ISOLATION OF *SALMONELLA* FROM FLIES ON CATTLE FARMS AND HORIZONTAL TRANSFER OF INTEGRON-MEDIATED ANTIBIOTIC RESISTANCE GENES ON MICROBIOLOGICAL MEDIA AND SELECTED FARM SAMPLES

by

YUMIN XU

(Under the Direction of Jinru Chen)

ABSTRACT

Flies can be transmission vehicles of *Salmonella* from cattle to human. This study determined the prevalence of *Salmonella* in/on flies captured from 33 dairy/beef cattle farms in Georgia and characterized antibiotic resistance profiles of, and integron structures in, isolated *Salmonella*. *Salmonella* was isolated from 26/33 cattle farms (79%) and 185/1,650 flies (11%). Among 185 selected *Salmonella* isolates, 29% were resistant to ampicillin, 28% to tetracycline, 21% to amoxicillin/clavulanic acid, 20% to cefoxitin, and 12% to streptomycin. Two out of the 185 isolates, 438 and 442 harbored class 1 integrons. Isolate 438 carried a gene cassette of *aadA7* (*ca.* 1.1 kb), and 442 carried *drfA12-orfF-aadA2* (*ca.* 2.0 kb). Both integrons were transferrable through conjugation on tryptic soy agar while the *drfA12-orfF-aadA2* was transferrable on 3/8 farm samples used. Study suggests that flies could be vectors of antibiotic-resistant *Salmonella* on cattle farms to potentially disseminate resistant genes, imposing risks to public health.

INDEX WORDS: antibiotic resistance, cattle, conjugation, integron, *Salmonella*, fly

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DEDICATION

I would like to dedicate this thesis to my loving mom and dad for their unfailing support throughout my study, research and writing. This accomplishment would have not been possible without them.

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CHAPTER 1

INTRODUCTION

BACKGROUND INFORMATION

Foodborne salmonellosis has become a serious threat to global public health. In the United States, the annual incidence of *Salmonella* infection was 15.2 per 100,000 people in the last decade *(6)*. In 2012, the numbers of reported *Salmonella* outbreaks (n=113, 20%), outbreak-associated illness (n=3,394, 28%), and hospitalizations (n=454, 61%) were all ranked the highest compared to the disease outbreaks caused by other foodborne pathogens *(3)*. *Salmonella* isolates responsible for the reported outbreaks have been traced to farm environments, including cattle farms *(5)*. Studies have shown that transmission of *Salmonella* from cattle to human is facilitated by direct contact with contaminated cattle or by consuming water or food contaminated by *Salmonella* from animal sources *(12)*. As one of the major reservoirs of *Salmonella*, cattle may shed 10^2 to 10^7 CFU of *Salmonella* per gram of feces *(10)* and flies that feed on cattle manure have played a critical role in disseminating *Salmonella* in farm environments *(15)*. *Salmonella* may attach to the body, mouth, leg hair, and sticky pad of feet of flies *(16)*. So far, there is no evidence that demonstrates the proliferation of *Salmonella* within flies *(20)*. However, research has shown that *Escherichia coli* is able to multiply on the mouthparts of houseflies *(7, 13)*.

Salmonella isolated from cattle farm-related outbreaks of infection has been confirmed to be antibiotic-resistant *(4, 19, 21)*. One well-known bovine *Salmonella* strain isolated from several outbreaks of human gastrointestinal infection is *S.* Typhimurium DT 104, which was

confirmed to resist multiple antibiotics, including ampicillin, chloramphenicol, tetracycline, streptomycin *(21)*, trimethoprim, and ciprofloxacin *(19)*. Another example is a *S.* Heidelberg strain, recently isolated from a multistate outbreak, which had resistance to amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftriaxone, streptomycin, sulfisoxazole, and tetracycline *(4)*. Extensive use of antibiotics as veterinary medicine and growth promoters during animal production is speculated as one of the contributing factors for the development of antibiotic-resistant strains in farm environments *(11)*. Antibiotic residue in cattle might provide a selective pressure during the invasion of *Salmonella* into the host and only isolates that have acquired antibiotic resistance are able to survive and proliferate.

Horizontal gene transfer is a common approach of acquiring genes encoding antibiotic resistance among bacteria. There are three approaches of horizontal gene transfer, transformation, transduction, and conjugation, among which conjugation is the primary mechanism of antibiotic-resistant gene transfer in living organisms *(17)*. During conjugation, the transfer of mobile genetic elements is mediated by physical contact between donor and recipient cells through bacterial surface appendages such as pili. The efficiency of conjugation depends on the characteristics of transmissible plasmids and the compatibility of the plasmids in donor and recipient cells *(18)*. Other factors, such as environmental temperature and pH, as well as the ratio and density of donor and recipient cells involved, may also affect the efficiency of conjugation *(1)*.

One of the mobile DNA elements that carry antibiotic resistant genes is the integron. Integrons themselves are not mobile, but they are transferable *via* transposon or conjugative plasmid. A integron has a 5'-conserved segment (5'-CS) (including integrase gene *intI*, *attI* site and promoters), 3'-CS segment (including *qacEΔ1* gene and *sul1* gene), and variable gene

cassette in the center of the integron often encoding antibiotic resistance *(9)*. Integrons are classified into three classes, class 1, 2, and 3, and *Salmonella* are often found to carry class 1 integrons *(2, 14)*. Class 1 integrons are frequently associated with Tn*21* and Tn*21*-related transposons that generally locate on conjugative plasmids *(8)*.

Numerous niches in cattle farms could be reservoirs of antibiotic resistant *Salmonella*, but few studies have been performed to determine if flies could be an active carrier of integron-mediated antibiotic-resistant *Salmonella* in farm environments. The objectives of this study were to:

Determine the incidence of *Salmonella* carried by flies in cattle farms and characterize the antibiotic resistance pattern of isolated *Salmonella* strains.

Characterize the integrons of *Salmonella* from flies captured from cattle farms, including the composition of integron gene cassettes and the transferability of integron-mediated antibiotic resistance genes on both microbiological media and cattle farm samples.

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CHAPTER 2

LITERATURE REVIEW

SALMONELLA

General characteristics

Salmonella are rod-shaped, primarily motile, non spore-forming, flagellated, gram-negative facultative anaerobes in the family *Enterobacteriaceae*. They are chemoorganotrophs in the presence of oxygen, which means that they obtain energy through oxidation of electron donors from organic sources. At low oxygen levels, usually during their invasion of hosts, nitrate, nitrite, fumarate, or dimethylsulphoxide are potential terminal electron acceptors *(41)*.

Besides essential substrates for respiration, several other environmental factors affect the growth of *Salmonella*, including temperature, pH, and water activity. Cells of *Salmonella* are able to grow over the temperature range 5 - 47℃ *(50)* while the optimal temperature is 35-43 ℃ *(171)*. It is debatable if *Salmonella* could multiply under refrigeration temperature (4℃) *(115, 139, 141)*. However, it is known that *Salmonella* are usually not heat-resistant and can be killed by pasteurization. The D-values of *Salmonella* are 151.5 to less than 1 min at 50-70℃ while Z-values are typically 5-7℃ *(126, 133)*. *Salmonella* can proliferate in a wide pH range from 3.8 to 9.5 but the optimal pH for their growth is 7-7.5 *(170, 171)*. *Salmonella* are reproducible at water activity above 0.93 with the optimal water activity being 0.99 *(171)*. It has been reported that *Salmonella* could survive at CO_2 levels up to 80% *(14)*. Noticeable, the effect of those factors on growth differs for each *Salmonella* strain and depends on the matrix where *Salmonella* colonize as well.

One of the common matrixes for *Salmonella* colonization is foods. Ingestion of *Salmonella* in contaminated foods might cause illness, depending on personal immune situation. Some populations are particularly at risk, including children, the elderly and people who are immunocompromised or whose gastric acidity is diminished. A high dose of this microorganism is necessary for inducing illness. According to a review by Kothary and Babu *(100)*, the infective dose varies from 10^5 to 10^{10} CFU/mL. One of the factors that affect the infective dose is the fat and protein content of the food vehicle, which might protect *Salmonella* from gastric acidity *(100)*. Symptoms such as diarrhea, fever (usually associated with *S. typhi*), and abdominal cramps *(56)* are frequently developed and last for 2-7 days among people infected by *Salmonella* after an incubation period of 12-72 h. More severely but rarely, *Salmonella* enter the human bloodstream leading to *Salmonella* septicemia of high mortality rate, which has been a problem in Africa *(43)*. A small portion of the population develops chronic disease after the infection, such as arthritis, appendicitis, and meningitis *(56, 85)* and some become long-term carriers of *Salmonella (90)*.

Classification

The genus of *Salmonella* was named in 1900 after Daniel Elmer Salmon, a veterinary pathologist working for the United States Department of Agriculture. *Salmonella* was initially classified based on where a *Salmonella* strain was isolated, for instance, *Salmonella* London and *Salmonella* Indiana *(148)*. However, this soon became problematic in tracking the origin of an outbreak during which specific characteristics of *Salmonella* were required for differentiating the isolates. Thus, other taxonomies that segregate *Salmonella* into species, subspecies, serovars, and subdivisions of serovars have thrived, which will be discussed in detail in the following paragraphs.

Currently, the genus of *Salmonella* has two different species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided into six subspecies, I, II, IIIa, IIIb, IV, and VI, based on their ability to utilize dulcitol, lactose, o-nitrophenol-β-D-galactopyranoside, salicin, D-tartrate, mucate, malonate, gelatinase, sorbitol, and potassium cyanide, etc. *(173)*, as well as their genomic relatedness *(24)*. *Salmonella enterica* subspecies I usually live within warm-blooded animals while the rest are usually isolated from cold-blooded animals and the environment.

Within each subspecies, *Salmonella* could be further separated into different serovars on the basis of their antigenic determinants: flagella (H) antigens, lipopolysaccharide (O) antigens, and, occasionally, virulence (V_i) capsular K antigens *(148)*. This taxonomy is known as Kauffmann-White scheme. The O antigen is differentiated dependent on composition of, and linkage between, sugar molecules. H antigen has two phases, phase 1 and phase 2. Phase 1 is universal among *Salmonella (119)*, while phase 2 is unique to *Salmonella enterica (128)*. Only one of the two phases are expressed at a time in a bacterial cell, *via* a phase variation mechanism *(159)*. The Vi antigen is found exclusively in three *Salmonella* serovars: Typhi, Paratyphi C, and Dublin *(89)*.

Although more than 2,000 serovars have been discovered, some are more pivotal epidemiologically since they are more frequently isolated clinically. Subdivision of those bearing clinical importance is on demand to facilitate investigations of outbreaks. Phenotypic and genotypic typings are commonly used for further classification. One phenotypic typing method is through phage typing, which tests the susceptibility of *Salmonella* to selected bacteriophages *(16)*. This method becomes a reproducible and systematic method, when sets of phages for typing are standardized internationally *(148)*. Other phenotyping methods comprise

biotyping, colicin typing, and resistance typing but they are not frequently applied *(173)*. Genotyping consists of plasmid fingerprinting, multilocus typing, and chromosomal fingerprinting *(173)*.

Pathogenesis

During invasion, *Salmonella* may encounter a series of distinct host environments and host innate immune systems. Successful invasion requires *Salmonella* cells to be able to sense the changes of the surroundings and adapt with responsive programs of gene expression for resisting host environmental stress, such as pH, temperature, nutrient deprivation, and manipulating host immune systems, such as epithelial barriers, phagocytic activity, and antimicrobial peptides.

After entering hosts, one of the significant initial barriers for *Salmonella* is gastric acidity *(137)*. However, *Salmonella* could survive in the acidic environment by promoting the acid-tolerance response *(137)*. After migrating to the small intestine and penetrating the mucosal layer, *Salmonella* produces several fimbriae that provide them the ability to adhere to intestinal epithelial cells *(137)*. Upon adhesion, *Salmonella* could activate its type III secretion system (TTSS) through which effector proteins are secreted to the host to facilitate the invasion of epithelial cells *(137)*. TTSS is a specialized virulence device consisting of a base structure and a needle that translocate effector proteins across the bacterial inner and outer membrane and the host plasma membrane *(87)*. The effector proteins assist *Salmonella* to manipulate the cytoskeleton of hosts leading to membrane ruffling, a primary mechanism for endothelial uptake *(137)*. The induced cytoskeletal rearrangement of host cell disrupts the normal brush border of epithelia and subsequently forms membrane ruffles that reach out to internalize *Salmonella* into

large vesicles *(137)*. *Salmonella* in large vesicles are transported to the basolateral membrane of epithelia while the brush border recovers.

Virulence genes necessary for *Salmonella* invasion of intestinal epithelial cells include *sopE*, *sipA*, *sipC*, and *SptP*, a cluster of genes on *Salmonella* pathogenicity island 1 (SPI-1). During adhesion to epithelia, effector protein SopE acts as a GDP/GTP exchange factor to activate *cdc42* and *rac1*, members of monomeric GTP-binding proteins that play a critical role in regulating the actin cytoskeleton in hosts, which leads to membrane ruffling and *Salmonella* uptake *(75, 78)*. Afterward, effector proteins SipA and SipC cooperate to change cytoskeletal structure. SipC nucleates actin and initiates actin polymerization, while SipA binds to and stabilizes actin filaments and modulates the actin bundling *(96)*. After *Salmonella* invasion, the brush border reconstitutes with the help of effector protein SptP that functions oppositely to SopE. SptP acts as a GDPase-activating protein to inactivate *cdc42* and *rac1 (137)*.

The two major syndromes of *Salmonella* infection are gastroenteritis and typhoid fever. Gastroenteritis is characterized by intestinal inflammation and watery diarrhea by an exudative mechanism. Severe inflammatory lesions result in the loss of permeability of epithelia, and fluids with rich serum proteins are secreted from blood to the intestinal lumen causing watery diarrhea, during which neutrophils are recruited and transmigrated along with the fluids into the lumen *(194)*. *SopA*, *sopB*, and *sopD* clustering on SPI-1 are responsible genes for neutrophil recruitment and fluid secretion *(96)*. It is suggested that SopB aggravates intestinal inflammation and fluid secretion by disturbing inositol phosphate signaling pathways *(137)*. Inositol polyphosphate is critical to the intimate association of plasma membrane and actin cytoskeleton *(96)*.

The syndrome of typhoid fever is a systemic infection caused exclusively by *S. typhi* and *S. paratyphi*. In such a situation, *Salmonella* that have crossed the epithelia enter macrophages by inducing micropinocytosis and are disseminated to other organs of the reticuloendothelial system. In macrophages, *Salmonella* would encounter a series of bactericidal defenses, such as low nutrients, reactive oxygen and nitrogen, and antimicrobial peptides *(96, 137)*. To avoid those bactericidal defenses, some virulence genes are upregulated while some are downregulated in *Salmonella (96, 137)*. This process is regulated by a two component system PhoP/PhoQ, of which PhoQ, a membrane-spinning sensor/kinase, transfers a phosphate to PhoP, the second cytoplasmic component and PhoP serves as a transcriptional regulator *(130)*. Although the exact stimuli in the host environment for the PhoP/PhoQ system are still unclear, simple physical and chemical cues are speculated to be the signals *(137)*. The activated genes by PhoP/PhoQ include a cluster of genes on SPI-2 the effector proteins of which are secreted into the macrophage cytosol, genes to modify the protein and lipopolysaccharide of bacterial membranes to confer resistance to antimicrobial peptides, and genes that assist to combat reactive oxidants *(73, 82)*. The repressed genes include components for constituting SPI-1 TTSS *(144)*.

Bovine *Salmonella*

Salmonella have a broad host range, including livestock, domestic and wild animals. One of its common reservoirs is cattle. Although most of the time bovine *Salmonella* infection is asymptomatic *(177)*, the clinical manifestation of bovine *Salmonella* includes septicemia, acute or chronic enteritis, and abortion *(180)*. From 1966 to 2007, USDA reported that the percent of cattle operations that had at least one animal that was positive for *Salmonella via* fecal culture increased from 20.0% to 30.9% while the percent of cattle positive for *Salmonella* escalated from 5.4% to 13.7% *(177)*. In 2011, FDA reported that among 340 non-typhoid *Salmonella* isolates of

cattle origin, 29.1% was *S*. Montevideo, followed by *S*. Dublin, *S*. Muenster, and *S*. Kentucky, among which *S*. Montevideo and *S*. Kentucky were also found in ground beef *(57)*. However, important clinical serotypes such as *S*. Enteritidis and *S*. Typhimurium were less commonly isolated from cattle *(57)*, although USDA reported that *S*. Typhimurium and *S*. Newport were the most abundant serotypes isolated from clinical cattle *(177)*.

A *Salmonella* outbreak on the cattle farm could last for a long period of time due to the persistence of *Salmonella* in the environment and continuous exposure to the risk factors causing reinfection cycle *(191)*. Drinking contaminated water, consuming contaminated feeds or grazing on contaminated pastures could potentially result in *Salmonella* infection. *Salmonella* found in animal drinking water on cattle farm could come from river, stream, sewage or recycled flushing water that has been used to clean the facility, etc. *(191)*. Although, commercial cattle feeds are usually heated to kill pathogens, *Salmonella* could be introduced into feeds through post-contamination, during mixing and milling *(191)*. *Salmonella* in contaminated pastures often come from *Salmonella*-contaminated soil, sludge and animal carcasses, and *Salmonella* could perpetuate in pasture for almost a year *(191)*. Mice, rats, flies, cats, dogs, and wild birds could also become vectors to transmit *Salmonella*. Even when sick cattle recover from salmonellosis, they might become active carriers of the pathogen, which could be another source of contamination to the farm environments. Especially in the case of *S*. Dublin, a positive carrier of this serotype could shed up to 10^6 CFU/g of feces on a daily basis *(98)*. Some of the chronically infected cattle excrete *Salmonella* in their milk *(161)*, which is a source of infection among calves. *Salmonella* on farm is of clinical importance to human health because people who have contact with contaminated farm environment or infected farm animals could acquire

salmonellosis. In 2015, the FDA listed animal contact as the second most common ways of catching *Salmonella* infection in humans *(31)*.

Additionally, bovine *Salmonella* could be transmitted through contaminated food. Carcasses of infected cattle could contaminate processing environment and other carcasses. Further processing and packaging of beef at wholesale or retail levels could increase the level of contamination *(98)*. This is of particular concern because beef sometimes is consumed raw or in smoked or undercooked, which are not sufficiently treated to kill *Salmonella* that has already colonized the meat ingredient.

Contamination of *Salmonella* in foods

In 2015, 7,719 and 2,104 people were infected and hospitalized, respectively, due to *Salmonella* infections, the number of which topped the other foodborne pathogens *(31)*. *Salmonella* Enteritidis, Newport, and Typhimurium were the top 3 *Salmonella* serotypes that caused outbreaks from 2010 to 2015, and Enteritidis continuously led the list over that period *(31)*. In 2015, the major cause of *Salmonella* outbreaks was foodborne *(31)*.

Private homes followed by restaurants were the most common places for contracting *Salmonella* infections in 2012 *(30)*. The most common food categories associated with *Salmonella* foodborne outbreaks in 2012 were fish, chicken, eggs, fruit, and seeded vegetables *(30)*. Inadequately low storage temperatures in restaurants or at home might allow proliferation of *Salmonella* in raw, contaminated ingredients. Studies have suggested that mishandling of contaminated raw ingredients at food preparation area could cross-contaminate other food ingredients *(34, 48, 102, 103)*. Kusumaningrum et al. *(103)* demonstrated that 1.4% of *Salmonella* on chicken carcasses were transmitted to a stainless steel surfaces when a 150-g piece of raw chicken breast with skin inoculated with 5 ml of approximately 10^6 CFU/mL of

Salmonella suspension was placed on the stainless steel surface for 5 min. In another study, the authors *(102)* found that *Salmonella* could persist on stainless steel surfaces for more than 96 h at an inoculum level of 10^7 CFU/100 cm^2 and less than 24 h at 10^5 CFU/100 cm^2 while less than 1 h at 10^3 CFU/100 cm^2. The authors recovered 34.8% of the *Salmonella* from lettuce that was in contact with the contaminated stainless steel surface for 10 s at 500-g pressure per surface *(103)*. Other routes of cross-contamination in food preparation area have also been studied, *e.g.* from chicken to hand to other ingredients, from chicken to cutting board to other food ingredients, and from chicken to utensils *(34, 48)*. Undercooking the contaminated foods, consuming raw, contaminated foods, and using cross-contaminated utensils are contributing factors for foodborne *Salmonella* outbreaks in humans.

Salmonella are able to colonize food contact surfaces and form biofilm. Compared to single bacterial cells, biofilm-embedded cells are more adaptive to stressful environment and more resistant to disinfectants. Thus, biofilm has become a serious problem of the food industry. For instance, in poultry processing industry, *Salmonella*, brought into a plant through dust, poultry feces, poultry feeds, could persist in the environment and on equipment forming biofilm *(163)*. Marin et al. *(123)* studied 823 *Salmonella* isolated from a poultry plant where 451 (54.8%) *Salmonella* isolates were able to form biofilm. Among the five common materials used in poultry plants, Teflon, stainless steel, glass, Buna-N rubber, and polyurethane, attachment of *Salmonella* to Teflon was significantly higher followed by stainless steel, and glass while the attachment to rubber and plastic was significantly lower *(35)*. Contradicted to this result, other studies found that *Salmonella* attachment to plastic was significantly higher than stainless steel and glass *(2, 164)*.

FLIES IN ASSOCIATION WITH *SALMONELLA*

Flies as carriers of *Salmonella*

Flies are able to transmit pathogens through direct contact with their wings, body, mouth, leg hairs, and sticky pads of feet *(149)* to which *Salmonella* have strong attachment *(86)*. Moreover, the colonization of pathogens in the digestive tract of flies could result in transmission of disease through feces *(71)* and vomitus *(86)*. The latter is considered as a more critical approach to transmit pathogens because 1) pathogens generally survive for a longer period on the internal surface than the external surface *(83)*, and 2) more *Salmonella* colonize internally than externally *(7, 143, 181)*. Almeida et al. *(7)* reported that 4.7% of the flies captured from dairy farms tested positive for *Salmonella* when the external surface of the flies was washed with a sterile saline solution using a vortex machine. However, 9.5% of the flies from the same farms tested positive for *Salmonella* when the internal tissue of surface-disinfected flies were sampled.

E. coli have been confirmed to multiply within the mouthparts of flies *(46, 99)*, but it is debatable whether flies could be a biological carrier of *Salmonella*. As a biological carrier, pathogens multiply inside the host. Greenberg et al. *(71)* fed 22 cells of *S.* Typhimurium to 45 *Musca domestica* L. and found that the number of *S.* Typhimurium in the excreta of 26 flies increased, suggesting that *Salmonella* could multiply within flies. The authors observed that the excretion of *S.* Typhimurium could last for at least 8 days, indicating the persistence of *Salmonella* in the gut of flies *(71)*. However, Greenberg et al. *(71)* pointed that *Salmonella* was primarily present in the mid- and hindgut of flies and did not persist in the crop after a few days, which revealed that the transmission of *Salmonella* through vomit was limited *(71)*. Nevertheless, Hawley et al.*(79)* did not observe the proliferation of *Salmonella* in flies even

when they challenged house flies with 900 cells of *S.* Schottmuelleri. *Salmonella* proliferation in flies depends greatly on fly species, pathogen challenge doses, and gut microbiota of flies *(71)*.

Flies as transmission vehicles of *Salmonella*

Although flies have been known to harbor *Salmonella*, epidemiological studies have been conducted to scrutinize the association between fly population and the morbidity of foodborne disease. This association has been reported historically in military camps, especially during war times *(83)*. A report from the commission conducting the investigation of typhoid fever, often caused by typhoidal *Salmonella*, in several United States military camps during the Spanish War in 1898 described flies swarmed around carelessly disposed filth and kitchens, sinks, tables and food in the camp *(83)*. Niven *(135)* conducted a 5-year epidemiological study on the co-relation between the number of diarrhea cases and the population of flies in which he found the intimate correspondence of the two and came to the conclusion that flies were the transmitting agents for summer diarrhea often caused by foodborne pathogens. Watt and Lindsay *(182)* and Lindsay et al. *(113)* found significantly positive correlations between fly populations and the incidence of salmonellosis in both south Texas and south Georgia before and after the application of pesticide, suggesting that flies were the transmitter of salmonellosis and suppressing the fly population was an effective way to control salmonellosis.

More recently, besides the correlation between fly population and salmonellosis, flies were proved as a transmission vehicle of *Salmonella*. Peppler *(145)* traced a group of flies that were breeding in sewage pond containing *Salmonella* three miles away from a bivouac area and isolated the *Salmonella* from those flies as well as from the foods and field kitchens in that bivouac area 36, 60 and 84 hours after fly emergence. Greenberg et al. *(70)* demonstrated that 7 out of 543 tagged house flies were able to transport *Salmonella* from a slaughterhouse to 3

housing areas of different hygienic levels, a dairy stable, a refuse tip, and an open space used as a dump located 0.1 to 3 miles from the slaughterhouse. Later, Greenberg et al. *(72)* proposed "fly-food-human cycle" as one of the routes for salmonellosis infection in one of their papers. To prove this hypothesis, Greenberg *(67)* exposed 20 sterile flies to dog feces challenged with 105 *Salmonella/*g for 2 h and later, 5 out of the 20 flies that became infected with 43 to 635 *Salmonella* were exposed to 10 milk samples (100 mL) for 100.5 h. They isolated 5,000 to 640,000 *Salmonella* from the milk samples *(67)*. Ten volunteers drank the fly-contaminated samples where the stool samples from 6 out of 10 volunteers tested positive for *Salmonella* during the following 4 days *(67)*.

Although there is lack of documented *Salmonella* outbreaks caused by flies carrying *Salmonella* to human foods *(138)*, the above studies illustrated that flies are capable of transmitting diseases on a large scale due to their indiscriminate travel among filth and foods, and thus, the risk on public health imposed by flies is not negligible.

Bionomic aspect of *Salmonella*-associated flies

Greenberg *(68)* summarized 17 fly species commonly harboring *Salmonella*. They are *Fannia canicularis, Fannia scalaris, Muscina stabulans, Musca domestica, Cochliomya macellaria, Stomoxys calcitrans, Chrysomya megacephala, Chrysomya putoria, Phormia regina, Protophormia terraenovae, Lucilia caesar, Phaenicia sericata, Calliphora vicina, Calliphora vomitoria, Cynomyopsis cadaverina, Sarcophaga carneria* and *Sarcophaga haemorrhoidalis*. *Fannia canicularis* are commonly known as little house flies, *Fannia scalaris* as latrine flies, *Muscina stabulans* as false stable flies, *Musca domestica* as house flies, *Stomoxys calcitrans* as blow flies, *Sarcophaga carneria* and *Sarcophaga haemorrhoidalis* as flesh flies and the rest are commonly known as blow flies. *E. coli* and *Shigella* are frequently isolated from these 17 fly

species and thus, they are characterized as disease-causing flies, flies associated with multiple types of foodborne pathogens, by FDA *(138)*. FDA has further classified them as disease-causing flies that threaten human health since they are synanthropic, that is, living around human residential areas *(138)*. Flies that live remote to human settlement could hardly impose risk to humans. Another criterion to evaluate the medical importance of certain fly species is whether they are attracted by both contaminated source of foodborne pathogens and human foods. Otherwise, they are not likely to transmit foodborne pathogens. Except for *Phormia regina, Protophormia terraenovae, Lucilia caesar*, the remaining 14 species among the 17 disease-causing fly species are all noted to be attracted by human food, excreta, and garbage *(21, 45, 69, 138)*.

Fly control on cattle farms

As one of the major reservoirs of *Salmonella*, cattle may shed 10^2 to 10^7 CFU of *Salmonella*/g of feces *(84)* and flies that feed on cattle manure played a critical role in disseminating *Salmonella* in farm environments and the surrounding residential areas, threatening both the public health and the wellbeing of cattle *(138)*. Therefore, FDA has strongly recommended appropriate preventive and corrective actions for controlling flies on cattle farms *(138)*.

Except for some large operations in the western United States, in most operations in other states cattle are confined for feeding and shelter with limited or no access to pasture *(11)*. This partially confined operation and feedlot operation of high-density management are characterized by fly problems *(11)*. Stalls, feeding areas and drinking areas are ideal locations for fly breeding where large accumulations of manure, spillage of nutrient-rich feeds and ample moisture from urine, drinking water and rain could be found *(11)*. Additionally, the storage area of feeds where

feeds spill around the facilities or proper cover and protection is lacking could also provide suitable habitat for fly breeding *(11)*. These problems could be aggravated on dairy farms since cows are more compacted for efficient and economical milk production. In addition to these locations, milking area is also of great concern for fly breeding *(11)*.

Cultural, biological, and chemical methods are commonly used to control flies on cattle farms. However, reliance on a sole method could be problematic, hence the combination of all three methods is a rational approach to control flies *(13)*. The cultural method involves manipulating the abiotic factors which means environmental factors such as conditions of breeding habitat, temperature and humidity, to reduce the fly population. Although the overall climate, such as the seasonal temperature and humidity of a state, is uncontrollable, the microclimate of the housing system and breeding medium within farms are the major factors for fly control. Preventing moisture is crucial for fly control. During the design of facilities, how efficiently rainwater will be removed from the fly breeding area and how well-designed and -constructed the watering systems are for the animals should be considered; appropriate use of sloped concrete floors with gutters and curbs assists smooth drainage *(13)*. Moreover, ventilation and airflow in stalls could enhance moisture removal *(13)*. Preventing feed spoilage could reduce the heat generated from fermentation that facilitates fly growth; proper protection and coverage of stored feeds could reduce feed spillage and keep feed dry making such environments unfavorable for fly breeding *(13)*. Likewise, proper management of manure, such as removing manure frequently and keeping it dry could also decrease fly breeding. This is usually completed by properly designed and functioning flushing and/or scraping systems. Ways to manage manure disposal includes spreading it immediately on fields in a very uniform thin layer and flushing it into a deep lagoon for anaerobic decomposition *(12)*. Nevertheless, even with proper

design, corners, edges, areas under railings and fences and areas under feed bunkers and drinking tanks could be missed for cleaning and flushing, which allows accumulation of manure and feed waste to retain water, creating ideal spots for fly breeding. Thus, entirely depending on cultural control of flies is insufficient.

Another way to control flies is to retain natural predators feeding on flies and parasites developing in fly pupae to kill flies, namely, the biological method. The primary predators in confined-animal operations consist of beetles (each feeding on 13-24 fly eggs per day) and mites (each feeding on up to 20 eggs per day) while the predominant parasites in confined livestock systems include small wasps *(13)*. The efficiency of this method could be maximized by dry manure since in wet manure, the predators are not able to move about agilely to find fly eggs and larvae and parasites are not capable of penetrating manure to locate pupae *(13)*. Thus, the cultural method which targets keeping manure dry concurrently benefits the predators and parasites to kill flies. However, the predators and parasites are not able to kill adult flies. Therefore, it is not wise to rely solely on biological control of flies.

The third method for fly control is to use insecticides to kill flies. Insecticides could be used as bait mixed with attractive substrates to flies, such as sugar, spoilages or fly sex pheromones which could be placed where flies frequently harbor *(13)*. Alternatively, insecticides could be applied as surface sprays to parts of the facility that are resistant to corrosive chemicals and where flies are of high-density *(13)*. Insecticides could be divided into adulticides and larvicides. The above mentioned two fly control methods are suitable for both adulticides and larvicides. Chemical spray of insecticides, offering effective, quick but temporary reduction in fly populations, could be applied to killing adult flies while, larvicides could also be incorporated into animal feeds. In such cases, larvicides will be excreted along with animal feces that become toxic for

flies to breed on *(13)*. Larvicides are usually combined with adulticides for the maximum insecticidal effect. However, many insecticides with broad spectrum could kill predators and parasites of flies as well. One solution is to apply the selective insecticide, such as cryomazine which is only toxic to fly larvae but not to beetles and mites; or spray insecticides only where flies are abundant but their predators and parasites are not, such as upper parts of the structure, and avoid spraying insecticides on manure *(13)*. Although insecticides are extremely effective against flies, relying exclusively on insecticides could lead to the development of resistance to insecticides among flies, resulting in the failure of insecticides. Additionally, resistance to one chemical is often characterized by cross resistance to other chemicals with similar structure or function *(13)*. To delay or avoid the development of resistance and to achieve the desired fly suppression effect, chemical control should be employed in conjunction with the other two methods.

ANTIBIOTICS AND ANTIBIOTIC RESISTANCE

Overview of antibiotics

Antibiotics are chemicals of low molecular weight produced by microbes or semi-synthesized from natural anti-bacterial products that hampers the growth of other bacteria at low concentration *(106)*. The "low molecular weight" of antibiotics refers to chemical structures with a relative mass of not more than a few thousand while "low concentration" means less than 1 mg/mL *(106)*. Strictly, antibiotics should be microbial metabolites the concept of which should be distinguished from antimicrobial substances which are usually active against different microorganisms including viruses, but nowadays, this term can also be used for anti-bacterial substrates modified from natural antibiotics or other bacterial metabolites *(106)*. Antibiotics are bacteriostatic when they inhibit the growth of bacteria temporarily while they are bactericidal

when the inhibitory effect is permanent *(106)*. At the molecular level, antibiotics disturb necessary metabolic process in bacteria to inhibit their growth. Based on the specific mechanisms of action, antibiotics could be classified into inhibitors of cell wall synthesis, inhibitors of replication or transcription of genetic material, inhibitors of protein synthesis, and inhibitors of cell membrane functions and antimetabolites *(108)*.

Although antibiotics possess heterogeneous chemical structure, 50% of the antibiotics are produced by *Actinomycetales*, particularly *Streptomyces*, only two fungal genera, *Aspergillus* and *Penicillium*, produces highly diverse antibiotics *(105)*. Certain antibiotics are not rigorously associated with certain microorganisms. Namely, the same antibiotics could be produced by different microorganisms while the same microorganisms could produce several different kinds of antibiotics. However, in general, the more distant strains by taxonomical definition are less likely to produce the same antibiotics *(105)*. Antibiotics are usually secondary metabolites of those producers that are biosynthesized by enzymes with relatively lower specificity. Therefore, in some cases, the same substrate could be catalyzed by different enzymes leading to multiple antibiotics with a common basic structure but different accessories, which is named as a family *(107)*. Even though the natural antibiotics have been divergent, the emergence of antibiotic resistant strains is still a problem. This requires chemical modification of natural antibiotics on the structural components that are indirectly involved in antibiotic activity to change the metabolic and pharmacokinetic properties, such as the solubility of the antibiotics, without profoundly altering the intrinsic antibiotic activity *(106)*.

To assess the medical importance of novel antibiotics, the following 3 aspects should be considered. Firstly, the spectrum of individual antibiotics should be examined *(109)*. Secondly, whether the antibiotics are easily absorbed by host should be tested *(109)*. Due to the structural differences in

bacterial cell walls, many antibiotics suppress the growth of gram-positive bacteria of which the cell wall is easy to be penetrated, compared to gram-negative bacteria. Potent antibiotics must be absorbed and directed to the infection sites and retained at effective concentration for an adequate period of time at the infection site *(109)*. It must be excreted from bodies in a rational time to avoid accumulative toxicity *(109)*. Thirdly, the toxicity of the antibiotics should be evaluated *(109)*. At effective dose, antibiotics must exert no intolerable toxicity to hosts with the exception to extremely severe or potentially lethal diseases *(109, 110)*.

Antibiotic use in animal agriculture

Introducing penicillin and sulfa drugs into clinic use in 1930s and 1940s respectively greatly reduced the fatal rate of infectious disease *(37)*. To meet the urgent need for treating bacterial infections in World War II, further development of novel antibiotics was prosperous between 1940 to 1960 *(193)*. During the latter stages of World War II, reconstituted penicillin was commercially available for treating bovine mastitis *(74)*. In 1946, Moore et al. *(132)* discovered that adding streptomycin to the diet of chicks could promote their growth, a prologue to the application of antibiotic in animal production to enhance animal performance. Later, multiple antibiotics were found to be effective for growth promotion on different livestock including swine and cattle *(28, 116)*, and this practice of catering to large-scaled confinement rearing of food animals soon sprang up *(74)*. Today, antibiotics are used in animal agriculture primarily in 4 ways: 1) therapeutic treatment to control occurring infectious diseases, 2) metaphylaxis treatment to treat infected animals and prevent contagion to other animals concurrently, 3) prophylaxis treatment for preventative purpose only and 4) growth promotion *(156)*.

In 2015, approximately 15.58 million kilograms of antibiotics for food-producing animals were sold and distributed in the United States, of which 62% were medically important

antibiotics *(58)*. Those antibiotics consisted of 71% of tetracyclines, 10% of penicillins, 6% of macrolides,4% of sulfonamides, 4% aminoglycosides, 2% of lincosamides, less than 1% of fluoroquinolones, less than 1% of cephalosporins and less than 1% of amphenicols *(58)*. World Health Organization (WHO) has listed penicillin, macrolides, and aminoglycosides as critically important for human medicine because they are the limited available therapies to treat serious human disease that can be acquired from non-human sources, among which macrolides were given the highest priority. WHO has also listed tetracycline as a highly important human antibiotic *(187)*.

In poultry production, bacitracin, bambermycin, chlortetracycline, penicillin, virginiamycin, arsenical compounds are typically used for growth promotion and feed efficiency in egg layers, broilers, and turkeys *(136)*. Antibiotics, such as fluoroquinolones, have replaced older antibiotics, such as tetracycline, for treating *E. coli* infection due to the emerging antibiotic resistance *(127)*. Hatching eggs may also be submerged in gentamicin to reduce mycoplasma and bacterial infection *(127)*. In the swine industry, enteritis caused by *E. coli* and *Clostridium perfringens* is controlled by gentamicin, apramicin, and neomycin while pneumonia is treated by antibiotics such as sulfonamides, tetracycline, ceftiofur, and tiamulin *(52)*. Macrolides and lincomycin are used to treat swine dysentery caused by *Serpulina hyodysenteriae (127)*, monensin, lasalocid, chlortetracycline, chlortetracycline-sulfamethazine, oxytetracycline, and tetracycline are frequently used for prophylaxis and enhance performance in cattle industry *(127)*. The prevailing infectious disease among unweaned heifers are diarrhea and respiratory diseases and 74.5% and 95.4% of affected unweaned heifers were treated with antibiotics for the two diseases, respectively *(176)*. The predominant antibiotics used to treat diarrhea among them are tetracycline, beta-lactam, and sulfonamide, while for respiratory disease, florfenicol,

macrolide, and beta-lactam are used *(176)*. Florfenicol and tetracycline, are applied to treat typical respiratory diseases among weaned heifers *(176)*. On dairy farms, intramammary antibiotics, mostly cephapirin (benzathine) and penicillin G (procaine)/dihydrostreptomycin, are used for treating and preventing infectious diseases during dry-off, a non-lactating period *(176)*.

Human health concern of antibiotics used in animals

Retrospectively, antibiotic residues in meat, milk and eggs were a concern but this has been limited to a tolerable amount by establishing withdrawal periods before slaughter, by prohibiting certain antibiotics in laying hens and by discarding milk produced right after intra mammary infusion of antibiotics *(74)*. Recently, it is of particular concern that the resistant strains compromise the treatment of human infectious disease with antibiotics because infections caused by antibiotic resistant strains often link to prolonged hospital stays, costly treatment, extra doctor visits, and higher mortality and morbidity rates *(25, 80, 81, 179)*. This concern leads to the speculation whether the extensive use of antibiotics on farm is the cause of antibiotic resistance in human pathogens.

Despite growing evidence of the correlation between antibiotic use in animals and resistance in human pathogens *(10, 54, 55, 92, 150, 162)*, very few papers have proved the transmission chain of antibiotic resistance from farm to table. The speculated routes of antibiotic resistance migrating from animal to human include 1) through transfer of antibiotic resistance genes generated in animal strains to human strains and 2) through acquiring resistant pathogens from animal products *(74)*.

Mathew et al. *(125)* verified the transfer of integrons carrying antibiotic resistance genes between *Salmonella* and *E. coli* on farms by typing integron and plasmid profiles of 571 *E. coli* and 98 *Salmonella* isolated from multiple farms in Thailand. Homologous integrons on a

common plasmid was found in both *E. coli* and *Salmonella* isolated from a single swine farm *(125)*. The study suggested the possibility of transmitting antibiotic resistance genes under farm environment, but the potential of the mobile elements to travel all the way to human clinic isolates is still unclear. In terms of the second speculated route as listed above, results from some papers contradicted to this hypothesis. Schmidt et al. *(155)* isolated *Salmonella* from various processing stages of a pork plant where they found *Salmonella* isolates decreased from 91.2% at the initial stage to 3.7% on the chilled final carcass and some of the serovars disappeared at the final sample point, indicating the low transmission rate of the foodborne pathogens originating from animals to the food products, let alone the transmission rate of antibiotic resistant foodborne pathogens. Mather et al. *(124)* compared *Salmonella* DT104 isolates from humans during an outbreak in Scotland to contemporaneous *Salmonella* DT104 isolates from animals by whole genome sequencing. Their results showed that the clade causing human diseases was separated from the clade isolated from animals and even the resistance genes from the two sources were different, indicating the limited transmission of both multidrug-resistant pathogens and their resistant genes from animals to human.

Flies as a link between farms and foods for antibiotic resistance

A new route for transmission of antibiotic resistance from farm to human has been proposed by studying the role of flies. Antibiotics applied on animals are sometimes poorly absorbed and could be excreted in their feces, which are common habitats for fly breeding *(17)*. Consequently, farm flies are highly likely to acquire bacteria with antibiotic resistance traits from animal manure, which has been supported by multiple studies *(3, 6, 19, 66, 114, 154, 178, 181)*. For instance, Literak et al. *(114)* and Rybarikova et al. *(154)* isolated *E. coli* with similar phenotypic and genotypic antibiotic resistance patterns from farm flies and the manure from the

same swine and dairy farm. In a 4-year study, Winpisinger et al. *(189)* demonstrated that the establishment of egg-layer facilities could significantly increase the fly population in its surrounding residential areas up to 6.4 km away from the facilities. Chakrabarti et al. *(32)* suggested that flies on the farms could disperse into city restaurants within approximately 125 km radius of farms. Based on the profile of enterococci including the antibiotic resistance profile of enterococci, the authors suspected that the flies harboring antibiotic resistance traits in the city restaurant of study came from the feedlots and dairy farms in the suburban areas of the city *(32)*, although the deduction is challengeable. In two related studies from this group, they implied the role of flies in contamination of foods served by a restaurant with antibiotic-resistant enterococci *(120, 121)*. The above studies pointed out the potential that flies could travel from farms to residential areas and if the dispersal flies possess antibiotic resistance traits, they could impose risks to human. Nevertheless, more profound research is required to elaborate the role of flies to transmit antibiotic resistance traits from farm to human.

Antibiotic-resistant *Salmonella* isolated from cattle

The extensive use of antibiotics on cattle farms has exerted selective pressure on common foodborne pathogens such as *Salmonella*. In 2011, National Antimicrobial Resistance Monitoring system (NARMS) reported that among 340 cattle *Salmonella* isolates, 30.6% were resistant to tetracycline, 20.0% to sulfisoxazole, 19.4% to streptomycin, 17.9% to chloramphenicol, 17.1% to ampicillin, 14.7% to β-Lactam14.4% to ceftriaxone, 13.8% to cefoxitin and 13.2% to ceftiofur *(57)*. Multi-drug resistant (MDR) isolates, isolates resistant to at least three different antibiotics, were most commonly found in serotypes Dublin, Typhimurium and Newport, of which Typhimurium and Newport were critical serotypes of human clinical isolates *(57)*.

MDR *Salmonella* originated from cattle farms have been involved in outbreaks in the past *(29, 175, 190)*. From 2015 to 2017, a *S.* Heidelberg strain originated from sick calves was involved in a multistate outbreak of infection *(29)*. The *Salmonella* strain was resistant to amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftriaxone, streptomycin, sulfisoxazole, and tetracycline *(29)*.

Another outbreak strain derived from cattle is *S.* Typhimurium DT 104, which was first discovered in the UK in 1984 *(174)* and later recognized globally *(63, 151, 183)*. This strain commonly exhibits resistance to five antimicrobial agents: ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (ACSSuT) *(63, 151)*. However, this resistance pattern has been observed less commonly after 2009 in US *(59)*. *S.* Typhimurium DT 104 has been found to acquire additional resistance to fluoroquinolone and higher-generation of cephalosporins *(95)*, which could be problematic since the two families were recommended to treat the infection caused by this microorganism *(183)*. Genes encoding the MDR, comprised of *aadA2*, *tet(G)*, $bla_{PSE\text{-}1}$ and *sul1* on a class 1 integron, *cmlA* and *floR*, cluster on *Salmonella* genomic island 1 located on the chromosome of *Salmonella* resulting in the stability of those antibiotic resistance genes even when selective pressure is absent *(23, 88, 151)*. The G+C content of these genes vary from that of *Salmonella* chromosome, indicating the acquirement of these genes through horizontal gene transfer *(23)* and this *Salmonella* genomic island 1 could be further transferred to other recipients through conjugation *(88)*.

ACSSuTAuCx (MDR-AmpC) is another typical MDR resistant pattern to cattle sources, with extra resistance to extended spectrum β-lactam *(57, 59)*. However, unlike the ACSSuT pattern, this extremely resistance pattern is characterized by a particular large plasmid, such as

MDR-AmpC IncA/C plasmids harboring resistant genes including *bla(CMY-2)*, *floR*, *aac(3)*,*aadA*, *aphA1, strA, strB, sulI, sulII, dfrA, dhf, tet(A)(B)(C)(D),* and *tetR (65, 160, 195)*.

MOLECULAR MECHANISMS OF ANTIBIOTIC RESISTANCE

Genes encoding antibiotic resistance

Three different mechanisms are known to mediate the resistance of bacteria to aminoglycoside: 1) reducing the uptake of aminoglycoside from the surroundings by limiting cell membrane permeability or activating cell efflux pumps, 2) through a point mutation at a single ribosome binding site of aminoglycoside on the 30S subunit, and 3) through enzymatic modification of aminoglycoside, which is the predominant mechanism responsible for high-level of aminoglycoside-resistance in *Salmonella (61)*. *StrA* and *strB*, encoding aminoglycoside phosphotransferases, and *aad* and *ant*, encoding aminoglycoside nucleotidyl-transferase, are both involved in enzyme modification of aminoglycoside.

The mechanism of β-lactam resistance in *Salmonella* is through hydrolyzation of the β-lactam ring by an enzyme known as β-lactamase *(61)*. Specificity to substrate of β-lactamase determines the spectrum of β-lactam resistance *(112)*. Among *Salmonella* of animal-origin, $bla_{\text{TEM-1}}$ and $bla_{\text{PSE-1}}$ are the most prevalent β-lactamase encoding resistance to the penicillin group of antibiotics, including ampicillin *(65)*. The $bla_{\text{CMY-2}}$ encodes resistance not only to the penicillin group, but also to the second generation of cephalosporin, including cefoxitin and β-lactamase inhibitors such as augmentin (a human medicine equivalent to amoxicillin/clavulanic acid) *(65)*.

Resistance to chloramphenicol is realized through two primary mechanisms: 1) through enzymatic modification by chloramphenicol acetyltransferase encoded by genes, such as *catA* and *catB*, to inactivate chloramphenicol *(140)*, and 2) by efflux pump encoded by genes *cmlA*

*(26)*and *floR (184),* between which *floR* is more frequently associated with *Salmonella* than *cmlA* *(1)*.

Several genes (*tet*, *otr*, and *tcr*) have been confirmed to encode resistance to tetracycline *(153)*. The products of tetracycline resistant genes protect resistant bacterial cells from tetracycline through mechanisms similar to those of aminoglycosides, such as efflux pump *(192)*, ribosomal protection *(153)*, and enzymatic modification of tetracycline in the presence of O_2 and nicotinamide adenine dinucleotide phosphate (NADPH) *(153)*.

Other antibiotic resistance genes include *dfrA* genes encoding trimethoprim-resistance, *ereA* encoding resistance to erythromycin, *qnr* and *qepA* encoding quinolone-resistance and *sat* encoding streptothricin-resistance.

Integrons

One of the mobile DNA elements that carry antibiotic resistant genes is integron. An integron is characterized by an integrase gene (*intI*) and a primary recombination site (*attI*) *(142)*. A typical integron structure contains a 5'-conserved segment (5'-CS) (including integrase gene *intI*, *attI* recombination site and promoters, Pc and P2), 3'-CS (including *qacEΔ1* gene, *sul* gene and *orf*5), and variable gene cassette array in the center of the integron, often encoding antibiotic resistance *(77)*. At 3'-CS, *qacEΔ1*confers resistance to ethidium bromide *(97)* and *sul* to sulfonamides *(62)* while the function of *orf*5 is unknown. However, not all the variable regions of integrons are flanked by a 3'-CS. Sometimes they are flanked by a complete *tni* region *(147)* or no 3'-CS or *tni* region, which could lead to the failure of amplifying the variable region by PCR reaction *(185, 186)*.

Classification of integrons is based on the sequence of integrase genes *(60)*. Integrons carrying *intI1* are categorized as class 1 integrons, *intI2* as class 2 and *intI3* as class 3, and

sequences of the three differ. For instance, both *intI1* and *intI3* encode a fully functioning recombinase while *intI2* is truncated by a stop codon causing it to be dysfunctional *(64)*, while the sequential difference at the recombination site of *intI1* and *intI3* leads to a higher recombination frequency of class 1 integrons than class 3 integrons *(9)*. For these reasons, the gene cassettes and arrays carried by class 1 integrons is more abundant and diverse than the other two classes. *Salmonella* often carry class 1 integrons *(8, 101)*. Class 1 integrons are frequently associated with Tn*402*- and Tn*21*-related transposons that generally locate on conjugative plasmids *(47)*. The association with Tn*402*-like transposon in the evolutionary history of class 1 integron contributes to the wide spread of class 1 integrons, compared to the other classes of integrons *(64)*. Tn*402* is extremely adaptable and specifically translocates into the *res* sites of plasmids. As a result, integrons would be able to harbor various plasmid vectors and would further be disseminated through plasmid transfer among bacteria *(131)*.

Gene cassettes inserted or excised at the variable regions of integrons are compact DNA elements consisting of a single ORF, such as ORF encoding antibiotic resistance, and a recombination site, *attC (76)*. *AttC* is composed of a series of inverted repeats called R″, L″, L′ and R′ in order from the 5′ end to 3′ end that are binding sites for integrase *(91, 166, 167)*, among which only R″ and R′ are highly conserved with DNA sequence 5′-RYYAAC and 5′-GTTRRRY, respectively *(64)*. During insertion, circular gene cassettes are cleaved into single strained DNA between G and T of R′ of *attC* since only the one strain is required for recombination *(22, 91, 118, 166, 167)*. The targeted strain for recombination is marked by a protruding base in L″ of that strain to ensure the correct orientation of the gene cassette during insertion *(22, 27)*. Regardless of the fact that except for R″ and R′, the rest of *attC* sequence lacks homology, *attC* could fold into a highly conserved hairpin structure by pairing R″ with R′ and L″ with L′ *(117,*

167). This secondary structure is crucial for the recognition by integrase and for recombination. Like *attC*, *attI* also has integrase binding sites named as L and R, of which the R site is characterized by the same DNA sequence as that of R′ site in *attC* and a gene cassette is inserted between G and T base *(64).* Although recombination between *attI* and *attC* for insertion and recombination between two *attC* sites of integrons for deletion have preferred tendency *(38, 39, 77)*, insertion between two *attC* sites or recombination between two *attI* sites may happen as well but with much lower efficiency *(38, 77).*

Promoters are absent from gene cassettes and thus, the expression of antibiotic resistance genes is regulated by two external promoters, Pc1 and Pc2, located on *intI1* and *attI1* respectively *(40).* This is a beneficial system since the newly acquired gene cassettes that locate closest to the *intI* and *attI* could be expressed right after insertion, conferring advantageous phenotypes*(64).* The strength of promoters of integrons has been extensively studied *(42, 64, 94).* Integrons with weaker promoters often experience higher excision rate, suggesting the unstableness of the gene array of the integrons *(94)*; the strength of expression declines in positive relation to the distance of gene cassettes to promoters *(42, 64)*, which might be the reason why class 1 integrons barely possess more than six cassettes *(64).* With those exceptionally long gene arrays of integrons, such as those observed in *Vibrio*, internal promoters may be identified for further expression of distal cassettes *(129).* Internal promoters have been described in a few antibiotic resistant gene cassettes, such as *cmlA* conferring resistance to chloramphenicol *(18, 165)* and *qnrVC* family encoding resistance to quinolone *(44).*

Other mobile elements

Integrons themselves are not mobile, but they are transferrable *via* transposons or conjugative plasmids. Transposons are DNA sequences that can translocate themselves from one

position to another within a genome mediated by recombinase enzymes transposases and often, the dispersal of transposons is not at the same position. They do not possess self-replication systems and must integrate into chromosomes or plasmids for replication. An insertion sequence is small DNA encoding only a transposases and a regulatory protein for transposition activity which is flanked by inverted repeat termini. Insertion sequences may occur as parts of composite transposons, *e.g.* Tn*10*, in a structure that has two insertion sequences flank one or more accessory genes, such as antibiotic resistance genes *(33, 111)*, but in a unit transposon, such as Tn*A*. Instead of insertion sequences, it has its own transposase and resolvase for excision and integration function *(49, 51)*. Plasmids are small circular double-stranded DNA separated from chromosomal DNA in bacteria that can replicate independently and control their copy numbers. Conjugative plasmids possess *tra* gene complex that allows them to develop necessary conjugal apparatus to mediate their transfer *(157)*. Conjugative plasmids can carry non-conjugative transposons and integrons *(158)* while non-conjugative small plasmids can utilize the conjugative apparatus that is encoded by co-existing conjugative plasmids to transfer as long as they possess *ori*T or *mob* genes *(157)*.

Horizontal gene transfer

Integrons, plasmids and transposons are the most important mobile elements in horizontal dissemination of antibiotic resistance traits *(156)*. There are three approaches of horizontal gene transfer: transformation, transduction, and conjugation. Transformation means the bacterial cells directly uptake the exogenous genetic materials from its environment and incorporate the genetic materials into their genomes *(93)*. After the uptake, the new genes must develop a replication-proficient form, in order to persist in the new host *(172)*. However, natural transformation happens when bacteria are in a competence state, such as starvation and high cell-density, and

thus, natural transformation plays limited role in disseminating antibiotic resistance genes *(15, 93, 172)*. In transduction, the foreign resistant genes are injected from bacteriophage to bacterial cells. Although, the size of DNA that could be transferred through transduction is limited and the process needs matches between phage types and bacteria serotypes, phage transduction of antibiotic resistance genes has been observed previously *(20, 122, 188)*. Transduction of chloramphenicol resistance was observed in *E. coli* inside the gut of house fly *(146)*.

During conjugation, the transfer of mobile genetic elements, including conjugative plasmids and chromosomally integrated conjugative elements and conjugative transposons, is mediated by the physical contact between donor and recipient cells through bacterial surface appendages such as pili. Conjugation is the primary mechanism of antibiotic-resistant gene transfer in living organisms *(157)*. Transfer of antibiotic resistance genes through conjugation have also been observed in flies. Petridis et al. *(146)* observed that transfer of a plasmid containing chloramphenicol resistant genes between *E. coli* strains in house fly gut and fly crop occurred at a frequency of 10^{-2} per donor cell in 1 h post-feeding. Akhtar and Zurek *(4)* demonstrated that plasmid pCF10, embedded with a tetracycline resistance gene, could be transferred through conjugation between *Enterococcus faecalis* strains in house fly mouth parts and digestive tract at a conjugation efficiency of 8.5×10^{-5} to 4.5×10^{1}.

Since integrons are immobile themselves, transfer of integrons depends on conjugative plasmid or transposons where they locate. Therefore, transfer of integrons is often associated with co-transfer of antibiotic resistance genes that are not encoded by integrons but are embedded on the same mobile elements as integrons. Szmolka et al. *(169)* observed the co-transfer of *tet(A)* and *catA1* on a IncI1 plasmid encoding resistance to tetracycline and chloramphenicol, respectively, along with a class 1 integron containing an *aadA* gene located on

the same plasmid. The most frequently co-transferred antibiotic resistance gene with integron is tetracycline *(36, 134, 168, 169)*, which is not surprising since tetracycline resistance genes have been extensively found on mobile DNA elements, such as transposons, integrative and conjugative elements, and transferrable SGN1 *(53, 152)*.

In a designed experiment, the methodology used in the conjugation experiment could influence the efficiency of conjugation. Lampkowska et al. *(104)* found that the population of transconjugants derived from two *Lactococcus lactis* strains on GM17 agar could be 4-log higher than that in GM17 broth under the same incubation condition because compared to liquids, the solid materials offer limited space for cell movement which increases the interaction among bacterial cells. Other factors, such as the characteristics of transmissible plasmids, the compatibility of the plasmids in donor and recipient cells, temperatures, pH, as well as ratio and density of donor and recipient cells involved may also affect the efficiency of conjugation *(5, 172)*.

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CHAPTER 3

SALMONELLA, INCLUDING ANTIBIOTIC-RESISTANCE *SALMONELLA*, ISOLATED FROM FLIES ON CATTLE FARMS IN GEORGIA, U.S.A.[1]

[1] Xu, Y., S. Tao, and J. Chen. To be submitted to *Journal of Food Protection*.

ABSTRACT

Flies can be transmission vehicles of *Salmonella* from cattle to humans. This study determined the prevalence of *Salmonella* in/on flies captured from 33 different cattle farms including 5 beef farms and 28 dairy farms in Georgia, USA, and characterized antibiotic resistance profiles of the isolated *Salmonella* strains. *Salmonella* were isolated from 26 out of the 33 cattle farms (79%) and 185 out of the 1,650 flies (11%) in the study. The incidence of *Salmonella*-positive flies varied from farm to farm ranging from 0 to 78%. Among the 185 *Salmonella* strains isolated from flies, 29% were resistant to ampicillin, 28% to tetracycline, 21% to amoxicillin/clavulanic acid, 20% to cefoxitin, and 12% to streptomycin. Incidences of resistance against other tested antibiotics were low, ranging from 0 to 3%. Furthermore, 28% of the *Salmonella* isolates were multidrug resistant, demonstrating resistance to 3 or more antibiotics. The minimal inhibitory concentrations of ampicillin, cefoxitin, streptomycin, and tetracycline against the *Salmonella* isolates ranged from 32 to >2,048, 64 to 2,048, 128 to 1,024, and 32 to 1,024 µg/mL, respectively. These data suggest that flies could be transmission vehicles of antibiotic resistant *Salmonella* on cattle farms to potentially disseminate resistance genes, imposing risks to public health.

INTRODUCTION

Foodborne salmonellosis has become a serious threat to global public health. In the United States, the annual incidence of *Salmonella* infection was 15.2 per 100,000 people in the last decade *(2)*. In 2012, the numbers of reported *Salmonella* outbreaks (n=113, 20%), outbreak-associated illness (n=3,394, 28%), and hospitalizations (n=454, 61%) were all ranked the highest comparing to the disease outbreaks caused by other foodborne pathogens *(3)*. *Salmonella* isolates responsible for the reported outbreaks have been traced to farm environment, including cattle farms *(5)*. Studies have shown that transmission of *Salmonella* from cattle to human is facilitated by direct contact with contaminated cattle or by consuming water or food contaminated by *Salmonella* from animal sources *(21)*. As one of the major reservoirs of *Salmonella*, cattle may shed 10^2 to 10^7 CFU of *Salmonella* per gram of feces *(19)* and flies that feed on cattle manure played a critical role in disseminating *Salmonella* in farm environments *(28)*. *Salmonella* may attach to the body, mouth, leg hair, and sticky pad of feet of flies *(30)*. So far, there is no evidence that demonstrates the proliferation of *Salmonella* within flies *(39)*. However, research has shown that *Escherichia coli* is able to multiply on the mouthparts of houseflies *(10, 22)*.

Salmonella isolated from cattle farm-related outbreaks of infection has been shown to carry antibiotic resistance genes *(4, 36, 41)*. One well-known bovine *Salmonella* strain isolated from several outbreaks of human gastrointestinal infection is *S.* Typhimurium DT 104, which was confirmed to resist multiple antibiotics, including ampicillin, chloramphenicol, tetracycline, streptomycin *(41)*, trimethoprim, and ciprofloxacin *(36)*. Another example is a *S.* Heidelberg strain, recently isolated from a multistate outbreak, which had resistance to amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftriaxone, streptomycin, sulfisoxazole, and tetracycline

(4). Since both of these isolates originated from cattle farms, misuse of antibiotics during cattle production was speculated to have exerted a selective pressure for the development of antibiotic resistant *Salmonella* strains *(41)*.

Numerous niches in cattle farms could be the reservoir of antibiotic resistant *Salmonella*, but few studies have been performed to determine if flies could be active carriers of antibiotic resistant *Salmonella* in farm environments. The objectives of this study were to determine the incidence of *Salmonella* carried by flies on cattle farms and to characterize the antibiotic resistance pattern of isolated *Salmonella* strains.

MATERIALS AND METHODS

Sample collection

Flies were captured, using Revenge sticky tapes (Bonide Products Inc., Oriskany, NY; 2-3 m) , from 5 beef farms and 28 dairy farms in Georgia, U.S.A. which were designated randomly by two-lettered codes, from June to September, 2016. Farms AA, AB, BD, BE, and BF were pasture beef farms and the rest were dairy farms. Approximately 3-5 pieces of sticky tapes (~3 m per piece) were set up on each farm. Each piece of tape was knotted to structures approximately 1.7 m above the ground. The sticky tapes were placed where flies populated, such as barns, storage rooms, and milking parlors. After approximately 50 flies landed within 10-60 min, the sticky tapes were cut into short segments (5-30 cm) and taped onto a piece of cardboard using vinyl tape (3M, St. Paul, MN) before being transported to our laboratory in an electric car cooler (Igloo Products Corp., Katy, TX). The 50 flies captured from each farm were placed individually, using sterile forceps, into a Whirl-Pak sample bag (Nasco, Fort Atkinson, WI), containing 1 mL of 0.2% peptone water (Becton Dickinson, Sparks, MD). The flies in sample bags were crushed using a pestle to expose their inner tissues to the peptone water. Each sample

was mixed with an equal volume of 30% glycerol, and 0.5 mL of sample was stored at 4 ℃ and analyzed within 48 h while the remaining 0.5 mL was stored at -18 ℃.

Salmonella isolation and identification

Salmonella was isolated from collected flies by following the protocols outlined in Bacteriological Analytical Manual (BAM) *(14)* and the U.S. Department of Agriculture (USDA) Microbiology Laboratory Guidebook *(11)* with some modifications. For pre-enrichment, 0.5 mL of fly tissue samples described above was added to 4.5 mL of buffered peptone water, and the mixture was incubated at 37 ℃ for 18 h. Thereafter, 0.1 mL of the pre-enriched sample was added to 10 mL of Rappaport-Vassiliadis (RV) broth, while another 1 mL of the same sample was transferred to 10 mL of tetrathionate (TT) broth. Both broth cultures were incubated at 42 ℃ for 24 h. Following the incubation, a loopful of RV and TT enrichment broth culture was streaked separately onto bismuth sulfite agar and xylose-lysine-tergitol 4 plates, which were incubated at 37 ℃ for 24 h. Presumptive *Salmonella* colonies from each plate were sub-cultured onto MacConkey agar plates, from which the presumptive colonies were transferred onto tryptic soy agar plates and incubated at 37 ℃ overnight. The resulting cultures were confirmed biochemically on triple sugar iron agar slants and serologically using *Salmonella* Poly A-I and Vi antiserum. All the reagents and media were purchased from Becton Dickinson (Sparks, MD).

Antibiotic susceptibility testing

One isolate from each *Salmonella*-positive fly was selected for antibiotic susceptibility testing, using the disc diffusion assay *(7)*. After the *Salmonella* cultures diluted to McFarland 0.5 were inoculated onto the plates with sterile cotton swabs, a total of 12 antibiotic disks, including those of amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, streptomycin, sulfisoxazole,

tetracycline, and trimethoprim (Oxoid, UK), were placed on 3 different Mueller-Hinton agar 2 plates (Sigma-Aldrich, St. Louis, MO) using sterile forceps. The agar plates were incubated at 37 ℃ for 18 h, and the diameters of zones of inhibition around the discs were measured and compared against the recommendations of Clinical and Laboratory Standards Institute (CLSI) to classify the strains as resistant, intermediate, or sensitive to a specific antibiotic *(9)*.

Minimal inhibitory concentrations (MICs)

Based on results of the disc diffusion assay, *Salmonella* strains having resistance to ampicillin, cefoxitin, and tetracycline (Sigma-Aldrich, St. Louis, MO), as well as streptomycin (MP Biochemicals, Solon, Ohio), were selected to determine the MIC of the 4 antibiotics following the protocols described by CLSI *(8)*. The antibiotics were diluted in two-fold series in Mueller Hinton broth (Sigma-Aldrich, St. Louis, MO), after being dissolved in appropriate solvents. The lowest concentration that did not have visible turbidity was regarded as the MICs. The breakpoints for classifying resistance of MICs were based on the CLSI and FDA standards *(9, 13)*.

RESULTS

Salmonella isolation

Among the 33 cattle farms surveyed in the study, flies captured from 26 farms (79%) carried *Salmonella* (Detailed data not shown). In total, *Salmonella* was isolated from 185 out of the 1,650 (11%) captured flies. The prevalence of *Salmonella* carriage by collected flies on individual farms ranged from 0 to 78% with an average prevalence of 11% (Fig. 3.1). The top three incidences of *Salmonella* carriage by flies were 78% (39 out of 50 flies), 52% (26 out of 50 flies), and 30% (15 out of 50 flies).

Antibiotic susceptibility

Among the 26 farms where *Salmonella* was detected, those from 21 farms (81%) had resistance to at least one antibiotic tested in the study (Table 3.1). Among the 185 antibiotic resistant *Salmonella* strains selected for antibiotic resistance testing, the most common resistance was to ampicillin (53 out of 185, 29%), followed by tetracycline (51 out of 185, 28%), amoxicillin/clavulanic acid (38 out of 185, 21%), cefoxitin (37 out of 185, 20%), and streptomycin (23 out of 185, 12%), while resistance against sulfisoxazole, chloramphenicol, trimethoprim, ciprofloxacin, and ceftriaxone were less common, in a range of 0.5 to 3% (Table 3.1). No isolates were resistant to gentamicin or nalidixic acid (Table 3.1). Resistance to amoxicillin/clavulanic acid, ampicillin, cefoxitin, streptomycin, and tetracycline was observed in *Salmonella* isolated from multiple farms. On each farm, 0 to 100% of isolates were resistant to ampicillin; 0 to 42% of isolates to amoxicillin/clavulanic acid and cefoxitin; 0 to 82% of isolates to tetracycline; and 0 to 51% of isolates to streptomycin (Table 3.1).

Among the 185 representative *Salmonella*, 42 isolates were resistant to 3 antibiotics, 26 isolates to a single antibiotic, 7 isolates to 2 antibiotics, and 5 isolates to 4 antibiotics (Table 3.2). The resistance to 5, 6, 8, and 9 antibiotics was observed in 1 isolate for each category. No isolate was resistant to 7 or more than 9 antibiotics. In total, 52 out of the 185 isolates (28%) were resistant to at least 3 antibiotics, which were multi-drug resistant isolates according to the definition set by National Antimicrobial Resistance Monitoring System (NARMS) *(13)*.

Minimum Inhibitory Concentrations

Results of disc diffusion assays showed that resistance to amoxicillin/clavulanic acid, ampicillin, cefoxitin, streptomycin, and tetracycline was predominant among isolated antibiotic resistant *Salmonella* strains (Table 3.1). Since amoxicillin/clavulanic acid is a combined

antibiotic, and the product for laboratory research use is commercially unavailable, only ampicillin, cefoxitin, streptomycin, and tetracycline were selected for the MIC determination. The MICs of ampicillin, cefoxitin, streptomycin, and tetracycline ranged from 32 to >2,048, 64 to 2,048, 128 to 1,024, and 32 to 1,024 μg/mL, respectively (Table 3.3).

DISCUSSION

Salmonella isolation from flies

Although commercial sticky tapes are commonly used for fly control on cattle farms, few research studies have used them for fly capture *(26)*. The most commonly used capture devices were sterile sweep nets *(25, 27, 38)* and baited fly traps *(1)*. Both sweep nets and baited traps share the common concern of potential cross-contamination among flies through external interaction, vomit drops, or defecation *(34)*, since flies are allowed to move freely in the devices after capture. On the contrary, sticky tapes restrict fly movement upon capture, which reduces the possibility of cross-contamination among captured flies.

Almeida et al. *(1)* reported that 4.7% of the flies captured from dairy farms tested positive for *Salmonella* when the external surface of the flies was washed with a sterile saline solution using a vortex machine. However, 9.5% of the flies from the same farms tested positive for *Salmonella* when the internal tissues of surface-disinfected flies were sampled. Although the efficiency of the washing in removing pathogen cells from the surface of flies is challengeable, it is commonly acknowledged that *Salmonella* colonized flies internally more than externally *(1, 29, 38)*. Since the organisms maybe internalized or externalized, the present study was designed to isolate *Salmonella* from both external surface and internal tissues of captured flies.

Results of the present study showed that flies from 79% of surveyed farms carried *Salmonella*, which was higher than what was reported in similar previous studies *(1, 25)*. For

instance, a study in California showed that flies from 67% of cattle farms carried *Salmonella* *(25)*. However, the sample size of this study was rather small and only 3 farms were surveyed. The only relevant study with a reasonable sample size that we could find took place in Brazil where flies from 14% of 30 cattle farms carried *Salmonella (1)*. However, the authors of the study left the fly trap on farms for 24 h before transporting captured flies to laboratory for analysis and the flies were killed by placing them at -12°C for 30 min. These handling practices might have adversely impacted the recovery rate of *Salmonella* from the flies.

Data from a single-farm study in Egypt *(26)*, Australia *(38)*, and Nigeria *(27)* revealed that 6.7% of 120 flies, 63% of 86 flies, and 28.4% of 223 flies carried *Salmonella*, respectively, different from the incidence of 11% reported in the present study. Since the incidence rate of *Salmonella*-positive flies varies drastically from farm to farm, the incidence rates reported in these studies might not well represent the situation in surveyed areas. In a study by Mian et al. *(25)*, 0.5% of 1,172 flies from 3 dairy farms tested positive for *Salmonella*, lower than the incidence rate reported in the present study. The flies were, however, stored at -51°C overnight before *Salmonella* were isolated *(25)*, the influence of which on *Salmonella* recovery is not known.

Flies from cattle farms could potentially serve as a vehicle for *Salmonella* transmission to foods. For instance, fly-borne *Salmonella* could contaminate cattle, and subsequently beef or beef products, if necessary precaution and sanitation protocols are not followed. On dairy farms, flies around milking parlors could transmit *Salmonella* into milk *(1)*. Alternatively, *Salmonella*-positive flies could disperse into households around farms contaminating cooked food in kitchens *(1, 20)*. Thus, fly control on cattle farms is critically important for preventing the

dissemination of fly-borne *Salmonella*. Improving environmental sanitation and hygiene might be a cost-efficient and sustainable method for fly control *(33)*.

Antimicrobial susceptibility

A Brazilian research team tested antibiotic resistance of 8 *Salmonella* strains isolated from flies of cattle origin *(1)*. Similar to results reported in the present study, the authors found that the incidences of resistance to ampicillin (3/8, 38%) and tetracycline (8/8, 100%) were high and none of the isolates had resistance against ceftriaxone *(1)*. The reported incidences of gentamicin and ciprofloxacin resistance (2/8, 25%) were higher than what was reported in the present study (Table 3.1). A Nigerian study also reported a high incidence of resistance to gentamicin (24% of 219 isolates), while the incidence of resistance to tetracycline was relatively low (16% of 219 isolates) compared to the results of the current study (Table 3.1) *(27)*. Cattle farms in various geographical areas must have different production and management practices, which might have contributed to the varied incidences of resistance to antibiotics in the cited studies.

Literak et al. *(24)* tested the effect of 12 different antibiotics against 198 *E. coli* isolates from piglets in a swine farm, along with 216 *E. coli* isolates from flies captured from the same farm, and a similar antibiotic-resistance profile was observed in the *E. coli* strains from both reservoirs. According to a report of NARMS *(13)*, the largest number of isolates among the 340 non-typhoidal *Salmonella* strains isolated from cattle had resistance to tetracycline and the incidences of resistance to amoxicillin/clavulanic acid, ampicillin, cefoxitin, and streptomycin were also high. These findings are in agreement with the results reported in the present study. Also similar to the finding of our study, the incidences of resistance to ciprofloxacin, gentamicin, nalidixic acid, and trimethoprim reported by NARMS were relatively lower.

The most common MDR pattern observed in our study is ampicillin-amoxicillin/clavulanic acid-cefoxitin (AAuFox) (Table 3.2). All three antibiotics in this pattern belong to β-lactam class, which is extensively used to treat navel infection in un-weaned heifers *(37)*. The mechanism of β-lactam resistance in *Salmonella* is through hydrolyzing the β-lactam ring by an enzyme known as β-lactamase *(17)*. Specificity to substrate of β-lactamase determines the spectrum of β-lactam resistance *(23)*. Among *Salmonella* of animal-origin, $bla_{\text{TEM-1}}$ and $bla_{\text{PSE-1}}$ are the most prevalent β-lactamase encoding resistance to the penicillin group of antibiotics, including ampicillin *(18)*. The $bla_{\text{CMY-2}}$ encodes resistance not only to the penicillin group, but also to the second generation of cephalosporins, including cefoxitin and β-lactamase inhibitors such as augmentin (a human medicine equivalent to amoxicillin/clavulanic acid) *(18)*. Both the occurrence of a single *bla* gene with broad spectrum and multiple *bla* genes with narrow spectrum could lead to the MDR pattern AAuFox.

Part of the resistance pattern of one MDR isolate obtained from farm DD in the present study included resistance to ampicillin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline (ACSSuT) (Table 3.2). ACSSuT is a pivotal MDR pattern of *Salmonella* isolated from cattle farms, indicative of a classical *Salmonella* genomic island 1 (SGN1) derived from *S.* Typhimurium DT104 *(13)*. However, this pattern has decreased sharply after 2009 *(16)*, which might explain why this pattern was not commonly distributed among *Salmonella* isolates in the present study. In addition to ACSSuT, ACSSuTAuCx (MDR-AmpC), with additional resistance to extended spectrum β-lactam, is another typical MDR pattern from cattle sources *(13, 16)*. This pattern was part of the resistance pattern of one isolate from farm CC in this study (Table 3.2).

The high incidence of tetracycline resistance observed in the present study (Table 3.2) might be associated with the fact that tetracycline is frequently used in food-producing animals in the United States *(15)*. Tetracycline is the primary antibiotic for treating diarrhea in un-weaned heifers, and it is also used for treating respiratory diseases in weaned heifers and lameness in cows *(37)*. Several genes (*tet*, *otr*, and *tcr*) have been confirmed to encode resistance to tetracycline *(32)*. The products of tetracycline resistance genes protect resistant bacterial cells from tetracycline through mechanisms such as energy-dependent efflux against the concentration gradient of tetracycline *(42)*, protection of bacterial ribosomes from binding with tetracycline *(32)*, and enzymatic modification of tetracycline in the presence of O_2 and nicotinamide adenine dinucleotide phosphate (NADPH) *(32)*. Tetracycline resistance genes have been found on mobile DNA elements, such as transposons, integrative and conjugative elements, and transferrable SGN1 *(12, 31)*, which might explain the wide distribution of these genes and high incidence of bacterial resistance to tetracycline.

Unlike tetracycline, aminoglycosides are not commonly used on cattle farms in the United States. However, resistance against streptomycin was still relatively high in the present study (Table 3.2). Three different mechanisms are known to mediate bacterial resistance to streptomycin: 1) to reduce the uptake of streptomycin from the surroundings by limiting cell membrane permeability or activating cell efflux pumps, 2) through a point mutation at a single ribosome binding site of streptomycin on the 30S subunit, and 3) through enzymatic modification of streptomycin, which is the predominant mechanism responsible for high-level streptomycin-resistance in *Salmonella (17)*. *StrA* and *strB*, encoding aminoglycoside phosphotransferases and *aad* and *ant*, encoding aminoglycoside nucleotidyl-transferase are both involved in enzyme modification of streptomycin. Interestingly, all *Salmonella* isolated in our

study that were resistant to streptomycin had co-resistance to tetracycline (Table 3.2), an observation which has been reported previously *(6, 31, 35)*. Genes encoding resistance for both antibiotics were previously found on the same mobile DNA elements *(6, 31, 35)*, which probably caused the dissemination of the two genes concurrently even when only selective pressure for tetracycline resistance existed. This might also explain why streptomycin resistance is common among the isolates of the present study.

Some of the antibiotics to which the *Salmonella* strains isolated from flies of cattle farm origin in the present study were resistant included amoxicillin/clavulanic acid, ampicillin, cefoxitin, streptomycin, and tetracycline, all of which are important medicines for treating serious infections in humans *(40)*. On farms with poor sanitation and management, populated flies can serve as vectors to disseminate antibiotic-resistant *Salmonella* and antibiotic-resistance genes. The development of increasing numbers of antibiotic resistant bacterial strains could potentially reduce or diminish the efficacies of antibiotic therapy in human medicine, imposing risks to public health.

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TABLE 3.1. *Number of antibiotic-resistant* Salmonella *among selected* Salmonella *isolates (1 isolate per* Salmonella*-positive fly) against 12 different antibiotics*

farms	n[a]	No. of isolates[b]											
		Au	A	Fox	Cx	C	Cip	G	N	Su	S	T	W
AA	2	2	2	2							1	1	
BD	1	1	1	1									
BE	5	5	5	5								2	
BF	2	2	2	2								1	
BG	8	1	1	1									
BH	26	2	2	2									
CA	5												
CB	3	1	1	1								1	
CC	39	3	21	2	1	1				2	20	24	1
CD	2											1	
CE	3	1	1	1									
CF	15											1	
DB	3	2		2								1	
DC	11	1	1	1								9	
DD	4	4	4	4		1	1			2	2	2	1
DE	2	2	2	2									
EA	11	1	1	1						1		8	
EB	12												
EC	2												
FA	5	2	2	2									
FC	4	1	1	1									
FD	2												
FE	4	2	1	2									
GA	1												
GC	5	3	3	3									
GD	8	2	2	2									
total	185	38	53	37	1	2	1			5	23	51	2

[a] number of isolates chosen per farm.

[b] Au, amoxicillin/clavulanic acid; A, ampicillin; Fox, cefoxitin; Cx, ceftriaxone; C, chloramphenicol; Cip, ciprofloxacin; G, Gentamicin; N, Nalidixic acid; S, streptomycin; Su, sulfisoxazole; T, tetracycline; W, trimethoprim

TABLE 3.2. *Number of antibiotic(s) resisted by* Salmonella *isolated from flies captured on Georgia cattle farms and their resistance patterns*

Farm	Number of isolates resistant to different numbers of antibiotic(s)									Resistant patterns[a]
	1	2	3	4	5	6	7	8	9	
AA			1		1					AAuFox, ASTAuFox
BD			1							AAuFox
BE			3	2						AAuFox, ATAuFox
BF			1	1						AAuFox, ATAuFox
BG			1							AAuFox
BH			2							AAuFox
CB	1		1							T, AAuFox
CC	6	3	16	2				1		A, T, ST, AAu, AST, AAuFox, ASTW, ASSuT, ACSSuTAuCxFox
CD	1									T
CE			1							AAuFox
CF	1									T
DB	1	2								T, AuFox
DC	9		1							T, AAuFox
DD			2			1			1	T, ASSuTAuFox, ACSSuTAuFoxCipW
DE			2							AAuFox
EA	7	1	1							T, ST, AAuFox
FA			2							AAuFox
FC			1							AAuFox
FE		1	1							AuFox, AAuFox
GC			3							AAuFox
GD			2							AAuFox
Total	26	7	42	5	1	1	0	1	1	

[a] Au, amoxicillin/clavulanic acid; A, ampicillin; Fox, cefoxitin; Cx, ceftriaxone; C, chloramphenicol; Cip, ciprofloxacin; S, streptomycin; Su, sulfisoxazole; T, tetracycline; W, trimethoprim.

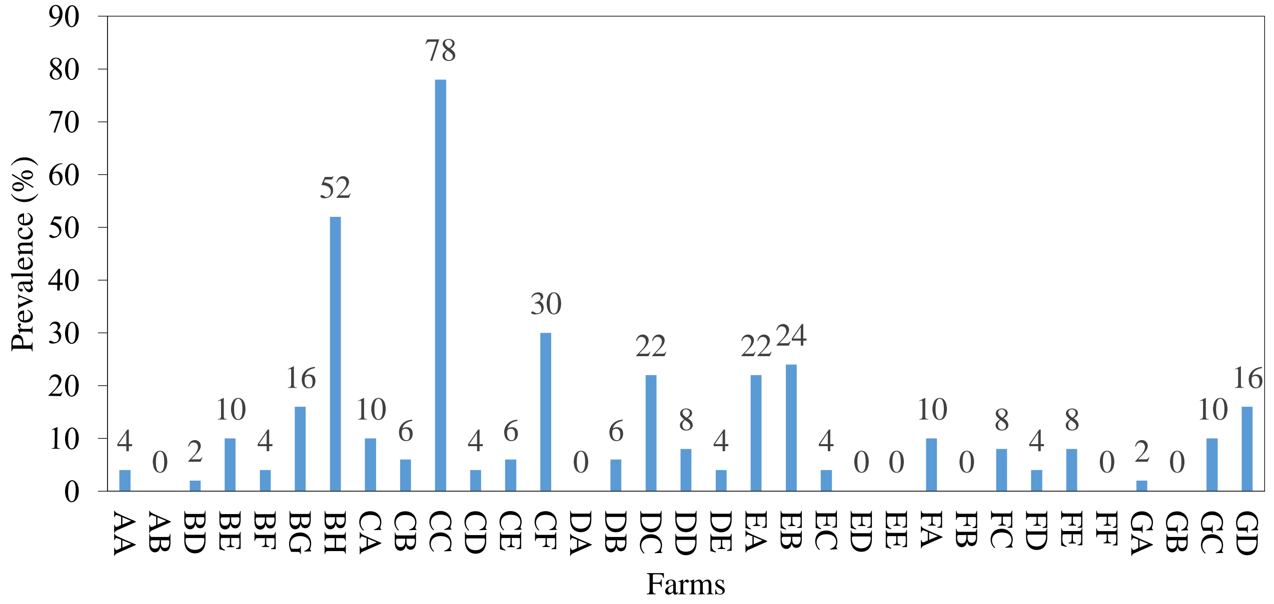
TABLE 3.3. *MICs of four different antibiotics against the* Salmonella *isolated from flies captured on Georgia cattle farms*

Farm	n[a]	Distribution of MICs to A (µg/mL)[b]								Distribution of MICs to Fox (µg/mL)						Distribution of MICs to S (µg/mL)				Distribution of MICs to T (µg/mL)					
		32	64	128	256	512	1024	2048	>2048	64	128	256	512	1024	2048	128	256	512	1024	32	64	128	256	512	1024
AA	2					1		1					2			1									1
BD	1			1								1													
BE	5				2	3					1	4								2					
BF	2					1	1					1		1											1
BG	8					1							1												
BH	26					1	1					2													
CB	3						1								1									1	
CC	39			1	2				18			2					2	8	10			1	13	11	
CD	2																					1			
CE	3					1						1													
CF	15																				1				
DB	3										1		1									1			
DC	11					1								1										3	6
DD	4				2	1	1			1			2	1			1		1						2
DE	2							2				1		1											
EA	11				1									1								5	1		2
FA	5			2								2													
FC	4				1								1												
FE	4				1							1		1											
GC	5		2	1								3													
GD	8	1				1								1	1										
total	185	1	2	5	9	11	4	3	18	1	2	18	7	7	2	1	3	8	11	2	1	8	14	15	12

[a] number of *Salmonella* iselected from each farm.

[b] A, ampicillin; Fox, cefoxitin; S, streptomycin; Su, sulfisoxazole; T, tetracycline.

FIGURE 3.1. *Prevalence of* Salmonella*-positive flies among the 50 flies captured on each Georgia cattle farm that were designated randomly by two-lettered codes.*



CHAPTER 4

TRANSFER OF CLASS 1 INTEGRON-MEDIATED ANTIBIOTIC RESISTANCE GENES FROM *SALMONELLA* OF FLY ORIGIN TO SUSCEPTIBLE *E. COLI* AND *SALMONELLA* STRAINS [1]

[1] Xu, Y. and J. Chen. To be submitted to *Journal of Food Protection*.

ABSTRACT

Integrons in pathogens such as *Salmonella* are critical genetic elements carrying antibiotic-resistance genes that could potentially be disseminated through horizontal gene transfer, imposing risks to public health. This study was undertaken to determine the structure of integrons carried by *Salmonella* isolated from flies on cattle farms and to examine the transferability of the integrons through conjugation. Results showed that 2 out of 606 (0.3%) isolated *Salmonella*, *Salmonella* 438 and 442, harbored class 1 integrons. The integron gene cassette in *Salmonella* 438 carried a single gene of *aadA7* (*ca.* 1.1 kb), and that in *Salmonella* 442 carried *drfA12-orfF-aadA2* (*ca.* 2.0 kb). The two integrons were transferrable through conjugation on microbiological media to the cells of a *Salmonella* strain isolated from fly and *E. coli* C600 at efficiencies ranging from 1.47×10^{-6} to 4.25×10^{-5}. However, only *Salmonella* 442 was able to transfer its integron to the recipient cells on 3 out of the 8 farm samples used in the study, namely cattle hair, bedding sand, and drinking water, with conjugation efficiencies ranging from 4.26×10^{-10} to 1.36×10^{-8}. Transconjugants were confirmed to harbor integron gene cassettes of similar sizes to those in the corresponding donor strains and expressed antibiotic resistance encoded by integron-mediated resistance genes. Antibiotic-resistance genes that are not carried by integrons, *e.g.* genes encoding resistance to tetracycline and chloramphenicol, were co-transferred with integron-mediated antibiotic resistance genes. These data suggest that *Salmonella* isolated from flies collected from cattle operations could carry integrons which could disseminate antibiotic resistance genes through horizontal gene transfer in farm environments.

INTRODUCTION

Salmonella were responsible for a large number of foodborne outbreaks in the United States during the period of 1998 to 2015 *(5)*. *Salmonella* isolates that are responsible for the outbreaks have sometimes been traced to farm environments, including cattle farms *(6)*. Many of these outbreak strains are resistant to antibiotics, resulting in increasing hospitalization and prolonged hospital stays *(39)*. Extensive use of antibiotics as veterinary medicine and growth promoters during animal production is speculated as one of the contributing factors for the development of antibiotic-resistant strains in farm environments *(19)*. Antibiotic residues in cattle might provide a selective pressure during the invasion of *Salmonella* into the hosts because only isolates that have acquired antibiotic resistance are able to survive and proliferate.

Horizontal gene transfer is a common approach of bacteria acquiring genes encoding antibiotic resistance. There are three approaches of horizontal gene transfer, transformation, transduction, and conjugation, among which conjugation is the primary mechanism of antibiotic-resistant gene transfer in living organisms *(33)*. During conjugation, the transfer of mobile genetic elements is mediated by physical contact between donor and recipient cells through bacterial surface appendages such as pili. The efficiency of conjugation depends on the characteristics of transmissible plasmids and the compatibility of the plasmids in donor and recipient cells *(37)*. Other factors, such as environmental temperatures and pHs, as well as ratio and density of donor and recipient cells involved, may also affect the efficiency of conjugation *(3)*.

One of the mobile DNA elements that carries antibiotic resistant genes is the integron. Integrons themselves are not mobile, but they are transferable *via* transposons or conjugative plasmids. An integron has a 5'-conserved segment (5'-CS) (including integrase gene *intI*, *attI*

recombination site and promoters), 3'-CS (including *qacEΔ1* gene and *sul1* gene), and variable gene cassette array in the center of the integron, often encoding antibiotic resistance *(15)*. Integrons are classified into three classes, class 1, 2, and 3, and *Salmonella* are often found to carry class 1 integrons *(4, 20)*. Class 1 integrons are frequently associated with Tn*21* and Tn*21*-related transposons that generally locate on conjugative plasmids *(11)*.

Several studies have identified integrons in *Salmonella* isolated from cattle farms *(1, 4, 36)*, yet little is known about integrons of *Salmonella* isolated from flies captured on cattle farms. The purpose of this study is to characterize the integrons of *Salmonella* from flies captured from cattle farms, including the composition of integron gene cassettes and the transferability of integron-mediated antibiotic resistance genes on both microbiological media and cattle farm samples.

MATERIALS AND METHODS

Bacterial strains and farm samples

In total, 606 *Salmonella* strains previously isolated from flies on 33 cattle farms (Xu et al., unpublished) were screened for integrons. Integron-positive strains were used as donor strains in the conjugation experiments. Nalidixic-acid-resistant *Salmonella* 439 previously isolated from a cattle-farm flies and *E. coli* C600 from our laboratory culture collections were selected as recipient strains. All strains were cultured on tryptic soy agar (TSA) (Becton, Dickinson and Co., Sparks, MD) plates before a single colony was transferred into tryptic soy broth (TSB) (Becton, Dickinson and Co., Sparks, MD). All the bacterial cultures were incubated at 37℃ for 18 h.

Milk powder for calves, feeds for calves and cows of high and low productivity, drinking water, tail hair, bedding sand, and bovine feces collected from the dairy farm on the University of Georgia Tifton Campus were used as matrices for the conjugation experiments. Powdered

milk was reconstituted based on manufacturer's instructions. Reconstituted milk and other farm samples were placed into 500 mL glass bottles and autoclaved at 121℃ for 15 min. The water activity of each sterilized sample was adjusted to the values before the samples were autoclaved.

Detection of integrase genes and gene cassettes

Presence of integrons in isolated *Salmonella* were detected using PCR and hep35 and hep36 primers (Table 4.1) targeting the conserved regions of integrase genes *intI1, intI2,* and *intI3 (41)*. Cassette regions of class 1 integrons were amplified with primers hep58 and hep59 whereas primers hep54 and hep71 were used for the amplification of gene cassette in class 2 integrons (Table 4.1) *(41)*. For all PCR amplifications, template was prepared by heating overnight cell cultures of *Salmonella*, which was pre-washed twice with sterile distilled water, in 100℃ water bath for 10 min. After the heated samples were cooled on ice for 10 min, supernatant containing crude DNA was obtained by centrifugation at 13,800 × *g* for 10 min using a bench top centrifuge (Brinkmann Instruments Inc., Westbury, NY). PCR amplifications were conducted in a 25 μL-reaction mix including 5 μL of template, 0.5 U *Taq* DNA polymerase, 0.2 mM dNTP, 1.5 mM $MgCl_2$, and 9.5 μL distilled water. All reagents, except distilled water, were purchased from Thermo Fisher Scientific (Waltham, MA). PCR was performed for 35 cycles, each of which was composed of 94℃ for 2 min, 55℃ for 1 min, and 72℃ for 1 min, and the final extension was at 72℃ for 10 min.

DNA sequencing and analysis of integron gene cassettes

The amplicons of class 1 integron gene cassettes were submitted to Eurofins Genomics, a Eurofins MWG Operon company (Louisville, KY) for purification using Exonuclease I and Shrimp Alkaline Phosphate and sequencing using the cycle sequencing technology (dideoxy chain termination/cycle sequencing) on a ABI 3730XL sequencing machine. The sequencing

results were compared against those in the NCBI database at https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome.

Conjugation on TSA

Both recipient and donor cells were diluted to 10^8 CFU/mL with TSB and equal volume (100 µL) of each diluted donor and recipient was mixed. Each donor and recipient mixture was pipetted onto the center of a TSA plate which was incubated at 37℃ for 18 h. Recipient cells in appropriate dilution were inoculated onto TSA plates and incubated at 37℃ for 18 h to determine the total number of recipient cells used in the experiment. Following incubation, the donor-recipient mixture on TSA plates was transferred to an Eppendorf tube with 1 mL of phosphate-buffered saline (PBS) (Becton, Dickinson and Co., Sparks, MD). After vortex, the mixture in PBS was centrifuged at 12,000 × *g* for 10 min. The supernatant was discarded and cell pellet was re-suspended into 1 mL of PBS. Transconjugants from the experiment using *Salmonella* 438 as the donor strain were selected on TSA supplemented with nalidixic acid (200 µg/mL) and streptomycin (50 µg/mL) and those from experiment using *Salmonella* 442 as the donor were screened on TSA supplemented with nalidixic acid (200 µg/mL) and streptomycin (50 µg/mL) as well as TSA supplemented with nalidixic acid (200 µg/mL) and trimethoprim (50 µg/mL). All the selective plates were incubated at 37℃ for 24 h. Conjugation efficiency was calculated as the number of transconjugants divided by the total number of recipients used in the experiment.

Conjugation efficiency on farm samples

Equal volume (5 mL) of donor and recipient overnight cultures in TSB were mixed and the resulting mixture was centrifuged at 12,000 × g for 10 min. Cell pellet obtained was re-

suspended in 100 µL PBS. The donor and recipient mixture in PBS (100 µL) was inoculated into 1 mL cattle drinking water or reconstituted powered milk in 15 mL Falcon tubes (Becton, Dickinson and Co., Sparks, MD). The same amount of donor and recipient cells were also inoculated onto solid cattle farm samples (1 g), including 3 types of feeds, fecal materials, bedding sand, and tail hair, in sterile petri dishes. The samples were incubated at 37℃ for 48 h. Following incubation, 3 mL of PBS were added to the solid farm samples on petri dishes to collect the conjugant mixtures. Collected conjugation mixtures along with the solid samples were transferred into Falcon centrifuge tubes. The solid samples and powdered milk in Falcon tubes were centrifuged at 700 × g for 10 min to precipitate undesired solids in the samples. The supernatant of those samples, along with the conjugation mixture in drinking water samples, were centrifuged at 12,000 × *g* for 10 min. Harvested pellets were re-suspended in 100 µL of PBS, which were streak-inoculated onto TSA supplemented with nalidixic acid (200 µg/mL) and streptomycin (50 µg/mL). All the plates were incubated at 37℃ for 24 h. Conjugation efficiency was calculated as described above.

Analysis of transconjugants

The integrase and gene cassettes on the integrons of transconjugants were screened by PCR using the conditions and primers described above. Antibiotic resistance profiles of the transconjugants and their parent strains were compared using the disc diffusion assays based on standard protocols provided by Clinical and Laboratory Standards Institute (CLSI) *(8)*. Twelve antibiotic disks, including those of amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim (Oxoid, UK) were placed on 3 separate Muellller-Hinton agar II (Sigma-Aldrich, St. Louis, MO) plates, which were incubated at 37℃ for 18 h. The

measurement of zones of inhibition around the discs were compared against the recommendations of Clinical and Laboratory Standards Institute (CLSI) to classify the strains as resistant, intermediate or sensitive *(9)*.

RESULTS

Integron

Two out of the total of 606 isolates (0.3%) tested positive for *intl* (Fig. 4.1A). Class 1 integrons were found in 2 *Salmonella* isolates, 438 and 442, in a single farm. *Salmonella* 438 carried an approximately 1.1-kb class 1 integron gene cassette, whereas *Salmonella* 442 carried a *ca*. 2.0-kb class 1 integron gene cassette (Fig. 4.1B).

DNA sequencing results indicated that the class 1 integron gene cassette in *Salmonella* 438 carried the gene for aminoglycoside adenylyltransferase (*aadA7*) (GenBank accession no. AF224733.1) conferring resistance to streptomycin. The gene cassette array in *Salmonella* 442 contained three genes, dihydrofolate reductase (*dfrA12*), open reading frame F (*orfF*), and aminoglycoside adenylyltransferase (*aadA2*) (GenBank accession no. HQ840942.1), conferring resistance to trimethoprim and streptomycin to the *Salmonella* isolate.

Conjugation on TSA

Transfer of the class 1 integrons from the 2 donors to *Salmonella* 439 was observed when TSA supplemented with nalidixic acid/streptomycin was used as a selective medium (Table 4.2). However, when TSA supplemented with nalidixic acid/trimethoprim was used, no transconjugants were recovered from the conjugation between 442 and 439 (Table 4.2). The efficiencies of conjugation between *Salmonella* 438 and *Salmonella* 439 or *E. coli* C600 were similar (4.25×10^{-5} and 1.24×10^{-5} respectively) (Table 4.2). The transfer of antibiotic

resistance genes was more frequent from *Salmonella* 442 to *E. coli* C600 (1.44×10^{-5} and 2.35×10^{-5}) than to *Salmonella* 439 (1.47×10^{-6} and $< 6.43 \times 10^{-8}$) (Table 4.2).

Conjugation on farm samples

Neither *Salmonella* 438 nor 442 were able to transfer class 1 integrons to their recipient cells on feces, feeds for cows of high or low productivity, feeds for calf, and reconstituted milk (Table 4.3). However, transconjugants were recovered from the conjugation between *Salmonella* 442 and *E. coli* C600 (6.38×10^{-9}) in drinking water and between *Salmonella* 442 and each of the two recipients on tail hair (3.25×10^{-9} and 1.36×10^{-8} respectively) and bedding sand (5.00×10^{-10} and 4.26×10^{-10} respectively) (Table 4.3). Similar results were not observed with the transfer of class 1 integron of *Salmonella* 438. Moreover, the efficiencies of antibiotic resistance gene transfer on the 3 farm samples were much lower (*ca*. 10^{-10} to 10^{-9}) than those on TSA (*ca*. 10^{-6} to 10^{-5}).

Analysis of transconjugants

Integrase was detected from all the transconjugants recovered from the study. The size of class 1 integron gene cassette in the transconjugants was similar to that in their corresponding donor strains (Fig. 4.1C). The antibiotic resistance profiles revealed that in addition to the antibiotic resistance encoded by integrons, some of the transconjugants were also resistant to other antibiotics that were characteristics of their donors but were not encoded by integrons (Table 4.4). For the transconjugants derived from *Salmonella* 438 and 439 or *E. coli* C600, the resistance genes of tetracycline were transferred along with the integron (Table 4.4). In transconjugants derived from *Salmonella* 442 and 439 conjugation, genes encoding resistance to tetracycline, chloramphenicol, and ciprofloxacin were co-transferred with the class 1 integron (Table 4.4). For transconjugants derived from the conjugation between *Salmonella* 442 and *E.*

coli C600, genes encoding resistance to amoxicillin/clavulanic acid, ampicillin, cefoxitin, chloramphenicol, ciprofloxacin and tetracycline were transferred together with the class 1 integron (Table 4.4).

DISCUSSION

Integrons and gene cassettes on integrons

Results of the present study indicated that flies on cattle farms carried *Salmonella* that were positive for integrons. Few researches have been performed on the carriage of integrons by *Salmonella* originated from farm flies. Previous studies have, however, found that *E. coli* isolated from farm flies possessed integrons. Rybarikova et al. *(32)* isolated 147 *E. coli* from 240 flies on a dairy farm, among which 18 (12%) were integron-positive. Literak et al. *(22)* found that 11 out of the 216 *E. coli* (5.1%) isolated from 236 flies on a swine farm carried integrons. These incidences are much higher than the incidence of 0.3% reported in the present study. It is uncertain whether this is due to the fact that strains of different bacterial species were used in the two studies.

Only class 1 integrons were found in this study, suggesting that class 1 integrons were more prevalent than class 2 integrons among the *Salmonella* isolates originated from cattle source. This finding is in agreement with what was reported in several previous studies *(1, 4, 36)*. Tabe et al. *(36)* reported that 29 out of 58 *Salmonella* from a feedlot cattle farm in North Dakota possessed class 1 integrons and only 2 isolates carried class 2 integrons. Ahmed et al. *(1)* reported that 28 out of 40 *Salmonella* of cattle source in Japan had integrons while none of the isolates carried class 2 integrons. It is known that the integrase of class 2 integrons is dysfunctional due to an internal stop codon, limiting their ability to acquire and rearrange new gene cassettes, which exerts constraints on the spectrum and evolution of class 2 integrons *(14)*.

Both gene cassettes identified in this study contained *aadA*, suggesting the predominance of *aadA* among class 1 integrons. This finding concurs with results of Antunes et al. *(4)* who found that 7 out of 8 different gene cassette arrays of class 1 integrons from 154 *Salmonella* isolates contained *aadA*. While acknowledging that the extensive use of streptomycin- and spectinomycin-related veterinary medicine might have contributed to the observed phenomenon, the authors believed that the structural association of *aadA* with other genes, such as *sul1,* the selective pressure of which were more frequently present in the environment, probably promoted the conservation of *aadA (4)*.

AadA encodes the aminoglycoside adenylyltransferase family that protects bacterial cells from spectinomycin and streptomycin through enzymatic modification of the antibiotics *(29)*. Although *aadA1* and *aadA2* are more commonly reported in the literatures *(29)*, *aadA7* was detected in one of the *Salmonella* isolates used in this study. Integrons carrying *aadA7* gene cassette are mostly seen in clinical *Salmonella* isolates *(7, 10)*, as well as isolates of some other sources, such as swine *(31)*. Molla et al. found this gene cassette in *Salmonella* originated from slaughtered cattle in Ethiopia *(25)*. The gene cassette has also been identified in other microorganisms, such as *E. coli*, isolated from cattle source *(2)*.

A common gene array of class 1 integron from *Salmonella* of cattle origin *(12, 42)*, *drfA12-orfF-aadA2* was also detected in this study. The *drfA12-orfF-aadA2* array has also been observed in *Salmonella* from other sources, including human *(13)* and swine *(18)*. Partridge et al. *(29)* suggested that the broad distribution of this array might be related to successful mobile elements, such as transposons, associated with this cassette array.

Transfer of antibiotic-resistant genes

Both class 1 integrons identified in this study are transferrable on TSA by conjugation (Table 4.2). Siqueira et al. *(34)* successfully transferred *aadA7* from an *E. coli* isolate to *E. coli* TOP10 with a conjugation efficiency of 8.5×10^{-6} while Yu et al. *(43)* demonstrated that the efficiency of *drfA12-orfF-aadA2* transfer from an *E. coli* donor to *E. coli* J53 was 6.1×10^{-6}, similar to the results of the present study. It has been reported that *AadA7* and *drfA12-orfF-aadA2* are located on transferrable plasmids *(16, 40)*.

From the same conjugation mix, a higher number of transconjugants carrying the *drfA12-orfF-aadA2* gene cassette were detected when TSA supplemented with nalidixic acid/streptomycin was used as a selective medium, compared to TSA supplemented with nalidixic acid/trimethoprim (Table 4.2). Perez-Valdespino et al. *(30)* observed that the transcription of integron was elevated 7 times when plasmid-borne *drfA12-orfF-aadA2* in an *Aeromonas* strain was under the selective pressure of streptomycin but similar induction was not observed under the selective pressure of trimethoprim.

Conjugation on some of the farm samples was successful in the present study (Table 4.3), suggesting that horizontal transfer of antibiotic resistance genes within and cross bacterial species in farm environment is possible. Mathew et al. *(24)* verified the transfer of integrons between *Salmonella* and *E. coli* on farms by typing integron and plasmid profiles of 571 *E. coli* and 98 *Salmonella* isolated from multiple farms in Thailand. Homologous integrons on a common plasmid was found in both *E. coli* and *Salmonella* isolated from a single swine farm *(24)*. It was observed in the present study that the efficiencies of conjugation taking place on farms samples were much lower than that on TSA (Table 4.3), an observation which has also been made by Nagachinta and Chen *(26, 27)*. TSA is a nutrient-rich medium to support the

growth of both donor and recipient cells while farm samples, such as bedding sand and feeds, were complex matrixes that limited cell movement, as well as the accessibility of nutrients and water, which are critical for bacterial conjugation *(26)*. Additionally, the nutrients of TSA benefit the formation of F pili, a pivotal structure for conjugation *(26)*, and possibly promote the reproduction of transconjugants.

Conjugation was unsuccessful on feeds for cows and calves or reconstituted milk for calves (Table 4.3). Possible presence of antimicrobials in these materials might have prohibited the growth of recipients and/or donors. For instance, lincosamides, an antibiotic often added to cow feeds for treating mastitis *(38)* and tetracycline, commonly used to treat diarrhea of calves *(38)*, might be present in the feed and powdered milk samples. Both of the antibiotics are stable at high temperature *(17)*, residues of which might have adversely impacted the viability of donor and/or recipient cells in this study. Furthermore, the low pH of feeds made from corn silage (pH *ca*. 3.5) could also affect the fate of bacterial cells and conjugation efficiency *(26, 28)*. Unlike the results of the present study, Nagachinta and Chen *(26)* reported that the efficiency of conjugation between two *E. coli* strains on bovine fecal materials ranged from 10^{-8} to 10^{-6}. The two studies used different bacterial strains as donors and recipients. It is possible that the fecal samples used in the two studies had different chemical compositions.

The efficiency of successful conjugation varied on different farm samples used in the study (Table 4.3). Higher efficiencies were associated with solid (bedding sands and tail hair) than liquid (drinking water) samples. Lampkowska et al. *(21)* found that the population of transconjugants derived from two *Lactococcus lactis* strains on GM17 agar could be 4-log higher than that in GM17 broth under the same incubation condition. The authors believed that,

compared to liquids, the solid materials offer limited space for cell movement which increases the interaction among bacterial cells *(21)*.

Analysis of transconjugants

Results of PCR analysis on transconjugants suggested that all the transconjugants obtained the entire gene cassettes from their donors (Fig. 4.1C). Martinez-Freijo et al. *(23)* also observed that the *aadA* gene cassette and *drfA-aadA* gene array were often transferred as part of the complete integron rather than as individual genes. Partridge et al. *(29)* confirmed the stableness of *drfA12-orfF-aadA2* through horizontal transfer when they noticed *drfA12* almost always dispersed as part of *drfA12-orfF-aadA2* array.

The antibiotic-resistant profiles of transconjugants suggested that the genes encoding resistance other than those encoded by integrons were transferred along with the integrons (Table 4.4). This is likely due to the co-transfer of integrons and integron-bearing mobile DNA elements during conjugation. Szmolka et al. *(35)* observed the co-transfer of *tet(A)* and *catA1* on a IncI1 plasmid encoding resistance to tetracycline and chloramphenicol, respectively, along with a class 1 integron containing an *aadA* gene located on the same plasmid.

Unlike *Salmonella* 442, *Salmonella* 438 failed to co-transfer β-lactam (amoxicillin/clavulanic acid, ampicillin and cefoxitin) resistant genes (*bla*) along with the integron into recipient cells (Table 4.4). Although the exact reasons for this phenomenon is unclear, Siqueira et al. *(34)* observed that in multi-plasmid isolates, *bla* genes are sometimes located on a small plasmid which is different from the large integron-borne plasmid and is often non-conjugative. In such a case, *bla* genes might not co-transfer with the integrons. It is not clear whether this is what happened in the situation of *Salmonella* 438.

This study showed that *Salmonella* isolates from flies of cattle source could carry integrons encoding genes for antibiotic resistance. The integrons can be co-transferred with other antibiotic-resistant genes within and cross bacterial species through conjugation on some of the farm samples used in the study. Possible dissemination of antibiotic-resistant genes through horizontal gene transfer in farm environment could cause the emergence of antibiotic resistant bacterial strains and failure of antibiotic treatment of infectious diseases.

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TABLE 4.1. *Oligonucleotide primers used in PCR amplification of integrase gene and resistance gene cassette*

Primer	Sequences[a]	Target genes	Size of amplicons	References
Hep35	TGCGGGTYAARGATBTKGATTT	integrase genes *intI1*, *intI2*, and *intI3* (conserved region of integron)	491 bp	
Hep36	CARCACATGCGTRTARAT			
Hep58	TCATGGCTTGTTATGACTGT	Variable region of class 1 integron	varied	*(27, 41)*
Hep59	GTAGGGCTTATTATGCACGC			
Hep 51	GATGCCATCGCAAGTACGAG	Variable region of class 2 integron	varied	
Hep 74	CGGGATCCCGGACGGCATGCACGATTTGTA			

[a] B indicates C, G, or T; K indicates G or T; R indicates A or G; Y indicates C or T.

TABLE 4.2. *Efficiency of transfer of integron-mediated antibiotic resistance genes on tryptic soy agar plates*

Donors	Recipients	Selective media[a]	Conjugation efficiency
Salmonella 438	*Salmonella* 439	TSA+ N+ S	4.25×10^{-5}
Salmonella 438	*E. coli* C600	TSA+ N+ S	1.24×10^{-5}
Salmonella 442	*Salmonella* 439	TSA+ N+ S	1.47×10^{-6}
Salmonella 442	*Salmonella* 439	TSA+ N+ W	$< 6.43 \times 10^{-8}$
Salmonella 442	*E. coli* C600	TSA+ N+ S	1.44×10^{-5}
Salmonella 442	*E. coli* C600	TSA+ N+ W	2.35×10^{-5}

[a] TSA+ N+ S, TSA supplemented with 200µg/ml nalidixic acid and 50µg/ml streptomycin; TSA+ N+ W, TSA supplemented with 200µg/ml nalidixic acid and 50µg/ml trimethoprim

TABLE 4.3. *Conjugation efficiency of integron-mediated antibiotic resistance genes on farm samples selected on tryptic soy agar supplemented with 200µg/ml of nalidixic acid and 50µg/ml of streptomycin*

Farm samples	Donors	Recipients	Conjugation efficiency[a]
Tail hair	*Salmonella* 442	*Salmonella* 439	3.25×10^{-9}
		E. coli C600	1.36×10^{-8}
Bedding sand	*Salmonella* 442	*Salmonella* 439	5.00×10^{-10}
		E. coli C600	4.26×10^{-10}
Drinking water	*Salmonella* 442	*E. coli* C600	6.38×10^{-9}

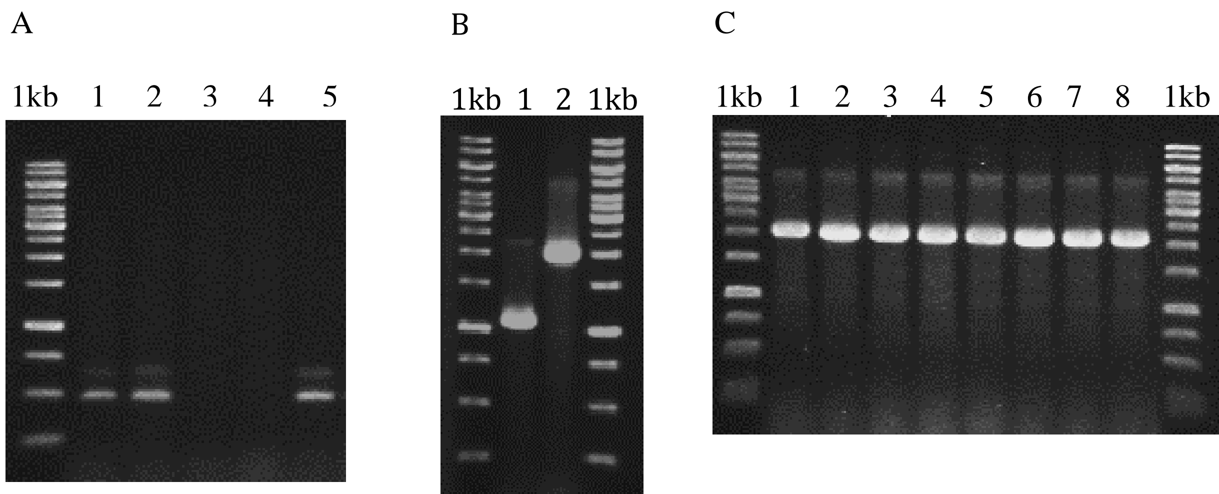
[a] detect limit of conjugation efficiency when *Salmonella* 439 was used as a recipient was 2.50×10^{-10} and when *E. coli* C600 was used as a recipient was 4.26×10^{-10}

TABLE 4.4. *Antibiotic-resistant profile of the donors, the recipients and the transconjugants*

Donors[a]	Recipients[a]	Matrixes[b]	Selective media[c]	Antibiotics[d]											
				Au	A	Fox	Cx	C	Cip	G	N	S	Su	T	W
438				R	R	R	S	S	I	S	S	R	R	R	S
442				R	R	R	S	R	R	S	I	R	R	R	R
	439			R	R	R	S	S	I	S	R	S	S	S	S
	C600			S	S	S	S	S	I	S	R	S	S	S	S
438	439	TSA	NA + S	R	R	R	S	S	I	S	R	R	R	R	S
438	C600	TSA	NA + S	S	I	S	S	S	I	S	R	R	R	R	S
442	439	TSA	NA + S	R	R	R	S	R	R	S	R	R	R	R	R
442	439	BD	NA + S	R	R	R	S	R	R	S	R	R	R	R	R
442	439	TH	NA + S	R	R	R	S	R	R	S	R	R	R	R	R
442	C600	TSA	NA + W	R	R	R	S	R	R	S	R	R	R	R	R
442	C600	TSA	NA + S	R	R	R	S	R	R	S	R	R	R	R	R
442	C600	DW	NA + S	R	R	R	S	R	R	S	R	R	R	R	R
442	C600	BS	NA + S	R	R	R	S	R	R	S	R	R	R	R	R
442	C600	TH	NA + S	R	R	R	S	R	R	S	R	R	R	R	R

[a] 438, *Salmonella* 438; 442, *Salmonella* 442; C600, *E. coli* C600.
[b] Matrix for conjugation. TSA, tryptic soy agar; BD, bedding sand; TH, Tail hair, DW, drinking water.
[c] NA + S, TSA supplemented with nalidixic acid (200 µg/mL) and streptomycin (50 µg/mL); NA + W, TSA supplemented with nalidixic acid (200 µg/mL) and trimethoprim (50 µg/mL).
[d] Au, amoxicillin/clavulanic acid; A, ampicillin; Fox, cefoxitin; Cx, ceftriaxone; C, chloramphenicol;Cip, ciprofloxacin; G, Gentamicin; N, Nalidixic acid; S, streptomycin; Su, sulfisoxazole; T, tetracycline; W, trimethoprim; R, resistant; I, intermediate; S, sensitive.

FIGURE 4.1. *PCR amplicons of* Salmonella *from flies and representative transconjugants using primers targeting integrase and class 1 integron gene cassettes. (A) Amplification of the 491-bp integrase from* Salmonella *438 (lane 1),* Salmonella *442 (lane 2),* Salmonella *439 (lane 3),* E. coli *C600 (lane 4), positive control (lane 5). (B) the 1.1-kb class 1 integron gene cassette from* Salmonella *439 (lane 1) and the 2.0-kb class 1 integron gene cassette from* Salmonella *442 (lane 2). (C) The class 1 integron of transconjugants resulted from* Salmonella *442* × Salmonella *439 on TSA supplemented with 200µg/ml nalidixic acid and 50µg/ml streptomycin (lane 1),* Salmonella *442* × Salmonella *439 on bedding sand (lane 2),* Salmonella *442* × Salmonella *439 on tail hair (lane 3),* Salmonella *442* × E. coli *C600 on TSA supplemented with 200µg/ml nalidixic acid and 50µg/ml streptomycin (lane 4),* Salmonella *442 × E. coli C600 on TSA 200µg/ml nalidixic acid and 50µg/ml trimethoprim (lane 5),* Salmonella *442* × E. coli *C600 in drinking water (lane 6),* Salmonella *442* × E. coli *C600 on bedding sand (lane 7),* Salmonella *442* × E. coli *C600 on tail hair (lane 8).*



CHAPTER 5

CONCLUSION

Results from the study described in Chapter 3 showed that *Salmonella* were isolated from 26 out of the 33 cattle farms (79%) and 185 out of the 1,650 flies (11%) in the study. The incidence of *Salmonella*-positive flies varied from farm to farm ranging from 0 to 78%. Among the 185 *Salmonella* strains isolated from flies, 29% were resistant to ampicillin, 28% to tetracycline, 21% to amoxicillin/clavulanic acid, 20% to cefoxitin, and 12% to streptomycin. Incidences of resistance against other tested antibiotics were low, ranging from 0 to 3%. Furthermore, 28% of the *Salmonella* isolates were multidrug resistant, which had resistance to 3 or more antibiotics. The minimal inhibitory concentrations of ampicillin, cefoxitin, streptomycin, and tetracycline against the *Salmonella* isolates ranged from 32 to >2,048, 64 to 2,048, 128 to 1,024, and 32 to 1,024 μg/mL, respectively. Some of the antibiotics, resisted by the *Salmonella* isolated from cattle flies are important medicines for treating serious infections in human. On farms with poor sanitation and management, populated flies can serve as vectors to disseminate antibiotic-resistant *Salmonella* and antibiotic-resistant genes. The development of increasing number of antibiotic resistant bacterial strains could potentially reduce or diminish the efficacies of antibiotic therapy in human medicine, imposing risks to public health.

Results from the study described in Chapter 4 indicated that 2 out of 606 (0.3%) isolated *Salmonella*, 438 and 442, harbored class 1 integrons. Integron gene cassette in *Salmonella* 438 carried a single gene of *aadA7* (*ca.* 1.1 kb), and that in *Salmonella* 442 carried *drfA12-orfF-aadA2* (*ca.* 2.0 kb). The two integrons were transferrable through conjugation on

microbiological media to the cells of a *Salmonella* strain isolated from fly and *E. coli* C600 at efficiencies ranging from 1.47×10^{-6} to 4.25×10^{-5}. However, only *Salmonella* 442 was able to transfer its integron to the recipient cells on 3 out of the 8 farm samples used in the study, namely cattle hair, bedding sand, and drinking water with conjugation efficiencies ranging from 4.26×10^{-10} to 1.36×10^{-8}. Transconjugants were confirmed to harbor integron gene cassettes of similar sizes to those in the corresponding donor strains and expressed antibiotic resistance encoded by the integron-mediated resistant genes. Antibiotic-resistant genes that are not carried by integrons, *e.g*. genes encoding resistance to tetracycline and chloramphenicol, were co-transferred with integron-mediated antibiotic resistance genes. This study showed that *Salmonella* isolates from flies of cattle source could carry integrons encoding genes for antibiotic resistance. The integrons can be co-transferred with other antibiotic-resistant genes within and cross bacterial species through conjugation on some of the farm samples used in the study. Possible dissemination of antibiotic-resistant genes through horizontal gene transfer in farm environment could cause the emergence of antibiotic resistant bacterial strains and failure of antibiotic treatment of infectious diseases.