Salmonella and antimicrobial resistance in broilers: A review

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Primary Audience: Researchers, Extension Services and Veterinarians

SUMMARY

Salmonella enterica is a zoonotic pathogen which can readily pass from animal to man through the consumption of contaminated food. The prevalence of *Salmonella enterica* associated with poultry and poultry meat products has been well-documented and this prevalence has both public health and economic implications. The estimated total cost for nontyphoidal *Salmonella* is in excess of 14 billion dollars/year in the United States alone. Almost 41,930 cases of nontyphoidal foodborne salmonellosis are confirmed annually with an estimated total number of 1 million cases of foodborne salmonellosis not reported. The emergence of antimicrobial resistant *Salmonella* recovered from meat products has heightened concerns regarding antimicrobial use in food animal production. This review will cover the history and taxonmy of *Salmonella enterica*, *Salmonella* in poultry and poultry products, colonization factors, transmission, detection and characterization, antibiotics, antimicrobial resistance, mechanisms of resistance in *Salmonella* by class, transmission of antimicrobial resistance, and the global implications of antimicrobial resistance.

Key words: *Salmonella*, antimicrobial resistance, colonization, broilers, antibiotics

2015 J. Appl. Poult. Res. 24:408–426
http://dx.doi.org/10.3382/japr/pfv038

SALMONELLA

History and Taxonomy

In the 19th century, the causative agent for typhoid fever was identified, which eventually became known as *Salmonella* [1]. Salmon and Smith [2] first isolated *Bacillus cholera-suis*, now called *Salmonella enterica* (***S. enterica***) subspecies *enterica* serovar Choleraesuis, from swine diagnosed with hog cholera [3]. While Smith was the first to actually identify the organism, Salmon was credited with the discovery which came to bear his name.

Bacteria of the genus *Salmonella* are Gram-negative, facultatively anaerobic, nonspore forming, usually motile rods (peritrichous flagella) belonging to the Enterobacteriaceae family, which are associated with the alimentary tract of animals. Salmonellae reduce nitrates to nitrites, carbon dioxide and hydrogen gases are usually produced from D-glucose, and hydrogen sulfide is typically produced by most salmonellae. Nearly all salmonellae are aerogenic except for *Salmonella* serovar Typhi which never produces gas. Tests for indole production (tryptophanase), oxidase, and urease are negative and 16S rRNA

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sequence analyses indicate that *Salmonella* belong to the Gammaproteobacteria [4]. The 2 *Salmonella* species, *S. enterica* and *Salmonella bongori*, were further separated by 16S rDNA sequence analysis and found to be closely related to the *Escherichia coli* and *Shigella* complex by both 16S and 23S rDNA analyses [4]. *Salmonella* species have an optimal growth temperature of 35 to 40°C with a growth range of 2 to 54°C depending on the serotype and growth matrix involved.

Most of the *Salmonella* isolates recovered from cases of human infection belong to *Salmonella enterica* subspecies *enterica*. Mazzotta [5] determined the D- (decimal reduction time or the time required at a certain temperature to kill 90% of the organisms being studied) and z-(thermal reduction time or the temperature required for the thermal destruction curve to move 1 log cycle) values of the commonly isolated *Salmonella* serotypes in ground chicken breast meat and determined that a thermal process of 3 s at 71.1°C is necessary for a 7 log reduction (7D) of *Salmonella* at a z-value of 5.7°C. Salmonellae do not grow well at low temperatures [6]. However, salmonellae are hardy and not always killed by freezing [7]. Most salmonellae survive well in acidic foods [pH $\leq$ 4.6, Food and Drug Administration (**FDA**); 8] and resist dehydration. They have long been considered some of the most important causal agents of foodborne illness throughout the world. Foodborne salmonellosis still occurs in developed, developing, and under-developed countries, giving testimony to the importance of this bacterial genus in terms of human morbidity and mortality contributions [9]. Many reports of salmonellosis are recognized as being sporadic in nature and often occur as isolated cases. However, improved methods for investigating foodborne disease combined with advancements in the collection and sharing of data on foodborne illnesses has enabled the identification of the etiologic agent linking individual illnesses into larger outbreaks.

Salmonella enterica subspecies *enterica* (serotypes) are antigenically differentiated by agglutination reactions with homologous antisera, and the combination of antigens possessed by each strain is referred to as its antigenic formula; this antigenic formula is unique for each *Salmonella* serotype. Presently, the Kauffman–White scheme is used for assigning the serotype name to the unique antigenic formula [10]. The antigens present on the surface of the bacterial cell include the somatic (O) or outer membrane antigens, the flagella (H) antigens; and the capsular (Vi) antigens [4]. More than 2,500 *Salmonella* serotypes are recognized and this number increases every year [11]. Additional methods for further differentiating *Salmonella* strains include phage typing [12], pulse-field gel electrophoresis (**PFGE**) analysis [13], PCR ribotyping [14], antimicrobial resistance patterns [14], and multilocus sequencing of DNA [15].

The ability of *Salmonella* species to cause human infection involves attachment and colonization of intestinal columnar epithelial cells and specialized microfold cells overlying Peyer's patches [16]. Symptoms of salmonellosis include diarrhea, abdominal pain, nausea, and vomiting lasting 1 to 7 d, and the illness is generally self-limiting in healthy adults with a mortality rate of <1% [1]. In severe cases, infection may progress to septicemia and death, unless the person is promptly treated with the appropriate antimicrobials, presently fluoroquinolones, macrolides, and third-generation cephalosporins [17]. Individuals who are immune-compromised, children, infants, and elderly are most likely to require antimicrobial treatment. Infections with antimicrobial-resistant strains may compromise treatment outcomes thus resulting in increased morbidity and mortality [18]. In rare instances, some individuals can develop chronic conditions including reactive arthritis, Reiter's syndrome, and ankylosing spondylitis [19].

The infective dose for salmonellosis in adult humans is estimated to be in the range of 10^4 to 10^6 cells or higher, but can be as low as 10^1 to 10^2 cells in highly susceptible individuals or if contained in a food with a high fat matrix (i.e., chocolate, cheese, salami, or peanut butter) [9, 20]. The prevalence of *Salmonella enterica* associated with raw poultry and poultry meat products have been well-documented [9, 21–24], and have both public health and economic implications.

Salmonella enterica is a zoonotic pathogen which can readily pass from animal to humans through the consumption of contaminated meat, animal products or other food products after

contamination with animal fecal material. Salmonellosis can also be acquired through direct or indirect contact with colonized animals as well as through consumption of contaminated water [24–27]. Salmonellae can also be considered a common commensal of the gut microflora of animals, including mammals, birds, reptiles, amphibians, fish, and shellfish [22, 28]. Fecal contamination is the main source of food and water contamination playing a large role in the dissemination of salmonellae in the environment and subsequently the food supply chain. Meat animals can be infected and act as reservoirs of salmonellae.

Scallan et al. [29] estimated that of the 9.4 million cases of foodborne illnesses, 5.5 million (59%) were caused by viruses, 3.6 million (39%) by bacteria, and 0.2 million (2%) by parasites in the United States. Nontyphoidal *Salmonella* accounted for approximately 1.0 million (11%) of these illnesses, resulting in approximately 42,000 laboratory-confirmed illnesses, 19,000 hospitalizations, and approximately 400 deaths [29]. Scallan et al. [29] estimated that cases of salmonellosis were reported only half of the time and under-diagnosed by a factor of 29.3. Using these factors combined with the confirmed case reports gives an estimate of almost 1.3 million cases of foodborne salmonellosis in the United States each year. The annual cost associated with salmonellosis in the United States has been estimated to be approximately $14.6 billion [30]. Scharff [30] estimated that the health-related economic cost of each foodborne illness in the United States is approximately $2,000, taking into account quality of life (pain and suffering) calculations.

Salmonella *in Poultry and Poultry Products*

Among *Salmonella*-contaminated poultry carcasses, total numbers of *Salmonella* are generally low [31]. From the 2007 to 2008 baseline survey for young chicken, upon enumeration of the 1,500 rehang carcass samples qualitatively confirmed as positive, 11% were below the limit of detection, 42% ranged from 0.0301 to 0.3 Most-Probable-Number (**MPN**)/mL, 34% ranged from 0.301 to 3.0 MPN/mL, and only 11 (0.007%) samples were above 30 MPN/mL. From the 170 postchill samples (n = 3,275) qualitatively confirmed as positive, none exceeded 30 MPN/mL, 46% of the positives ranged from 0.0301 to 0.3 MPN/mL, 14% ranged from 0.301 to 3.0 MPN/mL, and 5% were in the 3.01 to 30 MPN/mL range [32]. However, human salmonellosis is often attributed to small numbers of *Salmonella* replicating through temperature abuse during storage, poor handling, or improper cooking techniques and temperatures which are insufficient to kill the salmonellae prior to ingestion. The *Salmonella* serotypes most often isolated from young chicken during the 2007 to 2008 Nationwide Microbiological Baseline Data Collection Program: Young Chicken Survey were *Salmonella* Kentucky, Heidelberg, Typhimurium, and Typhimurium (*var* 5-) [32].

Salmonella accounted for 1,335 foodborne outbreaks and 36,490 associated illnesses in outbreaks reported to Food Disease Outbreak Surveillance System from 1999 to 2008. Poultry accounted for a higher percentage of *Salmonella* outbreaks of infection compared to other food commodities. A single food source was reported in 35% (468) of the outbreaks; 29% (137) were due to poultry with 71% (97) of those due to chicken. Most reported cases of *Salmonella* infection are sporadic and outnumbered outbreak-associated cases by more than 15 to 1 [29]. *Salmonella* Enteritidis and *Salmonella* Typhimurium were the serotypes most commonly reported in human illness and the first and second most common serotypes recovered from human cases, respectively [33, 34]. *Salmonella* Kentucky is the serotype most frequently recovered from carcass surveillance programs [34].

Exposure to poultry meat has also been linked to *Salmonella* illness. A review of the Centers for Disease Control and Prevention (**CDC**) outbreak data from 2006 to 2011 shows that 10 out of 25 outbreaks were related to live poultry, shell eggs, or further processed poultry products (Table 1). All of these outbreaks occurred over multiple states and Canadian provinces, infecting more than 6,000 individuals and created multiple public health incidences which led to recalls and corrective actions. These outbreaks represent the individuals actually linked to an outbreak of salmonellosis but did not include unreported individual cases of salmonellosis, which were not officially linked to the outbreak because either

Table 1. Reported salmonellosis outbreaks in the United States and Canada 2006 to 2011.

Source	Year	Location[1]	No. cases	Serotype	Reference
Ground turkey	2011	Multistate (26)	78	*Salmonella* Heidelberg	[161]
Cantaloupe	2011	Multistate (9)	20	*Salmonella* Panama	[162]
Chicks and ducklings	2011	Multistate (16)	49	*Salmonella* Altona	[163]
Chicks and ducklings	2011	Multistate (12)	22	*Salmonella* Johannesburg	[163]
Turkey burgers	2011	Multistate (10)	12	*Salmonella* Hadar	[164]
Alfalfa sprouts	2010 to 2011	Multistate (27)	140	*Salmonella* I 4,[5],12:i:-	[165]
Alfalfa sprouts	2010	Multistate (11)	44	*Salmonella* Newport	[166]
Alfalfa sprouts	2009	Multistate (14)	234	*Salmonella* Saintpaul	[167]
Shell eggs	2010	Multistate (11)	≥ 1,939	*Salmonella* Enteritidis	[168]
Frozen entrée	2010	Multistate (18)	44	*Salmonella* Chester	[169]
Red and black pepper/Italian style meats	2009 to 2010	Multistate (44)	272	*Salmonella* Montevideo	[170]
Peanut butter and peanut butter products	2008 to 2009	Multistate (46) and Canada	714 (United States) 1 (Canada)	*Salmonella* Typhimurium	[171]
Raw produce (jalapeno peppers)	2008	Multistate (43), Washington, D.C., and Canada	1,442 (United States) 5 (Canada)	*Salmonella* Saintpaul	[172]
Malt-O-Meal rice/wheat cereal	2008	Multistate (15)	28	*Salmonella* Agona	[173]
Cantaloupes	2008	Multistate (16)	51	*Salmonella* Litchfield	[174]
Banquet pot pies	2007	Multistate (35)	> 272	*Salmonella* I 4,[5], 12:i:-	[175]
Veggie booty	2007	Multistate (20)	65	*Salmonella* Wandsworth	[176]
Peanut butter	2007	Multistate (44)	425	*Salmonella* Tennessee	[177]
Live poultry (chicks)	2007	Multistate (2)	65	*Salmonella* Montevideo	[178]
Live poultry (chicks)	2007	Multistate (23)	64	*Salmonella* Montevideo	[178]
Live poultry (chicks)	2006	Michigan	21	*Salmonella* I 4, 5, 12, i:-	[179]
Live poultry (chicks)	2006	Multistate (21)	56	*Salmonella* Montevideo	[179]
Live poultry (chicks)	2006	Oregon	4	*Salmonella* Ohio	[179]
Tomatoes	2006	Multistate (21)	183	*Salmonella* Typhimurium	[180]
Poultry vaccine production	2006	Maine	21	*Salmonella* Enteritidis	[181]

[1]Number in parenthesis indicates the number of states involved in the outbreak.

the individuals did not seek medical care or no organisms were cultured by medical providers. Inclusion of these missed cases would increase the total numbers overall. Therefore, we can conclude that while poultry and poultry products are not the only vehicle for *Salmonella* infections in the United States, they are an important vehicle for these infections.

Factors Affecting Salmonella *Colonization in Chickens*

Factors known to affect *Salmonella* colonization include 1) age of the chicken, 2) environmental and physiological stressors (e.g., feed and water deprivation, dramatic temperature changes, and so on), 3) survival of *Salmonella* through the gastric barrier, 4) animal health and disease status of the chicken, 5) use of antimicrobials and or coccidiostats, 6) diet, and 7) genetic background of the chicks. Bacterial colonization and invasion are influenced by parameters specific to *Salmonella* and the effects of environmental stimuli (avian gastrointestinal tract) on gene expression [35].

One of the most important factors is the age of the birds. Newly hatched chicks are most susceptible to *Salmonella* colonization because they lack mature gut microflora or feed in the alimentary tract [36]. While very low doses of *Salmonella,* as low as 10 cells, can readily infect 1-day-old chicks, the susceptibility of chicks to infection with *Salmonella* tends to decrease with age [37]. Cox et al. [38] found that 38% of intracloacally inoculated 1-day-old chicks could be colonized with as few as 2 *Salmonella* cells.

Similarly it was determined that through oral and intracloacal inoculation, the number of cells required for a colonizing dose$_{50}$ was 100 times fewer than that of 3-day-old chicks that had been fed. Gast and Holt [39] challenged 1-day-old chicks to evaluate the persistence of *Salmonella* Enteritidis through maturity (24 wk age) and demonstrated that although *Salmonella* Enteritidis was usually cleared from internal organs within 8 wk postinoculation, the production of internally contaminated eggs by a hen that was not shedding *Salmonella* Enteritidis in her feces suggest that extended persistence in internal organs can occur at a low frequency. Beal et al. [40] determined that age and genetics affect the ability of chickens to resist *Salmonella* colonization.

One approach used to help control *Salmonella* colonization in chicks, particular those which lack mature intestinal microflora, is competitive exclusion (**CE**). First reported by Nurmi and Rantala [41], CE as a treatment involves the oral administration of intestinal microflora from healthy, salmonellae-free adult chickens to newly hatched chicks. This CE intestinal microflora is used to accelerate the maturation of the chick's gut and can be either defined (known bacterial strains) or undefined (a complex of unknown bacterial strains from an adult chicken's intestinal tract). Both defined and undefined CE cultures increase subsequent resistance to *Salmonella* colonization. The concept behind the use of probiotics is similar to that of competitive exclusion with the distinction that probiotics are intended to enhance the functions of the existing microflora [42, 43].

A second factor that can affect colonization is the ability of *Salmonella* to survive the passage through the pH of the gastrointestinal tract. Natural infection occurs mainly through the oral route and, in poultry, *Salmonella* encounter the acidic (pH ~4.5 to 5) environment of the crop [44]. *Lactobacillus* strains present in the crop assist in maintaining the low pH associated with the crop environment, but upon feed withdrawal, a decrease in the lactobacilli population causes the crop pH to increase to approximately pH 6.0 to 6.3 [45, 46], providing a more suitable environment for survival of *Salmonella*.

Salmonella must survive passage through the proventriculus and gizzard which are also acidic environments. The pH of the proventricular contents becomes acidic (pH 2.0 to 4.0) about the 20th d of egg incubation and is indicative of the considerable secretion of hydrochloric acid by the proventricular glands with the actual onset of secretions beginning between d 11 and 13 of egg incubation in response to the ingestion of albumin by the embryo [47]. In an in vitro study, Cox et al. [48] reported a decreased survival rate for *Salmonella* spp. at pH 4.4 which corresponds to the proventriculus, with limited survival at pH 2.6 which is encountered in the gizzard. Finally, the pH of the small intestine (6.2) and large intestine (6.3) are closer to neutral and therefore more suited for *Salmonella* survival and proliferation in 3-week-old chickens [49]. As with lactobacilli colonization, antimicrobial or anticoccidial feed additives may also influence *Salmonella* colonization by altering or reducing normal intestinal microflora [50]. Regardless of what initiates the change, alterations in the protective gut microflora can increase a chicken's susceptibility to *Salmonella* colonization.

A third factor associated with colonization includes both the dose and strain of *Salmonella* to which the chickens are exposed [37, 51], including the ability of the strain to attach, colonize, and invade the various intestinal tissues [52]. Higher levels (10^4 to 10^5 cfu) of *Salmonella* are more likely to colonize chickens, and some *Salmonella* serotypes can colonize the avian intestinal tract more efficiently at lower levels than others [53]. However, *Salmonella* must first attach themselves to the host epithelial cells to initiate the processes of colonization and invasion [54, 55]. Attachment is mediated by cell surface proteins known as adhesins, with the *Salmonella enterica* serovars possessing several fimbrial and nonfimbrial adhesins that are capable of binding to intestinal epithelial cells [56]. The *Salmonella* Pathogenicity Island (**SPI**) 1 (discrete genetic units) contributes to colonization of the chicken with *Salmonella*, while SPI2, in the absence of SPI1, inhibits colonization [57]. *Salmonella* invasion is mediated by genes located on SPI1 [58]. Several studies have shown that mutations in these SPI1-specific genes

can affect the intestinal colonization of young chicks [59–61].

Rabsch et al. [62], Callaway et al. [63], and Foley et al. [64] all analyzed epidemiological data collected through surveillance studies from the last half of the 20th century in the United States and Europe to explain the reduction of host specific *Salmonella*, specifically *Salmonella* Gallinarum and *Salmonella* Pullorum, in poultry production. These 3 studies support the theory that the increase in the prevalence of *Salmonella* Enteritidis and other nonhost-specific *Salmonella* serotypes in poultry and poultry products might be the result of the reduction and/or elimination of the host-specific *Salmonella* serovar Gallinarum which includes the 2 biovars, Gallinarum and Pullorum. Rabsch et al. [62] proposed that the increase in prevalence of *Salmonella* Enteritidis was a result of the industry's actions which resulted in the reduction in the prevalence of *Salmonella* Gallinarum and *Salmonella* Pullorum. Since *Salmonella* Gallinarum has no animal reservoirs other than domestic and aquatic fowl, the eradication left a niche which was filled by nonhost-specific *Salmonella* serovars; Heidelberg, Typhimurium, and Enteritidis in particular [64]. Thomson et al. [65] sequenced the genomes of *Salmonella* Enteritidis PT4 isolate P125109, a host-promiscuous serovar, and *Salmonella* Gallinarum isolate 287/91, a chicken-restricted serovar. Genomic comparisons between these 2 genomes indicate that *Salmonella* Gallinarum 287/91 is highly related to and likely a direct descendent of *Salmonella* Enteritidis, which has undergone extensive degradation through deletion and pseudogene formation, which might explain the increase in *Salmonella* Enteritidis colonization of chickens following the reduction and/or elimination of *Salmonella* Gallinarum in the poultry industry [65].

Other studies looking at the competition between *Salmonella* serotypes in the gut of broiler chicks are almost nonexistent. Nógrády et al. [66] examined the growth suppression of *Salmonella* Hadar, in vitro under strict anaerobiosis and in vivo in the intestine of 1-day-old chicks. Four strains were selected for evaluation of their ability to suppress the growth of *Salmonella* Enteritidis, Typhimurium, Virchow, and Saintpaul. Nógrády et al. [66] were able to show that precolonization of the chicken with *Salmonella* Hadar prevented the super-infection with any of the 4 mentioned serotypes. Ngwai et al. [67] looked at the in vitro growth suppression of antibiotic-resistant *Salmonella* Typhimurium DT-104 by non-DT104 strains. The non-DT104 strains were able to prevent the multiplication of the antibiotic-resistant DT104 strain when the DT104 strain was added in low numbers to 24-h cultures of the non-DT104 strains. The implication is that one *Salmonella* serotype might be able to prevent the colonization of another *Salmonella* serotype.

Horizontal Transmission of Salmonella *in Poultry*

Horizontal transmission of salmonellae among broiler and layer chickens has been demonstrated in studies conducted worldwide [68–72]. Byrd et al. [68] found that after colonizing a minimum of 5 chicks per treatment pen with as few as 10^2 cfu/chick of *Salmonella* Typhimurium, approximately 57% of the remaining birds became colonized with $\log_{10}$ 2.2 cfu *S.* Typhimurium per gram of cecal contents by d 17 of grow-out. This population of salmonellae in the ceca increased when the seeder chicks were orally gavaged with larger concentrations of *Salmonella* Typhimurium. Byrd et al. [68] also recovered *Salmonella* Typhimurium from litter samples at d 17, which indicates the potential for horizontal transmission of salmonellae from seeder chicks to contact chicks through the litter.

Liljebjelke et al. [69] recovered *Salmonella enterica* from 2 integrated poultry systems over 7 consecutive flocks isolating 15 different serotypes. *Salmonella* Typhimurium and Enteritidis isolates, respectively, from poultry carcasses shared the same PFGE pattern as those isolated from the rearing environment and from rodents caught in the same house implicating horizontal transmission as one means of spread of these *Salmonella* serotypes [69]. However, indistinguishable PFGE types of *Salmonella* Typhimurium, Enteritidis, and Heidelberg were isolated from carcasses, the broiler chicken environment and chick-box liners which also implicate the hatchery as a source for these persistent serotypes on this farm [69].

Detection and Characterization of Salmonella

PFGE has been used and widely accepted as the gold standard for tracking outbreaks of foodborne illness since 1995 when the CDC selected 4 state public health laboratories for a national molecular subtyping network for foodborne bacterial disease surveillance [13, 73–75]. This network later became known as PulseNet [76] and has expanded to include countries all over the globe, from northern Canada to islands in the Pacific [77].

For over 80 yr, subtyping of *Salmonella enterica* for epidemiological surveillance has been performed by serotyping [75, 78]. Serotyping is a method in which surface antigens are used to indentify *Salmonella* serotypes based on agglutination reactions with specific antibodies. This typing method has allowed for the long-term epidemiological surveillance of *Salmonella* in the food chain and in public health investigations [75]. However, in epidemiological investigations, identification and tracking of salmonellosis outbreaks require the use of more sensitive methods for determining the causative strains at a taxonomic level than is achieved by serotyping alone [74, 75, 79, 80]. PFGE profiling is a DNA fingerprinting method based on the restriction digestion of purified genomic DNA and is currently considered the gold-standard for the subtyping of foodborne pathogens, especially *Salmonella* [81–83]. PFGE is the platform used by PulseNet, a national molecular subtyping network that was established in 1996 by the CDC [76, 81]. PulseNet is now utilized by all state public health laboratories and food safety laboratories at the FDA and the USDA [84]. Currently, PFGE data are considered a reliable and sensitive way to detect differences between closely related strains [75]. Isolates with indistinguishable PFGE profiles can be classified as epidemiologically linked with a high degree of confidence [83, 84]. PFGE can be used to assess relatedness within *Salmonella* serotypes and has been useful during outbreak investigations [82]. The ability to track *Salmonella* serotypes through an animal model gives researchers the ability to follow the adaptations of *Salmonella* strains and to answer questions regarding the complex interactions between *Salmonella* serotypes in the animal hosts and/or the environment.

ANTIMICROBIAL RESISTANCE

Definition of Antibiotics and Antimicrobial Resistance

Antibiotics (chemical substances produced by various microorganisms), synthetic chemicals, disinfectants, or drugs, collectively referred to as antimicrobial agents, have been used since the time of antiquity to treat patients with a variety of bacterial diseases [85]. Since the 1940s, antibiotics have greatly reduced morbidity and mortality from infectious diseases. During the Second World War, the use of penicillin and sulfa drugs greatly improved the survival rate of injured and ill soldiers, sailors, and Marines fighting in less-than-hospitable locations [85, 86]. Penicillin was the first used antibiotic to be discovered by Fleming in 1928 [87]. Since that time, scientists have discovered and developed a number of different classes of antimicrobials exerting bactericidal or bacteriostatic effects [88].

Although heralded as wonder drugs, antimicrobials can lose some level of efficaciousness as resistance develops. Antimicrobial resistance is a result of microbes changing to reduce or eliminate the effect of an antimicrobial to which it had previously been susceptible. Soon after Fleming's discovery, he cautioned everyone that resistance to penicillin might not be long in developing and within 1 yr of widespread use, he was proven correct as a number of strains developed resistance [86]. The pharmaceutical industry easily kept pace with the rapidly evolving resistant microorganisms that emerged during the middle part of the 20th century by developing new forms of the existing antibiotics and/or entirely new classes of antimicrobial drugs [86, 88].

Antimicrobial resistance can be intrinsic (part of the normal architecture of a bacterium) or acquired through exchange of DNA [88]. Intrinsic resistance results through spontaneous mutation of genetic material which confers some new adaptation allowing the organism to resist the lethal effects of the antimicrobial agent. Spontaneous mutations can be either base-substitutions, frame shift mutations, deletions of genetic material, or insertions of large DNA elements and can occur naturally at an average frequency of 1×10^{-6} per base pairs

[89–91]. In acquired resistance, resistance factors in the form of plasmids, transposons, or integrons move between bacteria either through conjugation, transformation, or transduction [92]. Common drug-resistant microorganisms include methicillin-resistant *Staphylococcus aureus* [93, 94], multidrug-resistant *Salmonella* spp. [95, 96], and multidrug-resistant *Mycobacterium tuberculosis* [86], all of which can be linked to increases in morbidity and mortality, especially in immune-compromised patients. This resistance can lead to longer, more expensive hospital stays, and increased mortality from bacterial infections [97].

Some important factors in the development of resistance include selective pressures, proliferation of multiple resistant clones, and the inability to detect emerging phenotypes. These selective pressures can include overuse or misuse of antimicrobials in the treatment of human disease, in agriculture, and in-home disinfectants [98].

In the past 60 yr or so, physicians and pharmaceutical companies have been constantly challenged to stay one step ahead of bacteria which are adapting rapidly to antimicrobial drugs which have been developed for their control. While initially expected to virtually wipe out infectious diseases and deaths related to these pathogenic organisms by the middle part of the 20th century [88], overuse and misuse of antimicrobials have resulted in their decreased efficacy. More and more of these pathogens have acquired or are acquiring the genetic material (either chromosomal DNA or plasmids) to effectively block the actions of these drugs and some bacteria have even become resistant to multiple drugs and classes of drugs, making them almost "pan-resistant" [86]. Infections resulting from resistant organisms once only found in hospitals and health care facilities are now commonly found in the community, creating a potential crisis for the future control of these pathogenic species (e.g., methicillin resistant *Staphylococcus aureus*) [99]. Additionally, the development of new antimicrobial drugs and classes of drugs by the pharmaceutical companies has virtually ceased due to 1) the increased cost associated with development, 2) the ethics and negative public opinion of animal and/or human testing, and 3) an increase in government regulations required for the approval of any new antimicrobial drug or new use for an existing drug [88].

According to the CDC, over 47 million cases of domestically acquired foodborne illness occur annually in the United States, of which at least 70% of the pathogenic organisms involved are resistant to at least one antimicrobial drug. Approximately 3,000 people die in the United States each year from these illnesses. According to the CDC's website, drug-resistant infections lead to longer hospital stays and more expensive treatments which may be less effective and even toxic to the patient [33]. This problem appears to be increasing rather than decreasing as more bacteria acquire multiple drug resistance (**MDR**).

In the mid to late 1980s, the medical community and consumers realized antimicrobial drugs might not be the "magic bullet" for control of bacterial infections and illnesses as once believed. Public and scientific interest in the administration of therapeutic and sub-therapeutic antimicrobials to animals increased due to the emergence and dissemination of MDR zoonotic bacterial pathogens [100]. The definition for MDR varies by laboratory and has been reported as resistance to 3 or more antimicrobials [101]. Currently, the National Antimicrobial Resistance Monitoring System defines MDR as resistance to 2 or more classes of antimicrobials [102]. Regardless, treatment of resistance to multiple classes of antimicrobials, particularly those involving the cephalosporins and fluoroquinolones [20], has severely limited treatment options.

Mechanisms of Drug Resistance

The 2 primary routes which bacteria use for the development of antimicrobial resistance are spontaneous (natural) and acquired. Both mechanisms are forms of genetic modification of a microorganism for survival; Darwinism at work. In spontaneous mutation, a genetic mutation naturally occurs conferring on the organism the ability to resist the lethal effects of an antimicrobial; the trigger for spontaneous mutations is unknown but exposure to the antimicrobial agent may provide selective pressure for antimicrobial resistance [86]. Acquired resistance results from

the uptake of genetic material from other bacteria [88].

Mechanisms of bacterial resistance vary and can be described by 3 mechanisms. The oldest known mechanism of resistance is for the bacteria to produce specific proteins, usually enzymes, which alter the antimicrobial into a form which no longer has the intended mode of action. One example is the production of β-lactamases by *Salmonella* which inactivate the β-lactam class of antimicrobials [103]. A second mechanism of resistance is the efflux pump which actively pumps antimicrobials out of the bacterium such that antimicrobial concentrations in the cell never reach the threshold necessary to interfere with the cell's metabolic processes [88]. Tetracycline and chloramphenicol resistance in *Salmonella* isolates are examples of energy-dependent efflux pumps which remove the tetracycline and chloramphenicol from the bacterial cell before it can prevent the binding of tRNA to the A site of the 30S ribosomal subunit, thus inhibiting protein synthesis [103, 104]. A third mechanism of resistance is to chemically change or mutate the target which the antimicrobial works on, preventing binding of the antibiotic to the target, also known as receptor modification [88]. This mechanism is observed for vancomycin-resistant enterococci which mutate the terminal peptides from D-Ala-D-Ala to D-Ala-D-Lac which have a lower affinity to vancomycin [88]. One thing is certain; bacteria have demonstrated an extraordinary capability to survive.

Antimicrobial Resistance Mechanisms in Salmonella *by Antimicrobial Class*

Aminoglycosides. Aminoglycosides were first discovered in 1943 when streptomycin was isolated from *Streptomyces griseus* [105]. Other commonly known compounds in this class of drugs include gentamicin, neomycin, amikacin, and kanamycin [105]. These drugs are effective for treating infections caused by Gram-negative bacilli and are usually used in combination with glycopeptides and β-lactams to ensure a broad spectrum of action [105, 106]. Aminoglycosides bind to conserved sequences within the 16S rRNA of the 30S ribosomal subunit [104] which leads to codon misreading and translation inhibition. Most aminoglycosides are bactericidal with the exception of spectinomycin, which is bacteriostatic [104]. Primary mechanisms for nontyphoidal *Salmonella* to resist aminoglycosides are 1) decreased drug uptake, 2) drug modification, and 3) modification of the ribosomal target of the drug [96].

Beta-lactams. Penicillins, cephalosporins, and carbapenems are the 3 major groups of beta-lactams. The antimicrobial effects of these drugs are mediated by their ability to interfere with a group of proteins known as penicillin-binding proteins, which are involved in the synthesis of peptidoglycan, a component of the bacterial cell wall. Beta-lactams are generally bactericidal, but the activity varies among beta-lactams, organisms, and target penicillin-binding proteins [96]. Beta-lactams must cross the bacterial outer membrane to reach their penicillin-binding protein targets. This passage is facilitated by two porins, OmpC and OmpF [96]. While changes or loss of the porins are uncommon mechanisms of resistance, some cases have been documented where a decrease in either OmpF or OmpC porin concentrations resulted in observable increases in resistance to beta-lactams such as ampicillin, cefoxitin, and other cephalosporins [96].

In *Salmonella*, inhibition of the essential penicillin-binding proteins leads to bactericidal activity. With the widespread use of penicillins, resistance to ampicillin, methicillin, and other penicillin drugs is common [20]. The most common mechanism of resistance is the secretion of beta-lactamases into the periplasmic fluid for Gram-negative microorganisms and into the environment for Gram-positive microorganisms. These enzymes hydrolyze the beta-lactam rings into beta-amino acids which have no antimicrobial activity. The genes encoding for beta-lactamase production are typically carried on plasmids [104]. *Staphylococcus* resistance to methicillin has become particularly worrisome as methicillin-resistant *Staphylococcus aureus* has emerged as a serious problem [99]. In response to beta-lactam resistance, a second class of beta-lactams, the 6-member ringed cephalosporins was developed. Carbapenems are the latest group of beta-lactams containing a 5-member ring without sulfur bound to the 4-member beta-lactam ring [104]. These beta-lactamases have become particularly

important in treatment of acute otitis media, an important health problem in early childhood and the most frequent condition for which antimicrobials are prescribed for children in the United States [107, 108]. Beta-lactams have a broad range of activity against Gram-negative and Gram-positive bacteria, with the later generations having the broader spectrum of activity.

Phenicols. Chloramphenicol, once the drug of choice for the treatment of typhoid fever, and florfenicol, the newest phenicol, are included in this class of antimicrobial drugs [104]. Chloramphenicols produced by *Streptomyces venezuelae* were discovered in 1947 and work by binding to the peptidyltransferase center of the 50S ribosomal unit, preventing the formation of peptide bonds [104]. Chloramphenicols have a broad range of activities against both Gram-positive and Gram-negative bacteria, and are able to cross the blood–brain barrier, making them a powerful choice in systemic infections [96]. However, chloramphenicols are limited in use except in developing countries due to the widespread resistance and toxicity.

Resistance in *Salmonella* isolates is conferred by two mechanisms: 1) enzymatic inactivation of the antibiotic by chloramphenicol O-acetyl-transferase, and 2) removal of the antibiotic by an efflux pump. Neither chloramphenicol acetyltransferase, the enzyme responsible for most of the plasmid mediated resistance to chloramphenicol [109], nor the known nonenzymatic chloramphenicol resistance genes (*cmlA* and *cmlB*) confer resistance to florfenicol [110, 111]. However, both mechanisms are known to be effective in conferring chloramphenicol resistance in *Salmonella* serotypes, especially Typhimurium and Agona [112]. Development of florfenicol for use in animal husbandry was intended to decrease the resistance to chloramphenicol in humans. Florfenicol was approved by the FDA in 1996 for the treatment of bovine respiratory pathogens and is not currently approved for use in humans [113]. Chloramphenicol was banned from veterinary use in Europe in 1994, while florfenicol was approved for use in 1995 in France [114].

Quinolones and fluoroquinolones. Quinolones and fluoroquinolones are synthetic bactericidal drugs and nalidixic acid was the first medically approved quinolone [104]. The early quinolones targeted DNA gyrase, while the later generations of quinolones target DNA gyrase and DNA topoisomerase IV [115]. The mode of action for quinolones is complex and not fully understood [104]. High-level resistance to quinolones is still rare [116, 117], but some *Salmonella* isolates with resistance to nalidixic acid and low-level resistance to other quinolones have been documented [118, 119].

Two mechanisms of resistance occur. The first mechanism is mediated by target mutations in the quinolone resistance determining region of *gyrA* and *gyrB* in the *parC* subunit of topoisomerase IV [120, 121]. The second mechanism involves alterations in the expression of the *AcrAB–TolC* efflux system through mutations in the genes encoding the system regulators resulting in the over-expression of this efflux system and decreasing quinolone sensitivity [121, 122]. No single mutation confers high-level resistance to the quinolones; instead, it is the result of an accumulation of various mutations [123].

When fluoroquinolones were first licensed for human therapy, no immediate rise in *Salmonella* resistance was observed. After the licensing of fluoroquinolones for animal use, the rates of fluoroquinolone-resistant *Salmonella* in animals and food and subsequently in human infections rapidly increased in several countries [18]. Currently, 6 fluoroquinolones have been approved for animal use in the United States, i.e., enrofloxacin, danofloxacin, orbifloxacin, difloxacin, marbofloxacin, and sarafloxacin [124]. However, 2 of these drugs, sarafloxacin and enrofloxacin, which were licensed for treatment of respiratory diseases in poultry, have been removed from the approved list due to increased antimicrobial resistance in *Campylobacter* and *Salmonella* species recovered in human illnesses [125].

Tetracycline. Chlortetracycline was isolated from *Streptomyces aureofaciens* in the 1940s and this family of drugs became popular because of their broad spectrum of activity with minimal adverse effects [96]. Tetracyclines act by preventing the binding of tRNA to the A site of the 30S ribosomal subunit, thus inhibiting protein synthesis [104]. Tetracycline resistance in *Salmonella* isolates is generally attributed to the production of an energy-dependent efflux pump,

which removes tetracycline from the bacterial cell. Other mechanisms of tetracycline resistance have been documented in other bacterial species but are not yet reported among *Salmonella* isolates [126].

There are at least 32 different genes that confer resistance to tetracycline and oxytetracycline with *tet*(*A*), *tet*(*B*), *tet*(*C*), *tet*(*D*), *tet*(*G*), and *tet*(*H*) found most often in *Salmonella* isolates [104, 126]. The most commonly reported of these is *tet*(*A*) which is located within *Salmonella* genomic island 1 [127], on integrons [128], and on transferrable plasmids [129–131]. The *tet*(*B*) gene is also relatively common and is located on transferable plasmids [132]. These genes appear to be easily transferred and widespread among *Salmonella* isolates and are almost always present in isolates that display multidrug resistance [127, 130, 133], which might make them important markers enabling the identification of potentially serious *Salmonella* infections.

Tetracycline and 31 other antimicrobials were approved in 1951 for use in broiler feeds in the United States without a veterinary prescription for the treatment of coccidiosis, growth promotion, and other purposes [134]. Beginning in the late 1950s and 1960s each European state has approved its own national regulations concerning the use of antibiotics in animal feeds [135]. Diarra et al. [136] found that isolates recovered from broiler chickens over a 35-d grow-out period showed some degree of multiple antibiotic resistances. The consequences of poultry production for environmental, food safety, and animal welfare issues are now part of consumers' opinions and demands [137]. Decreased use of antimicrobial growth promoters is both consumer- and legislative-driven [136–138].

Sulfonamides and trimethoprim. These 2 classes of antimicrobials have been used in combination for the treatment of bacterial infections since the late 1960s. They are bacteriostatic and competitively inhibit enzymes involved in synthesizing tetrahydrofolic acid [96]. Sulfonamides are structural analogues of *p*-amino benzoic acid and compete with *p*-amino benzoic acid in the synthesis of dihydrofolic acid effectively inhibiting dihydrofolate synthetase in bacteria which synthesize folate [139]. As a result, sulfonamides do not affect mammalian cells because mammals do not synthesize folate; instead, folate is taken up directly from food [140]. Trimethoprim inhibits dihydrofolate reductase [104]. Sulfonamide resistance in *Salmonella* isolates has been attributed to the presence of an extra *sul* gene, which expresses an insensitive form of dihydrofolate synthetase [104, 141]. Trimethoprim resistance is attributed to the expression of dihydrofolate reductase which does not bind trimethoprim [104].

Combinations of trimethoprim and sulfonamides have been used in veterinary practice since 1970 because of their wide spectrum of activity, clinical efficacy and relatively low cost [140]. Trimethoprim/sulfonamides combinations are used in the treatment of diseases caused by Gram-positive and Gram-negative bacteria to include infections of the respiratory tract, urogenital tract, alimentary tract, skin, joints, and wounds [139].

Transmission of Antimicrobial Resistance in Salmonella

Two mechanisms are implicated in the spread of antimicrobial resistance in *Salmonella* populations: 1) horizontal transfer of genes for antibiotic resistance, and 2) clonal spread of antimicrobial drug-resistant *Salmonella* isolates [118, 142]. Resistance genes can be horizontally transferred between *Salmonella* strains or from other bacterial species to the *Salmonella* strains [132]. In *Salmonella,* plasmids, and Class I integrons are primarily responsible for horizontal transmission [57, 132]. Other species can contribute resistance genes not currently found in the *Salmonella* gene pool through this mechanism. Resistance genes for the various antimicrobial drug classes can be found on several different plasmid types and many of these plasmids carry multiple antimicrobial resistance genes which can be transferred to other *Salmonella* and other bacterial species [143–145]. Integrons are elements that contain the genetic determinants of components of a site-specific recombination system that recognizes and captures mobile gene cassettes [146]. Integrons contain the gene for an integrase (i.e., *int*) and an adjacent recombination site. Although gene cassettes are not necessarily part of the integron once incorporated, they become part of the integron [145]. Two integron classes exist, i.e., resistance and

super-integrons. Nearly all gene cassettes from resistance integrons encode resistance to antibiotics or disinfectants [146]. Class I and Class II integrons have been found in *Salmonella*. Class I integrons are primarily in the *Salmonella* genomic islands [146] while Class II integrons are embedded in the TN7 transposon family but have not been fully described [147].

Antimicrobial Resistance as a Global Problem

Antimicrobial resistance is widespread according to the American Academy of Pediatrics [148]. Resistance has been elevated by major world health organizations as one of the top health challenges of the 21st centure [101, 149]. Antimicrobial resistance is also increasing among human pathogens. Bacteria resistant to multiple antimicrobials are of particular concern. In some cases, few or no antibiotics are available to treat resistant pathogens [118, 150]. The escalating resistance has raised concern that we are entering the "postantibiotic era," meaning we may be entering a period where there would be no effective antimicrobials available for treating many life-threatening infections in humans [151]. If this is true, deaths due to infection will once again become a very real threat to substantial numbers of children, young adults, sick, and elderly individuals.

Overuse and/or misuse of antimicrobials in both veterinary and human medicine is responsible for the increasing crisis of antimicrobial resistance [151]. In 2001, the Union of Concerned Scientists estimated that over 11.2 million kg antimicrobials were used as growth promoters in animals compared to 1.4 million kg antimicrobials for human medical use [152]. Volumes have been written on direct and indirect evidence linking animal use of nontherapeutic antimicrobials to the antimicrobial resistance now confronting humans [153].

One of the most effective ways to select for resistance genes in bacteria is to expose bacteria to low doses of broad-spectrum antimicrobials [148]. Levy et al. [154] examined the effect of low-dose tetracycline in feed on the intestinal flora of chickens. When comparing the antimicrobial resistance of bacteria isolated from chickens fed low doses of tetracycline to bacteria isolated from birds fed a diet without tetracycline, resistance increased after 36 h on a diet with low levels of tetracycline and after 2 wk approximately 90% of the chickens in the experimental group were excreting bacteria all of which were resistant to tetracycline [154]. Another trend observed was that feeding tetracycline to the chickens in the experimental group resulted in the development of multidrug resistance among the microorganisms recovered. Resistance to not only tetracycline, but also to sulfonamides, streptomycin, ampicillin, and carbenicillin developed through plasmid transfer [148]. This resistance extended over time to the control birds although at lower levels and subsequently to the farm workers. Six months after the removal of tetracycline from feed on the farm, no tetracycline-resistant bacteria were isolated from 8 of 10 farm workers tested [154].

When animals become colonized by resistant organisms, these organisms spread to other animals and eventually humans either through the food chain, direct contact or contamination of the environment with animal excreta [155]. The increasing industrialization of food animal production increases the stress on the animals which causes increased bacterial shedding and the inevitable contamination of hides, carcasses, and meat with fecal bacteria [156, 157]. There is also an increase in the amount of active antimicrobials detected near waste lagoons, surface waters, and river sediments [158]. The presence of these antimicrobials in the environment raises concerns that microbial populations might be under selective pressure stimulating horizontal gene transfer and amplifying the number and variety of organisms that are resistant to antimicrobials [148]. Chee–Sanford et al. [159] found resistance genes identical to those found in swine waste lagoons, in groundwater, and in soil microbes hundreds of meters downstream.

While it was hoped by many that the years of experience following the bans on antimicrobials as growth promotants in Europe would precede an end to the use of antimicrobials as growth promotants in the United States arguments continue based on the lines of cost-to-benefit ratios and perceived deficits in solid scientific evidence [153]. The European Common Market began by issuing a ban against the use of tetracycline in the mid-1970s and the bans continued until a total ban on the use of antimicrobials as

nontherapeutic growth promotants was enacted in 1999 by the European Union [153]. Industry voiced concern that the total withdrawal of antimicrobials from nontherapeutic uses would lead to an increase in the disease rate of the food animals and thus to an increase in the use of therapeutic antimicrobials [153]. In Denmark, a different result seems to have appeared after initial negative after-effects. Farmers have modified their animal husbandry practices accommodating for the loss of the banned antimicrobials resulting in improved immunity and reduced infection rates leading to fewer demands for therapeutic antimicrobials [153].

CONCLUSIONS AND APPLICATIONS

1. *Salmonella* species continue to be one of the major causes of bacterial illnesses in the United States causing an estimated 1.4 million cases/year. These cases are linked to foodborne outbreaks, live animal contact, poor hygiene, and environmental exposure. Much research has been conducted on virulence, pathogenicity, and invasiveness of the various serotypes in humans and animals. With the emergence of antimicrobial resistance, the pathogenicity and virulence of certain *Salmonella* serotypes have increased and treatment options are decreasing and becoming more expensive.
2. The effectiveness of antimicrobials, long considered "wonder drugs" and "silver bullets" for the treatment and control of bacterial infections, has rapidly been decreasing due to the development of resistance mechanisms. Bacteria are able to obtain genetic material which allows for the survival and selection of antimicrobial resistant cell lines. The acquisition of resistance has been linked to the selective pressure applied when antimicrobials are either overused (too often and in the wrong concentrations) or misused (the wrong antimicrobial selected for use) in animal production or human medicine. Politicians, farmers, scientists, and consumers are becoming more concerned with the increase in antimicrobial resistance and measures are being taken to reduce the amount of antimicrobials used in animal husbandry either through regulation or education of producers, doctors, and consumers.
3. In 2002, the Facts about Antimicrobials in Animals and Their Impact on Resistance made the following recommendations: 1) antimicrobial agents should not be used in agriculture in the absence of disease, 2) antimicrobials should be administered to animals only when prescribed by a veterinarian, 3) quantitative data on antimicrobial use in agriculture should be made available to inform public policy, 4) the ecology of antimicrobial resistance should be considered by regulatory agencies in assessing human health risk associated with antimicrobial use in agriculture, 5) surveillance programs for antimicrobial resistance should be improved and expanded, and 6) the ecology of antimicrobial resistance in agriculture should be a research priority [160]. Implementation of these six recommendations along with further research into the mechanisms and the ecology of antimicrobial resistant bacteria, especially *Salmonella* species, may provide a return to the effectiveness of antimicrobials in treating infections caused by pathogenic bacteria.

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