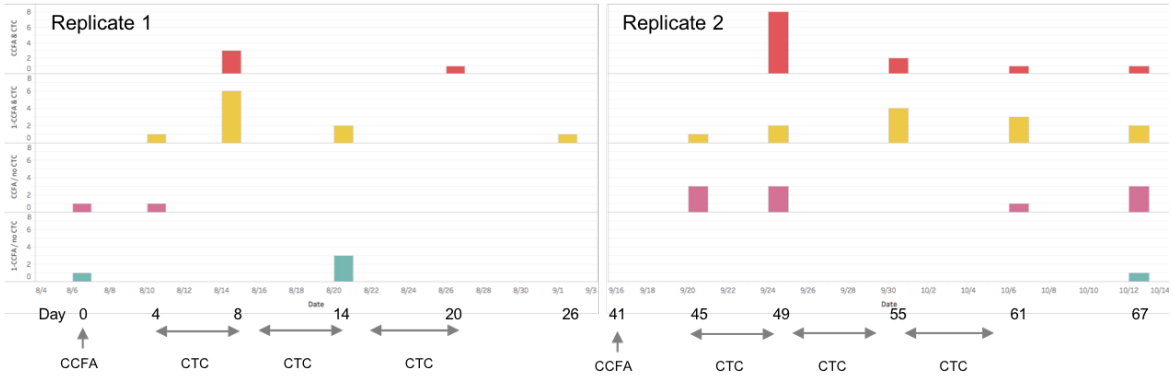


Figure 51. Epidemiologic curve of *Salmonella* Reading newly detected infections

5.3.7.2 Core-genome alignment of *S.* Reading with isolates from public database

The core-genomes of the *S.* Reading isolates were aligned and visualized into phylogenetic trees using maximum-likelihood methods. In *S.* Reading isolates, 97% of the

assembled genomic DNA were covered with the core genome. When 17 publicly available *S.* Reading isolates were included in the core-genome alignment, 3 isolates (97-0463 a U.S. human isolate from the CDC, CVM N42528 isolated from a pork chop in the U.S., and BCW-2763 from a human in Taiwan) belonged to the same phylogenetic cluster as our *S.* Reading isolates (Figure 52); however, they remained quite distant in terms of phylogenetic relations to our isolates.

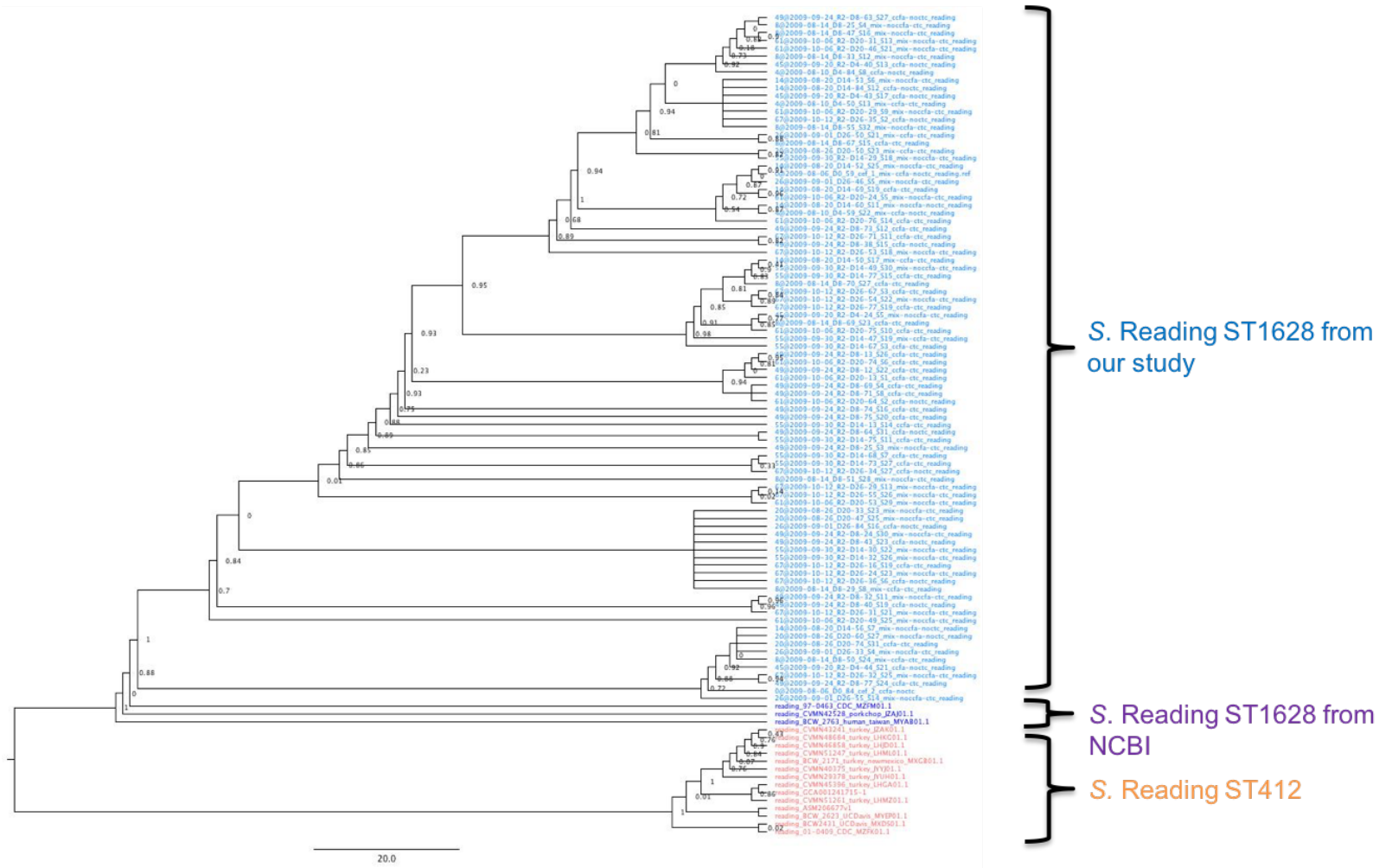


Figure 52. Maximum likelihood tree of core-genome SNPs of *S.* Reading with publicly available S. Reading contigs analyzed with Parsnp and visualized with FigTree.

5.3.7.3 Core-genome alignment of *S.* Reading isolates from this study

When only the *S.* Reading isolates from this study (88 isolates) were aligned, isolates grouped into 2 major clusters (Figure 53). The first cluster (Cluster 1) contained 76 isolates (86.3%) and the second cluster (Cluster 2) included 12 isolates (13.6%). Cluster 1 was further divided into 2 groups, 1a (68 isolates) and 1b (8 isolates). The composition of the clusters was evaluated by antibiotic treatment, pen, and day. All isolates that derived from animals that did not receive any treatment or else were treated with CCFA were in Cluster 1a. Isolates from animals treated with CTC were largely comprised of Cluster 1a (84.8%) Cluster 1b (6%), and Cluster 2 (9.1%) (Figure 54). Isolates from animals treated with both CCFA & CTC were in Cluster 1a (55.9%), 1b (17.6%), and Cluster 2 (26.5%) (Figure 54). CTC treatment was significantly associated with Cluster 2 by exact logistic regression (Odds ratio: 10.2, P=0.02); however, Cluster 1b was also composed of isolates from CTC treated animals (Figure 54) so the effect was not exclusive. Other variables including CCFA treatment, day, pen and most interestingly, replicate, were not significantly associated with phylogenetic clusters by exact logistic regression methods. The sample size was too small to allow for multivariable analysis. It is also interesting to note that the four isolates that carried both an IncA/C2 plasmid and IncI1 plasmid (green) were not associated with the cluster formation, since accessory genes are not included in core-genome analysis.

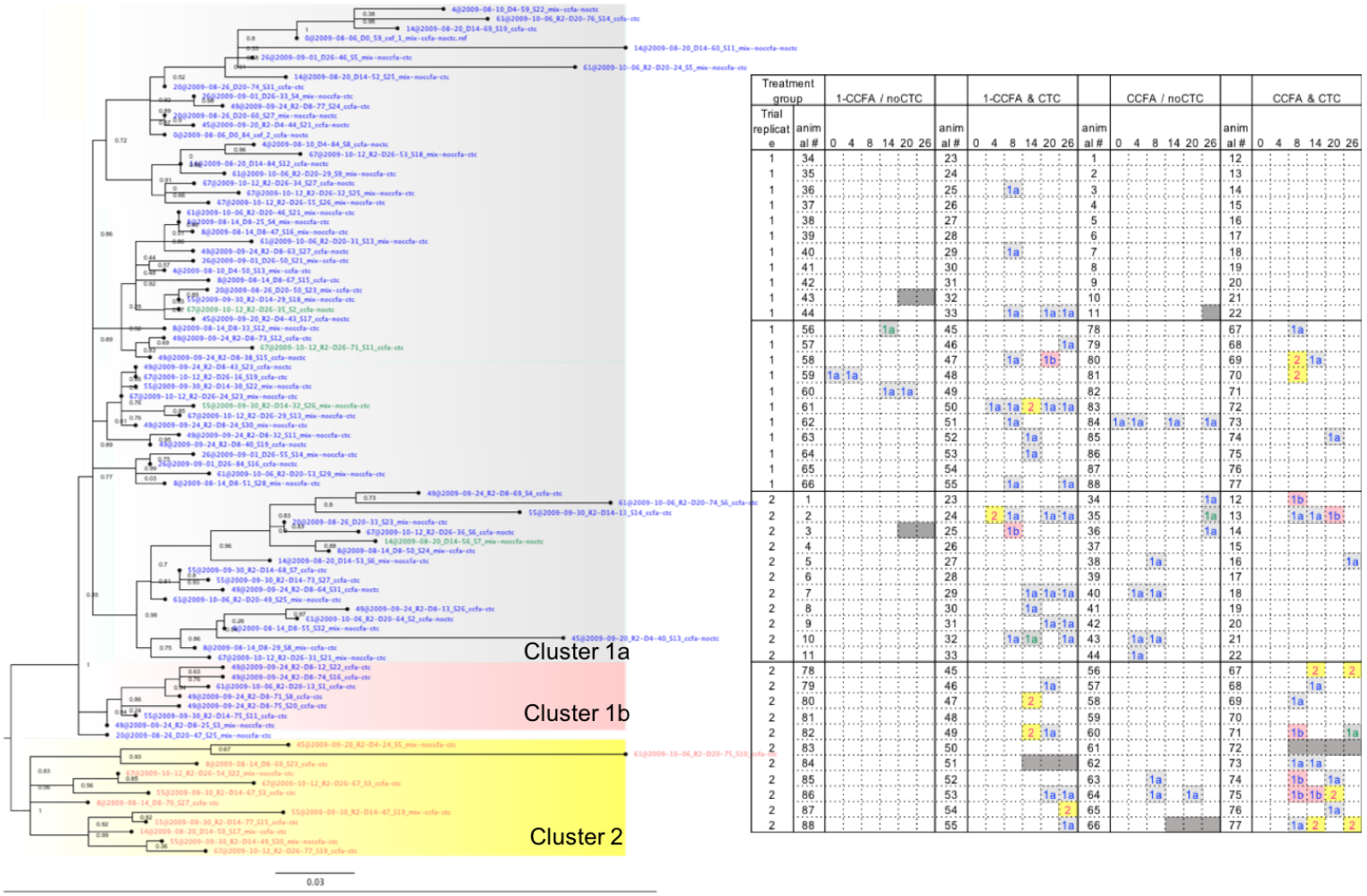


Figure 53. Maximum likelihood tree of *S.* Reading by core-genome SNP alignment (left) and corresponding isolate data per animal, treatment, replicate, and pen (right). Numbers in the right table corresponds to the clusters. Green isolates carried IncA/C2 and IncI1 plasmids. All other isolates carried only the IncA/C2 plasmid.

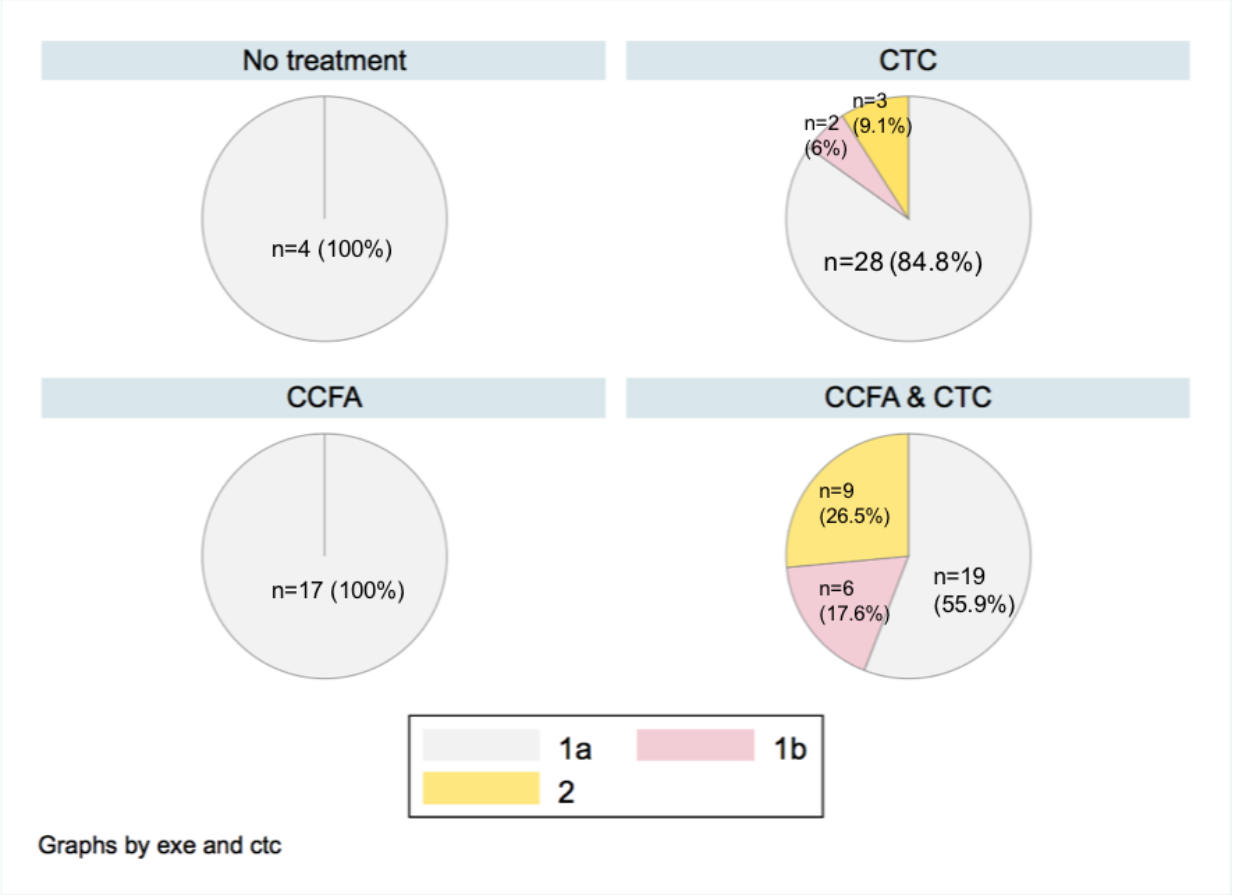


Figure 54. Distribution of phylogenetic Clusters 1a, 1b, and 2 by treatment group among the 88 *S*. Reading isolates.

5.3.7.4 SNP analysis of *S*. Reading

Single nucleotide polymorphisms (SNPs) of *S*. Reading isolates were visualized with Gingr (Figure 55). The SNPs were annotated using the GenBank file acquired by RAST annotation of our D0-59 isolate, which was used as a reference for the *S*. Reading analysis. In total, 200 SNPs were located among *S*. Reading isolates. Using the definition of a SNP as a nucleotide difference observed in > 1% of the population, those differences observed in only one isolate were not included and therefore only 53 SNPs were included in the analysis. When significant clusters of SNPs were observed, annotated genes were confirmed. The major difference between Cluster 1 and 2 was a polymorphism in the molybdenum transport ATP

binding protein *modC* gene. The *modC* gene forms the molybdenum transporter with the *modABC* genes. The adjacent *modB* and *modF* genes also had SNPs; however. no SNPS were identified in the *modA* gene.

The differences between Cluster 1a and 1b were in the phage terminase (ATPase subunit) gene. Out of 7 SNPs that were identified in this gene, 4 of them were nonsynonymous. Other genes that were detected with major SNPs were within the phosphotransferase system coding gene, mannose-specific IIC component coding gene (EC 2.7.1.69), intergenic region of the *yebE* inner membrane protein and FIG01200701 (possible membrane protein), biotin synthase gene (EC 2.8.1.6), putative molybdenum transport ATP-binding protein coding gene (*modF)*, and galactose-I-phosphate uridylyl transferase coding gene.

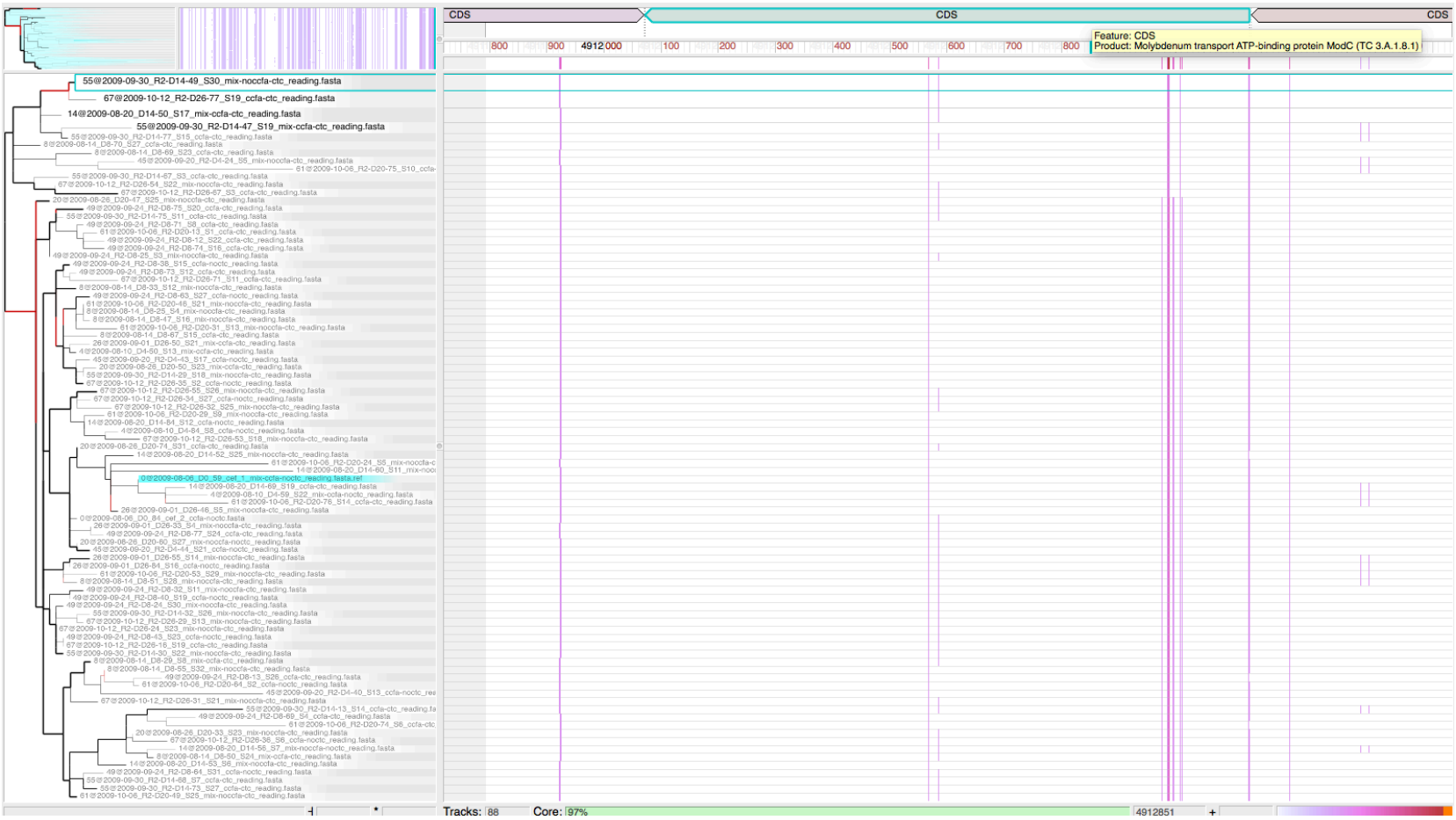


Figure 55. SNP differences identified in the *modC* gene between Cluster 1 and Cluster 2.

5.3.8 Salmonella *Mbandaka phylogenetic analysis*

For the isolates derived from this study, the total core-genome coverage was 94.6% among all the *S.* Mbandaka sequences. *S.* Mbandaka was divided into 4 major clusters among the isolates from this study (Figure 56). Overall, 64.8% of Clusters 1, 2 and 3 consisted of isolates from CCFA treated animals. Cluster 4 was divided into 4 sub-clusters, which consisted of 44.4% of isolates from animals without antibiotic treatments and 26.8% of CCFA treated animals. Six major SNPs were identified, corresponding with the cluster formations. Due to computational limitations, the genes corresponding to the SNPs were not analyzed for *S.* Mbandaka.

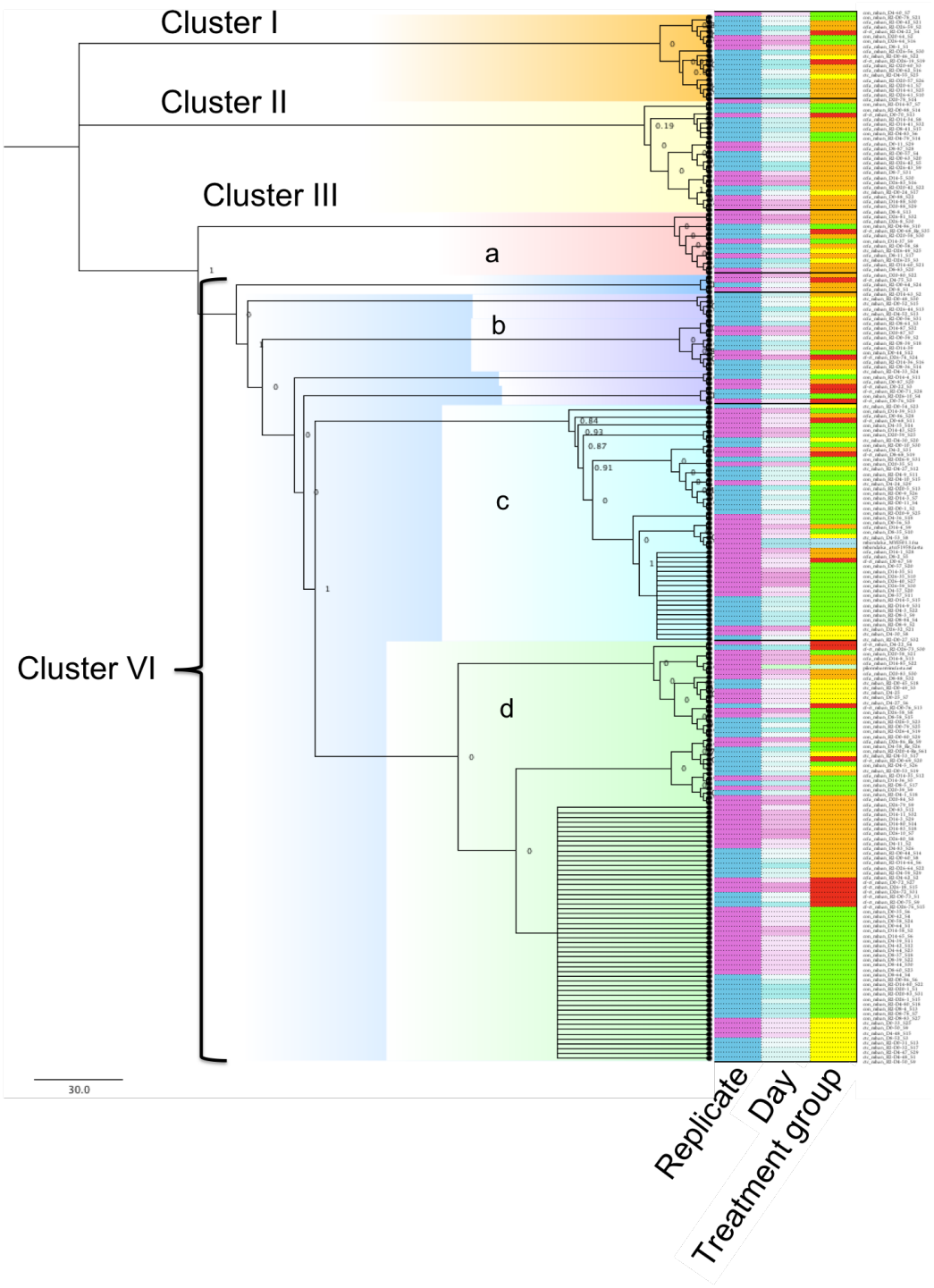


Figure 56. Maximum likelihood phylogenetic tree of *S.* Mbandaka isolates. Replicate (Pink: Replicate 1, Green: Replicate 2), Day (earlier day - lighter color), Colors of treatment group. Green: 1-CCFA / no CTC, yellow: 1-CCFA & CTC, orange: All-CCFA / no CTC, red: All-CCFA & CTC.

5.3.9 Salmonella *Kentucky phylogenetic analysis*

The core genome of each *S.* Kentucky isolate was aligned using Parsnp. *S.* Kentucky isolates shared 96% of the core genome within the sampled population and the phylogenetic analysis revealed 3 clusters. The *Salmonella* Kentucky serotype contains at least 15 different sequence types (STs) [109]. The most common ST isolated in the United States is ST152, largely from chickens, retail meat, and dairy cattle. All of our *S.* Kentucky were ST198. Occasionally, ST198 has been isolated from dairy calves, ground beef, and human clinical isolates. Therefore, in the core-genome alignment, other ST198 sequences available from public sources were included. When one isolate of ST152 (CVM 29188) and 10 isolates of ST198 were included, 89% of the core genomes were shared and all our isolates were clustered with the publicly available ST198 (Figure 57). Isolates from this study were classified into two clusters shown in pink and teal (Figure 57). It has been reported that ST198 has 2 groups: 198.1 and 198.2 [109]. ST198.1 is largely comprised of ground beef and dairy calf derived isolates while 198.2 is largely comprised of isolates from human clinical cases [109]. When both 198.1 and 198.2 were included in the Parsnp alignment, and one of our *S.* Kentucky isolates was used as a reference genome (D0-3), the sequences shared 75.1% of the core genome. All the *S.* Kentucky isolates from this study formed a clustered in 198.1 together with isolates derived from dairy calf feces in Florida (ARS-CC273, ARS-CC274, ARS-CC938), ground beef from New Mexico (CVM N51290), and ground turkey from California (CVM N42453) (Figure 57). One ground beef derived isolate (CVM N41913) clustered in 198.2 along with ATCC9263 isolates from human clinical cases; however, none of our isolates was found in ST198.2.

When isolates from only our study were aligned (Figure 58, left side) the total core-genome coverage among all isolates was 96.7%. Isolates were clustered into three groups. The major SNP differences between Clusters 1 and 2 in comparison to Cluster 3 were with the Heme exporter protein C. Multiple other SNPs differentiated Clusters 1 and 2, including the Thiol:disulfide interchange protein DsbE, 16S ribosomal RNA, a few hypothetical proteins, and several intergenic regions. No clear differences in the distribution of the isolates were found among the treatment groups (Figure 58, right side).

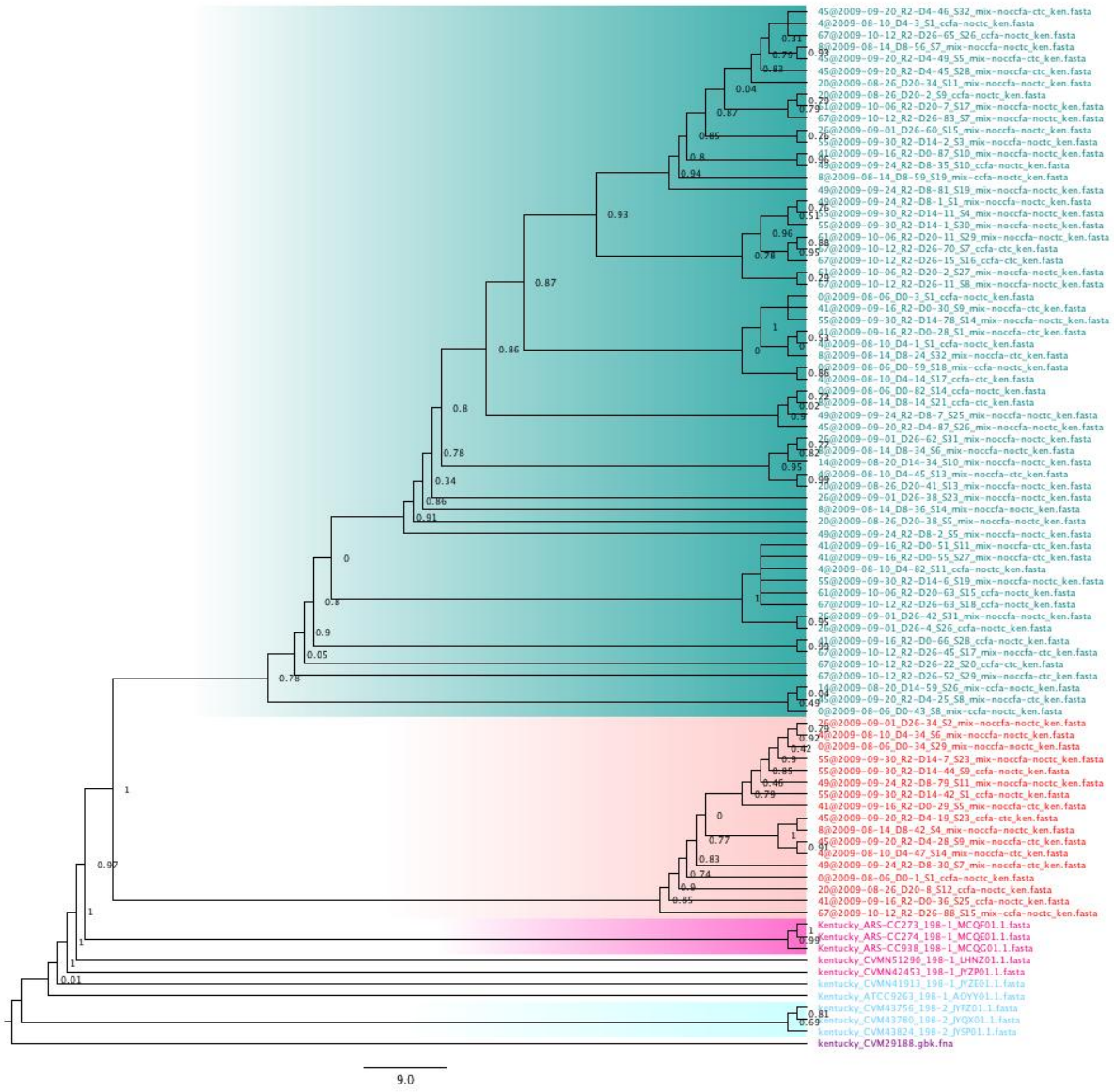


Figure 57. Maximum likelihood phylogenetic tree of *S.* Kentucky isolates. S. Kentucky core-genome was aligned using CVM 29188 (ST152) as a reference genome. Tree was rooted at the midpoint and branches are proportionally transformed.

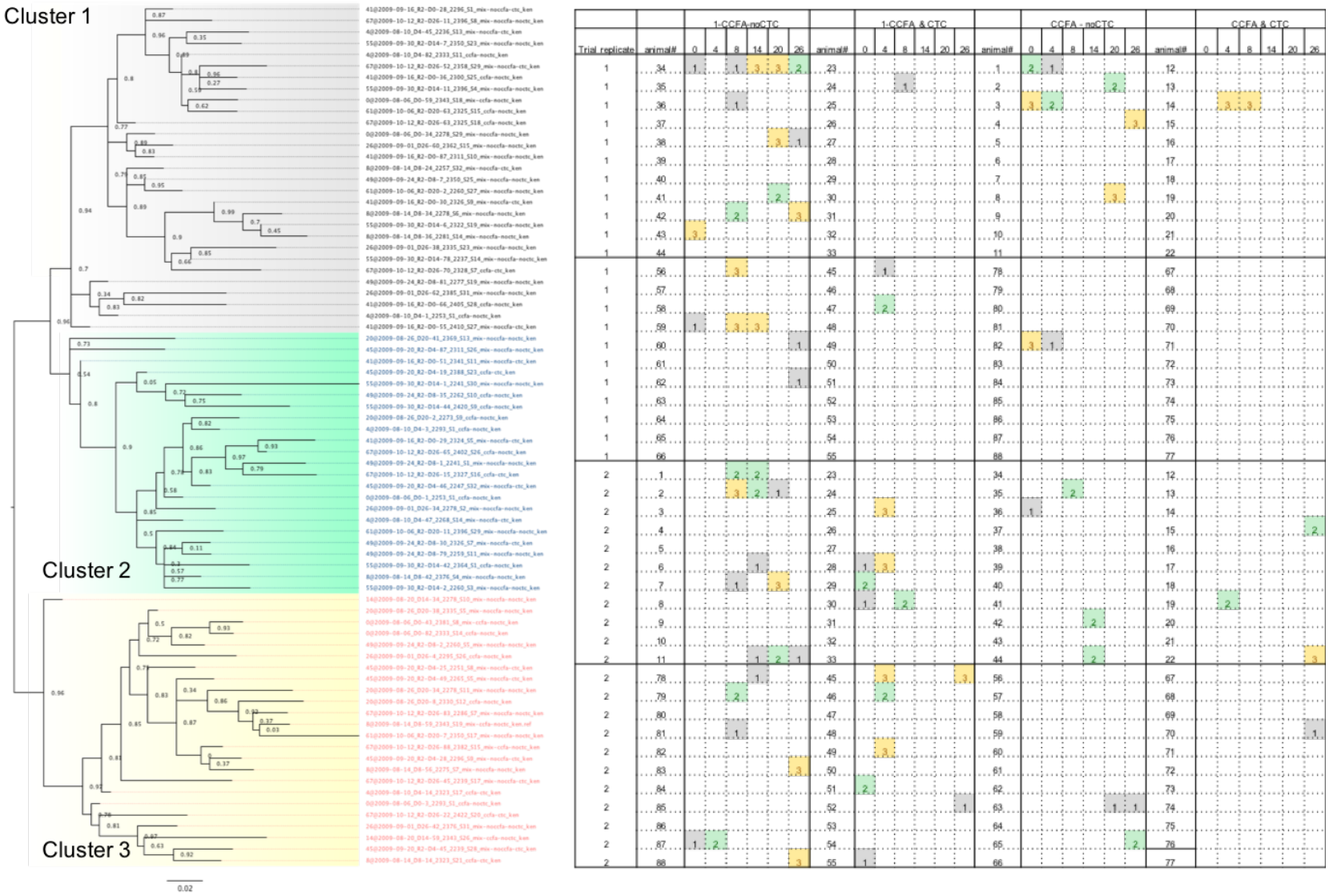


Figure 58. Phylogenetic analysis of *Salmonella* Kentucky from this study aligned with Parsnp (left) and corresponding table of isolates per replicate, treatment group, day, and animal (right). Grey: cluster 1, green: cluster 2, yellow: cluster 3. Numbers in the cells corresponds to the cluster.

5.3.10 Salmonella *Give phylogenetic analysis*

In total, 108 *S.* Give were isolated from our study. From NCBI Bioproject PRJNA59703, PRJNA186035, and PRJNA224224, 16 publicly available assembled contigs were included in the phylogenetic analysis. The additional sequences were included in the analysis to determine the relationship between our *S.* Give isolates to other *S.* Give isolates since *S.* Give is known to be polyphyletic [108]. One public isolate (GCA_000505465.1_CFSAN004343_01.0) was of ST 19, which was from a composite cattle fecal sample and belonged to different cluster from the other isolates (Figure 59). In the other cluster, one isolate was ST3969 and one ST was unknown (ST allelic gene pattern: 301, 11, 16, 343, 748, 71, 2), and 4 others were ST516. Within ST654 from the public database, 2 isolates (ASM48721v1 and ASM187889v1) clustered with our isolates. These isolates were derived from a filter on a dairy farm and river water in Mexico, respectively.

The isolates from our study were aligned using one of the isolates from this population as reference genome. Total coverage of the core genome among all our sequences was 95.6%. At least 6 well-supported clusters were formed by these isolates (Figure 60). However, the cluster formation was not associated with treatment groups. Cluster 4 had multiple SNPs in different genes, including inosine-5'-monophosphate dehydrogenase, Ded A family inner membrane protein (YqjA), Ferric reductase, and an intergenic region between ATP dependent protease HslV and HslU (Figure 61).

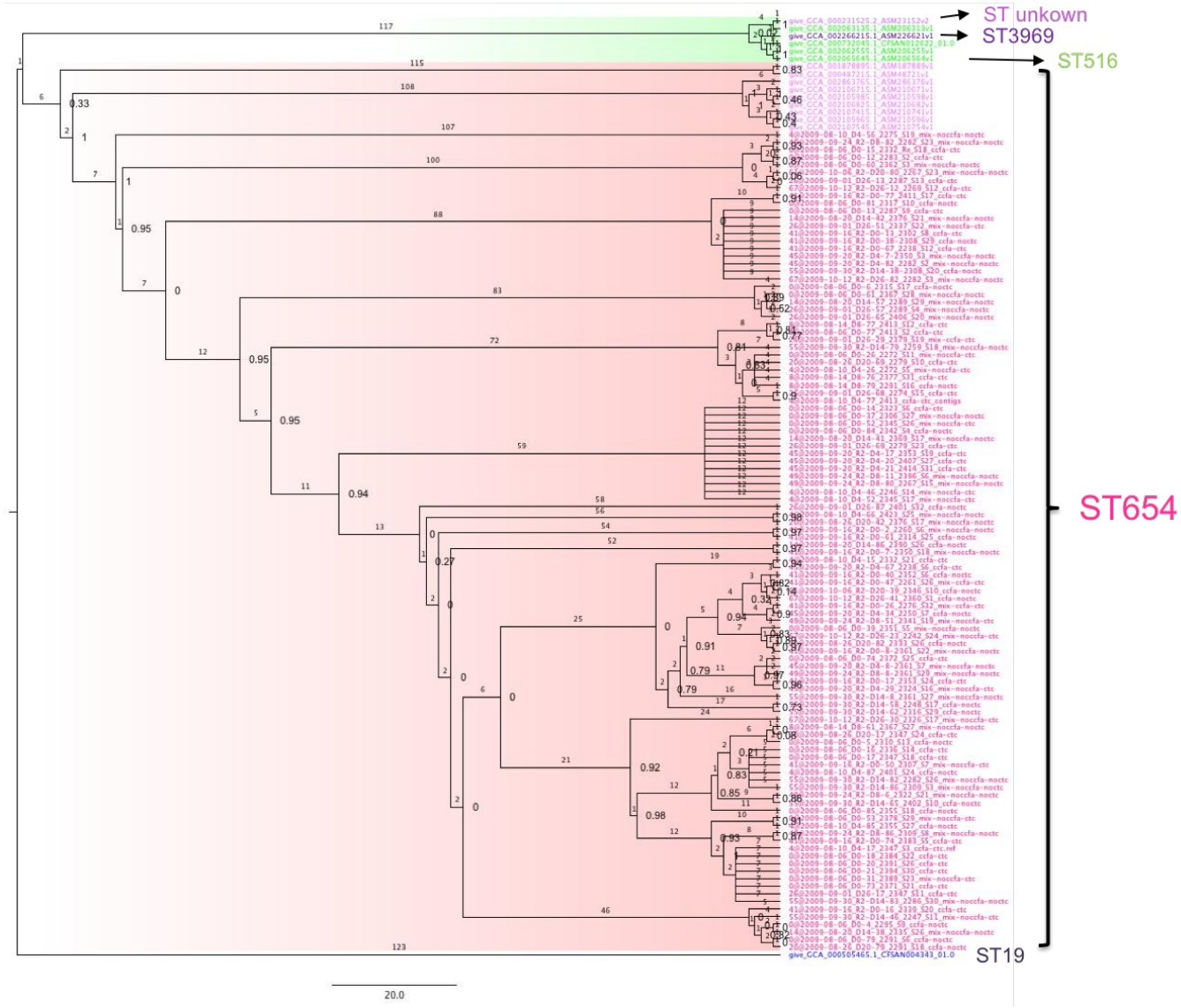


Figure 59. *Salmonella* Give core-genome phylogenetic tree including publicly available sequence data.

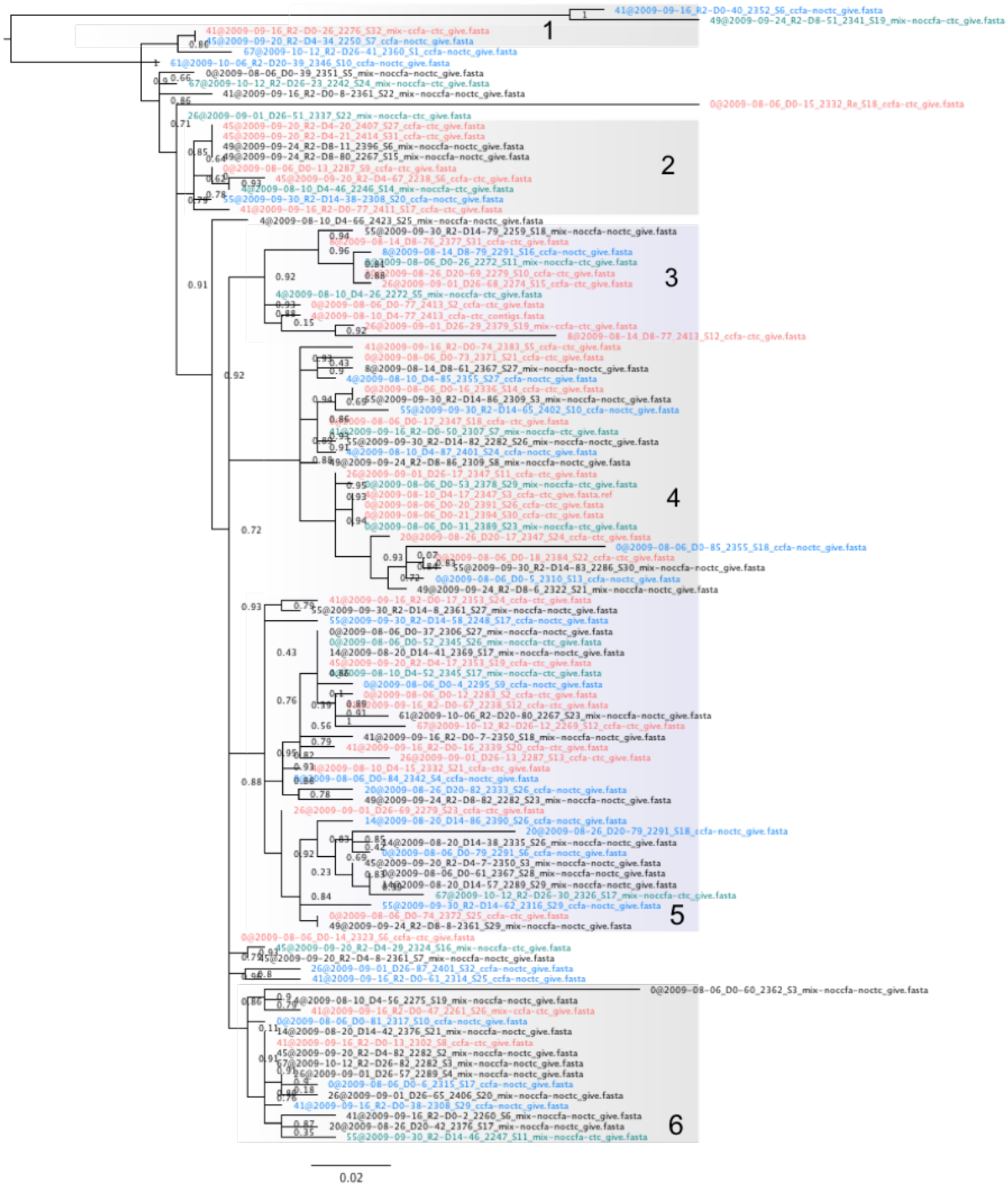


Figure 60. Phylogenetic maximum likelihood tree of *S*. Give from our study. Colors are coded by individual-animal treatments. Black: no treatment, blue: CCFA treatment, green: CTC treatment, red: CCFA & CTC treatment.

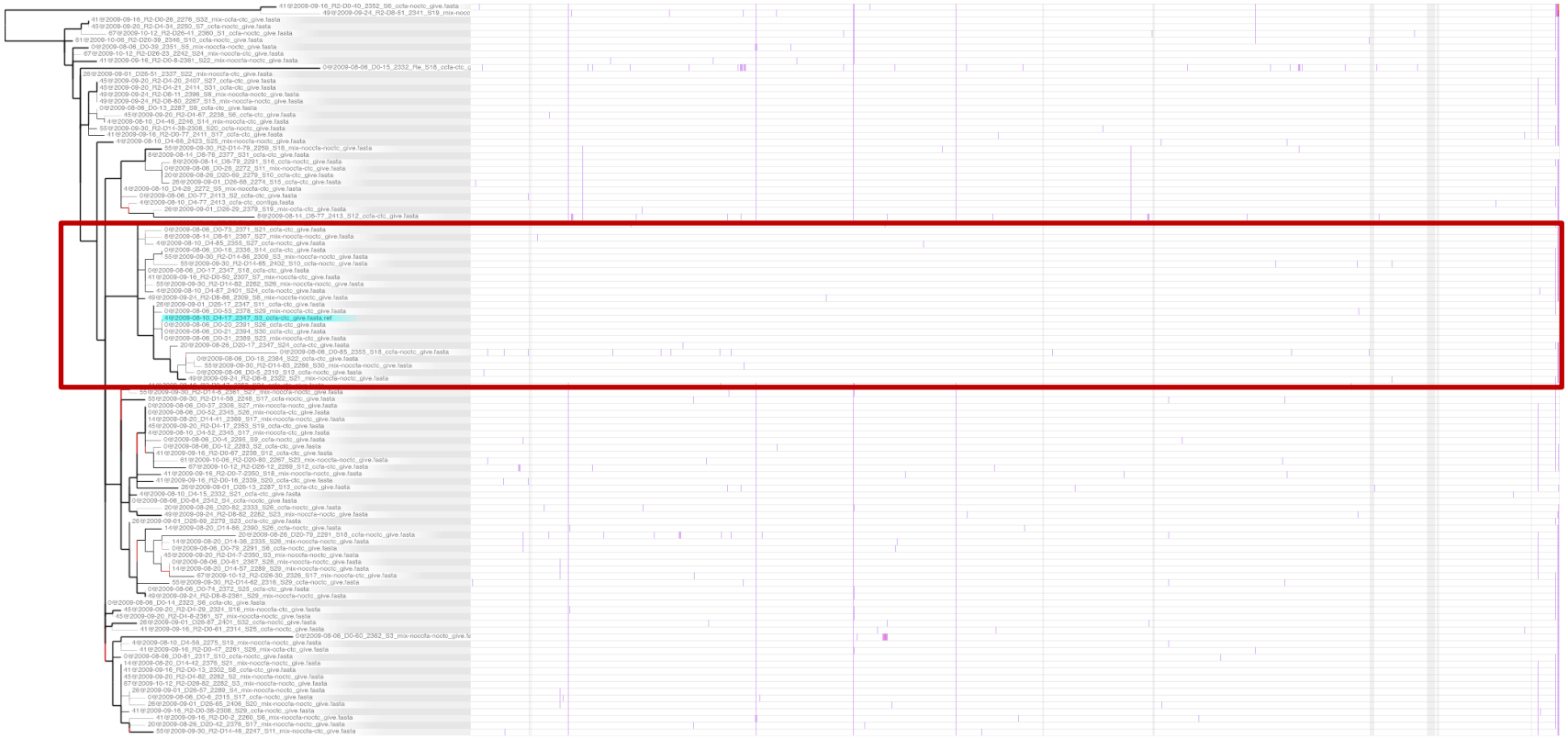


Figure 61. Visualized SNPs of *S*. Give within the sampled population.

5.3.11 Salmonella *Montevideo*

Among *Salmonella* Montevideo isolates, the total coverage of the core genome was 96.4% among all sequences observed in studied population. Two major clusters were formed for *S.* Montevideo. A few sub-clusters included only isolates from CCFA treated animals (Figure 62). The major difference identified between Cluster 1 and 2 was in an intergenic region. Eight more major SNPs were found within *S.* Montevideo. In Cluster 1 and 2, one cluster each was composed by the isolates treated with CCFA (highlighted in grey and in blue color in Figure 62). The SNPs that clustered them were located at an intergenic region. Other cluster formation was due to SNPs in DNA gyrase subunit A, diguanylate cyclase/phosphodiesterase with PAS/PAC sensor coding gene, *clpB*, putative inner membrane protein coding gene. These SNPs were not associated with antibiotic treatments.

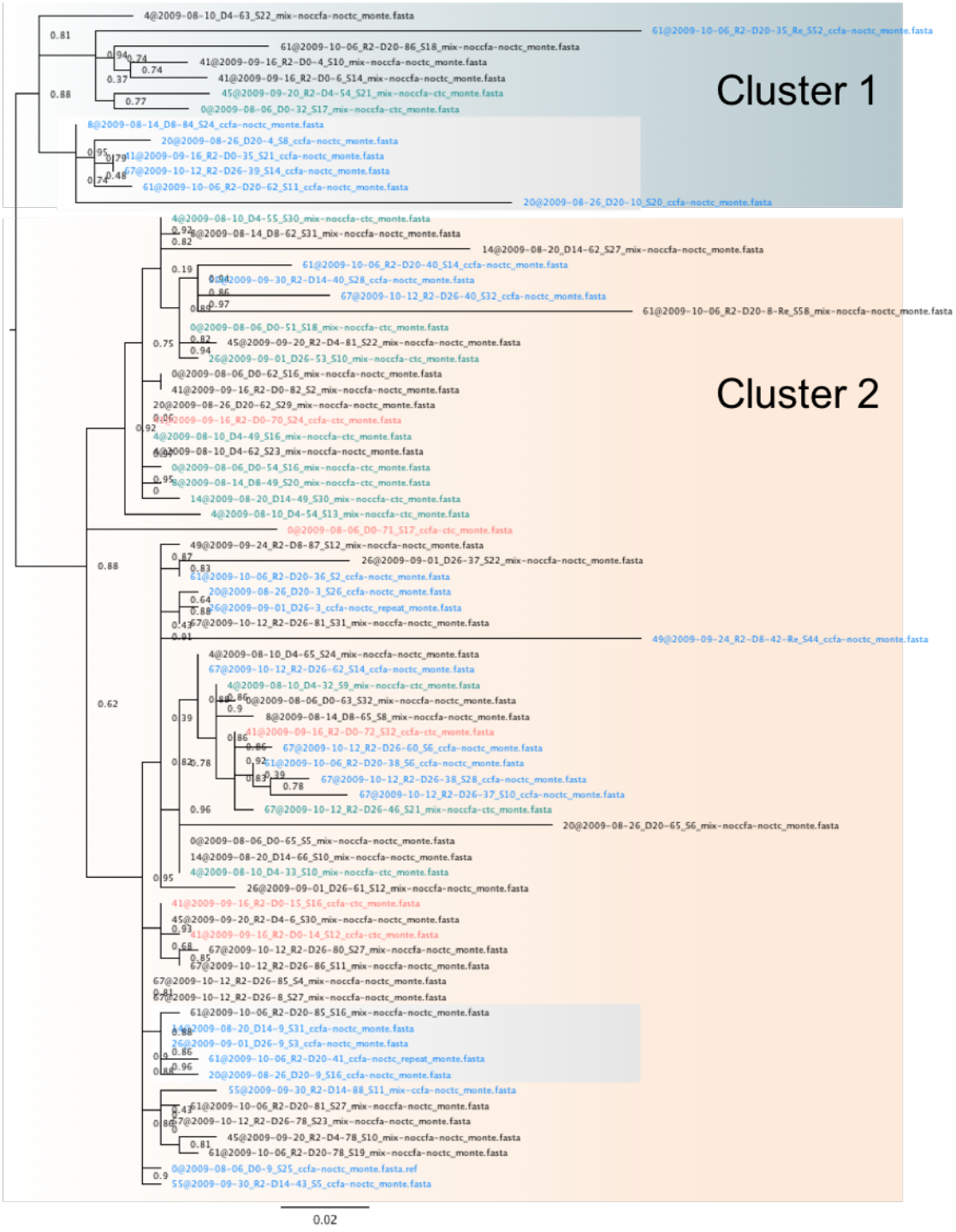


Figure 62. *S.* Montevideo phylogenetic maximum likelihood tree. Color-coded by treatments: Black: no treatment, blue: CCFA treatment, green: CTC treatment, red: CCFA & CTC treatment. Grey shades are where isolates consisted of mostly CCFA-treated animal-derived isolates.

5.4 Discussion

Whole-genome sequencing of *Salmonella* is becoming a common practice in bacterial molecular epidemiology. We successfully sequenced 568 isolates of *Salmonella* from a single cattle population arising from 2 replicates each studied for 26 days.

The total sequence length of each serotype differed, in order from *S*. Give through *S*. Montevideo, *S*. Anatum, *S*. Reading, *S*. Mbandaka, and *S*. Kentucky as the largest. The length also differed by the presence or absence of plasmids. It is known that genome size differs by serotype in *Salmonella*, and also it differs by the number of accessory genes [127, 340]. The number of CDS was between 4,200 and 4,560, which agrees with previous studies. A study by Laing *et al*. found that *S*. Anatum has on average 401.5 *Salmonella* species-specific genomic regions, which was one of the highest numbers followed by *S*. Kentucky (380.3), *S*. Mbandaka (374.5), *S*. Reading (370.4), and *S*. Montevideo (360.1) [127]. *S*. Give was not included in their study.

An accurate assembly is crucial for downstream analysis, which we achieved by SPAdes assembly. SPAdes assembly returned median contig numbers of 45 and a median total length of 4.7 Mb, both of which were consistent within serotypes. Initially, the assemblies were performed in Velvet and the assemblies were searched for the presence of antibiotic resistance genes and plasmid replicons. For the most part, we found good agreement among resistance-gene and plasmid data when comparing Velvet and SPAdes assemblies. However, IncY plasmid replicons were not detected with Velvet assembled genomes but were identified with SPAdes assembled genomes. The reason for this may be due to differences in the assembly algorithm between the two software packages. Since the same database was used for plasmid replicon searches (PlasmidFinder) to compare between the Velvet and SPAdes assembled genome, these

differences could not due to database differences. The IncY plasmid may contain repeated regions that the Velvet assembler had difficulty assembling, whereas SPAdes could correctly assemble the repeats. Either way, the SPAdes assembler had a lower number of contigs and optimal total length. Therefore, genomes assembled by SPAdes had high detection of the genes and replicons, which is consistent with other reported studies [249, 341].

Serotyping by SeqSero based on WGS data and traditional serotyping match 100% among a small subset of the isolates. Since we did not conduct traditional serotyping on all of the isolates, we are unable to assess the accuracy of WGS based serotyping. However, based on the total length of the assembled genome, it is highly likely that most of the isolates were serotyped accurately. One isolate each from *S.* Montevideo, *S.* Mbandaka, *S.* Mbandaka with IncI plasmid, and *S.* Kentucky had a longer length than the other isolates in these serotypes, respectively, and no additional plasmids were detected. Assembly statistics were in an acceptable range, and therefore such extended genome isolates may be a variant of the serotype or else may be carrying an unknown plasmid that was not included in the PlasmidFinder database. In *S.* Reading, a few genomes were detected with more than 4,700 CDS, while others were below 4,700; despite this, only the IncA/C2 plasmid was detected among all of these isolates. These isolates may also carry additional plasmids or be a variant of *S.* Reading. Although we have not used SISTR serotyping in this study, the authors of SISTR showed that *S.* Reading had a relatively low concordance with reported serotypes [97]. However, SISTR uses assembled fasta files as an input instead of raw fastq reads like SeqSero, so the results are not directly comparable.

The hybrid assembled genomes using both Oxford Nanopore MinION long sequence reads and Illumina MiSeq short sequence reads resulted in a fewer number of contigs. The final number of contigs was 9 for *S.* Mbandaka and 10 for *S.* Reading isolates. The total length of the

genome was similar between the hybrid and Illumina assemblies, but the N50 and largest contig size of the hybrid assembly were each significantly larger than that of the assembly derived from only Illumina reads.

The *S*. Reading IncA/C2 plasmid from the isolate sequenced on the MinION was assembled into a single contig via the hybrid assembly with Canu, and was polished with Pilon. Similarly, George *et al*. compared the assembly of plasmids of *Klebsiella* spp, *Citrobacter fruendii*, *E. coli*, *Serratia marcescens*, and *Enterobacter cloacae* by multiple assembly methods, in which Canu alone or Canu and Pilon based assembly approaches showed the best recovery of plasmids when compared with plasmidSPAdes, hybridSPAdes, and npScarf [342]. Our study showed that this assembly method is feasible for *Salmonella* and may be a cost-effective method to assemble complete plasmid sequences.

In our studied population, 6 serotypes each were divided into one of two clades: *S*. Mbandaka, *S*. Kentucky, *S*. Reading, and *S*. Anatum were in the first clade, and *S*. Montevideo and *S*. Give were in the second clade. Previous studies have shown that *Salmonella enterica* generally are divided into 2 major clades, Clade A and B, which was consistent with our study [107, 108]. *S*. Reading and *S*. Anatum clustered with serotypes highly pathogenic to humans, including: *S*. Typhimurium, *S*. Dublin, *S*. Enteritidis, and *S*. Heidelberg. Several *Salmonella* serotypes are known as polyphyletic, such as *S*. Newport, *S*. Kentucky, and *S*. Give [110]. Among the serotypes that were recovered here, *S*. Give, *S*. Kentucky, and *S*. Reading are known or suspected to be polyphyletic and have multiple MLST within the serotype [107-109]. *S*. Mbandaka has not been reported as polyphyletic previously; however, our *S*. Mbandaka were clustering with a few other NCBI obtained strains and were not clustered with the standard *S*. Mbandaka strain ATCC51958. One *S*. Mbandaka that formed an isolated

branch following core-genome alignment was BLAST searched, and turned out to be *Salmonella diarizonae*. This was unexpected, and the reason for this misclassification is not known. This isolate was kept in the phylogenetic tree to be used as an outgroup. *S.* Give was the most divergent serotype and consisted of at least 5 STs when publicly available sequences were included into the phylogenetic analysis. The MLST of our *S.* Give isolates were identical to one another.

The phylogenetic tree of our isolates, when compared with the publicly available sequences, showed that all of our isolates were clustering together per serotype in the tree; importantly, this shows that they belong to the same lineage. In *S.* Anatum, *S.* Montevideo, *S.* Give, and *S.* Kentucky, our isolates clustered with other bovine-derived isolates. The clusters of *S.* Anatum and *S.* Kentucky included human-derived isolates, which may suggest a public health concern. *S.* Reading clustered with pork chop and human derived isolates and *S.* Mbandaka clustered with a chicken breast-derived isolate. However, the publicly available isolates that were included in this study are limited; therefore, further sequence data are needed to investigate comparisons with other isolates. The cattle in our study were all from the same source and therefore the same lineage of serotypes may have expanded in these cattle. The cattle were comingled prior to the study to normalize the microbiota between individuals. *Salmonella* can easily transmit between cattle within the same pen. In fact, core-genome coverage was approximately 95% within the same serotype. On the other hand, several SNPs were found among the isolates within serotypes and they were further divided into several clusters, suggesting at least some within serotype diversity emerged within the temporal and geographical limits of this controlled field trial.

All *Salmonella* Reading isolates were phenotypically and genotypically multidrug resistant (MDR). The whole-genome sequencing genotypic resistance data of the *Salmonella* isolates showed good agreement with phenotypic data. Complete agreement was observed for florfenicol, all tested beta-lactams, and tetracycline. Streptomycin and sulfisoxazole had nearly 100% agreement. The disagreements in sulfisoxazole typing were observed among susceptible phenotypes harboring *sul1* and *sul2* resistance genes. The NARMS cutoff value of sulfisoxazole resistance is >= 512 mg/L, and susceptible is <= 256 mg/L; meanwhile, the three isolates which were classified as susceptible had MIC values = 256, which is on the border of resistant and susceptible. Tyson *et al*. studied the genotypic cutoff values of *Salmonella*, and they too had a single isolate that was detected with sulfisoxazole resistance mechanisms, but had a MIC value <= 256 mg/L [132]. The possibility is that these three isolates had lower expression of resistance phenotypes. The other possibility is that there was an error reading the microbroth dilution plates. Overall, the agreement between resistance genotype and phenotype was very high, which supports the previous studies that suggest that WGS can be used as a surveillance alternative for phenotypic resistance determination [132, 136, 315].

All of the *S*. Reading isolates were carrying IncA/C2 plasmids and four isolates were detected with an additional IncI1 plasmid. The resistant genes detected in *S*. Reading were located on the IncA/C2 plasmid, which is consistent with previous studies suggesting that the ACSSuT+cef phenotype encoding genes are commonly carried on IncA/C2 plasmids [200, 343]. We closed an IncA/C2 plasmid (contig_04) and had a 99% match with plasmid pSN254b by BLASTn search, originally isolated with an *Aeromonas salmonicida* [336]. Our plasmid also matched multiple other reported plasmid sequences isolated from *E. coli*, *S*. Anatum, and *S*.

Newport, to list a few. The dot plot analysis showed almost complete match of sequences between our plasmid and pSN254b, when compared with another two plasmids derived from *S.* Newport and *S.* Anatum [200]. This result showed that this plasmid has broad bacterial host range (*A. salmonicida*, *E. coli* and *Salmonella enterica*) among those Enterobacteriaceae which are commonly associated with mammals and fish.

In a previous study, Cottell *et al*. reported that the $bla_{\text{CTX-M-32}}$ gene was detected from an IncN plasmid in *E. coli* from the same cattle population [202]. However, in our *Salmonella* population, neither the $bla_{\text{CTX-M-32}}$ gene nor IncN plasmids were detected. Even though IncN is an average sized plasmid (30-70kb) and has a broad bacterial host range [344], our results suggest that these IncN plasmids were not shared among *Salmonella* and *E. coli* among these cattle, assuming that the *Salmonella* we have detected are representative of the *Salmonella* population. Six isolates were carrying an IncY plasmid. It is worth noting that, recently, an IncY plasmid was detected with the plasmid-mediated colistin resistant gene (*mcr*-1) from *Salmonella* in China [345]. When the genome assembly was conducted with the Velvet assembler, the IncY plasmid was not detected; however, the plasmid was detected by SPAdes assembly. This can be due differences in the assembler algorithms.

Salmonella Reading is commonly reported in cattle but not in humans; however, occasional outbreaks have been reported in North America from alfalfa sprouts and dog food [346-348]. Few cases have resulted in hospitalization and septicemia [346, 348]. The most common animal reservoirs are turkeys and pigs. Isolates from our study clustered separately from *S.* Reading derived from turkey, but clustered closely to the one isolate from a pork chop. Interestingly, the isolates also clustered more closely to a human-derived strain from Taiwan. *S.* Reading is suspected to be polyphyletic as was reported in a previous study [107],

which is also supported by our data. Polyphyletic serotypes are those derived from a genetically distinct ancestor. In *Salmonella*, this can occur easily by the recombination between different serotypes. As our *S*. Reading isolates were distinct from those from derived from turkey, it is important to investigate the phylogenetic relationship of isolates, especially in outbreaks.

The core-genome coverage of our *S*. Reading isolates was 97%, which implies clonal expansion. We identified two clusters (Cluster 1 and 2) and two sub-clusters (Cluster 1a and 1b) within Cluster 1. The major differences between Cluster 1 and Cluster 2 was a polymorphism in the *modC* gene. When isolates were classified by treatment group, Cluster 1a and 2 contained only isolates derived from animals treated with CTC or CCFA & CTC. CTC treatment was significantly ($P < 0.05$) associated with cluster. Isolates from Cluster 1b and 2 were detected from multiple pens and from both replicates, which implies the *Salmonella* were not proliferating only in one animal or pen, or at a single period of time. Additionally, a few animals carried both Cluster 1 and 2 isolates, and therefore it is not likely that mutations occurred *in situ* because of the antibiotic treatment. Therefore, two clusters of *S*. Reading with polymorphisms in *modC* may have resided in the cattle or the environment initially and were selected for by the CTC treatment.

Further analyses, by including larger datasets of *S*. Reading sequences, are needed to explore whether the isolates with *modC* polymorphisms were favored by CTC treatment. It is also necessary to confirm the mutations by analyzing with different assemblers and variant callers to remove the possibility of artifact. Cluster 1b and 2 isolates may have a fitness advantage over Cluster 1a in the microbiota affected by the CTC treatment; however, both Cluster 1 and 2 were detected without temporal association. The animals carrying *S*. Reading

were not always detected with *S.* Reading even after antibiotic treatment; in a similar manner, after CTC treatment, Cluster 2 isolates were not always detected from animals that carried both Cluster 1 and 2 isolates. Mao *et al.* have shown that when mutant phenotypes were in the population at 0.001% rate, after selection by antibiotics, they proliferated to 0.5%; further, with two or more successive selection, they were able to proliferate to 100% [349]. The CTC treatment may have been confounded by other factors which helped expand both clusters 1b and 2.

A detailed investigation of the potential association between *modC* and antibiotic selection was not performed here. However, previous research shows that tetrathionate, which *Salmonella* utilize as respiratory electron acceptor, contains a molybdopterin cofactor. This molybdopterin cofactor derives from molybdenum that is metabolized in the bacteria. Since the *modC* gene is part of the *modABC*, a molybdenum transporter, the intake of isolates in Cluster 2 may provide an advantage over other isolates when the microbiota is disrupted by CTC. Although our results are limited by isolate numbers, the genomic comparisons suggest that antibiotics may be selecting for a subpopulation within those resistant isolates that have survived the antibiotic treatments. If the CTC treatment continued, or else the animals were given other antibiotics for disease treatment at a later date, this subpopulation may increase.

Salmonella Kentucky is most common serotype isolated from poultry sources in the United States [109]. ST152 and ST198 are the most common sequence types [109, 350, 351]. Haley *et al*. compared 119 isolates of poultry and cattle derived *S.* Kentucky, which showed that the majority of them were ST152 while only 12 were ST198. In the EnteroBase database, only around 5% of the *S.* Kentucky isolates are ST198 and the sources varied from poultry, humans, the environment, and livestock. In our study, only ST198 strains were identified,

which is interesting considering that most of the *S.* Kentucky from food animals in the United States are ST152. In previous study, within ST198, the authors identified two clusters based on the XbaI-PFGE profiles and phylogenetic tree analysis (198.1 and 198.2) [109, 350, 351]. The ST198.1 cluster contained isolates derived from dairy cattle feces, a milk filter from Florida, and ground turkey from California; meanwhile, ST198.2 contained isolates from human clinical cases [109]. All our *S.* Kentucky derived from cattle clustered with ST198.1, which corresponds with the previous result. Recently, there has been concern with an increase of *S.* Kentucky with high-level resistance to ciprofloxacin in Europe, Africa, and the Middle East, The source of this increase has been attributed to international travel to Europe from Africa and the Middle East, with poultry identified as a potential major vehicle [350, 352]. All ciprofloxacin resistant isolates belonged to ST198-X1 in the previously described study, which had mutations in the *gyrA* and *parC* genes leading to ciprofloxacin resistance [350]. Although it is not clear if ST198-X1 belongs to 198.1 or 198.2, it may be a public health concern.

Cattle in our study were derived from a single source and only one *S.* Kentucky isolate was a multidrug resistant phenotype carrying the corresponding resistance genes (*aadA7*-truncated*, sul1*, and *tet*(A)). The isolate carried an IncI1 plasmid and it is likely that the resistance genes were carried on the plasmid based on plasmidSPAdes results and annotation. Although this isolate did not have ciprofloxacin resistance, further caution may be needed for acquiring other resistant genes including ciprofloxacin resistant genes.

Salmonella Give was detected with distinct 6 clusters. *S.* Give has been isolated from dairy farms, water, opossum feces, and soil [353]. It is important to note that these antibiotic susceptible serotypes survived treatment by CCFA and CTC, absent the selective

advantages provided by antibiotic resistant genes. *Salmonella* can become persistent and survive inside infected host cells by forming *Salmonella*-containing vacuoles. Even after *S.* Reading dominated the population, we did not observe any other serotypes carrying IncA/C2 plasmids nor resistant genes that *S.* Reading were carrying. It may simply because most of other susceptible serotypes were killed (or, suppressed) by the antibiotics and *S.* Reading did not have conjugal partners with whom to transfer the plasmids. Even if other serotypes with resistance determinants existed post-treatment, they might not have been detected because *S.* Reading was the dominant MDR strain. Antibiotics are known to induce recombination and lateral transfer of the genes *via* SOS response [354]. For example, in *E. coli* O157:H7, fluoroquinolones trigger the expression of prophage genes and promote the transfer of prophages [354]. However, it has also been shown that some antibiotics can inhibit conjugation and stimulate plasmid curing [354]. Although the mechanisms are not known yet, *Salmonella* can survive inflammatory response caused by antibiotics in the host. The mechanisms of how these susceptible *Salmonella* survived the antibiotic treatments may be answered by analyzing the gene expression data.

There was no clear temporal relationship among the genomes of the isolates, which implies no mutations had occurred as a result of the antibiotic treatments within this studied period. Since the mutation rate is estimated as $5.3\text{-}3.9 \times 10^{-7}$ substitutions per site per year in *S.* Kentucky and *S.* Agona, this was not surprising [355]. Among wild type *E. coli*, the mutation rate is 1×10^{-3} per genome per generation [356]. It has been shown that exposure of *E. coli* to long-term sub-lethal levels of norfloxacin enhances the mutation rates and accelerates adaptation to the environment. Further, among *E. coli* exposure to β-lactams induces the *dpi*BA operon and the *dpi*A effector binds to the chromosomal replication origin and inhibits replication [354]. This

process induces a SOS response which increases genetic variability [354]. Antibiotics can increase reactive oxygen species (ROS) in the bacteria, which can damage proteins, lipids, and DNA and accumulate mutations in the bacteria. However, *Salmonella* rapidly change the outer membrane permeability by opening or closing the OmpA and OmpC proteins to defend against oxidative stress caused by antibiotic treatments [357]. Subinhibitory concentrations of tetracycline do not induce an SOS response in *E. coli* but do induce a response in *Vibrio cholerae* [357].

These previous studies were conducted *in vitro*, where direct effects of ROS were measured. Our *Salmonella* resided naturally in the cattle where various and complex host and environmental factors, such as the presence of background microbiota, could confound the effects of ROS. We also did not follow a single strain of *Salmonella* as in the inoculation studies, and we collected only a single isolate per animal per day, which limits our ability to investigate the mutation rate in this study. In order to further investigate the effects of antibiotic exposures on the mutation rates of *Salmonella* in cattle, we would need to collect multiple isolates per animal per day and explore the genetic differences between isolates before and after treatment.

In this chapter, we explored the genomic characteristics of the isolates derived from this study population. The resistance genotypes matched with the resistance phenotypes. S. Reading was the MDR strain that increased as a result of the antibiotic treatments. Via the hybrid Oxford Nanopore MinION and Illumina assembly, we found the resistance genes were located on an IncA/C2 plasmid. The IncA/C2 plasmid almost completely matched with an *Aeoromonas salmonicida* derived IncA/C2 plasmid, pSN254b. The genomic comparison among the isolates from this study and from publicly available sequences on NCBI showed that the *S.* Reading isolates from this study are clustered differently from those isolated from

turkeys and other sources. The *S.* Reading population was divided into 2 major clusters by core-genome-alignment based SNPs, and the cluster membership of isolates was associated with CTC treatment. The SNPs responsible for the differences in forming these two clusters were located on the *modC* gene. Most of other serotypes were clustered into 3 to 4 groups. *S.* Give was divided into 6 clusters, which was the most diverse serotype in our study. However, in these serotypes the antibiotic treatments were not associated with cluster formation. No temporal relationships were observed among all the isolates within the same serotypes in our studied period. Mutation induced by antibiotics use was not observed in this study; however, antibiotics may be selecting for certain pre-existing populations of resistant *Salmonella*.

CHAPTER VI

SUMMARY, CONCLUSIONS, AND FUTURE DIRECTION

In this research, we evaluated the effects of ceftiofur crystalline-free acid (CCFA) and chlortetracycline (CTC) treatment on *Salmonella* populations in feedlot cattle by examining the prevalence (both overall and multidrug resistant (MDR)), antimicrobial susceptibility, quantification, and genomic comparisons of isolates.

The prevalence of *Salmonella* in the studied population was around 70% at the beginning of the study. The prevalence was suppressed by both CCFA and CTC treatments. Especially evident with the CTC treatment, the prevalence of *Salmonella* decreased but the proportion of MDR *Salmonella* increased. Among 566 isolates, from 1,040 fecal samples, 87 isolates were of MDR *Salmonella*. Among them, 86 MDR *Salmonella* isolates were phenotypically resistant to aminoglycoside, sulfonamides, chloramphenicol, tetracycline, and β-lactams, which were all *S.* Reading. One *S.* Kentucky was also an MDR strain. Most of the phenotypically resistant isolates were carrying corresponding resistance genes (*aadA7*, *strA*, *strB*, *sul1*, *sul2*, *floR*, $bla_{\text{CMY-2}}$, *tetA*). All of the *S.* Reading isolates were detected with an IncA/C2 plasmid and a few harbored an IncI1 plasmid. The resistant genes detected in *S.* Reading were located on the IncA/C2 plasmid. Five other serotypes *S.* Anatum, *S.* Give, *S.* Kentucky, *S.* Mbandaka, *S.* Montevideo also were detected, which were determined by WGS. One to five serotypes were detected per studied animal. However, serotype distribution was not associated with treatment group and day.

Similar to prevalence, quantity of *Salmonella* decreased following the antibiotic treatments. Phenotypically, ceftriaxone and tetracycline resistant *Salmonella* were isolated from Day 0 fecal samples, which illustrated that resistant isolates were present before the

antibiotic treatment started. The quantities of phenotypically ceftriaxone- and tetracycline-resistant isolates were approximately 10^4 in the animals that were detected on Day 0, and remained at the similar level throughout the study period. Together with the prevalence study, we have shown here that antibiotic treatments decrease the number of animals detected with *Salmonella* but increase those detected with MDR *Salmonella*. These results raise a public health concern in that resistant *Salmonella* can possibly enter the slaughterhouse, especially considering the withdrawal period of CCFA is 13 days and CTC is 7 days in feedlot cattle. However, one of the pens in the All-CCFA & CTC treatment group had more animals shedding resistant *Salmonella* constantly, while in the other pens, no animals were shedding over LOQ. This shows that pen level differences in shedding resistant *Salmonella* could be very important.

The genomic study revealed that the serotypes detected in our study cluster with publicly available bovine derived sequences in *S.* Anatum, *S.* Montevideo, *S.* Give, and *S.* Kentucky. *S.* Reading clustered with pork chop- and human-derived isolates. *S.* Mbandaka clustered with a chicken breast-derived isolate. However, the publicly available isolates were limited. Even though 95% of the genomes were shared within the same serotypes, isolates further clustered into 2 to 6 clusters within the same serotype. In *S.* Reading, two major clusters were associated with CTC treatment. The SNPs responsible for the cluster formation in *S.* Reading were located on the *modC* gene, which encodes the molybdenum import ATP-binding protein. Although the correlation between this gene and antibiotic use is not known yet, this result indicates unknown effects of antibiotics on genes unrelated to antibiotic resistance, which may lead to new treatment strategy. Among the other serotypes, no associations between antibiotic treatments and cluster formations were observed.

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