GENOTYPIC DIVERSITY AND ANTIMICROBIAL RESISTANCE IN *SALMONELLA* ISOLATES FROM FOOD ANIMALS AND ANIMAL PRODUCTS

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EXTENDED ABSTRACT

Salmonella infection is the second leading cause of foodborne illness in humans worldwide. *Salmonella* spp. as one of the foodborne pathogens are widely distributed in food animal production systems. Food animal products, therefore, are considered as the major reservoir for the pathogens. *Salmonella* spp. have the potential to contaminate food across the production chain. Most strains of commonly occurring serotypes, such as Typhimurium, have been shown to exhibit multi-drug resistance. Previous studies on antimicrobial resistance have shown the emergence of multi-drug resistant (MDR) *Salmonella* isolates in food of animal origin even when there was no history of using antimicrobials, as therapeutics or as growth promoters. This suggests the presence of other co-selective pressure risk factors, including the use of heavy metal micronutrients and different types of biocides that may play a role in persistence of MDR.

The main objective of the thesis was to investigate the role of the potential co-selective agents, such as heavy metal micronutrients and biocides in the emergence and persistence of MDR *Salmonella* isolates in the swine production systems in the United States. In addition, the thesis study determined the prevalence of antimicrobial resistance and diversity of *Salmonella* isolates detected from food animal production in Tanzania as a baseline for future investigation of co-selective agents and risk factors. In this thesis, the feed and faecal levels of Cu^{2+} and Zn^{2+} were measured. The agar plate-dilution method was used to determine the minimum inhibitory concentrations (MIC) of $CuSO_4$, $ZnCl_2$, Quaternary ammonium compound (Biosentry®) and Quaternary ammonium compound (QAC) with gulteraldehyde (Synergize®) against *Salmonella* isolates. Antimicrobial susceptibility was tested using the Kirby-Bauer disc diffusion method against 12 antimicrobial agents representing various antibiotic classes. Genetic markers that are

known to confer tolerance against heavy metals and biocides such as *pcoA, czcD*, and *qacEΔ1* that encode for Cu^{2+}, Zn^{2+} and quaternary ammonium compounds (QAC) tolerance, respectively, were amplified. In the present study, the isolates of faecal origin were found more likely to be Cu^{2+} tolerant than those of feed origin (OR, 27.0; 95% CI, 2.8 to 250; $P = 0.0042$) or environmental origin (OR, 5.8; 95% CI, 3.1 to 11.1, $P = 0.0002$), suggesting the significance of gastrointestinal ecosystem and the role it may play as facilitating co-selective pressure. The PCR results on the level of *czcD* gene carriage by *Salmonella* isolates were concordant with the increase in the level of the Zn^{2+} MIC of *Salmonella* isolates (OR, 5.2; 95% CI, 2.4 to 11.5, $P < 0.0001$). Isolates with high zinc tolerance were more likely to carry the *czcD*, the zinc tolerance gene. The PCR results on the level of *pcoA* gene carriage by *Salmonella* isolates were also concordant with the increase in the level of the Cu^{2+} MIC of *Salmonella* isolates (OR, 5.83; 95% CI, 3.07 to 11.09, $P < 0.0001$), indicating that isolates with high copper tolerance were more likely to carry the *pcoA*, the copper tolerance gene.

Salmonella isolates were detected from 13.9% and 6.7% of environmental drag swab samples at pre- and post-disinfection, and from 17.2% and 7.1% of the faecal samples from early and late finishing pigs, respectively. Barn-level prevalence of antimicrobial resistance among *Salmonella* isolates detected from floor swabs was 92.3%, whereas, the prevalence of antimicrobial resistance among *Salmonella* isolates recovered from the faeces was 98.02%. The odds of *qac* genes carriage for *Salmonella* isolates were much higher for MDR *Salmonella* isolates with high MICs of the biocide than non MDR isolates with low MICs. There was a strong association between the level of MDR *Salmonella* isolates and the level of disinfectant resistance genes detected from the MDR *Salmonella* isolates, indicating the involvement of *qacEΔ1* as the one of the MDR efflux pumps

commonly known to extrude a wide range of structurally dissimilar toxic compounds across the bacterial cell membranes.

A cross-sectional design was carried out in a pastoral region of Tanzania with a large population of livestock and a principal meat and milk producer in the country. *Salmonella* isolates were recovered from 4.2% of the total of 1540 samples from apparently healthy animals, animal products, floor swabs and sewage. *Salmonella* isolates detected belonged to two subspecies, consisting of *Salmonella enterica* subsp. *enterica* (95.3%) and *S. enterica* subsp. *salamae* (4.7%). The predominant serotypes were *Salmonella* I 8, 20:i:- (32.8%), *S.* Hadar (10.9%), *S.* Colindale (6.3%), *S.* Anatum (6.3%) and *S.* Heidelberg (6.3%). About one-third of the *Salmonella* isolates were phenotypically resistant to at least one antimicrobial, of which 82.6% were multi-drug resistant (MDR). Two MDR *Salmonella* isolates were found to carry integrons (*intI1*) with gene cassettes *aac(3)-Id-aadA7* and two pansusceptible *Salmonella* isolates were found to carry the non integron-borne resistance genes such as *sul2*, *aadA1* and *blaTEM-1B*, *blaTEM-1A*. The pulsed field-gel electrophoresis (PFGE) DNA fingerprint patterns strongly indicated that the majority of *Salmonella* isolates were clonal.

The occurrence of clonal *Salmonella* isolates in food animals and animal products from agro-pastoral communities indicates that animal products are important source of pathogens to the agro-pastoralist communities and public at large. This finding is of great public health concern since consumption of uncooked meat and unpasteurized milk is common in Tanzania. In addition, occurrence of antimicrobial resistance to different classes of antibiotics has led to emergence of MDR *Salmonella* isolates which are becoming a serious public health menace to the contemporary world. Therefore, the

current study recommends a coordinated regular surveillances and monitoring on the use and sale of antimicrobials particularly in Tanzania. The laws and bylaws regarding the stocking, distribution and dispensing of the antimicrobials should be strengthened and enforced.

DECLARATION

I, **Julius John**, do hereby declare to the Senate of Sokoine University of Agriculture that, this thesis is my own original work done within the period of registration and that it has neither been submitted nor concurrently being submitted in any other institution.

_____ _____

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The above declaration is confirmed by

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(Supervisor)

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Prof. Wondwossen A. Gebreyes Date

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DEDICATION

This thesis is dedicated to my beloved wife, Margareth, my sons, John and Jasonpeter and my whole family, including my father John Medardus and my mother Crispina Sareá. Thank you for being part of this achievement.

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LIST OF ABBREVIATIONS

Am	Ampicillin
An	Amikacin
Ax	Amoxicillin-clavulanic acid
Ce	Ceftriaxone
Cp	Cephalothin
CIP	Ciprofloxacin
Cl	Chloramphenicol
Gm	Gentamycin
Km	Kanamycin
S	Streptomycin
Su	Sulfisoxazole
Te	Tetracycline
TMP	Trimethoprim
XLN	Ceftiofur
rRNA	Ribosomal ribonucleic acid
tRNA	Transfer ribonucleic acid
DNA	Deoxyribonucleic acid
DDH	DNA-DNA hybridization
NTS	Non-typhoidal *Salmonella*
TTSS	Type three secretion systems
MDR	Multi-drug resistant
MIC	Minimum inhibitory concentration
SPIs	*Salmonella* pathogenicity islands

SGI-1	*Salmonella* genomic island-1
QAC	Quaternary ammonium compounds
PBPs	Penicillin-binding proteins
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
MLST	Multi-locus sequence typing
QRDR	Quinolone resistance determining region
PMQR	Plasmid mediated quinolone resistance
FAE	Follicle-associated epithelium
GALT	Gut-associated lymphoid tissues
SCV	*Salmonella*-containing vacuole
U.S.	United States
CDC	Centers for Disease Control and Prevention
WHO	World Health Organization
%	Percentage
µl	Microlitres
bp	Base pairs

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Salmonella species are enteric organisms that are widely distributed in nature (Barros *et al*., 2015). These organisms can reside as commensals in the gastrointestinal tracts of humans and animals (Newell *et al*., 2010). *Salmonella* spp. infection is established through a multiple of factors, including: bacterial epithelial adhesion, invasion, replication, and host defense mechanism suppression (Lopez *et al*., 2012). Other infection sites apart from the gastrointestinal tracts include: vascular structures, bones, meninges of the brain and spinal cord, or other localized sites (Kedzierska *et al*., 2008). *Salmonella* spp. are recognised as one of the major bacterial foodborne pathogens of zoonotic potential worldwide (Tadee *et al*., 2015), including such industrialized regions as the European Union (EU), where it is the most common cause of foodborne outbreaks (Forshell and Wierup, 2006). It is the second most reported zoonotic pathogen in the EU (EFSA, 2014).

Salmonella enterica is an enteric, ubiquitous, intracellular facultative anaerobe of worldwide importance causing a disease called salmonellosis at approximately 1.3 billion cases of disease annually (Coburn *et al*., 2007). *S. enterica* is typically orally acquired pathogen that cause disease whose manifestations depend on both host susceptibility and the infectious *S. enterica* serotype (Fierer and Guiney, 2001). Salmonellosis can cause significant morbidity and mortality in humans and animals and has a substantial global socio-economic impact (Coburn *et al*., 2007). In human, *S. enterica* causes two forms of a disease, namely, typhoidal and non-typhoidal salmonellosis (Coburn *et al*., 2007). The incidence of typhoid fever in low-income and middle-income countries, particularly in

Africa, Asia, Latin America and the Caribbean is very high because of ingestion of human excreta contaminated water and food (Crump *et al*., 2004; Mogasale *et al*., 2014).

S. enterica infection is one of the leading causes of foodborne illness in the United States (Mead *et al*., 1999) and has been estimated to cause 1.4 million cases of human salmonellosis resulting in 16,430 hospitalizations with almost 600 deaths each year (Voetsch *et al*., 2004). Non-typhoidal *Salmonella* [NTS] isolates account for an estimated 27% of all foodborne illnesses caused by known bacterial agents with the majority of human salmonellosis cases related to the consumption of the contaminated food products (Hald *et al*., 2004). The increase in NTS foodborne illness cases each year in the high-income countries such as the United States is perhaps due to overwhelming industrialization and mass food production, decreased trade barriers and human migration that are more frequently incriminated for increased incidence and severity of foodborne diseases worldwide (Galanis *et al*., 2006; Mogasale *et al*., 2014). Most individuals suffering from non-typhoidal salmonellosis experience mild gastrointestinal illness, which involve: diarrhoea, fever, chills, abdominal cramps, nausea and vomiting. NTS infections are usually self-limiting gastroenteritis, and therefore, antimicrobial treatment is not recommended for uncomplicated illnesses (Fierer and Swancutt, 2000; Gill and Hamer, 2001). Antimicrobial treatment is required essentially for enteric fever, invasive salmonellosis and in patients at risk of extra-intestinal illnesses, such as meningitis and osteomyelitis (Molyneux *et al*., 2009).

Antimicrobials such as chloramphenicol, ampicillin, and trimethoprim-sulphamethoxazole were drugs of choice for many years, until the recent years, where the antimicrobial resistance of *S. enterica* to commonly used antimicrobials has become a

matter of concern worldwide (Hammad *et al*., 2011). Of particular concern are those strains that have acquired multi-drug resistance against two or more antimicrobial agents (Lindsey *et al*., 2009) or drugs used as first line of defense in invasive systemic infections. Although fluoroquinolones such as ciprofloxacin and ofloxacin, and extended spectrum cephalosporins, including ceftriaxone and cefotaxime, were used as the alternatives, their usefulness have dwindled because of emergence of antimicrobial resistance due to extended use of antimicrobials in the various settings (Hammad *et al*., 2011; Rahman *et al*., 2014). Based on the socio-economic impact and the public health concern of salmonellosis, there is a worldwide interest to contain *Salmonella* foodborne infections (Forshell and Wierup, 2006). So, in order to track *Salmonella* infections and disrupt epidemic spread, many countries have established extensive surveillance systems (Sabat *et al*., 2013). A number of various phenotypic and genotypic methods have been developed to detect, track and distinguish *Salmonella* isolates from each other, so as to understand their epidemiology in animals, humans and their environment. Thus, typing to the strain level has been an important tool in surveillance and outbreak investigation of *Salmonella* infections (Liebana, 2002).

1.2 Literature Review

1.2.1 Historical perspective of *Salmonella*

The history of *Salmonella* isolation dated back to 1886 when Theobald Smith isolated the bacillus which he claimed was the cause of hog cholera. However, it was later shown that hog cholera was a viral infection and the bacillus [currently known as *S. enterica* serotype Choleraesuis] formally discovered by Smith was later reported as a secondary invader in the host (Salmon and Smith, 1886; Schltz, 2008). Although *Salmonella* was isolated from the study pioneered by Smith, but it was named after Daniel E. Salmon who was the

immediate Smith's supervisor in the Veterinary Division at the United States Department of Agriculture (USDA). So, it was Smith who carried out the study in the early application of Koch's postulates, a study which enlightened on the discovery of the Genus *Salmonella*. *Salmonella* is now a well recognized bacterial pathogen that causes foodborne illness worldwide. To date, there are over 2,600 *Salmonella* serotypes that are regarded as capable of causing salmonellosis in humans (Guibourdenche *et al*., 2010). Over the years, non-typhoidal salmonellosis remained to be one of the most frequently reported foodborne illness worldwide (WHO, 2002).

1.2.2 General characteristics, classification and nomenclature of *Salmonella*

Salmonella spp. are ubiquitous enteropathogens which commonly reside as commensals in the gastrointestinal tracts of humans, livestock, wild animals, reptiles, birds, and insects. *Salmonella* spp. are Gram-negative, non-spore forming, and facultative anaerobes, ranging 0.7 to 1.5 x 2 to 5 μm in size. They are rod shaped bacilli that belong to family Enterobacteriaceae of the class γ- Proteobacteria in the kingdom Bacteria (Euzeby, 1999).

Table 1.1: Classification of the *Salmonella*

Domain	**Bacteria**
Kingdom:	Eubacteria
Phylum:	Proteobacteria
Class:	γ-Proteobacteria
Order:	Enterobacteriales
Family:	Enterobacteriaceae
Genus:	*Salmonella*
Species:	*Salmonella enterica* and *Salmonella bongori*
Subspecies:	*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*.

Source: (Guibourdenche *et al*., 2010)

Classification (Table 1.1) and nomenclature of *Salmonella* has evolved over the years. The World Health Organization [WHO] and WHO Collaborating Centre hold the right to

define and maintain the antigenic formulae of *Salmonella* serotypes and list new serotypes in annual updates of the Kauffmann-White scheme (Popoff *et al*., 2000), however, the communication between scientists, health officials, and the public, has never been an easy task. As a result of the complex nomenclature of *Salmonella*, the scientists used different systems to refer to and communicate about the genus *Salmonella*. To overcome the scenario, the uniformity in *Salmonella* nomenclature was emphasized for communication between scientific communities. Since then, *Salmonella* nomenclature evolved from the scheme initially proposed that based on the serologic identification of somatic [O] and flagella [H] antigens (Kauffmann and Edwards. 1952; Kauffman, 1966). It was until 1973, when Crosa *et al*. (1973) demonstrated by DNA-DNA hybridization [DDH] that all serotypes and subgenera I, II, and IV of *Salmonella* and all serotypes of "Arizona" were related at the species level; thus, they belonged to a single species. This splendid work marked a defining development in *Salmonella* taxonomy and formed basis for further studies as a result of the DDH drawbacks, bacterial taxonomists actively researched on alternative methods that can replace DDH experiments (Gevers *et al*., 2005; Goris *et al*., 2007).

Currently, the nomenclature and classification of the members of genus *Salmonella* have changed and restructured. Traditionally, *Salmonella* spp. were named in accordance with the Kaufmann-White typing scheme as defined by different combinations of somatic [O], flagellar [H] and capsular [Vi] antigens. It was in 2005, *S. enterica* gained its official approval as the type species of the Genus *Salmonella* (Su and Chi, 2007). The Genus *Salmonella* comprises two species namely; *S. enterica* and *S. bongori*. *S. enterica* is further subdivided into six subspecies and five subgenera, namely, *S. enterica* subspecies [subspp.] *enterica* (I), *S. enterica* subspp. *salamae* (II), *S. enterica* subspp. *arizonae* (IIIa),

S. enterica subspp. *diarizonae* (IIIb), *S. enterica* subspp. *houtenae* (IV) and *S. enterica* subspp. *indica* (VI) [Table 1.2]. *S. enterica* have serologically defined names appended as serotypes, for instance, the current nomenclature of *Salmonella* Typhi is *S. enterica* subspecies enterica serotype Typhi (*S.* Typhi). To date, more than 2600 serotypes of *S. enterica* have been described (Guibourdenche *et al*., 2010). Certain serotypes of *Salmonella*, such as *S.* Typhi and *S.* Paratyphi A, B, and C, are host-restricted [host specialist], while the rest of *Salmonella* spp. have a broad host range [host generalist] (Brenner *et al*., 2000). Serotypes of *S. enterica*, other than *S.* Typhi, and *S.* Paratyphi, are also restricted to as NTS and they cause systemic infections in humans all over the word (Herikstad *et al*., 2002). *Salmonella enterica* subspp. *enterica* (subspp. I) is responsible for over 99.5% of the infection in humans and animals (McClelland *et al*., 2001). Although the Genus *Salmonella* includes over 2600 serotypes, only three serotypes; *S. enterica* subspp. *enterica* serotype Enteritidis (*S.* Enteritidis), *S. enterica* subspp. *enterica* serotype Newport (*S.* Newport) and *S. enterica* subspecies *enterica* serotype Typhimurium (*S.* Typhimurium), were responsible for about half of all human infections in the United States (CDC, 2011). *Salmonella* Enteritidis is the most common *Salmonella* serotype in humans especially in Europe, where it accounts for 85% of *Salmonella* cases (Galanis *et al*., 2006).

Table 1.2: Current *Salmonella* nomenclature

Genus	Species	Subspecies	No. of serotypes in each subspecies
Salmonella	*enterica*	enterica (subspecies I)	1504
		salamae (or subspecies II)	502
		arizonae (or subspecies IIIa)	95
		diarizonae (or subspecies IIIb)	333
		houtenae (or subspecies IV)	72
		indica (or subspecies VI)	13
	bongori	subspecies V	22

Source: Su and Chiu (2007)

1.2.3 *Salmonella* antigenic structures

Most of *Salmonella* spp. poses peritrichous flagella and hence they are motile except *S.* Gallinarum-pullorum. *Salmonella* spp. are non-capsulated except *S.* Typhi, *S.* Paratyphi C and some strain of *S*. Dublin (WHO, 2003). *Salmonella* are defined mainly by two sets of antigens, namely, somatic (O) and flagella (H) antigens. The other sets of *Salmonella* antigens which can be demonstrated by serological reactions include: virulence (Vi), the mucus (M), and the fimbrial (F) antigens (Old, 1996). The somatic antigens represent the side-chains of repeating sugar units projecting outwards from the lipopolysaccharide (LPS) O-antigen layer on the surface of the bacterial cell wall. The somatic (O) antigens are unaffected by heating (heat stable) for 2.5 hours at 100^{o}C), and are alcohol stable withstanding treatment in 96% ethanol at 37^{o}C (Old, 1996). Flagellar (H) antigens are formed from structural proteins, which make up the flagella that provide basis for motility. They are heat labile. Heating at a temperature above 60^{o}C can detach the flagella from the bacterial cell. *Salmonella* unlike other members of the family *Enterobacteriaceae*, have two distinct phases of H antigens, the phase 1 (protein product of the *fli*C gene) and phase 2 (protein product of the *fljB* gene), both are coordinately regulated (Smith and Selander, 1991). On the basis of the two H antigens (H1 and H2), *Salmonella* can be separated into monophasic and biphasic based on whether they express only one (H1) or both flagellar antigens (H1 and H2). Existence of O, H1 and H2 antigens is of great importance mainly in classical *Salmonella* serotyping which requires implementing of a battery of O and H antisera and a rapid multiplex polymerase chain reaction (PCR)- based typing scheme to screen for the prevalent *S. enterica* serotypes (Hong *et al*., 2008). The antigenic composition of the O, H1 and H2 antigens is a reflection of their unique DNA sequence alleles (Samuel and Reeves, 2003).

1.2.4 Salmonellosis

Genus *Salmonella* contains as many as over 2,600 serotypes, including *S. enterica* serotypes such as *S.* Typhi, *S.* Paratyphi, *S.* Typhimurium, *S.* Enteritidis, *S.* Newport, *S.* Infantis, *S.* Heidelberg and *S.* Virchow (Guibourdenche *et al*., 2010). *Salmonella* can infect also plants (Que *et al*., 2013). *Salmonella enterica* infection in the human host is commonly associated with *S. enterica* subspecies *enterica* (also termed subspecies I). Salmonellosis may occur as an acute, self-limiting gastroenteritis and or as systemic infection causing severe illness in children less than five years of age, the elderly and immuno-compromised individuals (DuPont, 2009). An acute salmonellosis can be manifested through one of the four different forms: enteric fever, gastro-enteritis, bacteraemia, and extra-intestinal focal infections. Bacteraemia complicates infection in approximately 8% of normal healthy persons (Fhogartaigh and Edgeworth, 2009; Fhogartaigh and Dance, 2013). As for other infectious diseases, the course and outcome of an interaction between *Salmonella* (pathogen) and its host depends on factors including the *Salmonella* serotype involved, host species, infecting dose, immunologic competence of the host, and gut flora (Cammie and Miller, 2000).

Salmonella spp. causing human infections can be subdivided into two groups: the enteric fever (typhoidal) and non-typhoidal salmonellosis. There are four serotypes of *S. enterica* associated with enteric fever (typhoid salmonellosis), and these include: *S. enterica* subspp. *enterica* serotype Typhi (*S.* Typhi), *S.* Paratyphi A, *S.* Paratyphi B, and *S.* Paratyphi C (Dougan *et al*., 2011). Typhoidal salmonellosis is characterised by systemic illnesses, whereas the non-typhoidal salmonellosis is a gut-associated gastroenteritis. NTS serotypes are capable of infecting different animals and such serotypes are referred to as promiscuous. These serotypes of NTS that can cause diseases to animals as well as to

humans are referred to as serotypes of zoonotic potential to public health. One of the important serotypes of some of the more promiscuous serotypes, such as *S*. Typhimurium has recently been implicated as a major cause of invasive NTS infections in humans in low and high-resource countries worldwide (Dougan *et al*., 2011; Feasey *et al*., 2012). Gastroenteritis due to NTS in human may be characterised with fever, nausea, abdominal pain and vomiting, whereas, the early symptoms of enteric fever are often indistinguishable, and these may include anorexia, severe headache, fever, and constipation (Parry *et al*., 2002). Enteric fever requires early medical intervention, and a delayed intervention may lead to serious complication such as haemorrhages from abdominal ulcers. Usually perforation of the Peyer's patches can cause generalized peritonitis and septicaemias, which are often the commonest cause of death in typhoid fever (Everest *et al*., 2001).

1.2.5 Pathogenesis of Salmonellosis

Salmonella infection process involves: bacterial adhesion, invasion, *Salmonella*-containing vacuole [SCV] maturation and replication. However, for all processes of infection to take place in the host, an invading pathogen must overcome the defense mechanisms of the host so as to develop an intracellular lifestyle (Lopez *et al*., 2012). All *Salmonella* infections usually results from ingestion of contaminated water, food or contact with a carrier (Kidgell *et al*., 2002). As *Salmonella* enter and move through the gastrointestinal tract of the host, it encounters changes in chemical and physical properties, such as temperature, pH, osmolarity, and nutrient availability (Ohl and Miller, 2001). *Salmonella* infections do not occur if the pathogens do not survive the gastric acidity which presents a significant initial barrier to infection by altering the bacterial infective dose. Gastric acidity is an important defense mechanism of the host to limit pathogen from reaching the

small intestine (Giannella *et al*., 1972). As it enters a host stomach, *Salmonella* senses the changing pH of the environment. A study by Ohl and Miller, (2001) has suggested that upon entry to the stomach, *Salmonella* exhibit an adaptive acid tolerance response [ATR] as it is exposed to low pH. After leaving the stomach, *Salmonella* traverses the mucosal layer overlaying the epithelium of the small bowel and adhere to epithelial cells of the small intestine. *Salmonella* spp. infection is only initiated when the invasive organisms passes through the epithelial surface of the small bowel and overcomes all associated host factors (Jones *et al*., 1994).

The invasive *Salmonella* strains initiate infection in mammalian hosts by penetrating the intestinal epithelium of the small bowel, selectively invading the microfold cells (M-cells) and move to access gut-associated lymphoid tissues [GALT]. The GALT is defined as the largest collection of lymphoid tissues in the body, consisting of both organized lymphoid tissues, such as mesenteric lymph nodes and Peyer's patches and more diffusely scattered lymphocytes in the intestinal lamina propria. The invading *Salmonella* preferentially interact with aggregates of lymphoid follicles making up the GALT. These collections of lymphoid follicles along the length of gastrointestinal tract is known as the Peyer's patches (Wood *et al*., 1998; Forchielli and Walker, 2005), and induce membrane ruffling within the follicle-associated epithelium (FAE) of the Peyer's patches. (Fhogartaigh and Dance, 2013).

Briefly, *Salmonella* spp. entry to the M-cells results to extrusion and death of these cells, and followed by elimination of the M-cells. Finally, the invading bacteria either move laterally along the basal lamina or deeper into the dome of the follicle. M-cells are specialized epithelial cells that are found exclusively in lymphoid FAE (Owen and Jones,

1974; Jepson and Clark, 2001). They form tight junctions with adjacent enterocytes. In addition, M-cells possess a flexible cytoskeletal structure that allows lymphoid cells migration towards the epithelial cells to deform their cytoplasm, and actively take up the invading bacteria. The pinocytosized materials including the invading bacteria from the lumen of the intestine are transported from the apical to the basolateral surface of the cell where they are delivered to underlying lymphoid cells (Fujimura, 1986; Pappo and Ermak, 1989). M-cells are sites for internalization and transport of *Salmonella* spp. to the underlying lymphoid tissue (Jepson and Clark, 2001; Parry *et al.*, 2002). After the organisms are internalized and transported into sub mucosal lymphoid tissue, where they migrate within infected macrophages to intestinal lymph nodes, and penetrate through lymphatics to infect the systemic reticulo-endothelial system, particularly, spleen, liver and bone marrow, and gall bladder (Fhogartaigh and Dance, 2013). Migration of infected macrophages to the organs of reticulo-endothelial systems facilitates the dissemination of bacteria in the host. After penetration of the reticulo-endothelial system, the invading *Salmonella* spp. are displaced to the intestinal lymphoid follicles and the draining mesenteric lymph nodes. Gall bladder as one of the commonest sites for secondary infection of *S*. Typhi facilitate to excrete the microorganisms in the bile where they may either reinvade the intestinal wall or shed out in the faeces (Parry *et al*., 2002).

1.2.6 Pathogenicity and virulence genes of *Salmonella*

Salmonella spp. have selective potentials to invade and persist in host cells and they are well adapted to intracellular lifestyle (Lopez *et al*., 2012). Their ability to colonize macrophages and other immune cells is considered vital in establishing the infection in the host. This property has been genetically linked to virulence as mutants are unable to survive in such cells; in general, and they have a reduced or no ability of causing infection

(Fields *et al*., 1986). Previous studies have reported on a large number of genes encoding virulence factors and the role they play in *Salmonella* pathogenesis. Most of these genes are in close contact to each other in the bacteria genome. These genes required for *Salmonella* virulence are found on the chromosome and on plasmids common to many *Salmonella* serotypes. Most are encoded within pathogenicity islands (Groisman and Ochman, 1996).

The genome of *S. enterica* is reported to harbor over 100 essential genes that have been implicated for *Salmonella* virulence (McClelland *et al*., 2001). These virulence-associated genes can be horizontally transmitted between *S. enterica* spp., and a number of regions (loci) harboring multiple virulence (pathogenicity genes) called Pathogenicity Islands (PIs) have been reported in *Salmonella* spp. *Salmonella* pathogenicity islands are defined as large gene cassettes within the *Salmonella* chromosome and the plasmid that encode for genetic determinants responsible for establishing specific interactions between the host and *Salmonella* spp. *Salmonella* pathogenicity islands (SPIs) are acquired by horizontal transfer from phages or plasmids and they are highly conserved between the different *Salmonella* serotypes. The SPIs are located adjacent to tRNA genes (Marcus *et al*., 2000; Schmidt and Hensel, 2004).

A total of 21 SPIs, namely, SPI-1 to SPI-21 have been identified from *Salmonella*, although the major SPIs include SPI-1, SPI-2, SPI- 3, SPI-4 and SPI-5. The SPI-1 and SPI-2 genes are the most extensively studied than other SPIs in the group, and they function to code for proteins forming the type three secretion system (TTSS) which enable the transport of *S. enterica* proteins from the bacterial cell directly into the host cells (Schmidt and Hensel, 2004; Rychlik *et al*., 2009). Generally speaking, the two large SPIs,

namely, SPI-1 and SPI-2, encode for TTSS that have a central role during *Salmonella* pathogenesis, which involves invasion and intracellular accumulation. The SPI-1 is required for the initial stages of salmonellosis, including: the entry of *Salmonella* into non-phagocytic cells by triggering invasion and the penetration of the gastrointestinal epithelium, and also SPI-1 is required for the onset of diarrhoeal symptoms during localised gastrointestinal infections (Galan, 2001; Schmidt and Hensel, 2004).

The SPI-1-associated proteins include the effector proteins such as Sop (SopA–E); proteins associated with invasion, *SipA* and *InvA*; translocon assembly protein including: *SipD* and flagella associated proteins, *FlgK*, *FljB* and *FlgL*. On the other hand, the SPI-2 is required for later stages of the infection, including: systemic spread, proliferation and the colonisation within host organs. The role of SPI-2 for survival and replication in host phagocytes appears to be essential for pathogenesis. So, pathogenesis of *S. enterica* is facilitated by a TTSS encoded by genes of SPI-2. The SPI-2-associated proteins, *SsaR* and *SifA*, are associated with survival and replication within the host cells (Figueira and Holden, 2012; Zou *et al*., 2012). In addition, there are virulence-associated plasmids that have the spv operon, which consists of five genes, namely, spvRABCD, associated with *Salmonella* survival and growth in macrophages (Rychlík *et al*., 2006).

Type three secretion systems (TTSS) are specialized organelles of Gram-negative bacterial pathogens (Schmidt and Hensel, 2004). TTSS is a needle shaped structure that spans the inner and outer membranes of the bacterial envelope and secretes translocon (translocation channel) and effector proteins (Mueller *et al*., 2008). Translocon proteins allow entry of effector proteins to the host cell, by either forming pores in the host cell membrane or in some cases act as a connecting channel between the bacterium and the host cell membrane

(Frankel *et al*., 1998; Mueller *et al*., 2008). These effectors proteins control a variety of host cell processes in order to successfully invade epithelial cells and to establish host cellular conducive environment permissive for pathogen replication. Type three secretion systems play a vital role in the *Salmonella*-host interaction. *Salmonella* serotypes possess two types of TTSS, namely, TTSS-1 and TTSS-2, which are encoded in distinct regions of the *Salmonella* chromosome (Hansen-Wester and Hensel, 2001). TTSS-1 is expressed when *Salmonella* are moving along the intestinal lumen before it is first encountered with the host cells, and therefore, it is required for initiating intestinal inflammation (Wallis and Galyov, 2000; Hapfelmeier and Hard, 2005). TTSS-1 triggers invasion of gut epithelial cells, enhances colonization of the lamina propria and spread of *Salmonella* to systemic sites. Upon entry into the host intestinal epithelium, *S. enterica* inhabits a vacuolar compartment of the host cells known as macrophages and dendritic cells. This process requires the TTSS-2, which speed up the maturation of the *Salmonella*-containing vacuole (SCV). The TTSS-2 also help to prevent oxidative killing and facilitates systemic spread of the infection (Kuhle and Hensel, 2004; Cheminay *et al*., 2005).

1.2.7 Epidemiology and distribution of Salmonellosis

1.2.7.1 Typhoid Salmonellosis

In the early 19th century, typhoidal salmonellosis (typhoid fever) was a major cause of morbidity and mortality worldwide, including high-income countries such as the United States and Europe in the 19th century (Kothari *et al*., 2008). However, there was a dramatic decrease in the incidence of typhoid in the high-income because of the provision of clean water and good sewage systems. Typhoid salmonellosis is a life threatening disease caused by a human-restricted typhoidal serotype of *S. enterica*, such as *S*. Typhi, which cause a disease called typhoid fever in human. Paratyphoid fever is another typhoid

fever-like disease, but often less severe, is caused by *S*. Paratyphi A, B, and C. *Salmonella* Typhi is a highly adapted human-specific pathogen that has evolved over 50 000 years, and has various mechanisms for persistence in its animal host (Kidgell *et al*., 2002; Merrell and Falkow, 2004). There is no other animal host apart from human that could develop a clinical disease. A recent study by Katani *et al*. (2015) reported a possible carriage of *S*. Typhi by Indian house crows (*Corvus splendens*) and confirmed the role of these birds as potential carriers of enteric *Salmonella* for humans.

Typhoid fever is a major health problem, especially in low-income and middle-income countries worldwide, where there is substandard water supply and lack of sanitation (Crump and Mintz 2010; Mogasale *et al*., 2014). Typhoid fever is a global health problem and its actual impact is difficult to estimate because of the similar clinical presentation to those of many other febrile infections. In recent studies, the incidence of typhoid fever was estimated to be more than 22 million illnesses each year with more than 200 000 deaths (WHO, 2003), and in another similar study, about 217,000 deaths due to typhoid fever were reported for 21.7 million illnesses, whereas, paratyphoid fever alone, caused an estimated 5.4 million illnesses worldwide (Crump *et al*., 2004; Bhan *et al*., 2005). During this time, the greatest burden of illnesses was observed mainly in infants, children, and adolescents in south-central and south-eastern Asia (Crump *et al*., 2004).

Typhoid and paratyphoid fever, most often present a clinically similar acute febrile illnesses, and accurate diagnosis due to the two infections in humans relies on laboratory confirmation (Crump *et al*., 2003). The two febrile enteric illnesses may co-infect individuals (Tankhiwale *et al*., 2003). Although *S*. Typhi and *S*. Paratyphi can be cultured in the laboratories, however, often no reliable data is available from which to estimate the

burden of disease in the low and middle-income countries. The main reason is that many hospitals lack facilities for blood culture and up to 90% of patients with fever are treated as outpatients (Kothari *et al*., 2008). Human acquisition of *S*. Typhi and *S*. Paratyphi A, B and C, most often occurs by ingestion of food or water contaminated with human excreta as a result of poor sanitation and hygiene in crowded and impoverished populations in disease endemic countries, particularly in Asia, Africa, Latin America and Caribbean regions (Miller *et al*., 2000; Mogasale *et al*., 2014). According to a recent web-based surveillance from 2000 to 2002, a total of five serotypes were reported among the 15 most common human serotypes from all geopolitical continents (Africa, Asia, Latin America and the Caribbean, Europe, North America, and Oceania). The top five serotypes reported were: *S*. Enteritidis, *S*. Typhimurium, *S*. Typhi, *S*. Infantis, and *S*. Montevideo, and in Latin America and the Caribbean, S. Typhi accounted for the greatest proportion of *Salmonella* spp. (13%), while in Africa, *S*. Typhi is accounted the third most common serotypes after *S*. Enteritidis and *S*. Typhimurium. *Salmonella* Typhi is also a pathogen of concern in the developing world, especially Asia. It was reported to be the ninth most frequent serotype in Asia in 2002 (Galanis *et al*., 2006).

In 1995, *S*. Typhi was the sixth most frequent serotype globally, and the trend of isolating this human enteric pathogen decreased significantly and it was ranked 14th in 2002. This significant decrease in the incidence of *S*. Typhi globally, can be explained by the fact that S. Typhi lacks animal reservoir, which makes it susceptible as a result of improvements in hygiene and sanitation as observed in many regions of the world, such as Latin America and the Caribbean (Herikstad *et al*., 2002; Galanis *et al*., 2006). Typhoid endemic countries are characterised by rapid population growth, increased urbanisation, inadequate waste treatment, limited water supply, and over-burdened health care systems. Typhoid

fever is also reported in high-resource countries, but it is predominantly related to the history of traveling to disease endemic regions of the world (Steinberg *et al*., 2004). The risk of transmission of the organisms to laboratory workers was previously reported (Blaser *et al*., 1980). The overall incidence of *Salmonella* infections in Great Britain was reported to be 0.137 per 1,000 persons and most of the persons affected were the microbiologists (Sewell, 1995). It should be noted that the main mode of transmission of typhoidal salmonellosis occurs by ingestion of food or water contaminated with human faeces [excreta] (Kothari *et al*., 2008), although the contamination could also come from the animal faeces, such as Indian house cows (Katani *et al*., 2015). Other established risk factors include recent contact with a typhoid patient or a carrier individual, eating contaminated raw fruits and vegetables grown in fields fertilised with sewage (Bhan *et al*., 2005).

1.2.7.2 Non-typhoidal Salmonellosis

Salmonella serovars other than those causing typhoid or typhoid-like in humans are referred to as Non-typhoidal *Salmonella* (NTS). This group of serovars, for example, *Salmonella* serovar Typhimurium and *Salmonella* serovar Enteritidis, can infect a wide range of hosts, from insects to reptiles, birds and mammals. These serovars are of high importance with respect to their epidemiology as they have developed mechanisms to invade different hosts without any greater resistance. Thus, these *Salmonella* serovars can pose a greater zoonotic potential to the public health (Singh, 2013).

The global incidence of foodborne infections has markedly increased, with nearly a quarter of the population at a high risk of illnesses (Oliver *et al*., 2005). Foodborne illnesses occur ubiquitously along the globe. On a global scale, foodborne and waterborne

diarrhoeal diseases kill about 2.2 million people, including 1.9 million children annually (Min and Hussain, 2014). According to Mead *et al*. (1999), about 76 million cases of foodborne illnesses resulting in 325,000 hospitalisations and 5000 deaths, are estimated to occur annually in the United States. In 2010, the overall incidence of laboratory confirmed *Salmonella* infection was found to be 17.6 cases per 100,000 persons, more than twice the U.S. Healthy People 2010 objective of 6.8 cases per 100,000 persons (Matyas *et al*., 2010). Population-based, active laboratory surveillance in the U.S, reported that *Salmonella* infection caused by NTS is the most common foodborne disease (CDC, 2011). The prevalence of NTS in humans in Tanzania is estimated to range from 7.6-28% (Mtove *et al*., 2010; Meremo *et al*., 2012).

Non-typhoidal *Salmonella* (NTS), an aetiological agent of non-typhoidal salmonellosis, is considered as one of the three top foodborne bacterial pathogens worldwide (Newell *et al*., 2010). Whereas non-typhoidal salmonellosis is a disease of worldwide distribution; in contrast, the typhoidal salmonellosis is vastly distributed in low-income countries than the high-income countries (Tassew *et al*., 2010; Mogasale *et al*., 2014). The NTS is one of the most important foodborne pathogens of public health significance (Scallan *et al*., 2011). The NTS can colonize all warm and cold-blooded animas, including humans, and in addition, can cause most of the zoonotic infections in humans (Yan *et al*., 2004). In one of the hospital settings in Tanzania, colonisation by NTS caused a nosocomial outbreak of neonatal *S*. Enteritidis meningitis that claimed life of two children who were admitted in the hospital with septicaemia and meningitis (Vaagland *et al*., 2004).

Most of non-typhoidal salmonelloses in humans are associated with various serotypes of *S. enterica* subspp. *enterica* (McClelland *et al*., 2001). The NTS serotypes have broad host

range including food animals and humans (Weinberger and Keller, 2005). Of the NTS serotypes, *S*. Enteritidis and *S*. Typhimurium are the most common serotypes with worldwide distribution (Brent *et al*., 2006), and in a two year [2000/02] web-based surveillance study, *S*. Enteritidis was found to be the highly occurring serotype in human *Salmonella* isolates, whereas, *S*. Typhimurium was the dominating serotype in non human isolates (Galanis *et al*., 2006). Food of animal origin is identified as the main vehicle of transmission of public health important NTS serotypes to humans (Li *et al*., 2013; Ahmed and Shimamoto, 2014). In addition, food animals with subclinical infection constitute a vast reservoir for disease (Gomez, *et al*., 1997). So, human salmonellosis is mostly foodborne and is contracted through consumption of contaminated food of animal origin such as meat, milk, poultry and eggs (Thong and Modarressi, 2011).

Non-typhoidal salmonellosis in humans may occur as an acute, self-limiting gastroenteritis or as systemic infection characterized by septicaemia and extraintestinal focal infections. The gastrointestinal form is often referred to as food poisoning syndrome (Edgeworth, 2005; DuPont, 2009). The course and outcome of the infection in humans are dependent on a variety of factors including the immune status of the host, inoculating dose, and genetic background of both host and infecting organism (Cammie and Miller, 2000). All *Salmonella* serotypes are presumably pathogenic to humans **(**Forshell and Wierup, 2006). Children, the elderly and immunocompromised individuals are the high risk groups with case fatality rates of 38% in children (Walsh *et al*., 2000) and 47% in the elderly (Gordon *et al*., 2002) were recorded.

Human non-typhoidal salmonellosis disease syndromes range from asymptomatic colonization to severe extra-intestinal illnesses, such as meningitis, septic arthritis and

osteomyelitis (Graham *et al*., 2000). The high case fatality of extra-intestinal non-typhoidal salmonellosis with meningitis is reported in Malawians as 52% in children and 80% in adults (Molyneux *et al*., 2009), and a 100% case fatality in children in Tanzania (Vaagland *et al*., 2004). In the vast regions of the sub-Saharan Africa, the invasive NTS serotypes are important public health problem since they cause the community-acquired bloodstream infections, which also accounted for hospital admissions in the region. The invasive NTS is the second-most common pathogens causing bacterial bloodstream infections in the elderly, HIV/AIDS individuals and children in sub-Saharan Africa (Graham, 2002; Sigauque *et al*., 2009).

In rural Kenya, the estimated incidence of invasive NTS bacteraemia was 505 cases per 100 000 person-years in children under five years of age, of which 88 cases per 100 000 person-years (Morpeth *et al*., 2009), whereas, in a rural setting of Mozambique, the incidence of childhood bacteraemia was 425 cases per 100 000 person-years among children under 15 years of age, and within this age category, NTS incidence accounted for 120 cases per 100 000 person-years (Sigauque *et al*., 2009). Moreover, another study conducted in sub-Saharan Africa by Kariuki *et al*. (2002) reported the multi-drug resistant (MDR) *S.* Typhimurium as the most predominant cause of bacteraemia in children. Also, other previous studies conducted in Africa suggested an ecological association between the invasive NTS and malarial parasites (Bejon *et al*., 2008; Lusingu *et al*., 2010) and between invasive NTS and schistosomal parasites (Feasey *et al*., 2012). This ecological association suggests that any strategies that control malaria and schistosomiasis will consequently decrease the rates of invasive NTS infections. The invasive NTS infections have also been more frequently reported from south-eastern Asian countries (Chen *et al*., 2007). The estimates of the burden of the invasive NTS in the sub-Saharan region of

Africa and the public health implications are uncertain like the other developing regions of the word (Stevens *et al*., 2006). The infections with other enteric bacterial pathogens such as *Campylobacter* spp. and *Escherichia coli*, are often indistinguishable from the *Salmonella* gastroenteritis and hence failure to estimate the burden of the NTS or other enteric pathogens if laboratory options are not considered.

1.2.8 Identification of *Salmonella*

1.2.8.1 Biochemical identification

The classification of the members of Genus *Salmonella* has been controversial for many years. According to the Kauffmann scheme of classification, *Salmonella* were subdivided into four subgenera, namely, subgenus I, II, III and IV **(**Lindberg and Le Minor, 1984). This subdivision was based on their biochemical characteristics. Later in 1988, Le Minor proposed that the genus *Salmonella* consisted of only a single species, *S*. *enterica* which is divided into subspecies based on a panel of biochemical reactions including dulcitol, lactose, Ortho-Nitrophenyl-β-galactosidase [ONPG], maltose, gelatinase, sorbitol, d-tartrate, mucate, and salicin (Le Minor, 1988). Moreover, there has been much development in classification of the members of Genus *Salmonella*, and more strains of *Salmonella* of different serotypes keep to adding up (Popoff *et al*., 2000). There are several commercial systems for identifying *Salmonella* that are available, ranging from phenotyping and genotyping methods, but some of these methods are often difficult to obtain or too expensive to use in developing countries (Table 1.3 and 1.4). This has therefore, demanded the use of an array of conventional biochemical tests which most of laboratories in low-income and middle-income countries will be able to perform. Though laborious, these biochemical tests have been helpful and remain to be indispensable in diagnosis of *Salmonella* and other bacterial microorganisms (Cheesbrough, 2006).

1.2.8.2 PCR-based method of identification

The food animals and natural environments are the known principal reservoirs of the foodborne pathogens to humans (Winfield *et al*., 2003). The conventional methods used to detect and identify *Salmonella* spp. are laborious, time-consuming, and require a number of biochemical and serological tests to confirm presumptive isolates (Malorny *et al*., 2003b). On the other hand, tests using molecular tools have been useful in reducing the steps and the time needed for the detection, identification, and characterization of specific pathogens (Gallegos-Robles *et al*., 2008). Generally, the conventional methods take longer time, since they give only presumptive results after 3-4 days and definitive results is obtained after 5-6 days (Malorny *et al*., 2003b). *Salmonella* spp. isolation by conventional culture methods, are based on non-selective pre-enrichment followed by selective enrichment and plating on selective and differential agars. Presumptive *Salmonella* colonies are further subjected to biochemical and serological tests (Van Kessel *et al*., 2003; Gebreyes *et al*., 2004) and confirmed with *invA* specific Polymerase chain reaction (PCR) method (Malorny *et al*., 2003a; Joshi and Deshpande, 2010). Besides *Salmonella* spp. confirmation, the *invA* specific PCR method is also used for rapid detection and identification of *Salmonella* spp. (Saleh *et al*., 2005; Eriksson *et al*., 2007). The *invA* gene is also commonly used for rapid quantification of *Salmonella* spp. using a quantitative real-time PCR assay (qPCR) (Csordas *et al*., 2004).

The *invA* gene of *Salmonella* spp. contains sequences unique to this genus and has been proved as a suitable PCR target, with potential diagnostic applications (Shanmugasamy *et al*., 2011). The amplification of *invA* gene has been recognized as a renowned standard for detection of *Salmonella* spp. (Malorny *et al*., 2003a). The *invA* gene encodes a protein of a type three secretion system (TTSS) in the inner membrane of bacteria (Mueller *et al*.,

2008). The protein encoded is responsible for *Salmonella* invasion of the epithelial cells of the host (Darwin and Miller, 1999). Several genes such as *invA* have been studied and were used to detect *Salmonella* spp. from food of animal origin as well as faecal samples (Eriksson and Aspan, 2007). The virulence genes including *invA* (Malorny *et al*., 2003b), *invE* (Feder *et al*., 2001), *himA* (Bej *et al*., 1994), and other potential genes such as fimbriae genes, fimY (Yeh *et al*., 2002), fliC (Aldridge *et al*., 2006), can be used as target genes for PCR amplification of *Salmonella* spp.

Table 1.3: Biochemical tests of *Salmonella* spp.

Genus *Salmonella*	**Urea**	**VP**	**ONPG**	**Lact**	**Man**	**Glu**	**Suc**	**Ox**	**Cit**	**Mot**	**Ind**	**LDC**
Salmonella Typhi	−	−	−	−	+	+	−	−	−	+	−	+
Salmonella Paratyphi A	−	−	−	−	+	+	−	−	−	+	−	−
Salmonella Paratyphi B	−	−	−	−	+	+	−	−	+	+	−	+
Salmonella Paratyphi C	−	−	−	−	+	+	−	−	+	+	−	+
Non-typhoidal *Salmonella*	−	−	−	−	+	+	−	−	+	+	−	+

Source: (Cheesbrough, 2006)

Abbreviations: LDC = Lysine decarboxylase test, VP = Voges-Proskauer, ONPG = Ortho-Nitrophenyl-β-galactosidase, Lact = Lactose, Man = Manitol, Glu = Glucose, Suc = Sucrose, Ox = Oxidase, Cit = Citrate, Mot = Motility, Ind = Indole, + = Positive reaction, - = Negative reaction

Table 1.4: Additional biochemical tests of *Salmonella* spp.

Genus *Salmonella*	TSI				LIA				KIA			
	Slant	**Butt**	**H_2S**	**Gas**	**Slant**	**Butt**	**H_2S**	**Gas**	**Slant**	**Butt**	**H_2S**	**Gas**
Salmonella Typhi	R	Y	*+	+	R	Y	*+	+	R	Y	*+	+
Salmonella Paratyphi A	R	Y	−	−	R	Y	−	−	R	Y	−	−
Salmonella Paratyphi B	R	Y	+	+	R	Y	+	+	R	Y	+	+
Salmonella Paratyphi C	R	Y	+	+	R	Y	+	+	R	Y	+	+
Non-typhoidal *Salmonella*	R	Y	+	+	R	Y	+	+	R	Y	+	+

Source: (Cheesbrough, 2006)

Abbreviations: KIA = Kliger iron agar, TSI = triple sugar iron agar, LIA = Lysine iron agar test, Urea = Urease test, R = Alkaline reaction [red for TSI, LIA and KIA, purple for LIA], Y = Acidic reaction [Yellow for TSI, KIA and LIA], H2S = Blackening due to Hydrogen sulphide, Gas = Gas production, + = Positive reaction, **-** = Negative reaction, *+ = weak positive reaction

1.2.9 Typing of *Salmonella*

Typing is defined as splitting of organisms into useful groupings. The strain typing methods are useful methods that play an important role in understanding infectious disease transmission, tracking and distribution (Abatcha *et al*., 2014). Typing is necessary to determine the distribution of the organism, its association with disease, exclusion of sources, identification of carriers, determination of route of infection and assessment of the efficiency of preventative measures (Aarts *et al*., 2001). However, tracing the source of *Salmonella* foodborne related outbreaks requires substantial effort in terms of studying case histories, tracking potential food sources of the outbreak as well as isolating and

characterizing the causative agent at the point of contamination (Ross and Heuzenroeder, 2008). Thus, *Salmonella* detection requires typing methods to distinguish clinical and non clinical isolates recovered from humans, animals and environment (Botteldoorn *et al*., 2004). A number of typing methods have been used for epidemiological surveillance and the monitoring of foodborne outbreaks (Liebana, 2002; Ross and Heuzenroeder, 2008). The classical typing methods of *Salmonella* spp. are divided into two broad categories, namely, phenotypic and the genotypic methods (Maslow *et al*., 1993).

1.2.9.1 Phenotypic methods

The phenotypic methods are traditional typing methods for discriminating between bacteria from a single species based on phenotypes. The phenotypic methods are those that characterize the product of gene expressions in order to differentiate the *Salmonella* spp. involved. The phenotypic properties since they involve gene expression they are subject to change based on the changes in the growth conditions, spontaneous mutations and growth phase. The examples of the phenotypic properties include bacteriophage types, biochemical characteristics, antigens present on the cell surface of *Salmonella* spp. and the antimicrobial susceptibility profiles (Foley *et al*., 2007; Campioni *et al*., 2012). Phenotypic methods are limited by their low ability to differentiate subtypes within the same species and they have relatively low reproducibility power (Foley *et al*., 2007). In addition, they have low discriminatory power to discriminate related from unrelated *Salmonella* strains (Liebana, 2002).

(a) Serotyping

Over the past years, studies have suggested that antigenic variation could be used to classify *Salmonella* isolates into a diverse serogroups. Classification of *Salmonella* was

subsequently refined over the years and this progression was generally attributed to the Kauffmann-White serotyping scheme, which based on the antigenic structure of the surface lipopolysaccharide [LPS] O-antigen, flagellar proteins [H antigens] and capsular proteins [Vi antigens] (Brenner *et al*., 2000). There are currently over 2,600 *Salmonella* serotypes recognized based on antigenic differences in the LPS O-antigen and flagellar antigens (Popoff *et al*., 2003). Additionally, *Salmonella* can be further separated into monophasic and biphasic based on whether they express only one (H1) or both flagellar antigens (H1 and H2) (Hong *et al*., 2008). Serotyping method is thus, based on the identification of the variable O and H antigens. The antigenic composition of the O, H1 and H2 antigens are a reflection of their unique DNA sequence alleles (Samuel and Reeves, 2003). In this method, a number of commercially available antisera were used to detect different antigenic determinants such as somatic (O), capsular (Vi) and flagellar (H) antigens on the surface of bacterial cell (McQuiston *et al*., 2004). Apart from using a battery of O and H antisera for classical (conventional) *Salmonella* serotyping, the existence of O, H1 and H2 antigens is also helpful in a polymerase chain reaction (PCR)-based rapid diagnosis called multiplex PCR- based typing scheme for screening the prevalent *Salmonella* spp. serotypes (Hong *et al*., 2008). Overall, serotyping method classifies *Salmonella* into distinct species based on their serotypes. Of the two species of *Salmonella*, *S. enterica* and *S.* bongori, over 99% of serotypes are classified as *S. enterica*, and nearly 60% of them belong to *S. enterica* subspp. *enterica* (Brenner *et al*., 2000). Besides its limitation, serotyping method is an important tool widely accepted in differentiating *Salmonella* strains of public health importance. Some of its limitations include use of expensive antisera, time consuming, requires well-trained technicians, and some isolates are not typeable (Abatcha *et al*., 2014).

(b) Phage typing

Phage typing of *Salmonella* is a typing method of differentiating *Salmonella* spp. on the basis of the susceptibility or resistance to lysis by each member of a panel of bacteriophages (Baggesen *et al*., 2010). Phage typing utilizes the selective ability of a bacteriophage to infect certain strains of *Salmonella* and thus enabling to differentiate unique isolates (Yan *et al*., 2004). This typing method is epidemiologically valuable in strain differentiation within a particular *Salmonella* serotype, whereby *Salmonella* strains are separated into different phage types based on their reactivity against a set of serotypes specific typing phages. This technique has been developed for some relevant *Salmonella* serotypes (Nygard *et al*., 2004). In principle, when an appropriate phage receptor is located on the surface of the cell, the phage infects the bacterium and lyses the cell. As a result of bacterial cell lysis, a phage type designation is then assigned to the specific strain of bacteria based upon the array of typing phages that are able to lyse the cells and form plaques in the bacterial lawns (Hald *et al*., 2007; Baggesen *et al*., 2010). The phage type designation assignment requires a person who is much acquainted with interpretation of results and the procedure is not always fully reproducible between laboratories (Ross and Heuzenroeder, 2005). Despites its limitation in the investigation of the same phage-types, phage typing still represents a valuable technique for an initial evaluation of the potential relatedness among *Salmonella* isolates (Hopkins *et al*., 2011).

(c) Antimicrobial Susceptibility Testing

Identification of *Salmonella* in the laboratory usually begins with growth and morphologic observation, followed by biochemical identification and serotyping according to the White-Kaufmann classification scheme (Popoff *et al*., 2001). These conventional methods, however, may call for additional methods that would normally be applied if serotyping

determines relationships between isolates and further differentiation is required. Thus, *Salmonella* serotypes can be further differentiated by antimicrobial susceptibility testing. This typing method is used to characterise *Salmonella* serotypes according to antimicrobial resistance profiles [R-types] towards a panel of antimicrobial agents (Dalla *et al*., 2010). Antimicrobial resistance in many *Salmonella* serotypes is increasing with a number of isolates showing multi-drug resistance (Mathew *et al*., 2007). Antimicrobial susceptibility testing is highly reproducible, but the discriminatory power may not differentiate the host strain, thus, its application to epidemiology is limited. Additionally, antimicrobial susceptibility testing also have relatively poor discriminatory power, because antimicrobial resistance occurrence is under tremendous selective pressure (Castanon, 2007; Dalla *et al*., 2010) in healthcare institutions and often is associated with mobile genetic elements such as transposons and plasmids, which can be lost or acquired over short periods of time (Tenover *et al*., 1997). Besides discriminatory ability, lack of reliability of antimicrobial susceptibility profiles in typing of *Salmonella* spp. is due to the fact that the phenotypic properties involve gene expression and are subject to change based on the changes in the growth conditions and spontaneous point mutations as seen in quinolone-resistant *Salmonella* spp. and other related species (Eaves *et al*., 2004; Randall *et al*., 2005b).

1.2.9.2 Genotypic methods

The genotypic methods are those that are based on the analysis of the genetic structure of *Salmonella* spp. The genotypic properties of *Salmonella* are less subject to variation as compared to phenotypic properties (Tenover *et al*., 1997). The genotypic methods are useful for typing of *Salmonella* strains worldwide, complementing to the phenotypic methods. The use of molecular subtyping techniques for the genetic fingerprinting and

grouping of *Salmonella* are based on their genotypic characteristics (Abatcha *et al*., 2014). The genotypic methods include plasmid profile analysis, ribotyping, repetitive sequence-PCR fingerprinting (REP-PCR), enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), amplified fragment length polymorphism (AFLP), multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) have been shown to distinctively discriminate *Salmonella* strains (Aktas *et al*., 2007; Rivoal *et al*., 2009). PFGE is considered as the gold standard and highly discriminatory method in typing of the foodborne pathogens such as *Salmonella* strains (Almeida *et al*., 2013). So, it is recommended that the ideal technique to assess genetic diversity of *Salmonella* spp. should meet a number of criteria such as discriminatory power, reproducibility of the method, ease of operation, speed, and cost (Lukinmaa *et al*., 2004; Lim *et al*., 2005).

(a) Plasmid profile analysis

A plasmid is an autonomous self-replicating extrachromosomal, covalently closed circular DNA that carries genes that impart selective advantage to a bacterium such as virulence and antimicrobial resistance. Plasmids are not essential for normal bacterial growth and survival. Plasmids can be infectious if their genomes carry antimicrobial resistance and toxin genes. They can be transferred between bacteria of the same or different genera. Plasmid transfer between bacteria requires synthesis of pilli which are encoded by genes on the plasmid. Plasmid profile analysis enables bacterial isolates to be differentiated according to the number and size of their plasmids, ranging from 0 to several plasmids (Dantas and Sommer, 2014; Ranjbar *et al*., 2014).

Salmonella plasmid DNA can be isolated and purified from pure cultures as described elsewhere (Olsen, 1990). The plasmid DNA can be separated using gel electrophoresis.

The number and size of the plasmids presented on the gel are then analyzed providing profile for the strains involved (Nauerby *et al*., 2000). Alternatively, plasmid restriction profile can be used to create restriction fragments digested with restriction endonuclease enzymes. So, in the strict sense, if *Salmonella* isolates come from a similar outbreak scenario, contain identical plasmid profile or plasmid enzyme digestion profile with the same biotype, serotype, and phagotype, they have to be considered as a clone. One of the limitations of plasmid restriction profile analysis is that the plasmids may lack restriction site and behave as an unrestricted plasmid. Another limitation of plasmid profiling is that not all strains of *Salmonella* carry plasmids. This is because of mobility characteristic of plasmids, due to the fact that plasmids can rapidly be acquired or lost. Therefore, plasmid profiling will not discriminate among the *Salmonella* strains (Marek, 2013; Ranjbar *et al*., 2014).

(b) Pulsed-Field Gel Electrophoresis (PFGE)

This is one of the two important restriction digestion techniques, the other being the fragment length polymorphism analysis (RFLP). These techniques are based upon DNA isolation and restriction fragment analysis (Yan *et al*., 2004). Pulsed-field gel electrophoresis (PFGE) DNA fingerprinting is a powerful technique for studying the size and organization of bacterial genomes. PFGE fingerprinting is performed according to Ribot *et al*. (2006). It is a useful tool to establish the degree of relatedness among different strains of the same species (Favier *et al*., 2013). It has great value in epidemiological analysis, in the differentiation of pathogenic strains of public health importance, and in the monitoring of their spread among communities (Gautom, 1997). PFGE is currently a method used by a national foodborne disease surveillance system (PulseNet) to track the spread of foodborne pathogens and assist to identify sources of *Salmonella* outbreaks (Swaminathan *et al*., 2001). PFGE is a typing technique with high reproducibility and

discriminatory ability between different strains of the same species as compared to other molecular typing techniques (Campioni *et al*., 2012). PFGE pattern interpretation is done according to a proposed scheme by Tenover *et al*. (1995), in which, bacterial isolates yielding the same PFGE patterns are considered the same strain, whereas, bacterial isolates differing by a single genetic event, reflected as a difference of one to three bands, are considered as closely related. On the other hand, isolates differing by four to six bands, representing two independent genetic changes, are possibly related, whereas, bacterial isolates containing six or more band differences, representative of three or more genetic changes, are considered unrelated (Correia *et al*., 1994). This typing technique is considered as the gold standard typing method for bacteria (Campioni *et al*., 2012).

(c) Multi-locus Sequence Typing (MLST)

Various studies have reported the genetic diversity within bacterial population using PFGE fingerprinting during epidemiological surveillance and outbreak investigations (Basim and Basim, 2001; Sabat *et al*., 2013). However, in endemic areas various PFGE genotypes circulate, which may not be informative for the evolutionary relationships of the isolates across the globe (Thong *et al*., 1994). The genetic diversity in public health important pathogens, such as *S*. Typhi, is distributed throughout the bacterial chromosomes (Feil and Spratt, 2001). Several studies have reported up to 20% genome variations in different isolates of *S*. Typhi across the globe (Urwin and Maiden, 2003). The bacterial genome variations were shown to be due to mutations and homologous recombination between rRNA genes (Urwin and Maiden, 2003).

In 1998, a molecular epidemiologic tool called multilocus sequence typing (MLST) was proposed as a nucleotide sequence-based approach that could be applied to many bacterial pathogens to investigate the genetic diversity within bacterial population across the globe.

The first multilocus sequence typing (MLST) scheme developed was for *Neisseria meningitidis* (Maiden *et al.,* 1998). Later on, the method gained recognition and was extended to other bacterial species. Since then, it has become a very useful tool for global epidemiological monitoring and surveillance, and also for studies on the molecular evolution of pathogens (Meats *et al*., 2003; Feil *et al*., 2004).

In the MLST scheme, the internal sequences of size ranging from 399-501bp of the seven "housekeeping" genes, namely, *thrA* (aspartokinase and homoserine dehydrogenase), *purE* (phosphoribosylaminoimidazole carboxylase), *sucA* (alpha ketoglutarate dehydrogenase), *hisD* (histidinol dehydrogenase), *aroC* (chorismate synthase), *hemD* (uroporphyrinogen III cosynthase) and *dnaN* (DNA polymerase III beta subunit) are amplified by PCR and sequenced (Kidgell *et al*., 2002; Noda *et al*., 2011). These housekeeping genes encode enzymes (proteins) involved in metabolic processes. The housekeeping genes are spread throughout the bacterial genome and allow the prediction of genetic variations occurring at different positions of the bacterial chromosome (Jolley *et al*., 2004). The MLST database for most of the pathogenic bacteria has been generated and is regularly updated (Jones *et al*., 2003). Therefore, MLST provides a new approach to molecular epidemiology that can identify and track the global spread of virulent or antimicrobial resistant *Salmonella* strains or other important foodborne bacterial pathogens using the internet (Enright and Spratt, 1999). This approach is useful in *Salmonella* studies with wider spatial or longer temporal settings. This is due to the fact that house keeping genes of *Salmonella* are highly conserved so in other settings, non clonal isolates may look similar. Therefore, in *Salmonella* and other bacteria with stable genome, the MLST is best used in evolutionary and population analyses to estimate recombination and mutation rates and also to investigate the evolutionary relationships among bacteria classified within the same genus (Urwin and Maiden, 2003; Noda *et al*., 2011).

(d) Whole Genome Sequencing (WGS)

Advances in whole genome sequencing (WGS) of microbial genomes have resulted in a cost effective sequencing of a typical bacterial genome (Köser *et al*., 2012). This typing method has also resulted in the ability to perform high throughput sequencing of bacterial genomes and further reduce diagnostic times (Hasman *et al*., 2014). The WGS of foodborne pathogens such as *Salmonella* have been of great use for enhancing diagnostic and public health microbiology (Aarestrup *et al*., 2012; Didelot *et al*., 2012). A clear application for WGS is the understanding of bacterial evolution, epidemiological typing to define transmission pathways of pathogens, detect laboratory cross-contamination, and to support outbreak investigations (Schürch and Siezen, 2010). To date, many *Salmonella* isolates are already been sequenced and their whole genome sequence data have been deposited in the GenBank with specific accession numbers. For example, the genome of *Salmonella* Typhimurium strain LT2 has been sequenced, having the chromosome size of 4,857-kb and 94-kb virulence plasmid (McClelland *et al*., 2001).

The WGS provides the ultimate resolution for epidemiological studies as opposed to the current bacterial genotyping techniques which have a limited resolution as they only interrogate small regions of the microbial genome (Rasko *et al*., 2011; Grad *et al*., 2012). One potential application of WGS in microbial typing is the accurate detection of difficult-to-culture organisms, including fastidious bacteria and anaerobes (Kuroda *et al*., 2012). The WGS is also a useful typing technique in cases where standard diagnostic tests consistently fail to identify the causative pathogen. In addition, WGS could replace current PCR-based methods involved in determination of plasmids, integrons, resistance genes, bacterial toxins, MLST, and phylogenetic analysis of bacterial isolates (Relman, 2011; Larsen *et al*., 2012).

(e) Other genotypic methods

Several other genotypic methods are currently available for studying genetic variability among strains of the same organisms. These methods include various modifications of the PCR (Khoodoo *et al*., 2002), such as the random amplified polymorphic DNA [RAPD] (Williams *et al*., 1990). RAPD-PCR is a useful molecular typing tool applied to detect genomic diversity among microbial organisms (Lin *et al*., 1996), parasites (Murphy and Pellec, 1994), plants (Wolff and Rijn, 1993) and animals (Rothuizen and Van Wolferen, 1994). RAPD analysis produces highly reproducible and distinctive sets of DNA fragments by subjecting intact purified genomic DNA to PCR, primed by short oligonucleotide primers (approximately 8 to 25 bp) of arbitrary sequences. Generally, application of RAPD typing method does not require knowledge of the sequence of the target organism because the method allows a very large number of arbitrary primers to detect genomic diversity among organisms. The primer binds somewhere in the sequence, but it is not certain exactly where. Amplified products are subsequently separated on an agarose-ethidium bromide stained gel (Nath *et al*., 2010; El-Sebay *et al*., 2012). So, in a nutshell, the genetic variation analysis based on RAPD unveils genetic diversity among organisms, because of its capacity to generate random markers from the entire genome (Albufera *et al*., 2009).

The other PCR based typing methods used to detect genomic diversity among microbial organisms include Enterobacterial repetitive intergenic consensus (ERIC-PCR), PCR-restriction fragment length polymorphism (PCR-RFLP), repetitive extragenic palindromic (REP-PCR) and amplified length fragment polymorphism (AFLP). ERIC-PCR relies on amplification of genomic DNA fragments using sets of primers complimentary to the short repetitive sequences (Nath *et al*., 2010). Bacterial genomes contain repeat sequences such

as ERIC sequences (Ramazanzadeh *et al*., 2013). These can be used as molecular biological tools to assess the clonal variability of many bacterial isolates including *E. coli* and *Salmonella* spp. (Chansiripornchai *et al*., 2001). REP-PCR is a genomic fingerprinting technique that generates specific strain patterns obtained by the amplification of repetitive DNA elements present along the bacterial genome (Busch and Nitschko, 1999). ERIC-PCR was found to have high reproducibility and discriminatory ability than RAPD-PCR and REP-PCR (Nath *et al*., 2010; Campioni *et al*., 2012).

PCR- RFLP is a rapid test with high reproducibility for molecular typing in bacterial epidemiological studies (Taddele *et al*., 2011). For *Salmonella* spp. subtyping, several studies have focused on flagellin genes. Most of the *Salmonella* spp. posses two structural genes, such as *flpA*, *fljB* and *fliC* that contain a hyper-variable central region and a conserved flanking DNA region. These flagellin genes encode proteins that are related to the serotyping scheme. The *fliC* gene encodes the phase 1 antigens, whereas, the *fljB* gene encodes the phase 2 antigens (McQuiston *et al*., 2004). The hyper-variable central region of *Salmonella* flagellin genes is highly useful to differentiate the *Salmonella* isolates by the PCR- RFLP technique (Hong *et al*., 2003; Jong *et al*., 2010). Thus, RFLP is a useful molecular technique that can reflect the flagellar antigenic diversity of *Salmonella* spp. at the genome level (Kilger and Grimont, 1993).

Amplified fragment length polymorphism (AFLP) is a powerful PCR-based fingerprinting method (Janssen *et al*., 1996). It has the capacity to reveal variation around the whole genome by selectively amplifying a subset of restriction fragments for comparison (Vos *et al*., 1995). The purified genetic DNA is digested with two restriction enzymes, such as with an average (rare) cutter [*Eco*RI] and a higher frequency (frequent) cutter [*Mse*I or

*Taq*I] (Savelkoul *et al*., 1999). The use of different sets of restriction enzymes or different primer combinations can generate large numbers of different AFLP fingerprints. AFLP is a useful technique for studies of epidemiology, pathogenicity, and genetic variation in natural populations of microbial organisms (Kokotovic *et al*., 1999).

1.2.10 Definitions, discovery and applications of antimicrobials

The discovery and applications of antimicrobials, that is, antibiotics, occurred in 1929 (Bisht *et al*., 2009), and in the latter half of the 20th century, allowed control over most infectious diseases caused by bacteria, where their associated mortality and morbidity were drastically reduced (Felmingham, 2002; Aarestrup, 2005). Antimicrobials (antibiotics e.g. penicillins and non-antibiotics e.g. sulphonamides) have been used in food animals to manage diseases, maintain herd health, promote growth and also to improve nutritional benefits of the animal feed (Gorbach, 2001; Schwarz *et al*., 2001b). Biocides e.g. quaternary ammonium compounds, were discovered since 1916, and since then, they have been used as disinfectants and antiseptics for control of bacterial growth in domestic households, health care settings, food animal production environments and the food industry (Bjorland *et al*., 2003; Chapman, 2003). Antimicrobials are defined as substances against life of microorganisms (Giguere *et al.,* 2006). They are naturally occurring, synthetic and semi-synthetic compounds which affect life of microorganisms more adversely than the host (i.e. excellent selective toxicity), though biocides may have a lower or higher level of selective toxicity depending on the concentration used. Antimicrobials can be administered orally, topically (surface), and parenterally, and are used in human, and veterinary medicine and agriculture (Giguere *et al.,* 2006; Ian *et al.,* 2004).

The groups of antimicrobials most often used in animal production environments include antibacterials [antibiotics (streptomycin and penicillin) and non antibiotics (sulphonamides and amoxicillin)], anticoccidials, antimycotics, antivirals, anticancers, and biocides [disinfectants, antiseptics, and preservatives] (DANMAP, 2010). An antibiotic is a chemical substance produced by a microorganism and that have the capacity, in dilute solution, to selectively inhibit the growth of and even to destroy other microorganisms. Thus, the term antibiotic only refers to substances of microbial origin acting on microorganisms. The term antibiotic strictly excludes semi-synthetic compounds such as amoxicillin and amikacin, and also synthetic compounds such as sulphonamides, quinolones and fluoroquinolones (Aarestrup, 2006). Since antibiotics, biocides (disinfectants) and heavy metal micronutrients have antimicrobial activities, they are all referred to as antimicrobials. In order to differentiate antibiotics from biocides and heavy metals in this thesis, an antibiotic is defined as a drug, not a disinfectant, which, at low concentration, exerts an action against microbial pathogens and exhibits selective toxicity towards them (Cerf *et al*., 2010). In addition, antibiotic is also defined as any substance of natural, semi-synthetic, or synthetic origin that at "in vivo" concentrations kills or inhibits the growth of microbial pathogens by interacting with a specific target (Cerf *et al*., 2010). Antibiotics are used in vivo to kill or inhibit the growth of pathogenic microorganisms within the human and animal tissues and organs so that the immune system of the host can gain control over the pathogens and eliminate them.

Biocides (antiseptics and disinfectants) depending on their in-use concentrations are toxic to both prokaryotic and eukaryotic cells. Biocides can be applied on inanimate objects (disinfectants and preservatives) or on the surface of living tissues (antiseptics). Biocides are diverse compounds used for eradication or inhibition of microorganisms in a wide range of applications (Fraise, 1999; Mcdonnell and Russell, 1999). The most common

classes of biocides used in animal production environments are such as glutaraldehydes and quaternary ammonium compounds [QAC]. The two groups of biocides are occasionally used in combination, and are commercially available to be used for disinfection purposes at the farm level and at other relevant stages of food production chain, in order to inhibit bacterial growth, load and colonization (Russell, 2002; Maillard, 2007). QAC can be used for both disinfection and antisepsis depending on the concentration of the active compounds (Bjorland *et al*., 2003). Many QAC (e.g. benzalkonium chloride) and glutaraldehydes are easily inactivated by organic materials such as urine, blood, faeces, thus making them unsuitable for applications where organic materials may be present (Mcdonnell and Russell, 1999; Aarestrup, 2006). Chlorhexidine and triclosan on the other side, is used to disinfect the skin before surgery to treat skin infections, and is also used in hand washes (Aarestrup, 2006).

Antimicrobials (antibacterials) used in animal production are usually administered to individual animals for disease intervention (therapy), or to groups of animals for therapy, prophylaxis, metaphylaxis and growth promotion (Aarestrup *et al*., 2001; Aarestrup, 2005). The most common antimicrobials used in food producing animals are either identical to or related to those administered to humans, including penicillins, tetracyclines, phenicols, cephalosporins, (fluoro)quinolones, avoparcin (a glycopeptide that is related to vancomycin), and virginiamycin [a streptogramin that is related to quinupristin-dalfopristin] (Schwarz and Chaslus-Dancla, 2001a).

1.2.11 Mechanisms of antimicrobial action

1.2.11.1 Mechanism of antibiotics action

Antimicrobials (antibacterials) are categorized according to their four major principal mechanisms of action. These mechanisms of action include inhibition of protein synthesis,

inhibition of a metabolic pathways, inhibition of cell wall synthesis and disruption of bacterial membrane structure, and interference with nucleic acid synthesis (Tenover, 2006; Jayaraman, 2009). The inhibition of bacterial cell wall biosynthesis is a common mechanism for broad spectrum antimicrobials such as the β-lactams including penicillins (e.g. penicillin, ampicillin, and methicillin), cephalosporins (e.g. ceftiofur and cefoxitin), and carbapenems (e.g. imipenem and meropenem) and glycopeptides groups such as vancomycin and teicoplanin. The β-lactams bind to transpeptidase enzymes, also called penicillin-binding proteins [PBPs] required for the synthesis of the peptidoglycan layer (McManus, 1997; Levy and Marshall, 2004).

Upon binding to the β-lactam ring by the side chain oxygen atom of a serine residue at the active site of the enzyme, a relatively stable lethal covalent penicilloyl-enzyme complex is formed in which the serine is covalently acylated by the hydrolysed β-lactam. This leads to inactivation of the enzyme and block the normal transpeptidation reaction and eventually cell lysis and death (Kahne *et al*., 2005; Wilke *et al*., 2005). On the other hand, the glycopeptides [vancomycin and teicoplanin] interfere with formation of peptidoglycan by binding to the D-Ala_4-D-Ala_5 (D-alanine residues) termini of the Uridine diphosphate (UDP)-muramylpentapeptide peptidoglycan precursors. Through this binding, the bound glycopeptide acts as a steric impediment and the substrates are kept away from transglycosidase, and hence no chain elongation and transpeptidase cross-linking. This substrate impounding leads to the failure of peptidoglycan cross-linking, making the cell wall susceptible to osmolysis (Lambert, 2005; Yoneyama and Katsumata, 2006).

Several classes of antimicrobials including macrolides, aminoglycosides, tetracyclines, phenicols, streptogramins, lincosamides, ketolides and oxazolidones express their

mechanisms of action by inhibiting bacterial protein biosynthesis. Aminoglycosides (e.g. tobramycin) and tetracyclines (e.g. doxycycline) bind to the conserved sequences of the 16S rRNA of the 30S subunit, whereas macrolides (e.g. erythromycin), phenicols (e.g. chloramphenicol), streptogramins (e.g. quinupristin), lincosamides (e.g. clindamycin), ketolides (e.g. azithromycin) bind to the conserved sequences of the 23S rRNA of the 50S subunit. The binding of the antimicrobials to the conserved sequences of the 16S rRNA of the 30S or 23S rRNA of the 50S leads to misreading or termination of translation, rendering bacterial protein biosynthesis incomplete (Bockstael and Van Aerschot, 2009). Two classes of antimicrobials such as fluoroquinolones and rifamycins are known to interfere with nucleic acid biosynthesis. The quinolones (e.g. nalidixic acid) and fluoroquinolones (e.g. ciprofloxacin) and rifamycins (e.g. rifampicin) target DNA synthesis and cause double-strand DNA to break during DNA replication by inhibiting DNA gyrase, also known as topoisomerase II, which is an enzyme required in folding and supercoiling of bacterial DNA (Schmitz *et al*., 2002).

Rifampicins are antimicrobials known as RNA transcription inhibitors. Transcription is an essential process for decoding of genetic information from DNA to mRNA in bacteria. RNA polymerase of bacteria catalyses the transcription process by self attachment to the DNA template (Yoneyama and Katsumata, 2006). Rifampicin is an important antimicrobial in the treatment of *Mycobacterium tuberculosis* infections. It inhibits bacterial DNA-dependent RNA polymerase by binding to the β-subunit of the enzyme. Thus, it blocks the entry of the first nucleotide, which is necessary to activate the polymerase, thereby blocking mRNA synthesis (Yoneyama and Katsumata, 2006; Bockstael and Van Aerschot, 2009). Sulphonamides and trimethoprim inhibit distinct steps in folic acid metabolism. Sulphonamides inhibit dihydropteroate synthase in a

competitive manner and trimethoprim act at a later stage of folic acid synthesis and inhibit the enzyme dihydrofolate reductase. The two antimicrobials competitively target the pathway for folic acid metabolism by inhibiting the synthesis of folic acid, which ultimately inhibit DNA synthesis. A combination of sulphonamides and trimethoprim acting at distinct steps on the same biosynthetic pathway shows synergy and a reduced mutation rate for resistance (Skold, 2001; Bockstael and Van Aerschot, 2009).

1.2.11.2 Mechanism of biocides [disinfectants] action

The mechanisms of the antibacterial action of biocides are not well understood. However, biocides are known to interact with bacterial cell walls or envelopes (e.g. glutaraldehydes), produce changes in cytoplasmic membrane integrity (cationic agents, e.g. QAC), inhibit membrane enzymes (e.g. silver compounds), act as alkylating agents (e.g. ethylene oxide and formaldehydes), cross-linking agents (aldehydes e.g. glutaraldehydes) and intercalating agents (e.g. acridines) (Denyer and Stewart 1998; Mcdonnell and Russell, 1999). In contrast to common antibiotics used in practice, biocides are reported to have multiple target sites within a bacterial cell (Denyer and Stewart 1998).

1.2.12 Public health implications of antimicrobial resistance in food animals

Antimicrobials are used in large quantities worldwide, and many countries do not have legislative measures to control antimicrobial consumption both in humans and animals (Gilbert, 2012; da Silva *et al*., 2013). There are important differences in the use of antimicrobials in humans and animals, in particular food animals. For instance, in human medicine, antimicrobials are administered individually to patients after occurrence of infection or, in rare cases, administered to a mass of people, to prevent emergence of infection [prophylaxis]. The use of antimicrobials to treat diseases in food animals started

in 1940s, whereas, the introduction of antimicrobials in the food animal commercial feed started in 1950s (Gorbach, 2001; Hammerum and Heuer, 2009). Since then, the use of antimicrobials has saved countless lives despite the rising trend of antimicrobial resistance in the infectious agents (Davies and Davies, 2010).

Heavy metals are defined as a group of metals whose atomic density is greater than 5g/cm^3. Heavy metals such as zinc (Zn), copper (Cu), cobalt (Co), nickel (Ni), manganese (Mn) and iron (Fe) have the nutritional characteristics and are necessary for living organisms but in high concentrations they produce toxic effects (Christopher *et al*., 2014). Zinc and copper are essential trace elements for prokaryotic and eukaryotic cellular metabolism. Zinc is a cofactor of more than 300 metallo-enzymes, including alkaline phosphatases, whereas copper is needed for activation of several oxidative enzymes required for normal cellular metabolism (Nies, 2003; Rutherford and Bird, 2004). Zinc and copper are among many other micronutrients included in swine feed and other livestock to achieve growth promotion and increase feed efficiency (Jacob *et al*., 2010; NRC, 2012). Besides growth promotion and increasing feed efficiency, in-feed supplementation of zinc oxide has been suggested to prevent post-weaning scouring (diarrhoea) by helping to maintain the stability of the intestinal flora in weaned pigs, thus preserving its protective ability, which would otherwise be lost due to weaning. Stability of the intestinal flora in weaned pigs renders the gut less susceptible to the establishment of pathogens, either indirectly, by competing for the same niche, or directly, by the growth inhibition of pathogenic organisms by the metals (Jensen-Waern, *et al*., 1998). Due to these beneficial effects of zinc and copper in swine and poultry production, in-feed supplementation of zinc and copper has been very common (Hill *et al*., 2000; Hollis *et al*., 2005).

It is worthwhile to enlighten on the issue of the over-the-counter sale of both human and veterinary medicines in many countries, especially, in the low-income countries. Previous studies have reported the clear infringement of law with regard to stocking, distribution and dispensing of the antimicrobials in the low-income countries. In Tanzania, there is a fast growing unlawful practice where violation of health-related regulation is extremely common, in which the most important antimicrobials, the prescription-only medicines, are stocked by the drug stores which lack qualified and certified personnel (Goodman *et al*., 2007; Viberg *et al*., 2010).

The trend shows that there is an increase in bacterial antimicrobial resistance worldwide over the last decades. The emergence and persistence of bacterial resistance to antimicrobials is partly caused by rapid evolution of the bacterial genome under selective pressure of antimicrobials (e.g. antibiotics, biocides and heavy metals) and by the selective pressure of the environment. A continuous selective pressure as a result of the increased accumulation of sub-optimal dose of routinely used antimicrobials in the pathogen environment potentially provides conditions that will favourably select bacterial variants with increased tolerance to antimicrobials such as biocides and antibiotics. The emergence of resistance to antimicrobials is often related to sub-optimal drug dosing and such sub-optimal dosing results in the preferential killing of drug-susceptible microbial sub-populations, and on other hand allowing amplification of drug-resistant microbial sub-populations. So, in any large bacterial population, there is co-existence of sub-populations with differing susceptibilities to the administered antimicrobials. Under antimicrobial selective pressure, sub-populations with reduced susceptibilities to antimicrobials have a survival advantage (Braoudaki and Hilton, 2004a; Braoudaki and Hilton, 2004b).

Selective pressure exerted by the use of antibiotics as growth promoters in food animals appears to have created a large pool of transferable antimicrobial resistance in the bacterial ecosystems. For instance, it was reported that after using the streptothricin antibiotic in animal husbandry for growth promotion, plasmid-borne resistance to streptothricin was observed in *E. coli* from nourseothricin fed pigs, employees in pig farms and their family members. Additionally, this rapid spread of antimicrobial resistance in bacteria is mediated by plasmids, phages, transposons and other mobile genetic elements such as integrons (Witte, 2000; Walsh, 2003).

Most of the antimicrobials used for animal production belong to the same classes as those used in human medicine. For instance, tetracyclines constitute the antimicrobial class used most often in animals, followed by macrolides, lincosamides, penicillins, aminoglycosides, sulphonamides, (fluoro)quinolones, cephaloporins and phenicols (Schwarz and Chaslus-Dancla, 2001a). The use, over-use and miss-use of antimicrobials in production animals act as a potential risk factor (selective pressure) for the occurrence and the emergence of antimicrobial resistance in public health important pathogens. So, the effect of antimicrobial use in food animals on the development of resistance in pathogenic bacteria has been a subject of prolonged debates worldwide (Lathers, 2002; Callie *et al*., 2012). The increased criticism of agricultural usage of antimicrobials has reached a climax, particularly in the United States, European countries and elsewhere, coinciding with the antimicrobial resistance crisis in human medicine (Sharma *et al*., 2005).

Occurrence of antimicrobial resistance to different classes of antimicrobials has emerged, and this has led to emergence of antimicrobial resistant bacteria, which are becoming a

serious public health menace (Nweneka *et al*., 2009; Rodriguez-Rojas *et al*., 2013). As a result of the intensive use of antimicrobials in food animal production, food animal products such as meat, eggs and milk are frequently contaminated with antimicrobial resistant foodborne pathogens such as *Salmonella* spp. and *E. coli* (Cosgrove and Carmeli, 2003; Hammerum and Heuer, 2009). Of more direct relevance to public health is the potential of resistant pathogens in food animals finding their way into humans (Howard *et al*., 2001). Hence, humans can be colonised with the antimicrobial resistant pathogens of animal origin, and because of resistance to commonly used antimicrobials, these bacteria may cause infections for which limited therapeutic options are available. This may lead to treatment failure in both humans and animals (Howard *et al*., 2001; Gilbert, 2012). Furthermore, the resistant bacteria of animal origin may potentially act as a source of antimicrobial resistance genes for other pathogenic bacteria in the microbial ecosystem. Thus, the intensive use of antimicrobials in food animal production may add to the burden of antimicrobial resistance in humans (Hur *et al*., 2012; Zhu *et al*., 2013).

1.2.13 Global efforts to combat antimicrobial resistance

Antimicrobial resistance is a complex problem to tackle and is driven by many interconnected factors. As such, antimicrobial resistance is occurring everywhere in the globe to the extent of compromising our ability to treat infectious diseases (Aminov and Mackie, 2007; Wright, 2007). Therefore, coordinated actions are required to minimize the emergence and spread of antimicrobial resistance. World Health Organization (WHO), is working closely with the Food and Agriculture Organization of the United Nations (FAO) and the World Organisation for Animal Health (OIE) in a One Health approach to promote optimal use of antibiotics in both humans and animals. The sixty-eight World Health Assembly in May 2015, the World Health Assembly endorsed a global action plan to

tackle antimicrobial resistance. The goal of the global action plan is to ensure the continuity of successful treatment and prevention of infectious diseases with effective and safe medicines (WHO, 2001). A number of issues have been raised to help in containing antimicrobial resistance, including, improving awareness and understanding of antimicrobial resistance, strengthening knowledge through surveillance and research, reducing the incidence of infection, optimizing the use of antimicrobial agents and developing the economic case for sustainable investment that takes into account the needs of all countries, and the need for increasing investment in new medicines, diagnostic tools and vaccines (WHO, 2000). In recognition of such efforts, international partners such as the WHO, FAO and World Organization for animal health and the national partners are engaged in set of activities, such as, improving national awareness and understanding of antimicrobial resistance through public communication programmes that target the different audiences in human health, animal health and agricultural practices (WHO, 2001).

1.2.14 Genetic elements of antimicrobial resistance dissemination

Antimicrobial resistance is a natural consequence of adaptation of infectious agents to exposure to antimicrobials used in humans, food animals, agriculture and use of biocides (disinfectants) in production farms (Wise and Soulsby, 2002; Beceiro *et al*., 2013). Antimicrobial use selects for resistant bacterial strains as well as genetic vectors specifying resistance genes (Barza *et al*., 2002). The bacteria in that sense have a remarkable ability to adapt to adverse environmental conditions. The major drivers of resistance have lead to the emergence, persistence and dissemination of resistant bacteria and resistance genes (van den Bogaard and Stobberingh, 2000; Barton, 2014). Thus, it appears that the emergence of antimicrobial resistant bacteria is inevitable to almost every

new antimicrobial and it is recognized as a major problem in the treatment of microbial infections (Bockstael and Van Aerschot, 2009). Antimicrobial resistance among gram negative bacterial populations, including *Salmonella* can be acquired through errors or spontaneous mutation (vertical transmission) in the gene locations of target proteins or through horizontal transfer by transformation, transduction, or conjugation of individual antimicrobial resistance genes or complex genetic elements, such as plasmids, bacteriophages, transposons, genomic islands and integrons (Walsh, 2003; Carattoli, 2003). Vertical transmission is an evolutionary process by which a bacterial cell can accumulate errors in its genome during replication, such that the resulting progeny differ genetically from their bacterial ancestors. Besides the low rate of genome copying, the growing bacteria may introduce a mutation into the genome. The mutations introduced into the bacteria will generate mutants that can grow faster and tolerate higher concentrations of antimicrobials than their predecessors, and such bacterial mutants will increase in prevalence to the point of taking over the entire population (Acar and Rostel, 2001; Madhavan and Murali, 2011).

Gene transfer is a process of swapping genetic material between neighboring bacteria. The plasmids, transposons and the integrons are the main vectors for transfer the antimicrobial resistance genes to other members of the same bacterial species, as well as to bacteria in another genus or species (Palmer et *al*., 2010; Kaplan, 2014). Transformation is the uptake of genetic material from dead bacteria, while conjugation is the transfer of genetic material between two living bacteria. Transformation involves uptake of short fragments of naked DNA by naturally transformable bacteria, whereas transduction is a process of transfer of DNA from one bacterium into another through bacteriophages. The process of conjugation involves transfer of DNA through a sexual pilus and it requires cell to cell

contact. Therefore, DNA fragments that contain resistance genes from resistant donors can render previously susceptible bacteria express resistance as coded by the newly acquired resistance genes (Beceiro *et al*., 2013; Dantas and Sommer, 2014). Plasmids are dispensable extra-chromosomal elements consisting of circular double-stranded DNA molecules. Plasmids are self-replicating elements. Some of the plasmids have the ability to transfer themselves to other bacterial species by conjugation, thus, causing outbreak of multiple resistance (Hawkey, 2009; Sherley *et al*., 2004). Transposons are DNA segments capable of inserting themselves at a new location in plasmid, chromosome, or bacteriophage. They may contain one or several genes that code for antimicrobial resistance (Barkay and Smets, 2005; Beceiro *et al*., 2013).

Antimicrobial resistance genes are found to be associated with genetic elements known as integrons, which can be located on the plasmids, transposons but also on the chromosome (Leverstein-van Hall *et al*., 2003; Zhu *et al*., 2013). They are able to integrate and express resistance genes coding for antimicrobial resistance (Stokes and Hall, 1989). Class 1 integrons are the most common integron type (Hall and Stokes, 1993; Carattoli, 2001). Class 1 integrons have been detected in *Salmonella* serotypes (Fluit, 2005) and may be located on the *Salmonella* genomic island 1 [SGI-1] (Meunier *et al*., 2002; Levings *et al*., 2005). The SGI-1 is an integrative 43 kb mobilisable chromosomal element on which antimicrobial resistance genes are clustered (Doublet *et al*., 2005; Carattoli *et al*., 2002). The SGI-1 is flanked by two class 1 integrons (Boyd *et al*., 2001). Bacteriophages are common in all natural environments and play an important role in bacterial evolution (Labrie *et al*., 2010). Bacteriophages have characteristics that make them suitable vectors between different biomes for transferring antimicrobial resistance genes from biome to biome (Muniesa *et al*., 2013).

1.2.15 Mechanisms of antimicrobial resistance in gram-negative bacteria

1.2.15.1 Mechanisms of heavy metals [$Cu^{2+}$and Zn^{2+}] resistance

Heavy metals are widespread in the environment due to industrial effluent and sewage. Studies have shown the influence of heavy metals by adversely affecting the growth of bacteria. Therefore, heavy metals in the environment can cause many changes to occur in the structure and function of bacterial communities. Consequentially, bacteria have developed mechanisms to tolerate such changes in the environment (Christopher *et al*., 2014). Heavy-metal tolerance in bacteria has been shown to be associated with single- or multiple drug resistance. Such studies have clearly demonstrated the correlation between resistance to antibiotics such as penicillin, erythromycin, and tetracycline and tolerance to heavy metals, including, mercury, lead, cadmium, and zinc (Allen *et al*., 1977).

Resistance to heavy metal such as copper [Cu^{2+}] and zinc [Zn^{2+}] among bacterial pathogens is mediated by different mechanisms, including multidrug efflux systems. The PCO and CZC operon systems and *tcrB* are examples of multidrug efflux systems, that have been shown to be important mechanisms of resistance against antimicrobials and other structurally unrelated compounds, including heavy metals (e.g. Cu^{2+}, Zn^{2+}, Co^{2+} and Cd^{2+}) and biocides (e.g. QAC). The mechanisms of heavy metal resistance to copper in *Enterococcus faecium* strains isolated from pigs have been associated with the carriage of a conjugative plasmid carrying copper resistance determinants such as *tcrB* (Hasman *et al*., 2006; Amachawadi *et al*., 2011).

In addition, another efflux system that has also been associated with copper resistance in Gram-negative bacteria is the PCO operon system, which mediates resistance to Cu^{2+}. The PCO operon system is often carried by a conjugative plasmid such as pRJ1004

isolated from *E. coli* that resided in the gut of pigs fed a diet supplemented with copper sulfate. The PCO operon gene cluster is composed of seven genes *pco*ABCDRSE (Huffman *et al*., 2002; Bondarczuk and Piotrowska-Seget, 2013). Resistance to Zn^{2+} and other metals such as Co^{2+} and Cd^{2+} is conferred by genetic determinants often carried by a plasmid such as pMOL30. The CZC operon system is composed of gene clusters, including *CzcA*, which functions as a cation-proton antiporter protein, *CzcB* as a cation funnel, *CzcC* as a modulator of substrate specificity, and *CzcD* as a protein involved in regulation of the operon (Nies, 1992; Anton *et al*., 1999)

1.2.15.2 Mechanisms of antibiotics resistance

Resistance to antimicrobials such as antibiotics in *Salmonella* spp. can be acquired (active) if it is the result of a specific evolutionary selection pressure to adapt a "counter-attack" mechanism against an antimicrobial or intrinsic (passive), if is a consequence of general adaptive processes that are not necessarily linked to a given antimicrobial, e.g. the nonspecific barrier achieved by low cell membrane permeability to many antimicrobials in *Pseudomonas aeruginosa*. *Salmonella* spp. like other Gram negative bacteria achieve active drug resistance through three major mechanisms such as efflux of the antimicrobial from the cell through a collection of membrane-associated pumping proteins, modification of the antimicrobial target (e.g., through mutation of key elements such as bacterial DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) genes, and through the synthesis of modifying enzymes that selectively target and destroy the activity of antimicrobials (Randall *et al*., 2005b; Wright, 2005). Furthermore, acquired resistance results from changes in the bacterial genome. When resistance in bacteria is acquired through mutation, it will be passed vertically by selection to subsequent progeny.

Quinolones target the bacterial enzymes DNA gyrase (topoisomerase II) and topoisomerase IV, which are essential for cell growth and proliferation. DNA gyrase is an enzymes comprising two subunits namely, *gyrA* and *gyrB*, whereas topoisomerase IV comprising two subunits namely, *parC* and *parE* (Drlica and Zhao, 1997; Guan *et al*., 2012). Resistance to quinolones and fluoroquinolones can be due to decreased permeability of the antimicrobial to the cell, efflux pumps, or mutations in DNA gyrase or topoisomerase genes (Hopkins *et al*., 2005; Frye and Jackson, 2013). However, resistance to quinolones in *Salmonella* spp. is largely attributable to interference with bacterial DNA metabolism mediated by chromosomal point mutations in bacterial DNA gyrase and topoisomerase IV. Quinolone resistance in Enterobacteriaceae family members was primarily attributed to point mutations in the *gyrA* gene encoding the A subunit of the DNA gyrase. Resistance mutations of *gyrA* gene occur in a region of the gene product between amino acids 67 and 106. This active, highly conserved region between amino acids 67 and 106 is called the quinolone resistance-determining region (QRDR). Previous studies have most frequently observed point mutations (amino acid changes) with ciprofloxacin and nalidixic acid-resistant strains at Serine-83 (Ser-83) (to Phenylalanine [Phe], Tyrosine [Tyr]), or Alanine [Ala] or at Aspartic acid-87 (Asp-87) (to Glycine [Gly], Asparagine [Asn], or Tyrosine [Tyr]) (Piddock, 2002; Giraud *et al*., 2006).

Apart from the single mutations in the *gyrA* gene at codon 83 or 87 reported before, also double mutations at both residues 83 and 87 have been identified in clinical isolates of *S. enterica* serotype Typhimurium showing high-level resistance to ciprofloxacin (Heisig, 1993). Other point mutations reported in the QRDR are *gyrB*, a gene encoding the B subunit of gyrase (Heisig, 1993), leading to amino acid change at Serine-464 [Ser-464] to Phenylalanine [Phe] (Baucheron *et al*., 2002), and the *parC* gene, encoding the *ParC*

subunit of topoisomerase IV, leading to amino acid change at Serine-80 [Ser-80] to Isoleucine [Ile] (Baucheron *et al*., 2002).

Alteration in efflux systems is another mechanism of antimicrobial resistance in Gram negative bacteria. Generally speaking, bacteria are able to increase the expression of nonspecific, energy-dependent efflux systems that prevent the accumulation of intracellular concentrations of quinolones by actively pumping the antimicrobial across the cell membrane. The multidrug efflux system such as *AcrA-AcrB-TolC* has been shown to mediate quinolones efflux in *Salmonella* spp. and *E. coli* (Giraud *et al*., 2000; Guan *et al*., 2012). Besides alteration in efflux systems, Gram-negative bacteria can also regulate cell membrane permeability, hence altering the levels of outer membrane porins that form the channels responsible for passive diffusion of antimicrobials across the membrane. The loss of or reduced outer membrane porins has been shown to confer antimicrobial resistance (Guan *et al*., 2012).

Plasmid-mediated quinolone resistance (PMQR) is another indispensable mechanism of quinolone resistance in *Salmonella* spp. PMQR was first reported in 1998. Plasmid-borne *qnr* genes have been discovered on plasmids and the bacterial chromosome (Robicsek *et al*., 2006; Jacoby *et al*., 2008). The *qnr* genes currently comprise five families and more than 30 alleles with 16% - 99.8% similarity, *qnrA*, *qnrB*, and *qnrS*, *qnrC* and *qnrD*. These *qnr* genes encode quinolone-inactivating enzymes that have also been shown to cause quinolone resistance (Cavaco *et al*., 2009; Xia *et al*., 2013). For example, *qnrA*, a gene responsible for quinolone resistance codes for a 218 amino acid protein belonging to the penta-peptide family that protects DNA from quinolone binding to DNA gyrase and topoisomerase IV (Robicsek *et al*., 2006; Beceiro *et al*., 2013).

The β -lactam antimicrobials act by binding to cell wall synthesis enzymes known as penicillin-binding proteins (PBPs), thereby inhibiting peptidoglycan biosynthesis (Ghuysen, 1991). PBPs catalyze the polymerization of the glycan strand [transglycosylation] and the cross-linking between glycan chains [transpeptidation] (Sauvage *et al*., 2008). So, inhibition of PBPs weakens the cell wall, resulting in inhibition of cell growth and finally cell death. The three mechanisms of β-lactam resistance are reduced access to the PBPs, reduced PBP binding affinity, and destruction of the antimicrobial through the expression of β-lactamase enzymes that bind and hydrolyze β-lactams (Wilke *et al*., 2005; Fisher *et al*., 2005). β-lactamases including TEM-1, PSE-1, and OXA-1, are described as the enzymes most frequently related to ampicillin and amoxicillin/clavulanic acid resistance (Güerri *et al*., 2004; Biendo *et al*., 2005). β -lactamases are hydrolytic enzymes that disrupt the amide bond of the typical β-lactam ring, before the antimicrobial can get to the site of cell wall biosynthesis; thus, rendering the antimicrobials ineffective to Gram-negative bacteria (Poole, 2004; Fisher *et al*., 2005). There are four classes of β-lactamases. Three of them are serine-dependent enzyme classes [A, C and D] and one metal-dependent class [B] (Fisher *et al*., 2005). The resistance of *Salmonella* spp. to extended-spectrum β-lactams (ESBL) such as third-generation cephalosporins [e.g. ceftriaxone and ceftiofur] (Klein and Cunha, 1995) is primarily mediated by the production of extended-spectrum β-lactamases of the TEM, SHV, and CTX-M types, which are associated with different mobile genetic elements (Güerri *et al*., 2004; Michael *et al*., 2006).

Salmonella resist aminoglycosides by modifying the active sites of the enzymes (Frye and Jackson, 2013). Inactivation by modification of the key hydroxyl and amine groups on the aminoglycosides is the most significant form of acquired resistance in Gram-negative

bacteria to prevent the aminoglycoside from binding to its ribosomal target (Jana and Deb, 2006; Shahid, 2007). Mechanism of aminoglycoside resistances in *Salmonella* spp. is primarily due to acetyltransferases, phosphotransferases, adenylyltransferases, and nucleotidyltransferases which modify and inactivate the aminoglycosides (Shaw *et al*., 1993; Ramirez and Tolmasky, 2010). So, the types of aminoglycoside modifying enzymes are: aminoglycoside acetyltransferases [*aac*], aminoglycoside phosphotransferases [*aph*], aminoglycoside nucleotidyltransferases [*ant*] and aminoglycoside adenylyltransferases [*aad*] (Yoneyama and Katsumata, 2006). The *aac* genes found in *Salmonella* spp. can confer resistance to gentamicin, tobramycin and kanamycin. Aminoglycoside phosphotransferases confer resistance to kanamycin and neomycin, and are usually named *aph*. Apart from the *aph* genes designation of the aminoglycoside phosphotransferases, some *aph* genes have other designations (names) such as *str* genes (e.g. *strA* and *strB*) which encode resistance to streptomycin. Aminoglycoside nucleotidyltransferases can confer resistance to gentamicin, tobramycin, or streptomycin and they include *aad* and *ant* groups of genes. The most common genes reported and their variants of *aac*, *aad*, *aph*, and *str* genes include: *aac*(3'), *aac*(6'), *aadA1*, *aadA2*, *aadA12*, *aphA1*, *aph*(3'), *strA*, and *strB* (Frye *et al*., 2011; Glenn *et al*., 2011).

Chloramphenicol and related compounds such as florphenicol have long been used as the drug of choice to treat salmonellosis in human and veterinary medicine, which has led to the selection of phenicol-resistant strains (Murti *et al*., 1962). Resistance to chloramphenicols is mediated by the plasmid-borne enzymes called chloramphenicol acetyltransferases, *cat* [e.g. *cat*A1] (Frye and Jackson, 2013), or by the non-enzymatic chloramphenicol resistance gene, *cml* [e.g. *cmlA*] (Aarestrup, 2006), that encodes an efflux pump. The *cat* enzymes encoded by the *cat* family of genes are widespread in Gram-

negative bacteria such as *Salmonella* spp. and *E. coli*. The *cat* genes (formally known as *cat*I to *cat*III), are sub-categorised into *cat*A and *cat*B groups (White *et al*., 1999; Nogrady *et al*., 2005). The *cat*B genes in *Salmonella* spp. have been reported to be located on mobile genetic elements called integrons (Bunny *et al*., 1995; Tosini *et al*., 1998). The use of chloramphenicol is banned in developed countries, but it is still widely used. The fluorinated analogue called florfenicol was developed and approved for use to replace chloramphenicol in developed countries. It was reported that neither the *cat* nor the *cml* genes confers resistance to florfenicol (Nogrady *et al*., 2005). The first resistance gene reported that encodes resistance to florfenicol is called *pp-flo* (Kim and Aoki, 1996) and later the *flo* gene [e.g. *flo*R] was also identified in different *Salmonella* serotypes (Doublet *et al*., 2003). The *flo* gene in *Salmonella* serotypes was found in the class I integrons located in the genomic island called SGI-1 and on the plasmids (Meunier *et al*., 2003; Glenn *et al*., 2012).

Tetracyclines are among the most widely used antimicrobials worldwide. The efficacy of tetracyclines seems to be dwindling as a result of the high frequency of antimicrobial resistance (Chopra and Roberts, 2001; Kim *et al*., 2004). Resistance to tetracycline is mediated by one of the three mechanisms namely, an energy-dependent efflux pumps (Taylor and Chau, 1996), ribosomal protection by a soluble proteins [ribosomal protection proteins] (Burdett, 1991), and enzymatic inactivation of tetracycline (Speer and Salyers, 1989). In *Salmonella* spp., tetracycline resistance is generally attributed to the production of energy-dependent efflux pumps, which remove tetracycline from the bacterial cell. Other mechanisms of tetracycline resistance are not yet reported in *Salmonella* spp. (Chopra and Roberts, 2001; Cosby *et al*., 2015). There are different genes that confer resistance to tetracycline and oxytetracycline including *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(G),

and *tet*(H) found most often in *Salmonella* spp. (Chopra and Roberts, 2001). However, the most commonly reported of these is *tet*(A) which is located within SGI-1 (Carattoli *et al*., 2002), integrons (Briggs and Fratamico, 1999), and plasmids (Pezzella *et al*., 2004; Gebreyes and Thakur, 2005) followed by the *tet*(B) (Guerra *et al*., 2002). These genes are widespread among *Salmonella* spp. and are almost always present in MDR *Salmonella* spp. (Pezzella *et al*., 2004).

Sulfonamides compete with *p*-aminobenzoic acid for binding to dihydropteroate synthase (DHPS) in the folic acid [folate] biosynthesis, thus, inhibiting the formation of dihydrofolic acid [dihydrofolate] (Skold, 2000). Acquired resistance to sulfonamides in gram-negative bacteria can result from mutations in the chromosomal DHPS gene [*folP*] (Aarestrup, 2006) or by acquisition of an alternative DHPS gene [*sul* genes] (Sundström *et al*., 1988), whose products have a lower affinity for sulfonamides (Swedberg and Skold, 1980). Three genes encoding resistance to DHPS in gram-negative bacteria such as *Salmonella* spp., *E. coli*, are known as *sul1*, *sul2* and *sul3* (Antunes *et al*., 2005). The *sul*1 gene is found in class 1 integrons linked to other resistance genes, while *sul2* is located on small non-conjugative and large transmissible multiple resistance plasmids (Enne *et al*., 2001), and *sul3* is another plasmid-borne sulfonamide resistance gene (Perreten and Boerlin, 2003).

Trimethoprim resistance is mediated mainly by acquisition of a *dfr* gene encoding a resistant dihydrofolate reductase enzyme (Aarestrup, 2006). There are more than 30 different trimethoprim-resistance-mediating dihydrofolate reductase (*dfr*) genes. These genes are subdivided into two major types, namely, *dfrA* and *dfrB* (Pattishall *et al*., 1977; White and Rawlinson, 2001). The *dfrA1* gene is one of the most frequently detected

determinants, probably caused by the successful dissemination of its carrier transposons, Tn7. PCR assays have been used for detection of *dfr* subtypes such as *dfrA12*, *dfrA5-14*, *dfrA7-dfrA17* (Guerra *et al*., 2001; Frech *et al*., 2003). A new trimethoprim resistance gene is defined when the dihydrofolate reductase protein (DHFR) encoded by the gene has <95% identity at the amino acid level to known DHFR proteins (Skold, 2001).

1.2.15.3 Mechanisms of biocide [disinfectants] resistance

The use of biocides is also implicated to contribute to the emergence of biocide tolerant foodborne and environmental bacterial pathogens (Russell *et al*., 1999; Fraise, 2002). Bacterial tolerance to biocides can be induced as a result of exposure to sub-optimal concentrations of biocides and other antimicrobials. The mechanism of resistance to biocides in bacterial population is divided into intrinsic and acquired resistance. The intrinsic resistance is a natural property of an organism, which includes impairing of cell wall properties such as the outer membrane of gram-negative bacteria. The outer membrane of the bacteria act as an impermeability barrier to uptake of chemically unrelated drugs to reach the target sites (Kolar *et al*., 2001; Randall *et al*., 2007).

Other examples of intrinsic resistance mechanisms that hinder diffusion of biocides across bacterial cell are loss or alteration of the porins, and degradation of biocides (Ayres *et al*., 1993; Denyer and Maillard, 2002). In contrast to intrinsic resistance, acquired resistance is achieved by acquisition of new properties through mutations at sensitive target sites, or by intracellular removal of antimicrobials by efflux-mediated systems and by horizontal transfer of genes conferring resistance (McDonnell and Russell, 1999). Previous studies have shown a close association between resistance to biocides and antibiotics. The resistance genetic determinants of these agents are commonly linked with each other, for

instance, the *qac* genes such as *qacE1* or *qacEΔ1* genes are often present on the plasmids together with other resistance genes. The *qacEΔ1* genes encode the multi-drug efflux pumps (Weigel *et al*., 2003). For instance, if antibiotic and *qac* resistance genes are both carried (co-located) on class 1 integron, then selection for *qac* resistance phenotypes may result to co-selection of antibiotic or heavy metal resistance phenotypes. Cross-resistance, on the other hand, has the potential to occur when different antimicrobials attack the same target site to initiate a common pathway to cell death (Gaze, *et al*., 2005; Jaglic and Cervinkova, 2012).

1.3 Problem Statement and Justification of the Study

Non-typhoidal *Salmonella* (NTS) are recognized as one of the major bacterial foodborne pathogens of zoonotic potential worldwide (Tadee *et al*., 2015). They are the most common cause of foodborne illness and outbreaks in the industrialized regions such as the United States and the European Union (Forshell and Wierup, 2006). *Salmonella* spp. infection in the United States has been estimated to cause over a million cases of human salmonellosis, resulting into a significant number of deaths each year. The majority of human salmonellosis cases are related to the consumption of contaminated food products of animal origin (Voetsch *et al*., 2004). Although little is known with regard to the incidence of NTS in Tanzania and other sub-Saharan African countries, colonization by NTS is common in humans and animals, for instance, the high case fatality of extra-intestinal non-typhoidal salmonellosis with meningitis in children and adults in Malawi and a nosocomial outbreak of neonatal *S.* Enteritidis meningitis in children in Tanzania (Vaagland *et al*., 2004; Molyneux *et al*., 2009).

The emergence and persistence of antimicrobial resistant gram-negative bacteria such as *Salmonella* spp. is becoming a serious public health concern to the contemporary world (Rodriguez-Rojas *et al*., 2013). Because of the intensive use of antimicrobials in food animal production, food animal products such as meat, eggs and milk are frequently contaminated with antimicrobial resistant foodborne pathogens (Hammerum and Heuer, 2009). Of significant importance to public health is the ability of resistant pathogens in food animals finding their way into humans (Howard *et al*., 2001). Thus, humans can be colonized with the antimicrobial resistant pathogens of animal origin, and if this occurs then this will limit therapeutic options available to treat foodborne bacterial infections in humans (Howard *et al*., 2001; Gilbert, 2012).

As recently reported, MDR *Salmonella* serotypes are common in food animal production regardless of the use of antibiotics on the farms (Gebreyes and Thakur, 2005). These findings imply that the presence of selective pressure in the production environment, other than antibiotics, could play a role in the emergence and persistence of MDR *Salmonella* isolates in both conventional and antibiotic-free systems (Gebreyes *et al*., 2006). The occurrence of bacterial resistance to antimicrobials is reported to be partly caused by the rapid evolution of the bacterial genome under selective pressure of routinely used antimicrobials (e.g. antibiotics, biocides and heavy metals). Resistance to disinfectants has been reported in *Salmonella* spp. and other pathogens. A study in poultry farms in Denmark reported possible association between persistence of *Salmonella* spp. and resistance to commonly used disinfectants (Gradel *et al*., 2005). In the United Kingdom, Randall *et al*. (2005a) also reported the association of cyclohexane resistance with multi-drug resistance in *E. coli* and also found that the use of biocides may lead to an increased selective pressure towards antibiotic resistance in *Salmonella* isolates (Randall *et al*.,

2004). In this study, the MDR *Salmonella* isolate is defined as that isolate resistant to two or more antibiotics belonging to different classes of antibiotics (Chiu *et al*., 2010).

Heavy metals micronutrients such as zinc (Zn), copper (Cu), cobalt (Co), nickel (Ni), manganese (Mn), iron (Fe) and others have the nutritional characteristics (NRC, 2012). The most common heavy metal micronutrients that are used in swine ration are copper and zinc. These are essential micronutrients [trace elements] for growth which also have cytotoxic effect on bacteria. Copper and zinc are relatively non-toxic to mammalian tissues and systems. They are used in agriculture as in-feed growth promoters and for enteric disease control, particularly in the pig and poultry sectors (Wales and Davies, 2015). Previous studies identified genetic elements among *Salmonella* isolates that render some strains resistant to copper (Lim *et al*., 2002; Aarestrup and Hasman, 2004) and zinc (Aarestrup and Hasman, 2004). Such resistant strains were shown to carry specific resistance genes that are also associated with multiple antimicrobial resistance factors (Ciraj *et al*., 1999). A recent study on enteric bacteria such as Enterococci also reported an association of a copper resistance gene with that of antimicrobial resistance to macrolides on a conjugative plasmid (Hasman and Aarestrup, 2002). A study in the United Kingdom also showed *Salmonella* resistance to copper in swine production which was shown to be transferable to other organisms through conjugation (Williams *et al*., 1993). Generally speaking, various studies have reported the high occurrence of antimicrobial resistance in foodborne pathogens in swine production environment, but the intricate relationship with various selective pressures that enable such MDR strains to persist is poorly understood. Therefore, the current study investigated the role of various chemical interventions in the emergence and persistence of heavy metal and biocide-tolerant *Salmonella* isolates and its co-selective association with MDR *Salmonella* isolates. The study further investigated the

molecular epidemiology and antimicrobial resistance of *Salmonella* isolates from food animals and animal products.

1.4 Hypotheses of the Study

There are two hypotheses in the current study. Firstly, it is hypothesized that the use of certain classes of biocides and specific heavy metal micronutrients [copper and zinc] is associated with the emergence and persistence of MDR *Salmonella*. Secondly, that the circulating *Salmonella* isolates in food animals and animal products in Tanzania exhibit genotypic diversity and different phenotypic expressions suggesting their zoonotic potential.

1.5 Objectives of the Study

1.5.1 Main objective

To identify and characterize the role of various chemical interventions in food animal production systems including heavy metal micronutrients and biocides in the emergence of heavy metal and biocide-tolerant *Salmonella* and their epidemiological association to emergence and persistence of multi-drug resistant *Salmonella* isolates and also to determine the molecular epidemiology and antimicrobial resistance of *Salmonella* isolates from food animals and animal products.

1.5.2 Specific objectives

a) To determine the role of heavy metal micronutrients in swine feed in emergence of heavy metal-tolerant and multi-drug resistant *Salmonella* isolates.
b) To determine the role of biocide in swine production environment in emergence of biocide-tolerant and multi-drug resistant *Salmonella* isolates.

c) To determine the prevalence and antimicrobial resistance profiles of *Salmonella* isolates from food animals and animal products.
d) To investigate the mechanisms of antimicrobial resistance in *Salmonella* isolates from food animals and animal products.
e) To describe the phenotypic and genotypic relatedness of *Salmonella* isolates from food animals and animal products.

1.6 Organization of the Thesis

This PhD thesis is prepared according to 'Publishable manuscript' format of the Sokoine University of Agriculture. The first chapter addresses introduction, literature review, problem statement and justification, hypotheses, objectives, and the organization of the thesis. The findings of one study conducted in fulfilling one specific objective have already been published in a peer-reviewed scientific journal, and these findings, therefore, form Chapter two of the thesis. This paper presents findings on "In-Feed Use of Heavy Metal Micronutrients in U.S. Swine Production Systems and Its Role in Persistence of Multi-drug Resistant Salmonellae". Chapters three and four contain two publishable manuscripts for the two more studies conducted in the United States and Tanzania, respectively. The manuscript for the study conducted in the United States presents findings on "In-Use Concentrations of Different Classes of Biocides in U.S. Swine Production Systems and Its Role in Persistence of Biocide-tolerant and Multi-drug Resistant Salmonellae". The manuscript for the study conducted in in Tanzania presents findings on "Multi-drug Resistant *Salmonella* Isolates from Food Animals and Animal Products in Livestock in Tanzania". Chapter five presents the general discussion, conclusion and recommendations.

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CHAPTER TWO

In-Feed Use of Heavy Metal Micronutrients in U.S. Swine Production Systems and Its Role in Persistence of Multi-drug Resistant Salmonellae

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Abstract

The study aimed to characterize the role of heavy metal micronutrients in swine feed in emergence of heavy-metal-tolerant and multidrug-resistant *Salmonella* organisms. We conducted a longitudinal study in 36 swine barns over a 2-year period. The feed and fecal levels of Cu^{2+} and Zn^{2+} were measured. *Salmonella* was isolated at early and late finishing. MICs of copper sulfate and zinc chloride were measured using agar dilution. Antimicrobial susceptibility was tested using the Kirby-Bauer method, and 283 isolates were serotyped. We amplified *pcoA* and *czcD* genes that encode Cu^{2+} and Zn^{2+} tolerance, respectively. Of the 283 isolates, 113 (48%) showed Cu^{2+} tolerance at 24 mM and 164 (58%) showed Zn^{2+} tolerance at 8 mM. In multivariate analysis, serotype and source of isolates were significantly associated with Cu^{2+} tolerance ($P< 0.001$). Fecal isolates were more likely to be Cu^{2+} tolerant than those of feed origin (odds ratio [OR], 27.0; 95% confidence interval [CI], 2.8 to 250; $P= 0.0042$) or environmental origin (OR, 5.8), implying the significance of gastrointestinal selective pressure. *Salmonella enterica* serotypes Typhimurium and Heidelberg, highly significant for public health, had higher odds of having >20 mM MICs of Cu^{2+} than did "other" serotypes. More than 60% of *Salmonella* isolates with resistance type (R-type) AmStTeKm (32 of 53) carried *pcoA*; only 5% with R-type AmClStSuTe carried this gene. *czcD* gene carriage was significantly associated with a higher Zn^{2+} MIC ($p< 0.05$). The odds of having a high Zn^{2+} MIC ($\geq$ 8 mM) were 14.66 times higher in isolates with R-type AmClStSuTe than in those with R-type AmStTeKm ($p< 0.05$). The findings demonstrate strong association between heavy metal tolerance and antimicrobial resistance, particularly among *Salmonella* serotypes important in public health.

Introduction

Nontyphoidal *Salmonella enterica* serotypes are among the most important food-borne bacterial pathogens, with a broad host range, including food animals and humans. *Salmonella enterica* remains one of the leading causes of food-borne illness (11%), hospitalization (35%), and death (28%) in the United States (1). In addition, most strains of the commonly occurring serovars, such as Typhimurium, have been shown to exhibit multidrug resistance, resistance to two or more antimicrobials (2–4). Previous studies on antimicrobial resistance have shown the emergence of multidrug-resistant (MDR) *Salmonella* in swine production systems even when there was no history of using antimicrobials, either as therapeutics or as growth promoters (5–7). The emergence and persistence of MDR *Salmonella* serovars in a swine production environment where there is no history of antimicrobial use suggest the presence of other risk factors such as selective pressure, including the use of heavy metal micronutrients in intensive swine production units.

Micronutrients such as copper and zinc, among many others, are included in swine feed and other livestock to achieve growth promotion and increase feed efficiency (8, 9). Zinc and copper are essential trace elements for prokaryotic and eukaryotic cellular metabolic functions. Zinc is a cofactor of more than 300 metallo-enzymes, including alkaline phosphatases, whereas copper is needed for activation of several oxidative enzymes required for normal cellular metabolism (10, 11). Due to the proven and anticipated beneficial effects of zinc and copper in swine production, in-feed supplementation of zinc and copper in commercial production systems has been very common (12, 13).

On the other hand, tolerance to various chemicals among bacterial pathogens, mediated by different mechanisms, has also been on the rise. Multidrug efflux systems have been shown to be important mechanisms of resistance against antimicrobial agents and other

structurally unrelated compounds, including heavy metals and biocides. The mechanisms of heavy metal resistance to copper in *Enterococcus faecium* isolates from pigs have been associated with the carriage of a conjugative plasmid carrying copper resistance determinants such as *tcrB* (14–16). Another efflux system that has also been associated with copper tolerance reported in Gram-negative organisms is the PCO operon, which mediates resistance to Cu^{2+}.

Resistance to Zn^{2+} and other metals such as Co^{2+} and Cd^{2+} is conferred by genetic determinants often carried by a plasmid such as pMOL30. In the CZC operon system, the products of the *czc* gene clusters function as a main component of an efflux protein. (CzcA), a cation funnel (CzcB), a modulator of substrate specificity (CzcC) (17–20), and a protein involved in regulation of the operon (CzcD). The CzcD gene is involved in the regulation of a zinc, cobalt, and cadmium efflux system, the Czc system, which mediates resistance to these heavy metal cations (19, 21).

Co-selection of heavy-metal-tolerant and antimicrobial-resistant phenotypes often occurs when the genes are co-located on genetic elements such as a plasmid, transposon, or integron (22– 24). The efflux pump in bacteria plays a major role in development of resistance to several classes of antimicrobial agents. The presence and significance of resistance factors against micronutrients among *Salmonella* strains in the United States are poorly understood, and their association with antimicrobial resistance in the production environment has not been investigated. The present study was conducted to characterize the role of heavy metal micronutrient interventions (such as those by copper and zinc) in the emergence of heavy-metal-tolerant *Salmonella* and also its co-selective association with multidrug-resistant *Salmonella*. Further, we investigated the association with carriage of specific efflux gene markers.

MATERIALS AND METHODS

Study design and sample collections. This study was part of a large longitudinal group-randomized controlled study designed to investigate the association of heavy metal micronutrients in swine feed with the occurrence and persistence of multidrug-resistant (MDR) *Salmonella*. The field part of this study was conducted Raleigh, North Carolina, United States and the laboratory part of the study was done in Columbus, Ohio, United States. Briefly, three vertically integrated commercial swine production systems (systems 1, 2, and 3) selected based on their history of *Salmonella* occurrence were included. From each system, three farms were selected (total of 9 farms). At each farm, four barns were randomly selected for further follow-up in this study, and all barns used standardized disinfection systems to limit introduction of additional potential confounding effects. We visited each farm at two stages (early finishing and late finishing) in four replicates (repeated visits to the same barns during the study period of October 2007 to November 2009). Each replicate visit consisted of sampling assigned barn floors before and after disinfection, pigs at early and late finishing stages, and pooled feed samples (25). Sampling was done from all the 36 barns for a period of more than 2 years. A total of 48 fresh fecal samples (25 g) were aseptically collected from each barn in four replicates at the early finishing stage (6 to 9 weeks of age) (n = 6,842) and at late finishing stages (26 to 28 weeks of age) of production (n = 6,093) from individual pigs. Some samples were lost/missed at different stages of the study (48 samples/ barn X 36 barns X 4 replicates = 6,912 samples). Approximately 100 g of pooled feed samples (1 sample per barn collected from all 36 barns at 2 stages and 4 replicates with 13 losses to follow-up) was aseptically collected from 36 barns (n = 275) over a period of 2 years. Each pooled feed sample per barn was aseptically collected from the feeder bin in sterile Whirl-Pak bags and shipped to the laboratory on the same day as collection. For each farm, a survey assessment including

questions about basic production, herd health management, biosecurity, and in-feed use of heavy metals (copper and zinc) was done.

***Salmonella* isolation and identification.** Salmonellae were isolated and identified according to conventional methods as described previously (26, 27). Briefly, a 10-g portion of each fecal and feed sample was pre-enriched in 90 ml of buffered peptone water (BPW; Becton, Dickinson, Sparks, MD), and 90 ml of BPW was added to each Whirl-Pak bag containing individual drag swabs and incubated at 37°C overnight. The remaining portions of fecal and feed samples were stored at -20°C. After overnight incubation, 100 µl of the pre-enriched suspension was added into 9.9 ml of Rappaport-Vassiliadis (RV) enrichment broth (Becton, Dickinson, Sparks, MD) and incubated at 42°C for 24 h. A 10-µl portion of the suspension was inoculated onto xylose-lactose-Tergitol 4 (XLT-4) agar (Becton, Dickinson, Sparks, MD) plates and incubated at 37°C for 24 h, and incubation was extended to 48 h in cases where colonies were doubtful. Three presumptive *Salmonella* colonies were selected from each positive plate for biochemical testing. Each selected presumptive *Salmonella* colony was then inoculated onto triple sugar iron (TSI) agar slants (Becton, Dickinson, Sparks, MD), lysine iron agar (LIA) slants (Becton, Dickinson, Sparks, MD), and urea broth (Becton, Dickinson, Sparks, MD) and incubated at 37°C for 24 h. All biochemically confirmed *Salmonella* isolates were then stored at -80°C until further testing.

Phenotyping. *Salmonella* isolates recovered from swine feed (n = 30), swine barn floors (n = 1,628), and swine feces (n = 4,504) were serogrouped using commercially available polyvalent O and group-specific antisera (Mira Vista, Copenhagen, Denmark) according to the recommendations of the manufacturer. Of all *Salmonella* isolates biochemically

confirmed (n = 6,162), 283 *Salmonella* isolates were systematically selected based on origin and phenotypic characteristics (serogrouping and antimicrobial resistance profiles) and submitted to the National Veterinary Services Laboratories (USDA-NVSL, Ames, IA) for serotyping. S*almonella* isolates were tested for antimicrobial susceptibility to a panel of 12 antimicrobials using the Kirby-Bauer disc diffusion method according to the guidelines of the CLSI (28). The antimicrobials used and their respective disc potencies were as follows: ampicillin (Am; 10 μg/ml), amoxicillin-clavulanic acid (Ax; 30 μg/ml), amikacin (An; 30 μg/ml), ceftriaxone (Ce; 30 μg/ml), cephalothin (Ch; 30 μg/ml), chloramphenicol (Cl; 30 μg/ml), ciprofloxacin (CIP; 5 μg/ml), gentamicin (Gm; 10 μg/ml), kanamycin (Km; 30 μg/ml), streptomycin (St; 10 μg/ml), sulfisoxazole (Su; 250 μg/ml), and tetracycline (Te; 30 μg/ml). We used *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853 as control strains. *Salmonella* isolates showing resistance to three or more antimicrobials were classified as multidrug resistant (MDR), and those isolates showing intermediate resistance profiles were considered susceptible.

Copper and zinc level determinations in swine feed and fecal samples. Pooled feed and fecal samples were shipped to the Ohio State University soil analytic laboratory for copper and zinc level determinations. The concentrations of copper and zinc in pooled feed and fecal samples were measured by inductively coupled plasma emission spectroscopy (PerkinElmer Optima 2000) using a method adapted from the work of Luo et al. (29). Quality control samples included a negative control (blank), positive control (two matrix spikes), and one standard reference material per batch of 20 samples. The methods have been modified from those of Lindsey et al. (30), Ollers et al. (31), Zhu et al. (32), and USEPA 3051b (33).

MIC of copper sulfate and zinc chloride. Three hundred forty-nine *Salmonella* isolates, from 283 samples, recovered from floor swabs (*n* = 179 samples), feces (*n* = 94 samples), and feed samples (*n* = 10 samples) with different antimicrobial resistance patterns were systematically selected for tests of tolerance to different concentrations of zinc chloride ($ZnCl_2$) and copper sulfate ($CuSO_4$). The agar plate-dilution method was used to determine the MIC against *Salmonella* as described before (34). The susceptibilities were determined on Mueller-Hinton II (MH-II) agar plates with a dilution range for zinc chloride of 0, 0.25, 0.5, 1, 2, 4, 8, and 16 mM, with the pH of the medium adjusted to 5.5. Copper sulfate solutions contained the dilution range of 0, 1, 2, 4, 8, 16, 20, 24, 28, and 32 mM with the pH of the medium adjusted to 7.2. Briefly, 25 ml of MH agar was aseptically dispensed and allowed to solidify. Bacterial suspensions were adjusted to 10^7 CFU/ml (100 µl of each inoculum at a 0.5 McFarland standard plus 900 µl of sterile 0.85% NaCl solutions). Each of the 400-µl suspension was aseptically aliquoted to a corresponding well of the replicator inoculum block. All test *Salmonella* isolates and control strains were tested in triplicate. The inoculated plates were incubated at 37°C for 16 to 20 h. Plates were assessed for growth, and the MIC was determined. The MIC was defined as the lowest concentration that inhibits the visible growth of *Salmonella*. *Enterococcus faecium* A17 sv 1 HHA 210, *S. aureus* C10682, *S. aureus* ATCC 29213, and *S. aureus* SO385 were used as reference strains. The reference strains were generously provided by Henrik Hasman (Technical University of Denmark).

Identification of heavy metal micronutrient (Cu^{2+} and Zn^{2+}) tolerance genes. The 283 *Salmonella* isolates were tested for the carriage of selected tolerance genes (*pcoA* for copper tolerance and *czcD* for zinc tolerance) using PCR. Briefly, *Salmonella* isolates were inoculated onto tryptic soy agar (TSA) plates and incubated at 37°C overnight. The

genomic DNA was extracted using the Qiagen DNeasy tissue kit according to the manufacturer's instructions (Qiagen Ambion, Austin, TX, USA). Primers used for amplification of the *pcoA* gene included the following primers: Forward (5'-CGTCTCGACGAACTTTCCTG-3') and Reverse (5'-GGACTTCACGAAACATTCCC-3'). The thermocycling conditions included Hot Start *Taq* activation at 95°C for 5 min, denaturation at 95°C for 1.5 min, annealing at 57°C for 1.5 min, and extension at 72°C for 2 min, and amplification was done in 34 cycles (35). Primers used for amplification of the *czcD* gene included Forward (5'-TTTAGATCTTTTACCACCATGGGCGC-3') and Reverse (5'-TTTCAGCTGAACATCATACCCTAGTTT-3') (36). The PCR amplification conditions were initial denaturation at 94°C for 2.5 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, and then the amplification cycle was repeated for a further 28 cycles and final extension was done at 72°C for 5 min. Ten microliters of the PCR product of each isolate tested was electrophoresed on a 1% agarose gel stained with 5 μl of 10-mg/ml ethidium bromide for 1 h at 120 V using 0.5X Tris-borate-EDTA (TBE) as running buffer. A 1-kb Plus DNA ladder was used as a molecular size marker.

Data analysis. Data were first summarized descriptively: levels of *Salmonella* prevalence in different samples, proportions of isolates that were resistant or were carrying heavy metal tolerance genes, and copper and zinc MICs were calculated. To explore the role of heavy metals and heavy metal tolerance in *Salmonella* in swine production, the data were analyzed with SAS v.9.3 (SAS Institute Inc., Cary, NC) using a generalized linear mixed model approach (PROC GLIMMIX), with binary distribution and logit link. The clustered data structure was accounted for by testing and including production company, farm, and barn as random effects in the models. Only one isolate per sample was included in the statistical modeling. Isolates were categorized based on their heavy metal (Zn and Cu)

MIC values into high- or low-MIC groups (Zn MIC equal to 8 = high; Cu MIC equal to 24 mM = high), as well as on carriage of the zinc (*czcD*) or copper (*pcoA*) tolerance gene (yes/no). These characteristics were used as outcomes in the statistical analysis, and separate models were run for Cu and Zn.

Isolates were categorized into three groups based on their serotype: Typhimurium, Heidelberg, and "other." Based on their resistance patterns (R-type), isolates were also categorized into four groups: AmClStSuTe, AmStTeKm, resistant to any other combination of drugs, or pansusceptible. Associations between the above-mentioned outcomes and feed and fecal levels of the metals, source of the isolates (fecal, feed, or floor swab), different serotypes, and resistance patterns were assessed. Initially, all factors were individually modeled against the outcome in a univariate screening, and all that were associated with the outcome with P of < 0.2 were included in a full model. Nonsignificant ($P > 0.05$) variables were dropped one at a time until all variables remaining in the model were significant. Of the 283 isolates selected for further characterization, 100% of serovar Heidelberg and Typhimurium isolates were multidrug resistant; thus, no further statistical modeling was performed on multidrug resistance.

RESULTS

Antimicrobial resistance of *Salmonella*. *Salmonella* isolates were detected in 17.2% (1,180/6,842) and 7.1% (431/6,093) of the pigs at early and late finishing stages, respectively. Interestingly, in each of the three production companies different *Salmonella* serovars appeared to dominate: Typhimurium was found most frequently in production system 1 (61.8% of all isolates), Heidelberg was found most often in production system 2 (50.3%), and "other" serovars were isolated most commonly in production system 3

(56.5%). Antimicrobial resistance was very common among the isolates, with 90% (27 of 30) of *Salmonella* isolates that originated from feed samples, 92.3% (1,503 of 1,628) of isolates from drag swabs, and 98.02% (4,415 of 4,504) of isolates from swine fecal samples showing resistance to one or more of the antimicrobials tested. Almost two-thirds, 60% (18 of 30), of the *Salmonella* isolates recovered from feed samples, 51.4% (836 of 1,628) of the isolates from barn floor drag swabs, and 76.1% (3,428 of 4,504) of the isolates from swine fecal samples were multidrug resistant (MDR). Table 1 summarizes the phenotypic properties of *Salmonella* serotypes (n = 283) further characterized using phenotypic and genotypic approaches.

Fecal and feed levels of heavy metal micronutrients (Zn^{2+} and Cu^{2+}). The level of zinc in the pooled feed samples varied between 77 mg/kg and 2,000 mg/kg of feed with a median of 139.8 mg/kg. The levels of Zn^{2+} in fecal samples were found to be significantly higher than the in-feed levels, ranging between 536.5 mg/kg and 12,557.2 mg/kg with a median of 941.1 mg/kg. Copper levels were found to range between 3.2 mg/kg and 365.2 mg/kg in pooled feed samples, with a median of 31.5 mg/kg. Copper levels in fecal samples were also higher, ranging from 71.2 mg/kg to 2,397 mg/kg with a median of 137.6 mg/kg. Table 2 depicts the levels of copper and zinc in feed and fecal samples. Overall, the Zn^{2+} levels in feed or fecal samples were not associated with the occurrence of high tolerance of Zn^{2+} ($\geq$ 8 mM). On the other hand, copper tolerance was significantly associated with the Cu^{2+} levels found in fecal matter but not in feed.

Analysis of copper (Cu^{2+} tolerance. Source of the isolates (fecal, feed, or floor swab sample), serotype, the resistance pattern that the isolates exhibited, and the copper level in fecal matter were associated with Cu^{2+} MICs in the univariate screening ($P < 0.2$). Forty-

seven percent (134 of 283) of the isolates showed tolerance to Cu^{2+} at 24 mM, while the remaining 53% showed tolerance ranging from 4 mM to 20 mM. Carriage of the *pcoA* gene ($P = 0.8473$), copper level in the swine feed ($P = 0.6501$), or stage of sampling was not significantly associated with copper tolerance of *Salmonella* isolates. The carriage of the *pcoA* gene versus Cu MIC in *Salmonella* isolates recovered from fecal matter, feed, and barn floors is shown in Tables 1 and 3. When using multivariate analysis, two variables, serotype ($P = 0.0006$) and source of the isolates ($P< 0.0001$), remained significant in the model (Table 4). Resistance pattern became nonsignificant, and its effect appeared to be explained by the serotype when the two were included in the model simultaneously.

The odds of *Salmonella* isolates having high Cu^{2+} MICs (≥ 20 mM) were 5.8 times higher if the isolates originated from fecal samples rather than from floor swabs (95% confidence interval [CI] for an odds ratio [OR] of 3.1 to 11.1, $P = 0.0002$), indicating the significance of selective pressure in the gut ecosystem. Isolates of fecal origin were also significantly more likely to be tolerant to Cu^{2+} (MIC, ≥ 20 mM) than were those originating from feed samples (OR, 27.0; 95% CI, 2.8 to 250; $P = 0.0042$) (Table 4).

We found that serotype Heidelberg had 5.6-times-higher odds (95% CI for OR, 2.3 to 13.5; $P = 0.0002$) of having a MIC higher than 20mMMIC than did "other" serotypes. Similarly, Typhimurium isolates were 1.3 times as likely to have high Cu^{2+} MICs (> 20 mM) as were the "other" serotypes, but the difference was not statistically significant (95% CI for OR, 0.7 to 2.4; $P = 0.4741$). Also, serotype Heidelberg was 4.4 (95% CI for OR, 1.8 to 10.5; $P = 0.0009$) times as likely to have a MIC higher than 20 mM as was serovar Typhimurium (results not shown).

The copper extrusion efflux gene, *pcoA*, was detected in 35% (99/283) of the *Salmonella* isolates (Table 3). More than 60% of *Salmonella* isolates with R-type AmStTeKm (32 of 53) carried the *pcoA* gene, while 5% of those with R-type AmClStSuTe (4 of 84) carried *pcoA*. The *pcoA* gene was detected in all three categories of serotypes regardless of their MDR status (Table 3). Of the detected *pcoA* genes, 40% were found in serovar Heidelberg and 22% were found in serovar Typhimurium. A lower proportion of these genes was also detected in the following serotypes: Senftenberg, Worthington, Derby, Ohio, Mbandaka, London, Agona, and Rissen. This finding implies the common occurrence of the gene in *Salmonella* (Table 3).

Analysis of zinc (Zn^{2+}) tolerance. In the univariate screening, carriage of the *czcD* gene, serotype, resistance pattern, and source of the isolates were all significantly associated with high Zn^{2+} MICs. However, neither Zn^{2+} levels in the feed ($P = 0.9613$) nor those in the feces ($P = 0.8043$) were significantly associated with high Zn^{2+} MIC levels. Sixty percent (171 of 283) of the isolates showed zinc tolerance at 8 mM, and the remaining 40% (112 of 283) showed tolerance at 4 mM. The carriage of the *czcD* gene versus Zn MIC in *Salmonella* isolates recovered from fecal samples, feed, and barn floors is shown in Tables 1 and 3. In the final model, carriage of the *czcD* gene, serotype, and source of the isolates remained significantly associated with high Zn^{2+} tolerance (Table 5). Isolates carrying the *czcD* gene had 10.6-times-higher odds of having a Zn^{2+} MIC of 8 mM or more than did those not carrying the gene (95% CI for OR, 4.0 to 27.8; $P < 0.0001$), adjusting for the serotype. "Other" serotypes had 4.5 (95% CI for OR, 2.2 to 9.5; $P < 0.0001$)- and 2.4 (95% CI for OR, 1.0 to 5.6; $P = 0.0504$)-times-higher odds than serotype Typhimurium of having a high Zn^{2+} MIC, whereas Heidelberg isolates were only approximately half as likely to have a high Zn^{2+} MIC as were Typhimurium isolates (OR =

0.52; 95% CI for OR, 0.211 to 1.289), but the difference was not statistically significant (P = 0.1578) (Table 5). In the model with serotypes, resistance pattern became nonsignificant and its effect appeared to be explained by the serotype. In contrast to the findings with copper tolerance, isolates from floor swabs had 6.5-times-higher odds (95% CI for OR, 3.2 to 12.9; $P < 0.0001$) of having high tolerance for Zn^{2+} than did fecal isolates. Also, isolates from feed samples had 3.0- times-higher odds of having a Zn^{2+} MIC of >8mM than did fecal isolates, even though the difference was not significant (95% CI for OR, 0.7 to 13.5; P = 0.1546) (Table 5).

Assessment of association between tolerance and gene carriage showed that isolates with a high Zn^{2+} MIC also were more likely to carry the *czcD* gene (Table 3). The odds of *czcD* gene carriage were 5.2 times higher for isolates with high Zn^{2+} MICs than for those with low Zn^{2+} MICs (OR = 5.2; 95% CI, 2.4 to 11.5; $P < 0.0001$). However, 9.8% of the *Salmonella* isolates (11/112) with low Zn^{2+} MICs also carried the *czcD* gene, suggesting that carriage of this gene is not always associated with a Zn^{2+} -tolerant phenotype. Of the high-zinc-tolerant isolates, 52% belonged to serovar Typhimurium and 12% belonged to serovar Heidelberg. No *Salmonella* serovar Heidelberg carried the *czcD* gene, indicating a different mechanism for tolerance, whereas 69% of the Typhimurium isolates carried the *czcD* gene. Interestingly, none of the pansusceptible *Salmonella* isolates (12 of 283, 4.2%) were found to carry the *czcD* gene even though 66.7% (8/12) were tolerant to Zn^{2+} at 8 mM (Table 3). This indicates the presence of other mechanisms apart from the *czcD* gene. While the finding is just in contrast to the carriage of the *pcoA* gene encoding copper tolerance, it shows the occurrence of a strong association between distinct heavy metal tolerance and antimicrobial resistance (R-types).

The Zn^{2+} tolerance gene, *czcD*, was detected in 30% (85 of 283) of the *Salmonella* isolates. None of the *Salmonella* isolates with R-type AmStTeKm carried the *czcD* gene, while 84% of those with R-type AmClStSuTe (71 of 84) carried *czcD*. The *czcD* gene was almost exclusively detected in serotype Typhimurium (84 of 85); none of the Heidelberg isolates carried the gene and only one of the isolates belonging to the "other" serotypes carried the gene.

DISCUSSION

The magnitude of multidrug resistance in *Salmonella* and other pathogens at the human-animal and ecosystem interface has been a major concern globally. As we previously reported (25), in addition to isolation of *Salmonella* from feces and barn floor swabs, in the current study, *Salmonella* was also detected in 3.6% (10/275) of the commercially processed swine feed samples. Besides the direct selective pressure of antimicrobial resistance, co-selection due to other structurally related or unrelated chemical agents has also been a concern for the rising trend in multidrug resistance. However, there have been very limited studies conducted in this area. The current study attempts to fill the knowledge gap, mainly focusing on the use of heavy metal micronutrients. Previous studies have identified genetic elements among *Salmonella* strains that render some strains resistant to heavy metal micronutrients, including copper (37–39) and zinc (39, 40). Such resistant strains were shown to carry genes associated with multiple antimicrobial resistance factors (34, 38).

Heavy metal micronutrients such as Zn^{2+} at relatively low concentrations are essential for microorganisms since they provide vital cofactors for metallo-proteins and enzymes (2, 41). The use of copper in swine feed has also been repeatedly shown to have a positive

effect in production performance (42, 43), especially when dietary Cu^{2+} is supplemented above the National Research Council (NRC) (8) requirement of 5 mg Cu^{2+} /kg (44). Copper and zinc are considered two of the most widely researched alternatives to growth promotion antibiotics to enhance swine performance and maintain health (45). In the current study, we found a wide range in the levels of copper and zinc used in swine feed and concentrations often much higher than the NRC recommendations, as depicted in Table 2. In addition, it is worth noting that the concentrations of both micronutrients were higher in the fecal samples than in the feed. This is expected, and it was previously reported that as the various feed ingredients are absorbed within the gastrointestinal tract, a few elements such as heavy metal micronutrients tend to be more concentrated in the feces, and as a result, a large percentage of the consumed dietary copper and zinc ends up in feces (8), indicating a relatively low retention in the intestine and significant excretion of these minerals in feces. Previous reports (46) also showed that this phenomenon is particularly more significant in swine and poultry feces and reported that the concentration of zinc in swine feces is 10 to 100 times higher than that in dairy manure and that liquid swine manure had six times as much copper as did liquid dairy manure. In addition, the recent NRC report (8) indicated that even though high levels of dietary copper and zinc have been shown to improve animal performance, a large percentage of consumed minerals such as copper and zinc (approximately 90 to 95%) is excreted in the feces and ends up as environmental contaminants. In this study, we observed that the level of micronutrients, particularly zinc, in the swine feed is much higher than the NRC daily requirements. While we cannot confirm it, the high level of use could potentially ameliorate the co-selective pressure and its association with antimicrobial resistance. This area may need further investigation.

A few studies have reported different tolerance levels of important foodborne pathogens such as *Salmonella* and *E. coli* to different concentrations of Zn^{2+} and Cu^{2+} (34, 47). This study has reported up to 8 mM as the highest level of tolerance of *Salmonella* isolates to Zn^{2+}, which is higher than the level reported by Aarestrup and Hasman (39). The highest level of tolerance of *Salmonella* isolates to Cu^{2+} reported in our study was 24 mM. This is smaller than the maximum tolerance reported by the same study (39). The increased tolerance level of *Salmonella* isolates to Zn^{2+} and Cu^{2+} is attributable to the use of the respective micronutrients in swine feed. Zinc is known to inhibit some of the bacterial populations in the intestinal tract and thereby improve the health or feed conversion ratio of the food animals (39, 48, 49).

Carriage of the *pcoA* gene or level of copper in swine feed was not significantly associated with copper tolerance of *Salmonella* isolates. This may have different implications. One clear reason might be the presence of various other mechanisms that may result in copper tolerance besides *pcoA* and associated operon systems. Other genes, including *cuiD* and *scsC*, and other mechanisms have also been reported to encode copper tolerance in *Salmonella* (50, 51).

The PCR results on the level of *czcD* gene carriage by *Salmonella* isolates were consistent with the increase in the level of the Zn^{2+} MIC of *Salmonella* isolates. The PCR results on the level of *pcoA* gene carriage by *Salmonella* isolates were also consistent with the increase in the level of the Cu^{2+} MIC of *Salmonella* isolates. The two multidrug-resistant R-types AmClStSuTe and AmStTeKm were observed to be the highest-occurrence resistance types in the *Salmonella* isolates in this study; however, their occurrence varied by the type of heavy metal tolerance gene carriage. It should be noted that *Salmonella*

enterica serovar Typhimurium strains of phage type DT104, often containing the R-type AmClStSuTe, are some of the major strains reported worldwide and are commonly isolated from humans and food animals (52, 53). While there have been very limited studies conducted to date investigating the association between heavy metal tolerance and specific antimicrobial resistance patterns, a study in 1984 in drinking water reported the overall association between copper and zinc tolerance and antimicrobial resistance (54, 55). Heavy metal tolerance was more common among isolates from the fecal samples than among those of environmental origin (drag swabs).

In summary, the findings in this study clearly demonstrated the presence of a strong association between decreased susceptibility to heavy metals and antimicrobial resistance among *Salmonella* serovars isolated from swine, swine feed, and barn floors. The detection of decreased susceptibility to heavy metal micronutrients (copper and zinc) and associated genetic determinants among various *Salmonella* strains has implications for the control of multidrug-resistant *Salmonella* strains, which are of public health and veterinary medicine significance. However, further studies investigating the role of co-selection and mechanisms of genetic linkage could shed further light on the relationship and its significance.

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Table 2.1: Summary of phenotypic properties of *Salmonella* isolates (*n* = 283)

			Zn				Cu			
Serotype	Resistance pattern		MIC	Presence of czcD (no. of strains)	Level in sample (mg/kg)		MIC	Presence of pcoA	Level in sample (mg/kg)	
(no. of strains)	(R-type), no. of strains	Source	(mM)		Feed	Fecal	(mM)	(no. of strains)	Feed	Fecal
Schwarzengrund (3)	AmStTeAxChKm (1)	Swab	8	0	196	1,436	24	0	14	260
	Pansusceptible (2)	Swab	8	0	196	1,436	24	0	14	260
Mbandaka (7)	AmClStSuTeAx (1); AmSuChCe (1); StSuTe (2)	Swab	8	0	196–527.3	1,436–4,167.2	20–24	0	14–194.7	260–1,209.8
	AmStTe (1); AmClStSuTeAxCh (1)	Fecal	4-8	0	109.0–144	674–9,027.2	20–24	0 (1), + (1)	157.7–173	1,142–1,704.2
	SuTe (1)	Feed	8	0	1,668	10,530	16	1	268	1,852
Anatum (9)	AmTeAxChCeXNL (1); AmTeAxChXNL (1);	Swab	8	0	140.1–171.3	774–1,383.1	20–24	0	11.9–97	137.8–742
	AmStTeAxCh/XNL (3)									
	Pansusceptible (2)	Swab	4-8	0	171.3–201	1,017–1,691	20	0	34	406
	AmStTeAxCh (2)	Fecal	8	0	96	787	24	0	146	1,496
Derby (17)	StSuTe (4)	Swab	4-8	0	132.7–201	939.9–11,631	20	0	9.4–34	112.9–406
	AmStSuTeAxChCe/XNL (6); AmClStSuTe (1);	Fecal	4-8	0	108.8–2,000	865.9–11,185	20–24	0	8.3–316	110.6–1,911
	AmStSuTe/ Km (2)									
	AmStSuTe (1); AmStSuTeAxCh (1)	Swab	4-8	0	1,389.1–1,393.2	8,714.8–10,146.2	20	1	249.3–268.3	1,645.5–1,875.3
	Pansusceptible (1)	Fecal	4	0	91.4–166.3	639.2–1,229.4	20–24	0 (6), + (1)	6.2–173	95–143.9
	Te (1)	Feed	8	0	147.6	719.7	16	0	146.2	919.2
Heidelberg (72)	AmStTeKm (1)	Feed	4	0	127.2	736.1	20	1	20.9	86.7
	AmStTeKm (12); AmStTeKmGm (2); AmStSuTeKmGm/ Ax (2)	Swab	4-8	0	93.4–7,383.7	710.8–8,494.7	20–24	0 (7), + (9)	18.7–1,384.3	106.6–1,564.3
	AmStTeKm/AxChkmXNL (31); AmStSuTeKm	Fecal	4-8	0	79–2,000	675.3–11,631	20–24	0 (17), + (25)	135.4–308	137.3–2,130.7
	/Gm (10); AmClStSuTeKm (1) AmStTeAxChKmXNL (5); AmStTeAxChKmGm (4)	Fecal	4-8	0	92.9–1,549.1	675.3–8,952	20–24	0 (4), + (5)	203.2–291.1	12,176–1,839.1

	AmStTeAxChKmAnXNL (1); AmStTeAxChKm (2)	Fecal	8	0	178–191	1,059–1,197	24	0	14–87	160–172
	AmStTeKm (1)	Feed	4	0	1,330.3	948.6	20	0	229.5	1,412.70
Typhimurium (122)	AmClStSuTe (28); AmStSuTeKm/Ax (4); AmCl-	Swab	4-8	0 (2), + (12)	109.5–183.1	541–970	16–24	0 (10), + (4)	7.8–173	81.9–1,142
	StSuTeAx/ Ch (3)									
	AmClStSuTe/Ch (2); AmClStSuTeKm/Gm (2)	Swab	4-8	0 (5), + (14)	97.5–1,685	541–11,365	16–24	0 (18), + (1)	7.3–271	89.3.1–2,001
	AmClStSuTe (39); AmClStSuTeAx (9); AmStSuTeKm (4)	Fecal	4-8	0 (7), + (45)	81.3–1,762.2	787–11,365	16–24	0 (47), + (5)	9.9–262	90.7–2,001
	AmStTeKm (8); AmClStSuTeKm (3)	Fecal	4-8	0 (11)	89.1–1,728.2	692.6–7,852.4	20–24	0 (2), + (9)	7.5–365.2	152.3–1,703
	AmClStSuTeAxCh (6); AmClStSuTeAxChKmXNL (2)	Fecal	4-8	0 (1), + (7)	140–161.1	674–1,383.1	20–24	0 (7), + (1)	7.6–173	119.1–1,142
	AmClStSuTeAxChXNL (4); AmClStSuTeAxChKmGm (1);	Fecal	4-8	0 (4), + (1)	144–160.9	674–1,063.6	20–24	0	14.4–173	123.8–1,142
	AmClStSuTeCh (1) AmClStSuTeAxKmGm (1); ClStSuTe (2); AmClStSuTe-	Fecal	4-8	0 (3), + (2)	99.1–1,347.5	541–8,952.0	4–24	0 (3), + (2)	3.2–203.2	101.8–1,543.4
	ChKmGm (2)									
	AmClStSuTe (1)	Feed	8	1	116.2	761.3	20	0	19.4	87.8
Worthington (8)	AmTeAxChCeXNL (2)	Fecal	8	0	115.2–134.4	1,066.7–1,463.5	24	2	9.3–31.4	126.8–177
	AmStTeAxChKmGm (1)	Swab	8	0	81.9	732.1	24	1	133	935.7
	AmStTeAxChKmXNL (1)	Fecal	8	0	1,645	12,325	24	1	302	2,397
	AmStTe (1)	Feed	8	0	1,685	11,365	24	1	271	2,001
	AmTeAxChCe (1); AmTeAxChCeXNL (1)	Swab	8	0	119.6	734.6	20	1	7.3	89.3
	AmStTeAxChKmGmXNL (1)	Swab	8	0	81.9	732.1	24	0	133	935.7
Rissen (1)	AmStTeKm (1)	Swab	8	0	129.6	1,254.70	20	1	15.7	138.7
Enteritidis (1)	AmClStSuTe (1)	Swab	8	0	140	774	16	0	97	742
Infantis (9)	AmClStSuTeAxChKmXNL (2); AmClStSuTe (1)	Fecal	4-8	0 (2), + (1)	86.4–146.9	1,383.10	16-20	0	11.9–16.2	135.6
	AmClStSuTeAxChKmGm (2); AmClStSuTeAxChKm-	Fecal	4-8	0	111–169.1	674–1,266	16-20	0	17–173	149.2–1,142
	GmXNL (1); AmAxChXNL (1)									
	AmClStSuTeChKmGmXNL (1); AmAxChXNL (1)	Swab	8	0	97.5–160.9	990.5–1,063.6	20	0	11.7–15.2	123.8–144.4

Agona (5)	StSuTeKmGm (2); AmStSuTeAxChKmGmXNL (1)	Fecal	4	0	105.1–177.3	991-.9–1,007.7	16-20	0	8.5–14.9	133.9–140.5
	AmStSuTeAxChKmGmXNL (1)	Swab	8	0	173.6	883.2	20	1	10.1	133.9
	Te (1)	Feed	8	0	1,375	6,895.00	16	0	279.2	1,391.40
Senftenberg (7)	AmClStSuTeAxChGmKmXNL (4); AmClStSuTeGm (1);	Fecal	4-8	0	178–1,636.2	657.8–8,364.9	20-24	0 (1), + (4)	14–291.1	172–1,643.1
	StSuTeKmGm (1)									
	Pansusceptible (1)	Swab	8	0	191	1,059	24	1	87	160
Amsterdam (1)	Pansusceptible (1)	Swab	8	0	198	1,048	20	0	21	161
London (1)	Te (1)	Feed	4	0	1,558	12,120	20	1	262	2,144
Ohio (2)	Pansusceptible (1)	Swab	8	0	191	1,059	24	0	87	160
	AmClStSuTeKmGm (1)	Fecal	4	0	155.2	1,043.7	24	1	10.9	107.8
Inverness (1)	Pansusceptible (1)	Feed	8	0	131	924	20	0	4.8	99
Muenchen (3)	AmStTeKm (1)	Fecal	8	0	147	1,691	24	0	138	406
	Pansusceptible (2); AmStTeKm (1)	Fecal	8	0	192	1,183	20	0	15	120
Rough_Ob:e,n,x (1)	Pansusceptible (1)	Fecal	8	0	192	1,183	20	0	15	120
Serogroup E (5)	AmStSuTeChCeGm (1); AmStTeKmGm (1);	Fecal	4-8	0	139.5–1,393.2	855.2–1,215.9	16-24	0 (2), + (2)	23.1–249.3	85.5–1,875.3
	AmClStSuTeChKmGm (1); ClStSuTe (1)									
	Te (1)	Feed	8	0	119.9	731.4	16	0	14.1	102
Serogroup A-I+ (3)	AmStTeKmGm (1); AmStTe (1)	Swab	8	0	81.9–1,552.1	732.1–8,629.2	16	+ (2)	133–229.2	935.7–1,498.1
	AmStSuTeKmGm (1)	Fecal	8	0	111	675.4	16	1	17	78.2
Serogroup B (5)	AmClStSuTeChKmGm (2); AmClStSuTe (1);	Fecal	4-8	0	116.2–1,393.2	761.3–10,146.2	16	+ (4)	8.8–249.3	87.8–1,875.3
	AmStTeKmGm (1)									
	StSuTe (1)	Swab	8	0	1,389.10	8,714.80	20	1	268.3	1,645.5
Serogroup A-S+ (2)	AmClStSuTe (1); AmStTeKmGm (1)	Fecal	8	0	119.6–155.2	734.6–1,142.1	16	+ (2)	7.3–10.3	89.3–127.8

Table 2.2: **Zinc and copper levels in swine feed and fecal samples**

Heavy metal	Type of sample	Level (mg/kg)				Dietary Zn and Cu requirement (mg/kg)
		Median	Minimum	Maximum	95% CI of $\bar{x}$	
Zinc	Feed	139.8	79	7,383.7	563.14 ± 79.57	50–100
	Feces	941.1	541	1,2325	3070.17 ± 393.47	
Copper	Feed	31.5	3.2	1,384.3	123.33 ± 13.64	3–6
	Feces	137.6	71.2	2,397	806 ± 73.89	

[a] Dietary requirements are based on the guidelines of the National Research Council, 2012 (8).

Table 2.3: **Heavy metal micronutrients (Zn^{2+}, Cu^{2+}) phenotypes, genotypes (*czcD*, *pcoA*) and association with resistance types (R-types)**

		Heavy metal tolerance genes[a]	**R-type, n (%)**									
Heavy metal micronutrient	**MIC mM**	Detected (+) Not detected (-)	AmClStSuTe/Ax/Ch	AmStTeKm/Gm	AmStSuTeKm/Gm	AmStTeAxChKm/Gm	AmStTeAxChKm-XNL	StSuTe	Te	Pansusceptible	Others	**Total**
Zinc	4	*czcD* (+)	11(9.1)									11(3.8)
		czcD (–)	4(3.5)	42(37.5)	4(3.5)	3(2.7)	5(27)	–	1(0.9)	4(3.5)	38(34)	101(35.6)
	8	*czcD* (+)	67(39.2)								7(4.1)	74 (31.1)
		czcD (–)	10(8.3)	18(14.9)	11(6.4)	2(1.2)	1(0.6)	7(4.1)	3(1.8)	8(4.7)	37(21.6)	97(34.2)
Copper	1	*pcoA* (+)										
		pcoA (–)										
	2	*pcoA* (+)										
		pcoA (–)										
	4	*pcoA* (+)									1(0.5)	1(0.3)
		pcoA (–)										
	8	*pcoA* (+)										
		pcoA (–)										
	16	*pcoA* (+)	1(0.5)	3(1.5)							94(47.5)	13(4.6)
		pcoA (–)	3(3.0)						3(3.0)		94 (47.5)	100(35.3)
	20	*pcoA* (+)		10(13.9)	2(2.6)		1(1.4)	2(2.8)	1(1.4)		12(16.7)	28(9.9)
		pcoA (–)	5(6.9)	6(8.3)	1(1.3)			5(6.9)		7(9.7)	20(2.8)	44(15.5)
	24	*pcoA* (+)	2(1.9)	25(24.8)	11(10.9)	4(3.9)	4(3.9)			1(1.0)	9(8.9)	56 (19.8)
		pcoA (–)	3(2.9)	16(15.8)	19(18.8)	2(1.9)	1(1.0)			4(3.9)		45 (15.9)

[a] +, detected; – not detected

Table 2.4: **Multivariable model with copper tolerance (Cu MIC = 24 mM) as outcome**

Serotype/ sample	Estimate	SE	OR (95% confidence interval)	*P*-value
Serotype				0.0006
Typhimurium	0.2373	0.3311	1.27 (0.66-2.43)	0.4741
Heidelberg	1.7212	0.4486	5.59 (2.31-13.52)	0.0002
Others	reference			
Sample type				<0.0001
Fecal	1.7633	0.3266	5.83 (3.07-11.09)	<0.0001
Feed	-1.5257	1.1477	0.22 (0.02-2.08)	0.1849
Floor swab	reference			

Table 2.5: **Multivariable model with copper tolerance (Zn MIC = 8 mM) as outcome**

Serotype, sample type, or carriage	Estimate	SE	OR (95% confidence interval)	*P*-value
Serotype				0.0003
Heidelberg	-0.6507	0.4594	0.52 (0.211-1.289)	0.1578
Others	0.8609	0.4379	2.37 (0.999-5.602)	0.0504
Typhimurium	reference			
Sample type				<0.0001
Feed	1.095	0.7672	2.99 (0.66-13.54)	0.1546
Floor swabs	1.8727	0.3493	6.51 (3.27-12.94)	<0.0001
Fecal	reference			
czcD carriage				<0.0001
Yes	2.3576	0.4912	10.57 (4.02-27.79)	<0.0001
No	reference			

CHAPTER THREE

In-Use Concentrations of Different Classes of Biocides in U.S. Swine Production Systems and Its Role in Persistence of Biocide-tolerant and Multi-drug Resistant Salmonellae

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Abstract

The aim of this study was to investigate the role of biocide interventions in the emergence of biocide-tolerant *Salmonella* and its co-selective association with multidrug-resistant *Salmonella*. This study further investigated the association of biocide use with carriage of specific efflux gene markers by the *Salmonella*. Of the *Salmonella* isolates biochemically confirmed (n = 6162), 283 were systematically selected based on origin and phenotypic characteristics for serotyping. *Salmonella* isolates were detected in 13.9% (208/1,497) and 6.7% (98/1,468) of swine barn drag swab samples at pre- and post-disinfection with biocide, while, *Salmonella* isolates were detected in 17.2% (1,180/6,842) and 7.1% (431/6,093) of the early and late finishing pigs, respectively. Barn-level prevalence of antimicrobial resistance among *Salmonella* isolates recovered from the floor swab samples was 92.3% (1,503 of 1,628), while, the barn-level prevalence of antimicrobial resistance among *Salmonella* isolates recovered from the fecal samples was 98.02% (4,415 of 4,504). A total of 348 and 428 *Salmonella* isolates were tested to establish the minimum inhibitory concentrations (MIC) of the Biosentry® and Synergize®, respectively. Molecular characterization was performed to determine the tolerance genes, including *qac* [$qacE_1$ or *qacEΔ1*], $qacE_1$, *qacEΔ1*,*intI1*, and the conserved segments (CS) of the class 1 integrons for the variable regions. Of 428 *Salmonella* isolates from the Synergize® treated group, who were screened for tolerance genes, 22.9% (98 of 428) of the isolates carried the *qac* genes, while, 86% (84 of 98) of the isolates which carried the *qac* genes showed the MIC of ≥160 µg/ml. Of 348 *Salmonella* isolates from the Biosentry® treated group, who were screened for tolerance genes, 31.3% (109 of 348) of the isolates carried the *qac* genes, while, 89% (97 of 109) of the isolates which carried the *qac* genes showed the MIC of ≥ 80 µg/ml. Of 109 *Salmonella* isolates that carried *qac* genes, 99.1% (108/109) of the isolates contained attenuated *qacEΔ1*gene, whereas 69.4% (75 of 108) of the isolates

carrying *qacEΔ1* gene were found to be associated with class I integrons and the resistance gene cassettes of different sizes and patterns. Biocides were shown to be effective in limiting the growth, load and the colonization of bacterial pathogens in animals of food origin. Despite the significance of biocide use in disinfection, they are also implicated as among the risk factors of increased selective pressure towards antibiotic resistance in *Salmonella*.

Introduction

Salmonella remains to be one of the most important foodborne pathogens worldwide. *Salmonella* infection is a global problem whose occurrence is not restricted to particular countries, however, the problem may be more complicated in developing countries where the use of biocides and antimicrobials in human and veterinary practices is not well regulated (Fraise et al., 2002; Okeke et al., 2005). The main reservoir of *Salmonella* spp. is the gastro-intestinal tract of the food animals. The Salmonellae are frequently recovered from food and animal products such as meat, milk and eggs (Zhao et al., 2008; Hue et. al., 2010). Control measures in terms of disinfection of food animal contact surfaces such as barn floors, are important for limiting the risk of contaminated animal products from reaching the consumers and public as a whole (Fraise, 2002; Gantzhorn et al., 2014). Reduction of *Salmonella* spp. load and colonization in food animals is achieved if proper and effective detection and control measures are put in place at all levels of food production and in particular the animal production farms as the primary production level. For centuries, biocidal substances (disinfectants) have been used to improve hygiene (Morente et al., 2013; Wales and Davies, 2015). Hygienic practices have been fundamental to control foodborne pathogens in farm environments. At the farm level, the control programmes include proper cleaning and disinfection (Lo Fo Wong et al., 2002;

Zewde et al., 2009). The participation of all sectors involved throughout the food production levels is critical in ensuring safe food. Various studies have demonstrated that carrier food-producing animals are the main source of *Salmonella* at the slaughterhouse (Botteldoorn et al., 2003; Arguello et al., 2013).

Several biocidal substances such as glutaraldehydes and quaternary ammonium compounds (QAC) are commercially available for disinfection at the farm level and at other relevant stages of the food production chain, in order to inhibit bacterial growth, load and colonization (Russell, 2002a; Maillard, 2007). However, the use of disinfectants is implicated to contribute to the emergence of disinfectant tolerant foodborne and environmental bacterial pathogens (Chapman, 2003; Carson et al., 2008). A number of studies on the use of different biocides, including glutaraldehydes, quaternary ammonium compounds, intercalating dyes and diamidines have shown that the biocides are implicated in selection of bacteria with low-level of antimicrobial resistance (Russell, 1999; Whitehead et al., 2011). Previous studies have also reported that bacterial resistance can be induced as a result of exposure to a low concentration of a biocide (Karatzas et al., 2007; Randall et al., 2005).

Mechanisms of resistance of biocides in bacterial population are of two types: intrinsic and acquired resistance. The intrinsic resistance is a natural property of an organism which include impairing of cell wall properties such as the outer membrane of gram-negative bacteria which act as an impermeability barrier so that uptake of chemically unrelated drugs is reduced to reach their target site. The other examples of intrinsic resistance mechanisms that hinder diffusion of biocides across bacterial cell are loss or alteration of the porins, degradation of biocides and the presence of efflux systems (McDonnell and

Russell, 1999; Denyer and Maillard, 2002). Acquired resistance is achieved by acquisition of new properties through mutations at sensitive target sites, by plasmid- or transposon-mediated antibiotic inactivation or by intracellular removal of antibiotics by efflux-mediated systems and by horizontal transfer of genes conferring resistance (McDonnell and Russell, 1999; Russell et al., 1999). It is also reported that more than one mechanism of resistance such as membrane impermeability and efflux system, may occur in combination to allow bacterial survival (Maillard, 2007).

The bacterial mechanisms of resistance through transferable resistance determinants to biocides may be located on the bacterial chromosome, transposons, integrons, plasmids, or found to be clustered in pathogenicity islands and transferred by mobile genetic elements (Fluit and Schmitz, 2004; Rychlik et al., 2006). Previous studies have shown a close association between resistance to biocides and other antimicrobial agents such as antibiotics, suggesting that the genetic determinants to these agents are commonly linked with each other. For instance, the *qac* genes such as *qacE1* and *qacEΔ1* are often present on the plasmids together with other resistance genes (Weigel et al., 2003; Jaglic and Cervinkova, 2012). If antibiotic and *qac* resistance genes are both carried on class I integrons, selection for *qac* resistance may result as co-selection of antibiotic resistance in microbial population. Co-existence of resistance determinants such as antibiotic and *qac* resistant genes, on mobile genetic elements, provides a potential reservoir of antibiotic-resistant bacteria in *qac*-polluted environments (Russell, 2000; Gaze et al., 2005).

Three brands of biocides (with their active ingredients in brackets) were used for disinfection of the swine barn floors. The first biocide used was the Biosentry® 904. It contained three different cationic quaternary ammonium compounds (QAC) as the active

ingredients which were didecyl dimethyl ammonium chloride (9.2%), dimethyl benzyl ammonium chloride [Alkyl C12, 61%; C14, 23%; C16, 11%; C18. 2.5%; C8 and C10, 2.5%] (9.2%); dimethyl benzyl ammonium chloride [Alkyl (C12; 40%; C14, 50%; C16, 10%] (4.6%) and bis-n-tributyltin oxide (1%). On the other hand, the active ingredients of the Synergize® were the quaternary ammonium compounds and glutaraldehydes, such as: dimethyl benzyl ammonium chloride [Alkyl (C12 67%, C14 25%, C16 7%, C18 1%)] (26%) and the glutaraldehydes (7%), whereas the two active ingredients of Virkon-S® were the potassium peroxymonosulfate (21.41%) and sodium chloride (1.50%). Therefore, the present study was conducted to investigate the role of biocide (Biosentry®, Synergize® and Virkon-S®) interventions in the emergence of biocide-tolerant *Salmonella* and its co-selective association with multidrug-resistant *Salmonella*. This study further investigated the association of biocide use with carriage of specific efflux gene markers by the *Salmonella*.

Materials and Methods

Study design and sample collections. This study was part of a large longitudinal group-randomized controlled study designed to investigate the association of different kinds of biocides with the occurrence and persistence of biocide tolerant and multi-drug resistant (MDR) *Salmonella*. The field part of this study was conducted Raleigh, North Carolina, United States and the laboratory part of the study was done in Columbus, Ohio, United States. Briefly, three vertically integrated commercial swine production systems (systems 1, 2 and 3) were selected based on history of *Salmonella* occurrence. From each of production system, three farms were selected and from each farm, four barns were randomly selected for further follow-up on this study. All barns used standardized electrostatic disinfection systems to limit introduction of additional potential confounding

effects. Temperature of the pressurized hot water was set at 100^0C, with an the application time of ten minutes. We visited each farm at two stages (early finishing and late finishing) in four replicates (repeated visits to the same barns during the study period of October 2007 to November 2009). Each replicate visit consisted of sampling assigned barn floors before and after disinfection, pigs at early and late finishing stages, and pooled feed samples (Molla et al., 2010). Sampling was done from all the 36 barns for a period of more than two years. Barn floor swab samples were aseptically collected (10 samples per barn in five replicates) pre-disinfection (n = 1,800) and post-disinfection (n = 1,795) from randomly selected pens (n = 10/barn) in 36 barns. A total of 48 fresh fecal samples (25 g) were aseptically collected from each barn in four replicates at the early finishing stage (six to nine weeks of age) (n = 6,842) and at late finishing stages (26 to 28 weeks of age) of production (n = 6,093) from individual pigs. Some samples were lost/ missed at different stages of the study (48 samples/ barn x 36 barns x 4 replicates = 6,912 samples). Approximately 100 g of pooled feed samples (1 sample per barn collected from all 36 barns at two stages and five replicates with 13 losses to follow-up) was aseptically collected from 36 barns (n = 275) over a period of two years. Each pooled feed sample per barn was aseptically collected from the feeder bin in sterile Whirl-Pak bags and shipped to the laboratory on the same day as collection.

Salmonella **isolation and identification.** *Salmonella* isolates were recovered and identified following conventional methods as described previously (Gebreyes et al., 2000; Molla et al., 2010). Briefly, a 10g portion of each fecal and feed sample was pre-enriched in 90 ml of buffered peptone water (BPW; Becton Dickinson, Sparks, MD) and 90 ml of BPW was added to each Whirl-Pak bag containing individual drag swabs, and incubated at 37°C overnight. The remaining portions of fecal and feed samples were stored at -20°C.

After overnight incubation, 100 µl of the pre-enriched suspension was added into 9.9 ml of Rappaport-Vassilliadis (RV) enrichment broth (Becton Dickinson, Sparks, MD) and incubated at 42°C overnight. A 10 µl of the suspension was inoculated onto Xylose-lactose-Tergitol 4 (XLT-4) agar (Becton Dickinson, Sparks, MD) plates and incubated at 37°C for 24 h and incubation was extended to 48 h in cases where colonies were doubtful. Three presumptive *Salmonella* colonies were selected from each positive plate for biochemical testing. Each selected presumptive *Salmonella* colony was then inoculated onto triple sugar iron (TSI) agar (Becton Dickinson, Sparks, MD) slants, lysine iron agar (LIA) slants (Becton Dickinson, Sparks, MD) and urea broth (Becton Dickinson, Sparks, MD) and incubated at 37°C overnight. All biochemically confirmed *Salmonella* isolates were then stored at -80°C until further testing.

Phenotyping. *Salmonella* isolates recovered from swine feed (n = 30), swine barn floors (n = 1628) and swine faeces (n = 4504) were serogrouped using commercially available polyvalent O and group-specific antisera (Mira Vista, Copenhagen, Denmark) following the recommendations of the manufacturer. Of the *Salmonella* isolates biochemically confirmed (n = 6162), 283 were systematically selected based on origin and phenotypic characteristics (serogrouping and antimicrobial resistance profiles) and submitted to the National Veterinary Services Laboratories (USDA-NVSL, Ames, IA) for serotyping. *Salmonella* isolates were tested for antimicrobial susceptibility to a panel of 12 antimicrobials using the Kirby-Bauer disc diffusion method following the guidelines of the CLSI (2002). The antimicrobials used and their respective disc potencies were as follows: ampicillin (Am; 10 µg/ml), amoxicillin-clavulanic acid (Ax; 30 µg/ml), amikacin (An; 30 µg/ml), ceftriaxone (Ce; 30 µg/ml), cephalothin (Ch; 30 µg/ml), chloramphenicol (Cl; 30 µg/ml), ciprofloxacin (CIP; 5 µg/ml), gentamicin (Gm; 10 µg/ml), kanamycin (Km; 30

µg/ml), streptomycin (St; 10 µg/ml), sulfisoxazole (Su; 250 µg/ml), and tetracycline (Te; 30 µg/ml). *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains. *Salmonella* isolates showing resistance to three or more antimicrobials were classified as multidrug resistant (MDR) and to those isolates showing intermediate resistance profiles were considered as susceptible.

Minimum Inhibitory Concentration (MIC) of the Synergize® and Biosentry®. For Synergize® tolerance test, a total of 428 *Salmonella* isolates were tested. These consisted of 222 isolates recovered from floor swabs pre-disinfection, (n = 96 isolates) post-disinfection, (n = 72 isolates) from swine at early finishing stage, (n = 27 isolates) from swine at late finishing stage and (n = 11 isolates) from swine feed. For Biosentry® tolerance test, a total of 348 *Salmonella* isolates were tested. These included (n = 176 isolates) from floor swabs pre-disinfection, (n = 112 isolates) post-disinfection, (n = 42 isolates) from swine at early finishing stage, and (n = 18 isolates) from swine at late finishing stage. The *Salmonella* isolates selected for Synergize® and Biosentry® tolerance tests contained different antimicrobial resistance patterns and had been recovered from swine barns disinfected with Synergize® and Biosentry®, respectively. The agar plate-dilution method was used to determine the MIC against *Salmonella* following the methods described before (Aarestrup and Hasman, 2004; Kawamura-Sato et al., 2010). The susceptibility were determined on Mueller-Hinton (MH)-II agar plates containing two fold serial dilutions of biocides (Synergize® and Biosentry®).

The *Salmonella* isolates were tested for the following dilution ranges of Synergize®: 0, 20, 40, 80, 160, 320, 330, 640 and 1280 µg/ml. Biosentry® solutions contained the following

dilution ranges: 0, 20, 40, 80, 160, 320, 330 and 640 µg/ml. Briefly, 500 ml of 20,000 µg/ml of biocide stock solutions and the 2L of MH agar were autoclaved and cooled to 60-70^{0}C in a water bath. The specific volumes of Synergize® and Biosentry® solutions to be added to each dilution tube of 100 ml of MH agar were calculated according to the desired concentrations. About 25ml of MH agar was aseptically dispensed and allowed to solidify. Bacterial suspensions were adjusted to 0.5 McFarland (equivalent to 1-2 x10^{8}CFU/ml) in 2 ml of sterile 0.85% NaCl solutions. The suspensions were diluted to approximately 10^{7}CFU/ml (100µl of each inoculum at 0.5 McFarland + 900µl of sterile 0.85% NaCl solutions) in 1.5 ml eppendorf tubes before adding to the inoculum replicator block. About 400 µl of each suspension was aseptically aliquoted to a corresponding well of the replicator inoculum block. The *Salmonella* isolates and control strains were tested in triplicate. The inoculated plates were allowed to stand at room temperature for 15-20 min and incubated at 37^{0}C for 16 to 20 h. After incubation, the plates were assessed for growth and MIC and *E. faecium* A17 sv 1 HHA 210, *S. aureus* C10682, *S. aureus* ATCC 29213 and *S. aureus* SO385 were used as reference strains. The MIC was defined as the lowest concentration that inhibits the visible growth of *Salmonella* after overnight incubation.

Identification of quaternary ammonium compound tolerance genes, class 1 integrase gene (*intI1*) and resistance gene cassettes. A total of 776 *Salmonella* isolates were tested for the carriage of quaternary ammonium compound (qac) tolerance genes (*qacE$_1$* for multi-drug efflux and *qacEΔ1* for attenuated variant *qacE*) and the presence of class 1 integrase (*intI1*), and gene cassettes integrated between conserved segments (5'-3'CS) of class 1 integrons were detected using PCR. Briefly, *Salmonella* isolates were inoculated onto Tryptic Soy agar (TSA) plates and incubated overnight at 37°C. The genomic DNA was extracted using Qiagen DNeasy tissue kit following the manufacturer's instruction

(Qiagen Ambion, Austin, Texas, USA). The sets of primers used to screen for the presence of *qac* [*qacE*$_1$ or *qacEΔ1*] were 5'-ATCGCAATAGTTGGCGAAGT-3' and 5'-CAAGCTTTTGCCCATGAAGC-3' as forward and reverse primers. Following screening for *qac* [qac*E*$_1$ or *qacEΔ1*], other sets of primers used for amplification of the *qacEΔ1* were 5'-ATCGCAATAGTTGGCGAAGT-3' and 5'-TTAGTGGGCACTTGCTTTGG- 3' as forward and reverse primers, while, for *qacE*$_1$ the set of primers used were 5'-ATCGCAATAGTTGGCGAAGT-3' and 5'-AACACCGTCACCATGGCGTCG-3' as forward and reverse primers.

The modified PCR thermocycling conditions included initial denaturation at 95°C for 5 min, denaturation at 95°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min and final extension was done at 72°C for 7 min and amplification was done in 30 cycles (Sandvang et al., 1997; Kazama et al., 1998). Primers used for amplification of the *intI1* were 5'-GCCTTGCTGTTCTTCTACGG-3' and 5'-GATGCCTGCTTGTTCTACGG-3' as forward and reverse primers (Levesque et al., 1995) and those for conserved segments were 5'-GGCATCCAAGCAGCAAG-3' and 5'-AAGCAGACTTGACCTGA-3' as forward and reverse primers (Ploy et al., 2000). The PCR cycling conditions included initial denaturation at 94°C for 5 min, followed by 25 cycles of 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, and the final extension done at 72^0C for 7 min (Lindstedt et al., 2003). About 10 µl of the PCR product of each isolate tested were run on 1% agarose gel stained with 5 µl of 10 mg/ml ethidium bromide for 1 hr at 120 volts using 0.5X Tris-borate EDTA (TBE) as running buffer. A 1-kb plus DNA ladder was used as molecular size marker.

Data analysis. Data were entered in Microsoft excel 2007 (Ms Corp., Redmond, WA, USA) and statistical analyses were conducted using MedCalc® software version 12.7.1.0 (Ostend, Belgium) and SAS software version 9.2 (SAS Institute Inc., Cary, NC), whereas construction of graphs were conducted with Microsoft excel 2007 (Ms Corp., Redmond, WA, USA).

To calculate *Salmonella* floor prevalence, three production systems were taken and within each of these three farm sites were enrolled in the study. On each farm site, four barns were included in the trial. In each barn, one of the four treatments (cleaning with hot water only [control] or disinfection with Biosentry®, Synergize® or Virkon-S®) was applied and replicated five times. Floor swab samples in the barns were collected before and after disinfection. Altogether this provided 360 (3 production systems x 3 farms x 4 barns x 2 stages x five replicates). For the assessment of the *Salmonella* prevalence in the environment, 10 floor swab samples were collected at each sampling time in each swine barn environment. Descriptive statistics were calculated to estimate the overall *Salmonella* prevalence in the environment. The change in environmental *Salmonella* prevalence between pre- and post-disinfection sampling was calculated and used as the outcome in mixed models approach, using PROC MIXED in SAS version 9.2 (SAS Institute Inc., Cary, NC) to determine the impact of three different biocides or pressurized hot water only, on *Salmonella* prevalence. Compound symmetry covariance structure was used to account for the correlated observations (replicates) within barns within a farm site. Production system was included in the model as a random effect. The pairwise comparisons of the least squares mean estimates of the prevalence change between different biocide treatments were made using Tukey-Kramer adjustment for the multiple comparisons.

Fecal samples were collected from 48 individual pigs at early and finishing stages of the production cycle in four barns in each of the three farm sites of the three production systems. Barn-level fecal *Salmonella* prevalence was calculated by dividing the number of *Salmonella* positive samples by the number of samples tested. Four replicates were run in each barn, theoretically providing 288 estimates for barn-level fecal *Salmonella* prevalence. However, in 13 barns, sampling at the finishing stage was missed. Descriptive statistics were run to obtain overall fecal *Salmonella* prevalence among growing pigs, at the two production stages and in the three different production systems. Change in barn-level *Salmonella* prevalence between finishing and early stages was calculated for each replicate and used as the outcome variable in Proc Mixed procedure of SAS to assess the impact of the different biocides, or pressurized hot water only, on barn-level *Salmonella* prevalence among pigs. The modeling procedure was as described above for floor *Salmonella* prevalence.

Results

***Salmonella* floor prevalence and antimicrobial resistance.** *Salmonella* isolates were detected in 13.9% (208/1497) and 6.7% (98/1468) of swine barn drag swab samples at pre- and post-disinfection with biocide, whereas, from control swine barns treated with pressurised hot water, *Salmonella* isolates were detected in 16.7% (75/450) and 27.6% (124/450) of drag swab samples between pre- and post-disinfection, respectively. *Salmonella* prevalence on swine barn floors varied from 0 to 100% in different replicate samplings, with 191 (53.1%) of the 360 barn-level estimates being 0 (no *Salmonella*-positive samples found) and 0.8% (3 of 360) being 100%, with all collected floor swabs in the barn at a particular sampling time found to be positive for *Salmonella* (Fig. 3.1). The change in environmental *Salmonella* prevalence between pre- and post-disinfection

samplings varied from -0.7 to 0.7. Most frequently, in 43.3% (78 of 180) of the replicates there was no change in the prevalence observed, however, in 33.9% (61 of 180) of the replicates the prevalence increased and in only 22.8% (41 of 180) of cases was prevalence decreased as a result of cleaning and disinfection (Fig. 3.2).

Of the 1628 *Salmonella* isolates from the drag swab samples, the most common antimicrobial resistance was found with tetracycline (79.9%, 1300 of 1628), followed by streptomycin (64.2%, 1045 of 1628), sulfisoxazole (44.9%, 731 of 1628), ampicillin (27.9%, 455 of 1628), chloramphenicol (14.7%, 239 of 1628), kanamycin (12.3%, 201 of 1628), cephalothin (4.3%, 70 of 1628), amoxicillin-clavulanic acid (3.6%, 59 of 1628), gentamycin (1.9%, 31 of 1628), ceftiofur (1.3%, 21 of 1628), ceftriaxone (1%, 16 of 1628), amikacin (0.2%, 4 of 1628), and ciprofloxacin (0.1%, 2 of 1628). Antimicrobial resistance was found to be common among the isolates, with 89.5% (401 of 448) of *Salmonella* isolates that originated from drag swabs from swine barns pre-disinfected with Biosentry®, 87% (287 of 330) of isolates from drag swabs from swine barns pre-disinfected with Synergize®, 93.9% (229 of 244) of isolates from drag swabs from swine barns pre-disinfected with Virkon-S®, and 96.7% (586 of 606) of isolates from drag swabs from swine barns pre-treated with pressurised hot water showing resistance to one or more of the antimicrobials tested. Almost 44% (195 of 448) of the *Salmonella* isolates recovered from drag swabs from swine barns pre-disinfected with Biosentry®, 43% (142 of 330) of the isolates from drag swabs from swine barns pre-disinfected with Synergize®, 69.3% (169 of 244) of the isolates from drag swabs from swine barns pre-disinfected with Virkon-S®, and 54.5% (330 of 606) of isolates from drag swabs from swine barns pre-treated with pressurised hot water were MDR *Salmonella* isolates.

Overall, barn-level prevalence of antimicrobial resistance (isolate resistant to at least one antimicrobial) among *Salmonella* isolates recovered from the floor swab samples was 92.3% (1503 of 1628) (ranging from 0 to 100%). The overall MDR *Salmonella* isolates originating from floor swab samples was 51.4% (836 of 1628 (Table 3.4). The barn-level MDR among the environmental isolates from floor swabs was 67.3%, with a median of 100% (range from 0 to 100%). Results from the mixed model approach suggested that the cleaning method or compound had a significant impact on the change in *Salmonella* prevalence in the swine barn environment from pre-disinfection to post-disinfection (P = 0.0003). The estimates for change in *Salmonella* prevalence for each biocide treatment are presented in Table 3.1. As a result of cleaning and disinfection of the swine barns with biocide, the percentage reduction in *Salmonella* prevalence between pre- and post-disinfection was 7.2%, significantly lower at post-disinfection than it was at pre-disinfection (% reduction in *Salmonella* prevalence = 7.2%, 95% CI, 4.99 to 9.42; $P < 0.0001$). Besides the reduction in the prevalence of *Salmonella* between pre- and post-disinfection stages of swine barns, the proportions of MDR *Salmonella* isolates significantly increased by 8.17% from pre- to post-disinfection of swine barns (% increase in MDR = 8.17%, 95% CI, 3.18 to 13.11; P = 0.0013). On the other hand, the average prevalence of Salmonella isolates increased by 10.9% following cleaning of swine barns with pressurised hot water (% increase in *Salmonella* prevalence = 10.9%, 95% CI, 5.34 to 16.4; $P < 0.0001$).

The odds of recovering *Salmonella* isolates from swine barns before disinfection were 2.26 times higher than after disinfection of the barns (OR = 2.26; 95% CI; 1.75 to 2.90; $P < 0.0001$), indicating the importance of cleaning and disinfection of swine barns before placement of pigs. *Salmonella* prevalence change estimates after all biocide treatments

were significantly different from those when cleaning with water only, Biosentry® against water, $P = 0.006$; Synergize® against water, $P = 0.0018$; Virkon-S® against water, $P = 0.0356$). The largest decrease in *Salmonella* prevalence between pre- and post-disinfection was observed for Biosentry® with 10.4% decrease, followed by Synergize® (8.9% decrease) and Virkon-S® (3.8% decrease). However, there were no statistical differences between the three biocide treatments in the change in *Salmonella* prevalence ($P > 0.6$), for all pairwise comparisons.

***Salmonella* fecal prevalence and antimicrobial resistance.** Barn-level fecal *Salmonella* prevalence varied between 0 and 100%, with an average prevalence of 12.4% (Table 3.2). *Salmonella* isolates were detected in 17.2% (1180/6842) and 7.1% (431/6093), respectively. Prevalence of *Salmonella* at late finishing stage (7.1%) was significantly lower than at early finishing stage (17.2%) at the time of pig placement in the barns (% reduction in *Salmonella* prevalence = 10.1%, 95% CI, 8.99 to 11.21; $P < 0.0001$). The odds of recovering *Salmonella* isolates from early finishing stage of placement of pigs to disinfected barns were 2.74 times higher than at late finishing stage of pigs (OR = 2.74; 95% CI; 2.44 to 3.08; $P < 0.0001$), indicating the significance of the long term effect of the disinfectant in the biocide treated swine barns. The effect of biocide use for disinfection of the swine barns was perhaps responsible for the reduced *Salmonella* prevalence by 10.1% points. The change in fecal *Salmonella* prevalence between early and late finishing stages among growing pigs was not significantly associated with biocide treatment in the barns ($P = 0.9119$).

Of the 4504 *Salmonella* isolates from the swine fecal samples, the most common antimicrobial resistance was found with tetracycline (96.6%, 4349 of 4504), followed by

streptomycin (75.2%, 3389 of 4504), ampicillin (57.5%, 2588 of 4504), sulfisoxazole (50.7%, 2284 of 4504), kanamycin (39.1%, 1760 of 4504), chloramphenicol (19.9%, 897 of 4504), amoxicillin-clavulanic acid (8.8%, 395 of 4504), cephalothin (8.2%, 369 of 4504), ceftiofur (4.2%, 187 of 4504), gentamycin (3.6%, 162 of 4504), ceftriaxone (1.4%, 61 of 4504), ciprofloxacin (0.1%, 4 of 4504), and amikacin (0.04%, 2 of 4504). Antimicrobial resistance was observed among *Salmonella* isolates, with 98.6% (1260 of 1278) of isolates that originated from fecal samples of swine placed in barns pre-disinfected with Biosentry®, 99.2% (970 of 978) of isolates from fecal samples of swine placed in barns pre-disinfected with Synergize®, 97.4% (1066 of 1095) of isolates from fecal samples of swine placed in barns pre-disinfected with Virkon-S®, and 97.1% (1119 of 1153) of isolates from fecal samples of swine placed in barns pre-treated with pressurised hot water showing resistance to one or more of the antimicrobials tested. Approximately 77% (984 of 1278) of isolates recovered from fecal samples of swine placed in barns pre-disinfected with Biosentry®, 77.1% (754 of 978) of isolates from fecal samples of swine placed in barns pre-disinfected with Synergize®, 80.5% (882 of 1095) of isolates from fecal samples of swine placed in barns pre-disinfected with Virkon-S®, and 70.1% (808 of 1153) of isolates from fecal samples of swine placed in barns pre-treated with pressurised hot water were MDR *Salmonella* isolates. Overall, barn-level prevalence of antimicrobial resistance (isolate resistant to at least one antimicrobial) among *Salmonella* isolates recovered from the fecal samples was 98% (4,415 of 4,504) (ranging from 0 to 100%). The overall fecal MDR *Salmonella* isolates were recorded to be as high to over a half of isolates, with 76.1% (3,428 of 4,504) of the isolates originating from swine fecal samples (Table 3.4). Multi-drug resistance was found in fecal *Salmonella* from 163 (90.6%) of the 180 replicate samplings. The barn-level MDR varied from 0 to 100%,

with median of 100% and average MDR prevalence being 79%. Fecal MDR prevalence was not associated with biocide treatment used in the barn (P = 0.9201).

Analysis of Biosentry® tolerance. A total of 348 *Salmonella* isolates were systematically selected, in which 328 isolates were obtained from the Biosentry® pre-disinfected swine barn floors and faeces from swine placed in those respective swine barns, and the rest 20 isolates were obtained from the control swine barns which were previously pre-treated with pressurised hot water. Of a total of 348 *Salmonella* isolates, about 176 (50.6%; 176 of 348) of the isolates selected for Biosentry® tolerance test were originated from drag swabs from Biosentry® pre-disinfected swine floors (Fig. 3.3). Of the 176 *Salmonella* isolates, a total of 156 (88.6%, 156 of 176) isolates were resistant to at least one antimicrobial, and 47.2% (83 of 176) of the isolates were MDR *Salmonella* isolates. Also, a total of 112 (32.2%, 112 of 348) *Salmonella* isolates selected for Biosentry® tolerance test, originated from floor drag swabs post-disinfection, in which 89.3% (100 of 112) of the isolates were resistant to at least one antimicrobial, and 50% (56 of 112) of the isolates were MDR *Salmonella* isolates. Overall, of the 348 *Salmonella* isolates, nearly ninety-one percent (315 of 348) of the isolates tested for tolerance to Biosentry® were resistant to at least one antimicrobial and 9% (33 of 348) of the isolates were pansusceptible.

On tolerance test, almost eighty-eight percent (305 of 348) of the isolates showed tolerance to Biosentry® at a breakthrough point of 80 µg/ml, whereas, the remaining 4.9% (17 of 348) and 7.5% (26 of 348) showed tolerance at MICs of < 80 µg/ml and > 80 µg/ml, respectively. Of the 331 *Samonella* isolates with Biosentry® MIC > 40 µg/ml, 90.3% (299 of 331) were resistant to at least one antimicrobial, whereas 9.7% (32 of 331) of the isolates were pansusceptible. Generally, the prevalence of pansusceptible

Salmonella isolates was 3.5% (217 of 6162). Of the 33 *Salmonella* isolates with pansusceptible phenotypes selected for Biosentry® MIC testing, approximately 97% (32 of 33) of the isolates showed high resistance to Biosentry® at the MIC breakthrough point of ≥ 80 µg/ml. The mean difference of Biosentry® MICs of *Salmonella* isolates obtained from floor drag swabs post-disinfecion was 21.0 higher than for *Salmonella* isolates obtained from floor drag swabs pre-disinfection ($\bar{x}$ difference = 6.64; 95% CI, 0.83 to 12.45; $P = 0.0253$).

Of the 348 *Salmonella* isolates screened for MDR efflux (e.g. *qacE1, qacEΔ1*) and *intI1* genes, about 31.3% (109 of 348) carried the *qac* gene, whereas 89% (97 of 109) of isolates that carried the *qac* gene showed the MIC of 80 µg/ml. The *qac* tolerance genes were also detected from 2% (7 of 348) and 1.4% (5 of 348) of *Salmonella* isolates at MICs of <80 µg/ml and > 80 µg/ml, respectively. Of the 109 *Salmonella* isolates that carried *qac* genes, 94.5% (103/109) of the isolates contained attenuated *qacEΔ1* gene, whereas 69.4% (75 of 108) of the isolates carrying *qacEΔ1* gene were found to be associated with class 1 integrons and the resistance gene cassettes of different sizes and patterns. The carriage of the *qac* gene versus Biosentry® MIC in *Salmonella* isolates recovered from fecal matter, feed, and barn floors is shown in Table 3.5. The odds of isolating resistant *Salmonella* from isolates with high MIC of Biosentry® (≥ 80 µg/ml) were 1.71 higher than for isolates with low MIC (<80 µg/ml) of Biosentry®, (OR = 1.71; 95% CI; 0. 22 to 13.34; $P = 0.6076$). Assessment of association between tolerance and gene carriage showed that isolates with a high Biosentry® MIC were more likely to carry the *qac* gene (Table 3.3). The odds of *qac* gene carriage were 1.57 times higher for isolates with high Biosentry® MICs than for those with low Biosentry® MICs (OR = 1.57; 95% CI, 0.58 to 4.25; $P = 0.3726$), whereas, the odds of *qac* gene carriage were 85.22 times higher for MDR

Salmonella isolates with high Biosentry® MICs than for those none MDR *Salmonella* isolates with low Biosentry® MICs (OR = 85.22; 95% CI, 26.41 to 274.98; $P < 0.0001$). We also found out that the odds of *intI1* gene carriage were 184.01 times higher for MDR *Salmonella* isolates with high Biosentry® MICs than for none MDR *Salmonella* isolates with low Biosentry® MICs (OR = 184.01; 95% CI, 11.29 to 2999.46; $P = 0.0003$). The analysis showed only weak correlation between MDR *Salmonella* isolates and *qac* gene carriage ($r = 0.55$; 95% CI for *r,* 0.47 to 0.62; $P < 0.0001$) and MDR *Salmonella* and *intI1* gene carriage ($r = 0.46$; 95% CI for *r,* 0.37 to 0.54; $P < 0.0001$).

Analysis of Synergize® tolerance. Nearly ninety-four percent (403 of 428) of the *Salmonella* isolates tested for tolerance to Synergize® were resistant to at least one antimicrobial and 6% (25 of 428) of the isolates were pansusceptible. Of 222 *Salmonella* isolates selected for Synergize® tolerance test, originating from floor drag swabs pre-disinfection, 89.2% (198 of 222) were resistant to at least one antimicrobial, whereas, 44.4% (88 of 198) of the isolates were MDR *Salmonella* isolates. A total of 96 *Salmonella* isolates selected for Synergize® tolerance test, originating from floor drag swabs post-disinfection, 96.9% (93 of 96) were resistant to at least one antimicrobial, whereas, 72% (67 of 93) of the isolates were MDR *Salmonella* isolates. Despite the reduced *Salmonella* prevalence between pre- and post-disinfection stages of swine barns, the proportions of MDR *Salmonella* isolates increased by 27.6% between pre- and post-disinfection stages of swine barns (Fig. 3.4). Eighty-percent (341 of 428) of the isolates showed tolerance to Synergize® at a breakthrough point of 160 µg/ml, whereas, the remaining 5.8% (25 of 428) and 17.8% (76 of 428) showed tolerance at MICs of <160 µg/ml and >1600 µg/ml, respectively.

Of the 417 *Samonella* isolates with Synergize® MIC > 80 µg/ml, 90.9% (389 of 417) were resistant to at least one antimicrobial, whereas 6.7% (28 of 417) of the isolates were pansusceptible to antimicrobials used. On the other hand, a total of 29 *Salmonella* isolates with pansusceptible phenotypes were selected for Synergize® MIC testing of which 96.6% (28 of 29) of the isolates showed high resistance to Synergize® at the MIC breakthrough point of ≥ 160 µg/ml. The MICs mean difference of *Salmonella* isolates obtained from floor drag swabs post-disinfecion was 21.0 lower than for *Salmonella* isolates obtained from floor drag swabs ($\bar{x}$, 21.0; 95% CI of difference, 3.38 to 38.61; $P = 0.0197$). Of the 428 isolates screened for tolerance gene, 22.9% (98 of 428) of the isolates that carried the *qac* gene showed the MIC of ≥160 µg/ml. Almost 86% (84 of 98) of isolates that carried the *qac* gene also carried attenuated *qacEΔ1* and 14% (14 of 98) did not carry *qacEΔ1* gene which is part of the 3' of the conserved segment (CS) of class 1 integrons. Of the 84 *Salmonella* isolates that carried attenuated *qacEΔ1* gene, 48.8% (41 of 84) of the isolates were found to be associated with class 1 integrons and the resistance gene cassettes of different sizes and patterns (Table 3.3).

Generally, for detection of genes such as *qac, qacE1*, *qacEΔ1* and *intI1*, a total of 348 *Salmonella* isolates, including 20 (5.7%, 20 of 348), 60 (17.2%, 60 of 348), and 268 (77%, 268 of 348) isolates detected from drag swabs from swine barns pre-treated with pressurised hot water (control barns), faeces from swine and drag swabs from swine barns pre-disinfected with Biosentry®, respectively, were initially subjected to a test to determine the MICs against different concentrations of Biosentry®. Also, two hundred eighty-eight (67.3%, 288 of 428), 102 (23.8%, 102 of 428), 30 (7%, 30 of 428), and 8 (1.9%, 8 of 428) *Salmonella* isolates whose Synergize® MICs were pre-determined and later tested for presence of *qac, qacE1*, *qacEΔ1* and *intI1*, originated from drag swabs and

faeces from swine placed in the barns pre-disinfected, drag swabs from swine barns pre-treated with pressurised hot water (control swine barns), and pooled feed samples from swine barns, respectively.

Genes such as *qac*, *qacEΔ1* and *intI1* were found among the *Salmonella* isolates originating from drag swabs and faeces from swine placed in the barns pre-disinfected with Biosentry®, with 29.6% (97 of 328), 27.7% (91 of 328), and 20.1% (66 of 328) of isolates carrying the genes, respectively. About 21% (83 of 398), 19.6% (78 of 398), 10.1% (40 of 398) of *Salmonella* isolates originating from drag swabs and faeces from swine placed in the barns pre-disinfected with Synergize®, were found to carry *qac*, *qacEΔ1* and *intI1* genes, respectively, whereas, 54% (27 of 50), 36% (18 of 50), and 20% (10 of 50), of *Salmonella* isolates originating from floor drag swabs and faeces from swine placed in the barns pre-treated with pressurised hot water were also found to carry *qac*, *qacEΔ1* and *intI1* genes, respectively. Overall, genes such as *qac*, *qacEΔ1* and *intI1* were detected from nearly 27% (207 of 776), 24.1% (187 of 776), and 14.9% (116 of 776), of *Salmonella* isolates systematically selected for Biosentry® and Synergize® MICs testing, respectively (Table 3.4). None of the *Salmonella* isolates harboured *qacE*. The *intI1* genes were observed in 62% (116 of 187) of the *qacEΔ1*-positive strains. Thirty eight-percent (71 of 187) of the *qacEΔ1*-positive strains did not contain the *intI1* genes. Although, the class 1 integrons may lack the typical 3′ CS, but in this setting, we strongly conclude that these *qacEΔ1*genes are not contained in class 1 integrons with or without typical 3′ CS end.

The odds of isolating resistant *Salmonella* isolates from isolates with high Synergize® MIC (≥160 µg/ml) was 1.39 times higher than for isolates with low MIC (< 160 µg/ml),

(OR = 1.39; 95% CI; 0. 17 to 11.25; *P* = 0.7580). Assessment of association between tolerance and gene carriage showed that isolates with a high Synergize® MIC were more likely to carry the *qac* gene (Table 3.3). The odds of *qac* gene carriage were 7.09 times higher for isolates with high Synergize® MICs than for those with low Biosentry® MICs (OR = 7.09; 95% CI, 0.41 to 121.42; *P* = 0.1765), whereas, the odds of *qac* gene carriage were 10.38 times higher for MDR *Salmonella* isolates with high Synergize® MICs than for those none MDR *Salmonella* isolates with low Synergize® MICs (OR = 10.38; 95% CI, 5.06 to 21.30; *P* < 0.0001). The odds of *intI1* gene carriage were 5.82 times higher for MDR *Salmonella* isolates with high Synergize® MICs than for none MDR *Salmonella* isolates with low Synergize® MICs (OR = 5.82; 95% CI, 2.24 to 15.15; *P* = 0.0003). Analysis of correlation showed weak association between MDR *Salmonella* isolates and *qac* gene carriage (*r* = 0.36; 95% CI for *r,* 0.27 to 0.44; *P* < 0. 0001) and MDR *Salmonella* and *intI1* gene carriage (*r* = 0.19; 95% CI for *r,* 0.10 to 0.28; *P* < 0. 0001).

Discussion

In the current study, nearly 25% of *Salmonella* isolates carried the *qacEΔ1* and about 15% of the *qacEΔ1* was disseminated among the *Salmonella* isolates by means of the class 1 integrons. Therefore, it is concluded that all of the *intI1*-positive isolates carried *qacE*Δ1 in their 3' conserved segments, confirming that the *qacE*Δ1 gene is linked to the integrons. As a whole, in the current study, no correlation between increased MIC values to Biosentry®, Synergize® *vis-à-vis* the presence of *qacE*Δ1 and *intI1* was observed. This finding was similar to the previous reports in clinical isolates of Gram-negative bacteria (Kucken et al., 2000) and *Salmonella* isolates from poultry and swine (Chuanchuen et al., 2007). Also, in the current study, the comparisons of abundances of class 1 integrons and disinfectant resistance genes (*qacEΔ1*) in *Salmonella* isolates from U. S. swine production

systems showed a significant correlation, suggesting that the MDR efflux pumps , such as the *qacEΔ1* in gram-negative bacteria such as *Salmonella* spp., *E. coli*, *Acinetobacter* spp. *Campylobacter* spp., *Pseudomonas* spp. and also in Gram-positive such as *Staphylococcus aureus*, are common transporters known as membrane-associated proteins that extrude a range of structurally dissimilar toxic compounds from the cytoplasm of the bacterial cells (Quinn et al., 2011; Wan and Chou, 2015).

The change in environmental *Salmonella* prevalence before and after disinfection samplings indicated that there was no change in 43% of the replicates. However, in nearly 34% of the replicates the prevalence increased and decreased in only 23%. This reduction in the prevalence of environmental *Salmonella* is attributable to cleaning and chemical disinfection of swine barns, however, there are various reasons for the increase in the *Salmonella* prevalence. Besides the factors affecting quality of disinfectants and efficiency of disinfection such as pH, temperature, humidity, surface organic matters and the active ingredients of the compounds, in this study one major attribute was observed which can greatly impend efficacious disinfectants. In this study, the prevalence of MDR *Salmonella* was observed to increase by 8.2%, despite a reduction by 7.2% of the environmetal *Salmonella* prevalence. While biocides play an important role in limiting the potential sources of infection, and also reducing the potential food contaminants such as *Salmonella*, as observed in the current study, there is a rising concern about the increasing use of biocides in the animal production environments, community and the attendant pressure *vis-à-vis* resistance development, as well as the potential for cross-resistance to clinically important antibiotics (Poole, 2002; Russell, 2003).

In the current study, the prevalence of pansusceptible *Salmonella* isolates was found to be 3.5%, and 97% and 96.6% of the isolates selected for Biosentry® and Synergize® MIC

testing, respectively, showed high resistance (tolerance) to different concentrations of the biocides used. These *Salmonella* isolates resisting different concentrations of biocides have potentials to tolerate different concentrations of the clinically important antibiotics in the environment as they continue to be exposed to the antibiotics. In the current study, almost all *Salmonella* isolates with MDR resistance phenotypes were also biocide-tolerant isolates. *Salmonella* prevalence change estimates after treatment with three classes of biocides were found to be significantly different from those when cleaning with hot water, and the highest decrease in *Salmonella* prevalence was observed for Biosentry® with 10.4% decrease, followed by Synergize® (8.9% decrease) and Virkon-S® (3.8% decrease). This observation suggests that the degree of efficacy of particular biocide depended on the active ingredients of certain classes of biocides and the in-use concentrations of their active ingredients. In the current study, the Biosentry® was found to have the highest activity than each of the Synergize® and Virkon-S®. Biosentry® was also found to have the minimum breakthrough point at the minimum inhibitory concentration (MIC) of 80 µg/ml, lower than that of the Synergize® (160 µg/ml). Biosentry® 904 contains three different cationic QAC as the active ingredients which are: didecyl dimethyl ammonium chloride (9.2%), dimethyl benzyl ammonium chloride [Alkyl C12, 61%; C14, 23%; C16, 11%; C18. 2.5%; C8 and C10, 2.5%] (9.2%); dimethyl benzyl ammonium chloride [Alkyl (C12; 40%; C14, 50%; C16, 10%] (4.6%) and bis-n-tributyltin oxide (1%). On the other hand, the active ingredients of Synergize® include a QAC and glutaraldehydes, such as: dimethyl benzyl ammonium chloride [Alkyl (C12 67%, C14 25%, C16 7%, C18 1%)] (26%) and the glutaraldehydes (7%), whereas the two active ingredients of Virkon-S® are potassium peroxymonosulfate (21.41%) and sodium chloride (1.50%). The two active ingredients of Virkon-S® used could most likely be the reason for least activity of Virkon-S® compared to each of Biosentry® and Synergize®.

Salmonella serovars are considered as a major cause of gastrointestinal illness in humans causing significant morbidity and mortality (Roberts et al., 2003). *Salmonella* serovars are predominantly zoonotic pathogens; as a result, their occurrence in the food chain can lead to human disease (Weill et al., 2004). Salmonellosis in humans is often associated with the consumption of food products which have been contaminated with *Salmonella* spp. (Carraminana et al., 2004). Increase in the magnitude and public health implications of antimicrobial resistance has been observed among many bacteria of public health importance, a phenomenon which can compromise the efficacy of antimicrobial therapy (Zhang et al., 2006; Rodriguez-Rojas et al., 2013). Over the years, biocides were broadly used to prevent microbial growth and thus, playing an important role in preventing the spread of pathogenic bacteria (Gantzhorn et al., 2015). Therefore, effective disinfection is crucial to contain spread of infectious pathogens, which often involves the measures to eradicate pathogens from a particular environment (Aiello and Larson, 2002; Cozad and Jones, 2003).

Different types of biocides have been in use since a long time for disinfection purposes, as a measure of controling the risk of contaminated food products from reaching the public at large (Gantzhorn et al., 2014). Thus, disinfection is well regarded as a crucial step in achieving a defined, desired hygiene status in food production and processing areas. Public health important foodborne pathogens, such as *Salmonella* serovars, may remain in food animal houses, including swine barn floors, abattoir lairages, floors and associated environments even after cleaning and disinfection, and this may pose a risk of transfer of foodborne pathogens and contamination food animal products such as animal carcasses. In a study conducted in France, approximately, 38% of the pre-disinfected poultry farms tested positive for *Salmonella* before placing day-old chickens (Rose et al., 2000).

Salmonella spp. were also detected from samples originated from pre-disinfected abattoir floors and lairages in which *Salmonella* prevalence from both sample origins were found to be 11.4% and 6.5%, respectively (Small et al., 2006; Gantzhorn et al., 2014). In the current study, the prevalence of *Salmonella* isolates detected from swine barn floors before disinfection was two times higher (13.9%) than after disinfection (6.7%). Similar observation was previously reported in Denmark in which the prevalence of *Salmonella* isolates before cleaning and chemical disinfection was found to be 17.7% and was then reduced to 5.2% after disinfection of abattoir (Gantzhorn et al., 2014). In contrast to observation during the chemical disinfection, the prevalence of *Salmonella* isolates observed in the swine barns pre-treated with pressurised hot water (control group) increased from 16.7% to 27.6% before and after disinfection, respectively, suggesting that the cleaning of the swine barn floors with pressurised hot water was the least efficient in clearing *Salmonella* isolates over the chemical disinfection of the swine barn floors and associated environments. In the current study, a significant difference in the *Salmonella* prevalence was found to be 7.2%, and the reduction in terms of the number of *Salmonella* isolates before and after disinfection was found to be nearly 53%, proving that disinfection with biocides caused a significant reduction of the number of *Salmonella* isolates in swine production environments.

Various studies have demonstrated the decreased susceptibilities to biocides, including quaternary ammonium compounds (QAC), heavy metals, and glutaraldehydes as a result of antibiotic and biocide cross-resistance (Randall et al., 2004; Gradel et al., 2005). For instance, QAC can induce leakage of intracellular components, which is an indicative of membrane damage (Takasaki et al., 1994; Tattawasart et al., 1999). Because of the membrane leakage, cross-resistance can potentially occur when different antimicrobials

(antibiotics and biocides) escape out of the bacterial cells and thus no cell death is initiated as the threshold required to kill the microorganisms is not attained (Guimaraes et al., 2000; Futoma-Koloch et al., 2013). In another study conducted to determine the frequency of mutation to triclosan resistance and to compare the sensitivity to triclosan and various antibiotics in the mutant strains obtained, the results showed that triclosan in the environment selects bacterial strains with reduced antibiotic susceptibility. So, this property depended on the multiple antibiotic resistance phenotypes of bacterial strains and on the triclosan concentration (Birosova and Mikulasova, 2009).

In view of the results obtained, it should be noted that detection of class 1 integrons in the biocide-tolerant and MDR *Salmonella* raise more concern on the public health and multi-drug resistance dissemination among foodborne important pathogens such as *Salmonella* spp. Interestingly, all of the *Salmonella intl1*-positive isolates were resistant to at least two antibiotics, and almost over a half (51%) of the *intl1*-positive isolates, showed a wide range of resistance patterns from pentaresistance to undecaresistance, mainly characterised with resistance to ampicillin, chloramphenicol, streptomycin, sulphonamides, tetracycline, and additionally resistance patterns such as amoxicillin/clavulanic acid, amikacin, ceftriaxone, ceftiofur, cephalothin, gentamycin and kanamycin were observed.

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Table 3.1: Least squares means estimates for the change in *Salmonella* prevalence between pre- and post-disinfection in the environmental samples.

Treatment	Estimate (post-pre prevalence)
Biosentry®	-0.1044[a]
Synergize®	-0.0889[a]
Virkon-S®	-0.0378[a]
Water	0.1089[b]

[a]Estimates with the same superscript did not differ significantly. [b]Estimates with different superscript differed significantly. Change in *Salmonella* prevalence after all biocide treatments significantly differed.

Table 3.2: **Barn-level fecal *Salmonella* prevalence among growing pigs in different production systems and at early and late finishing stages of the production cycle.**

	***Salmonella* prevalence [%]** **Mean, median (range)**	**F2-F1[1], %** **Mean, median (range)**
Overall	12.4, 4.2 (0, 100)	-10.1, -5.6 (-83.3, 50.0)
Production system 1	9.5, 4.2 (0, 61.7)	-10.9, - 8.3 (-61.7, 37.5)
Production system 2	23.5, 10.3 (0, 100)	-15.6, -6.2 (-83.3, 50.0)
Production system 3	4.3, 2.0 (0, 37.5)	-4.0, -2.1 (-37.5, 10.4)
Early finishing stage (F1)	17.3 8,3 (0, 100)	N/A
Late finishing stage (F2)	7.0, 2.1 (0, 93.7)	N/A

[1]Difference in *Salmonella* prevalence between late finishing (F2) and early finishing (F1) of production stage, calculated as [F2-F1]

Table 3.3: Distributions of MIC of biocides, multidrug resistance and the presence of *qac*, *qacEΔ1* and *intI1* genes among *Salmonella*.

Salmonella	**Biosentry® MIC (ug/ml)**						
	[n (%)]						
[n = 348)]	**320**	**160**	**80**	**40**	**20**	**0**	**Total**
Number		26 (7.5)	305 (87.6)	16 (4.6)	1 (0.3)		348 (100)
qac		5 (1.4)	97 (27.9)	7 (2)	0		109 (31.3)
qacEΔ1		5 (1.4)	92 (26.4)	6 (1.7)	0		103 (29.6)
intI1		4 (1.1)	70 (20.1)	1 (0.3)	0		75 (21.6)
MDR		13 (3.7)	177 (50.9)	7 (2)	1 (0.3)		198 (56.9)

Salmonella	**Synergize® MIC (ug/ml)**						
	[n (%)]						
[n = 428)]	**640**	**330**	**320**	**160**	**80**	**40**	**Total**
Number		58 (13.6)	18 (4.2)	341 (79.7)	11 (2.6)		428 (100)
qac		10 (2.3)	12 (2.8)	76 (17.8)	0		98 (22.9)
qacEΔ1		9 (2.1)	12 (2.8)	63 (14.7)	0		84 (19.6)
intI1		8 (1.9)	8 (1.9)	25 (5.8)	0		41 (9.6)
MDR		30 (7)	14 (3.3)	199 (46.5)	7 (1.6)		250 (58.4)

Table 3.4: Occurrence of antimicrobial resistance in swine and barn floors pre-disinfected with Biosentry®, Synergize®, Virkon-S® and hot water and the detection of the MDR efflux genes and class 1 integrons among *Samonella* isolates.

Type of sample	*Salmonella* resistance pattern	Classes of biocides used for disinfection of swine barns, [n (%)]					Detection of MDR efflux genes and class 1 integrons from selected *Salmonella* isolates								
							Biosentry® [n = 348]			Synergize® [n = 428]			Total [n = 776]		
		Biosentry®	Synergize®	Virkon-S®	Hot water	Total	*qac*	*qacEΔ1*	*intI1*	*qac*	*qacEΔ1*	*intI1*	*qac*	*qacEΔ1*	*intI1*
Swine feces	Pansusceptible	18 (0.4)	8 (0.2)	29 (0.6)	34 (0.8)	89 (2)									
	Resistant	1260 (28)	970 (21.5)	1066 (23.7)	1119 (24.8)	4415 (98)	30 (8.6)	26 (7.5)	15 (4.3)	35 (8.2)	31 (7.2)	9 (2.3)	65 (8.4)	57 (7.3)	24 (3.1)
	MDR	984 (21.8)	754 (16.7)	882 (19.6)	808 (17.9)	3428 (76.1)	30 (8.6)	26 (7.5)	15 (4.3)	31 (7.2)	29 (6.8)	7 (1.8)	61 (7.9)	55 (7.1)	22 (2.8)
Swine barns	Pansusceptible	47 (2.9)	43 (2.6)	15 (0.9)	20 (1.2)	125 (7.7)									
	Resistant	401 (24.6)	287 (17.6)	229 (14.1)	586 (36)	1503 (92.3)	79 (22.7)	77 (22.1)	60 (17.2)	63 (14.7)	53 (12.4)	32 (7.5)	142 (18.3)	130 (16.8)	92 (11.9)
	MDR	195 (12)	142 (8.7)	169 (10.4)	330 (20.3)	836 (51.4)	76 (21.8)	74 (21.3)	60 (17.2)	58 (13.6)	48 (11.2)	29 (6.8)	134 (17.3)	122 (15.7)	89 (11.5)
Swine feed	Pansusceptible				3 (10)	3 (10)									
	Resistant		9 (30)	6 (20)	12 (40)	27 (90)									
	MDR		6 (20)		6 (20)	12 (40)									

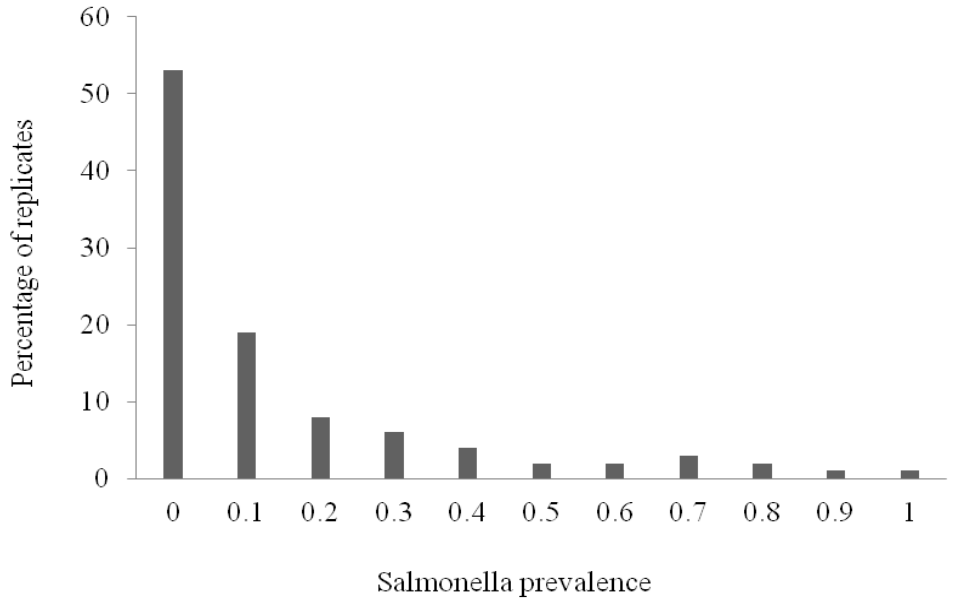

Figure 3.1: Distribution of environmental *Salmonella* prevalence at 360 samplings. Each prevalence estimate was based on 10 floor swab samples collected at a same time point in each swine barn.

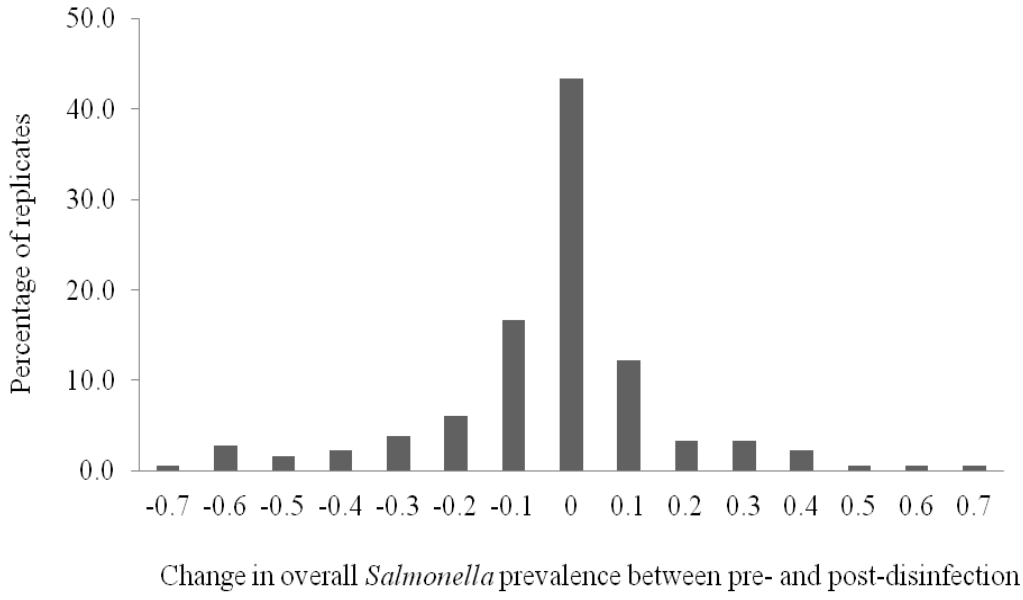

Figure 3.2: Distribution of the change in environmental *Salmonella* prevalence at 360 samplings. Change was calculated by subtracting pre-disinfection prevalence from the post-disinfection prevalence. Each prevalence estimate was based on 10 floor swab samples collected at a same time point in each swine barn.

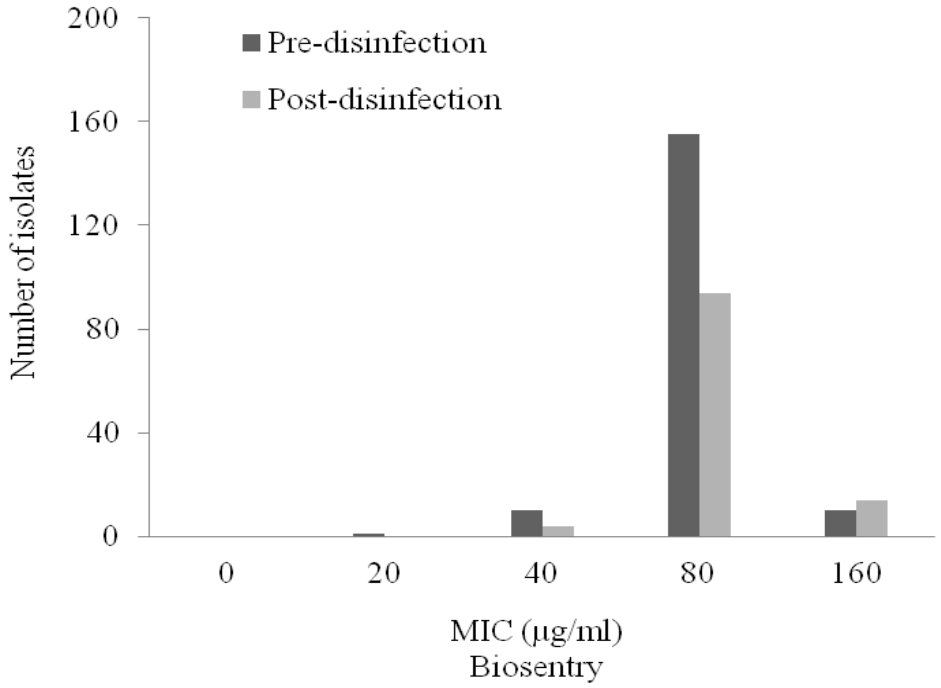

Figure 3.3: MIC of Biosentry® of *Salmonella* isolates obtained pre- and post-disinfection of barns

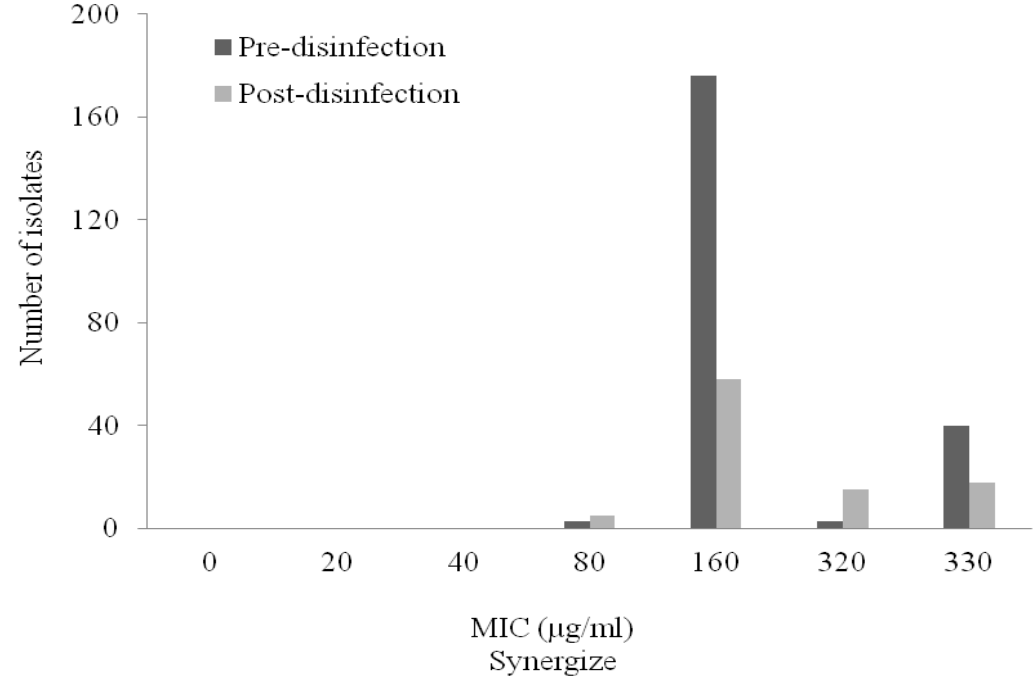

Figure 3.4: MIC of Synergize® of *Salmonella* isolates obtained pre- and post-disinfection of barns

Table 3.5: Biosentry® and Synergize® phenotypes and genotypes and the patterns of the associated resistance gene cassettes

Biocide	MIC (ug/ml)	[n (%)] *Salmonella*	*qac*[a]	*qacE1*[a]	*qacEΔ1*[a]	*intI1*[a]	Cassette pattern [n]	R-type
Biosentry®	0							
	20							
	40	1 (0.3)	+	−	+	+	1,1.2 kb [1]	AmStTeAxChKm
	80	39 (11.2)	+	−	+	+	1 kb [21]; 1,1.2 kb [7]; 1, 1.5 kb [1]; 1,1.2, 1.5 kb [10]	AmClStSuTe/Ax/An/Ch/Gm/Km
		3 (0.9)	+	−	+	+	1 kb [2]; 1,1.2 kb [1]	AmStTe/Ax/Ch/Gm/Km/Su
		28 (8)	+	−	+	+	1 kb [27]; 2 kb [1]	StSuTe/Gm/Km
	160	1 (0.3)	+	−	+	+	1 kb [1]	AmClStSuTe
		3 (0.9)	+	−	+	+	1 kb [3]	AmStSuTeKm/Ax/Gm
	320							
Synergize®	0							
	20							
	40							
	80							
	160	14 (3.3)	+	−	+	+	1 kb [6]; 1.2 kb [1]; 1.5 kb [2]; 1,1.2 kb [2]; 1,1.2, 1.5 kb [3]	AmClStSuTe/Ax/Ce/Ch/Gm/Km/XNL
		3 (0.7)	+	−	+	+	1 kb [3]	AmStSuTe/Ax/Ce/Ch/Gm/Km/XNL
		1 (0.2)	+	−	+	+	1 kb [1]	AmStSuTeKm
		7 (1.6)	+	−	+	+	1 kb [7]	StSu/Te
	320	6 (1.4)	+	−	+	+	1 kb [6]	StSuTe/Ax/Am/Ch
		2 (0.5)	+	−	+	+	1 kb [2]	StSu
	330	5 (1.2)	+	−	+	+	1 kb [2]; 1,1.2,1.5 kb [3]	AmClStSuTe
		3 (0.7)	+	−	+	+	1 kb [3]	StSu/Am/Su/Te

Abbreviations: [a]+ (plus), positive result; - (minus), negative result; Ax, amoxacillin-clavulanic acid; Am, ampicillin; Cl, chloramphenicol; CIP, Ciprofloxacin; An, amikacin; Gm, gentamycin; Km, kanamycin; S, streptomycin; Su, sulfisoxasole; TMP, trimethoprim; Te, tetracycline; XLN, ceftiofur; Ce, ceftriaxone; Cp, cephalothin.

CHAPTER FOUR

Multi-drug resistant *Salmonella* isolates from food animals and animal products in Tanzania

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ABSTRACT

Non-typhoidal *Salmonella* (NTS) serotypes are common causes of human bacteraemia in sub-Saharan Africa. NTS in animals may also play an important role in transmission of antimicrobial resistance to humans. The aim of this study was to determine the prevalence, antimicrobial resistance, the phenotypic and genotypic relatedness of non-typhoidal *Salmonella* isolates recovered from food animals. We investigated the occurrence of class 1 integrons, resistance gene cassettes mobilized in the class 1 integrons and plasmid incompatibility groups using Whole genome Sequencing (WGS). In order to achieve the objective of the current study, a cross-sectional design was carried out in a pastoral region of Tanzania with a large population of livestock, which is also a principal meat and milk source to the country. *Salmonella* isolates were recovered from 64 (4.2%) of a total of 1540 samples from apparently healthy animals, animal products, floor swabs and sewage. The results showed that *Salmonella* isolates were detected in 5.2% (26 of 496), 3.7% (30 of 818) and 3.8% (4 of 104) of the swine, cattle and chicken, respectively. Of the 64 *Salmonella* isolates, 61 belonged to *Salmonella enterica* subsp. *enterica* and 3 were *S. enterica* subsp. *salamae*. The predominant serotypes were *Salmonella* I 8, 20:i:- (32.8%, 21/64), *S*. Hadar (10.9%, 7/64), *S*. Colindale (6.3%, 4/64), *S*. Anatum (6.3%, 4/64) and *S*. Heidelberg (6.3%, 4/64). The three *S*. Heidelberg isolates belonged to the phage type 19, the most frequently encountered phage type among human sporadic cases and in outbreak cases. About one-third (23/64) of the isolates were phenotypically resistant to at least one antimicrobial, of which 82.6% (19/23) were multi-drug resistant (MDR). Two MDR *Salmonella* isolates were found to carry integrons (*intI1*) with gene cassettes *aac(3)-Id-aadA7*. Interestingly, two pansusceptible *Salmonella* isolates were found to carry the non integron-borne resistance genes such as *sul2*, *aadA1* and *blaTEM-1B*, *blaTEM-1A*. The PFGE DNA fingerprint patterns strongly indicated that the majority of *Salmonella* isolates

were clonal. The occurrence of clonal MDR *Salmonella* isolates in food animals and animal products from pastoral communities implicates *Salmonella* as an important source of pathogens to the pastoralist communities. This finding is of great public health concern particularly since unpasteurized milk and uncooked meat consumption is common in Tanzania.

Keywords: MDR *Salmonella*, Pastoralists, Animal products, Antimicrobial resistance, Class I integrons, Public health

INTRODUCTION

Foodborne diseases caused by non-typhoidal *Salmonella* (NTS) represent an important public health problem worldwide. NTS serovars are among the most important foodborne bacterial pathogens with broad host range including food animals and humans (Scallan et al., 2011; Elhadi et al., 2013). Previous studies conducted have implicated food animals to be the major reservoirs of *Salmonella* (Vo et al., 2006; Li et al., 2013). The *Salmonella* serovars isolated from food animals have significant overlap with those causing illness in humans. Of particular importance as far as the food animals are concerned is the food chain which has been shown to play an important role in the transmission of *Salmonella* from food animals to humans (Alcaine et al., 2006; Thong and Modarressi, 2011). The importance of the animal products in the dissemination of other important zoonotic disease pathogens, namely, *Mycobacterium bovis*, causing Bovine Tuberculosis and *Brucella* spp. causing Brucellosis was shown to be a key factor in pathogens transmission (Cleaveland et al., 2007).

In Tanzania, there is no official *Salmonella* surveillance data in place, however, there are a few studies conducted that have estimated the prevalence of NTS ranging from 7.6 - 28% in humans (Mtove et al., 2010; Meremo et al., 2012), whereas, the serological and cultural prevalence of *Salmonella enterica* subspecies *enterica* serovar Gallinarum (*Salmonella* Gallinarum) were recorded to range from 2.6 - 28% (Mdegella et al., 2000) and the overall prevalence of NTS in livestock was found to be 2.3% (Otaru et al., 1990). Majority of studies on Salmonellosis in Tanzania have largely addressed detection by isolation of *Salmonella*. A study conducted by Vaagland et al. (2004) has shown the exclusivity of *Salmonella* as a zoonotic pathogen of public health importance in the country for its incrimination in causing Enteritidis meningitis in children. Interestingly, another study

conducted by Mtove et al. (2010) has shown an appealing scenario in which children with invasive NTS infection were more likely to also have malaria. With the increasing trend in consumption of food products of animal origin, there is a need for more focused studies that investigate an increased potential for exposure to *Salmonella* through the food chain and the public health implications.

The magnitude of antimicrobial resistance in *Salmonella* as a significant threat to public health is not well documented in Tanzania. The excessive use and the uncontrolled over-the counter sale of antimicrobials, particularly β-lactams, tetracyclines and fluoroquinolones in developing countries is highly likely to aggravate the magnitude of antimicrobial resistance (Viberg et al., 2010; MacGowan and Macnaughton, 2013). The β-lactams and fluoroquinolones are reported to be important classes of antimicrobials used to treat complicated cases of salmonellosis in humans and veterinary medicine (Gonzalez-Sanz et al., 2009). The production of extended-spectrum β- lactamases has been shown as one of the main mechanisms of resistance to broad-spectrum β-lactams among the Enterobacteriaceae (Li et al., 2007; Bush and Jacoby, 2010). The β-lactamases genes have been detected worldwide in various serovars of NTS, located in plasmids or integrons, facilitating rapid transmission among *Salmonella* serovars (Cloeckaert and Schwarz, 2001; Frye and Jackson, 2013). Integrons are the DNA elements capable of capturing and mobilizing antimicrobial resistance genes among bacteria. The class I integrons are the most common type of integrons identified in multi-drug resistant (MDR) *Salmonella* known for the dissemination of resistance genes among pathogens in the microbial population (Thong and Modarressi, 2011). The aim of this study was to determine the prevalence, antimicrobial susceptibility patterns, the phenotypic and genotypic relatedness of *Salmonella* isolates recovered from food producing animals and food of animal origin.

In addition, we investigated the occurrence of class I integrons and resistance gene cassettes mobilized in the class 1 integrons.

MATERIALS AND METHODS

Study area and sample collections. Study was carried out in three regions of Tanzania mainland, namely, Morogoro, Iringa and Arusha. Morogoro and Iringa, are two main regions with relatively large populations of pastoral and agro-pastoral communities (Barabaig, Maasai and Sukuma) which own large populations of cattle herds, all originating from different zones of the country. The once Arusha region, now for administrative convenience split into Manyara and Arusha regions, was selected for the reasons that it is the home region for Maasai and Barbaig who keep on wandering with cattle herds in search for water and good pastures. Samples from live and slaughtered animals were collected to investigate the possibility of *Salmonella* species circulating in these animals as a result of the livestock mixing practices or livestock wandering on the communal lands.

The sample collection was conducted in farms, households and slaughterhouses of cattle, swine, sheep and goats and poultry from February 2013 to March 2014 in the three selected regions (Arusha, Iringa and Morogoro) of Tanzania. Sample size was calculated according to Charan and Biswas, (2013). Faecal (n = 136) and milk (n = 48) samples from cattle, faeces from goats (n = 103) from the pastoral and agro-pastoral communities, as well as from non pastoral communities, and faeces (n = 215) and milk (n = 238) from dairy cattle farms were collected. Other samples from live animals included: swine feces (n = 473), chicken cloacal swabs (n = 48), chicken eggs (n = 50) and feces (n = 6), from farms with and without mixed farming. Samples from slaughtered animals were cattle

carcass swabs (n = 181) and swine carcass swabs (n = 23). Environmental samples included the floor drag swabs of the slaughterhouses and slaughter slabs (n = 12). Also, one slaughterhouse pit latrine which is frequently used by workers was sampled. Briefly, a sterile cotton swab with a long hanging thread tied on a stick was submerged into the septic tank through its hole. The swab was kept in a sterile whirl-pak, kept in cool box and transported to the laboratory for processing. This sampling process of the slaughterhouse latrine was repeated at least twice a month for seven consecutive visits (Table 4.1). All samples were kept in a cool box before transporting to Sokoine University of Agriculture for further processing.

***Salmonella* isolation and identification.** Conventional methods of isolation were used as described previously (Gebreyes et al., 2004). These were used for isolation and identification of Salmonellae. Briefly, a 10g portion of each faecal and feed sample were pre-enriched in 90 ml of buffered peptone water (BPW; Becton Dickinson, Sparks, MD). In addition, about 90 ml of BPW was added to each Whirl-Pak bag containing individual carcass and floor drag swabs, and both incubated at 37°C for 24 h. A 100 µl of each pre-enriched suspension following overnight incubation was added into 9.9 ml of Rappaport-Vassilliadis (RV) enrichment broth (Becton Dickinson, Sparks, MD) and incubated at 42°C for 24 h. Following overnight incubation at 42°C, a 10 µl of each of the enriched suspension was inoculated onto Xylose-lactose-Tergitol 4 (XLT-4) agar (Becton Dickinson, Sparks, MD) or Xylose-lysine deoxycholate (XLD) agar (Himedia, Mumbai, India) plates and incubated at 37°C for 24 h. The incubation time was extended to 48 h in cases where colonies were doubtful. Three isolated presumptive *Salmonella* colonies were selected from each positive sample for biochemical tests. Each selected presumptive *Salmonella* colony was inoculated onto triple sugar iron (TSI) agar (Becton Dickinson,

Sparks, MD) slants, Lysine iron agar (LIA) slants (Becton Dickinson, Sparks, MD) and urea broth (Becton Dickinson, Sparks, MD) and incubated at 37°C for 16-24 h. Some atypical presumptive *Salmonella* isolates were observed after performing TSI, LIA and urea tests, as a result of biochemical indeterminacies, all presumptive *Salmonella* isolates were stored at -80°C until further testing using *invA* Polymerase Chain Reaction (PCR) and *16S rDNA* gene sequencing.

Phenotypic characterisation: The 64 *Salmonella* isolates were serogrouped by slide agglutination using commercially available *Salmonella* O polyvalent A-1 and vi antiserum (MiraVista Diagnostics, Indianapolis, IN) according to the recommendations of the manufacturer. The *Salmonella* isolates were serotyped at the Office International de Épizooties (OIÉ) Reference Laboratory for Salmonellosis of the Public Health Agency of Canada, Laboratory for Foodborne Zoonoses, Guelph, Ontario, Canada. Serovar Heidelberg isolates were also phage typed after assigning the serotypes of the *Salmonella* isolates. Briefly, the somatic (O) antigens were determined by slide agglutination tests (Ewing, 1986) and the flagellar antigens were determined using a microplate agglutination technique (Shipp and Rowe, 1980). In addition, the Grimont antigenic formulae were used to identify and assign the serotypes of the *Salmonella* isolates. Phage typing of *Salmonella* Heidelberg isolates was conducted as previously described (Demczuk et al., 2003). Briefly, the plates were incubated and lytic patterns were read and recorded (Amavisit et al., 2001). *Salmonella* isolates that reacted with the phages but did not conform to any recognized phage type were designated atypical (AT). The 64 *Salmonella* isolates and 25 *Citrobacter* isolates (initially identified as atypical *Salmonella* isolates) (Table 4.4) were tested for antimicrobial susceptibility to a panel of 14 antimicrobials using the Kirby-Bauer disc diffusion method (CLSI, 2002). The antimicrobial agents used and their

respective disc potencies were as follows: ampicillin (Am; 10 µg/ml), amoxicillin-clavulanic acid (Ax; 30 µg/ml), amikacin (An; 30 µg/ml), ceftriaxone (Ce; 30 µg/ml), cephalothin (Ch; 30 µg/ml), chloramphenicol (Cl; 30 µg/ml), ciprofloxacin (CIP; 5 µg/ml), gentamicin (Gm; 10 µg/ml), kanamycin (Km; 30 µg/ml), streptomycin (S; 10 µg/ml), trimethoprim (TMP; 5 µg/ml), sulfisoxazole (Su; 250 µg/ml), and tetracycline (Te; 30 µg/ml). *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains. *Salmonella* isolates showing resistance to three or more antimicrobial agents were classified as multi-drug resistant (MDR) and those isolates with intermediate resistance profiles were considered susceptible.

Determination of *16S rDNA* and *invA* gene in *Salmonella*. The 64 presumptively positive *Salmonella* isolates were tested for the carriage of invasion (*invA*) gene by PCR. Briefly, the isolates were inoculated onto tryptic soy agar (TSA) plates and incubated at 37°C for 16-24 h. The genomic DNA was extracted using the Qiagen DNeasy tissue kit according to the manufacturer's instructions (Qiagen Ambion, Austin, TX, USA). A set of forward primers (5'-TCGTCATTCCATTACCTACC-3') and reverse primers (5'-AAACGTTGAAAAACTGAGGA-3'), was used to amplify the *invA* gene. The thermocycling conditions included: Hot Start *Taq* activation at 94°C for 3 min, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and amplification was done in 35 cycles. The reaction mixture was kept at 72°C for 10 min after the final cycle (Hoorfar et al., 2000). A total of 25 phenotypically indeterminate *Salmonella* isolates (atypical *Salmonella* isolates) and three typical *Salmonella* isolates were selected for *16S rDNA* sequencing. Primers used for amplification of the *16S rDNA* included 27F (5'-AGAGTTTGAT**YM**TGGCTCAG-3') and

907R (5'-CCGTCAATTC**M**TTTGAGTTT-3') (Mao et al., 2012). The PCR amplification conditions were initial denaturation at 95°C for 4 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and then the amplification cycle was repeated for a further 35 cycles and final extension was done at 72°C for 7 min. About 10 µl of the PCR product of each isolate tested were run on 1% agarose gel stained with 5 µl of 10-mg/ml ethidium bromide for 1 h at 120 V using 0.5X Tris borate-EDTA (TBE) as running buffer. A 1-kb Plus DNA ladder was used as a molecular size marker. The PCR products generated for sequencing of *16S rDNA* gene were purified using ExoSAP-IT PCR clean-up method. Briefly, a 5 µl of each of the post- PCR reaction products and a 2 µl of ExoSAP-IT reagent (Miles Road, Cleveland, OH) were mixed together, followed by incubation at 37°C for 15 min and 80°C for 15 min. Following clean-up, a 10 µl of each purified PCR products were pre-mixed separately in the same tube with 5 µl of 5 pMol/µl of each of sequencing primers. The pre-mixing and the submission were done according to the organization guidelines (GENEWIZ, South Plainfield, NJ).

Detection of class 1 integron and resistance gene cassettes. The presence of class 1 integron and gene cassettes integrated between conserved segments of class 1 integrons were detected by PCR. Primers used for amplification of the *intI1* included IntI-F (5'-GCCTTGCTGTTCTTCTACGG-3') and *intI1*-R (5'-GATGCCTGCTTGTTCTACGG-3') (Levesque, et al., 1995) while those for conserved segments included 5'CS (5'-GGCATCCAAGCAGCAAG-3') and 3'CS (5'-AAGCAGACTTGACCTGA-3') (Ploy, et al., 2000). The PCR temperature profile included Hot Start *Taq* activation at 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, and then a final extension step at 72^0C for 7 min (Lindstedt et al., 2003). About 10 µl of the PCR product of each isolate tested were run on 1% agarose gel stained

with 5 µl of 10-mg/ml ethidium bromide for 1 h at 120 V using 0.5X Tris borate-EDTA (TBE) as running buffer. A 1-kb Plus DNA ladder was used as a molecular size marker.

Gene cassettes sequencing. The PCR products generated for sequencing of *16S rDNA* and variable regions of gene cassettes of class 1 integrons were purified using ExoSAP-IT PCR clean-up method. Briefly, a 5 µl of each of the post- PCR reaction products and a 2 µl of ExoSAP-IT reagent (Miles Road, Cleveland, OH) were mixed together, followed by incubation at 37°C for 15 min and 80°C for 15 min. Following clean-up, a 10 µl of each purified PCR products were pre-mixed separately in the same tube with 5 µl of 5 pMol/µl of each of sequencing primers. The pre-mixing and the submission were done according to the organization guidelines (GENEWIZ, South Plainfield, NJ).

Whole genome sequencing (WGS). Genomic DNA of the *Salmonella* isolates was extracted using the QIAamp DNA Mini Protocol (Valencia, CA). Extraction of genomic DNA was performed with the fully automated Qiagen QIAcube and samples were sequenced at the US Food and Drug Administration (FDA) using Illumina MiSeq (Hampton, VA) based on published methods (Hoffman et al. 2015). Fastq sequence reads from all sequences were deposited at the National Center for Biotechnology Information (NCBI) website under the BioProject ID: 275961 with the accession: PRJNA275961.

Pulsed-Field Gel Electrophoresis (PFGE) Genotyping. PFGE was performed following the PulseNet protocol (Ribot et al., 2006) of the Centers for Disease Control and Prevention (CDC). Selection criteria of *Salmonella* isolates for PFGE fingerprinting included isolates from different sources (cattle, swine, chicken, and environment). These represented different sample types (feces, milk, cloacal, carcass, and floor drag swabs)

showing phenotypic characteristics (serogroups and antimicrobial resistance profiles). The samples also represented isolates from different sampling dates and geographic locations. The isolates were selected systematically based on the source (swine, bovine, goats and chicken), type of samples, antimicrobial resistance profiles, and serogroup identity.

To determine the genotypic relatedness of *Salmonella* isolates recovered from the food animals and animal products originating from different farms and slaughterhouse, a total of 54 isolates (36 of 46 faecal isolates; 7 of 7 carcass isolates; 7 of 7 milk isolates; 3 of 3 slaughterhouse floor isolates and 1 isolate from sewage) with different phenotypic characteristics were subjected to PFGE DNA fingerprinting analysis. A total of 54 (84.4%) *Salmonella* isolates from epidemiologically related study, recovered from Arusha and Morogoro, from 2013 to 2014 were systematically selected. Two *Salmonella* outgroups, Kenya (n = 1) and North Carolina, United States (n = 2) were included in the PFGE fingerprinting for comparison.

Briefly, *Salmonella* isolates were grown on tryptic soy agar (TSA) plates (Becton Dickinson, MD) at 37°C for 16-24 h. The bacterial cell suspensions were prepared in sterile polypropylene culture tubes (Fisher Scientific, Hampton, NH) containing 2 ml of cell suspension buffer (CSB; 100 mM Tris and 100 mM EDTA [pH 8.0]). Each adjusted cell suspension with optical density [OD] between 1.3 to 1.4 at wavelength [λ] of 610 nm was transferred to a microcentrifuge tube containing proteinase K (20 mg/ml) and the combination was gently mixed and incubated at 54°C for 2 h.

The agarose-embedded cells were then lysed with cell lysis buffer (CLB; 50 mM Tris and 50 mM EDTA [pH 8.0], 1% Sarcosyl [Sigma, St. Louis, MO], 0.1 mg/ml proteinase K),

and the intact genomic DNA were digested with 20 U of the XbaI restriction enzyme (New England Biolabs, Ipswich, MA) for 2 h at 37°C. The fragments were then separated using the CHEF-DR III (Bio-Rad Laboratories, Hercules, CA). The universal standard marker strain, *Salmonella enterica* serovar Braenderup H9812, was used as a molecular reference marker. The PFGE agarose gels electrophoresis were run for 19 h at initial switch time of 2.2 s, final switch time of 63.8 s, voltage of 6V and included angle of 120°. The gels were stained with 40 ml of ethidium bromide solution (10 mg/ml) in 400 ml of double distilled water for 20 min with gentle shaking. The gels were then destained with 400 ml of double distilled water for 60-90 min with gentle shaking, changing water four times after every 20 min. The banding patterns were observed under ultraviolet (UV) illumination and a digital image of the PFGE patterns were acquired using the Gel Doc 1000 (Bio-Rad). PFGE images were analyzed with BioNumerics software version 4.6 (Applied Maths, NV, Belgium). Cluster analysis were performed using the unweighted-pair group method using average linkages (UPGMA), with 1.5% band position tolerances and 1.5% optimization values. All isolates with PFGE banding patterns having similarity indexes of >80% were grouped within the same cluster.

Discrimination index. The discriminatory power of serotyping and PFGE methods was evaluated using the Simpson's diversity index according to the formula previously explained by Hunter and Gaston (1988).

$$D = 1 - \left[\frac{1}{N(N-1)}\right]\sum_{j=1}^{s} nj(nj-1)$$

Where D is the Simpson's diversity index, N denotes the total number of isolates in this population, s is the total number of types described and nj is the number of isolates representing each type (j^{th} type). The Simpson's diversity index is based on the probability

that two unrelated strains from the test population will be placed into different groups. The index calculates values in a range of 0.0 (no diversity) to 1.0 (infinite diversity).

***16S rDNA* and gene cassettes sequence analysis.** All reverse sequences were converted to match the complement DNA forward sequences using the online reverse complement software available at www.bioinformatics.org/sms/rev_comp.html. Both forward and reverse complement sequences of each isolates were aligned using ClustalW2 software available at www.ebi.ac.uk/clustalw and trimmed to obtain the consensus DNA sequences. The consensus nucleotide sequences of *16S rDNA* were chimera checked using online DECIPHER software (Wright et al., 2012). None of the 28 nucleotide sequences of *16S rDNA d*eciphered chimeras. The consensus DNA sequences were compared with the best-matching sequences available on the NCBI databases using the GenBank BLASTN available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The phylogenetic relationships of *16S rDNA* sequences of *Salmonella* isolates were inferred by MEGA 6.0 using the Neighbor-Joining (N-J) method and the Kimura 2-P model of sequence evolution (Saitou and Nei, 1987). Using MEGA 6 software, the phylogenetic affiliations of the 28 isolates (*Salmonella* [n = 3] and *Citrobacter* [n = 25]), from different animal hosts from the current study area and five outgroups (*Salmonella* Typhimurium strain LT2 [NC003197.1], *Enterobacter asburiae* strain LF7a [NC015968.1], *Citrobacter rodentium* strain ICC168 [NC013716.1], *Salmonella* Anatum strain 315 [JQ694223.1] and *Escherichia coli* strain UMN026 [NC011751.1] were inferred.

Whole Genome Sequencing (WGS) analysis to identify MLST type, plasmids and antimicrobial resistance genes. The WGS is becoming increasingly accessible for complete characterization of antimicrobial resistance genes (Zankari et al., 2012),

detection of plasmids (Carattoli et al., 2014), and for multilocus sequence typing (Larsen et al., 2012). For WGS of *Salmonella* isolates in the current study, *Salmonella* isolates were grown on tryptic soy agar (TSA) tubes (Becton Dickinson, MD) at 37°C for 16-24 h and submitted to U.S. FDA for WGS under the BioProject name: ICOPHAI. Once the sequencing was completed, the sequence read archive (SRA) files were uploaded for public access on the NCBI website at http://www.ncbi.nlm.nih.gov/sra. The pair fastq format files were downloaded for each *Salmonella* isolate using http://www.ebi.ac.uk/ link. Two fastq files for each isolate were submitted to the Center for Genomic Epidemiology website (http://www.genomicepidemiology.org/) to identify MLST type, plasmids and antimicrobial resistance genes. Under a drop-down list we selected *Salmonella* as targeted organism, the type of the reads were Illumina-paired end reads with 85% threshold ID, 80% length.

SeqSero genotype-based serotyping. The WGS of *Salmonella* isolates was also utilized for *Salmonella* serotypes determination (Zhang et al., 2015). To determine the serotypes of the *Salmonella* isolates, we submitted the downloaded pair of fastq format files for each isolate into SeqSero website (http://www.denglab.info/SeqSero) by selecting reads (pair-end) in the option menu. After uploading the files the result was received through the email contact provided.

Data analysis. Data were handled using Microsoft excel 2007 (Ms Corp., Redmond, WA, USA) and statistical analysis was conducted with the MedCalc® statistical software package version 12.7.1.0 (8400 Oostende, Belgium). Prevalence of *Salmonella* and antimicrobial resistance profiles were analyzed at the animal and sample levels. A value of $P \leq 0.05$ was considered significant.

RESULTS

***Salmonella* prevalence and the serotypes.** *Salmonella* isolates were detected from 64 of 1540 apparently healthy animals, animal products, floor swabs, and sewage samples indicating a 4.2% prevalence. The results showed that *Salmonella* isolates were detected in 5.2% (26 of 496) of the swine; 3.7% (30 of 818) of cattle and 3.8% (4 of 104) chicken specimens. Twenty five percent (3 of 12) of slaughterhouse floor swabs and 14.3% (1 of 7) sewage samples were also found to be positive for *Salmonella*. *Salmonella* isolates were recovered from dairy cattle feces and milk and other samples as shown in Table 4.1. *Salmonella* recovery from swine, cattle and different sample types was not significantly different between any of the groups: swine versus cattle group (P = 0.245), Zebu cattle versus dairy cattle (P = 0.27), dairy cattle feces versus dairy cattle milk (P = 0.365) and Zebu cattle feces versus dressed cattle carcasses (P = 0.902).

Of the 64 isolates, 61 belonged to *S. enterica* subspp. *enterica* and three were *S. enterica* subspp. *salamae*. The predominant serovars were *Salmonella* ser. I 8,20:i:- (32.8%, 21/64), *S. enterica* subspp. *enterica* serovar Hadar (*Salmonella* Hadar) (10.9%, 7/64), *Salmonella* Colindale (6.3%, 4/64), *Salmonella* Anatum (6.3%, 4/64) and *Salmonella* Heidelberg (6.3%, 4/64). Interestingly, *Salmonella* serovar I 8,20:i:- isolates were widespread in different samples from different food animals and products, including the milk from a mastitic cow. The three *S.* Heidelberg isolates belonged to the phage type 19, the most frequently encountered phage type among human sporadic cases and in outbreak cases, whereas, one *S.* Heidelberg isolate did not conform to any recognized phage type and was considered atypical (Table 4.3).

Antimicrobial resistance phenotyping. According to the disc diffusion, the most common antimicrobial resistance was to tetracycline (25%), followed by sulfisoxazole (21.9%), trimethoprim (17.2%), amoxicillin-clavulanic acid (15.6%), cephalothin (14.1%),

ampicillin (14.1%), streptomycin (6.2%), ciprofloxacin (3.1%) and chloramphenicol (1.6%). No antimicrobial resistance was found to amikacin, gentamycin and ceftriaxone, however, intermediate resistance was found to ceftiofur (1.6%) and kanamycin (1.6%) (Table 4.2). Of the 64 *Salmonella* isolates resistant to one or more antimicrobials, 20.8% (5 of 64), 6.3% (4 of 64), and 1.6% (1 of 64) of the isolates were recovered from the dressed cattle swabs, cattle feces, and milk samples, respectively. In addition, 18.8% (12 of 64) and 3.1% (2 of 64) of the isolates were recovered from swine feces and chicken samples, respectively. Sixty-three percent (40 of 64) of the *Salmonella* isolates were pansusceptible, 37.5% (24/64) of the isolates were resistant to at least one antimicrobial, and 29.7% (19/64) were multi-drug resistant (MDR) *Salmonella.* Of the 19 MDR *Salmonella* isolates, 47.4% (9 of 19), 42.1% (8 of 19), 10.5% (2 of 19) of the isolates were recovered from the swine, bovine and chicken samples, respectively. The frequency of antimicrobial resistance of *Salmonella* spp. recovered from different animal species and sample types was not significantly different between any of the three groups, namely swine versus cattle ($P = 0.477$), Zebu cattle versus dairy cattle ($P = 0.338$) and dairy cattle feces versus dairy cattle milk ($P = 0.767$). However, the frequency of antimicrobial resistance of *Salmonella* spp. was significantly different between Zebu cattle feces and dressed carcasses ($P = 0.05$).

Polymerase Chain Reaction (PCR) of targeted genes. About 8.3% (2/24) of the resistant *Salmonella* isolates were found to also carry integrons (*intI1*) and 100% (2/2) of *intI1*-positive isolates contained resistance gene cassettes known as *aac(3)-Id-aadA7* of size 1500 bp showing high rate of MDR (Table 4.2). In addition, three of 17 (17.6 %) of the resistant *Citrobacter* isolates amplified *intI1* gene and 100% (3/3) of *intI1*-positive isolates contained resistance gene cassettes known as *dfrA1-orfC*, *dfrA7* and *dfrA15* of size 1250 bp, 800 bp and 700 bp, respectively (Table 4.3).

Analysis of Whole Genome Sequence findings. A web-based method, ResFinder for detection of acquired antimicrobial resistance gene profile from the whole-genome data identified *strB*, *sul2*, *dfrA*; *sul2, dfrA; strB, sul2*, *tet*(A), *dfrA14*; and *dfrA14* from *Salmonella* isolates with SuTeTMP resistance phenotypes. Interestingly, *Salmonella* isolate ID#s S16783 (*S.* Manchester) carrying incl1 plasmid and S16683 (I 8,20:i:-) carrying no plasmid, both with pansusceptible resistance phenotypes were observed to contain resistance genotypes such as *aadA1*, *blaTEM-1B*, *blaTEM-1A* and *sul2*, respectively. The Illumina sequence reads (paired-end reads) from 38 *Salmonella* isolates genomes were analyzed using PlasmidFinder. The PlasmidFinder analysis detected plasmid groups such as: incl1 (1 isolate), incFII (2 isolates), Colpvc (1 isolate), ColRNAI (3 isolates), and NKP (25 isolates) and none from six *Salmonella* isolates. The whole genome MLST (wgMLST) analysis of the sequence reads, using the web-based tool identified the unknown MLST sequence types (ST) from 35 *Salmonella* isolates and the three *Salmonella* isolates were untypeable (Fig. 4.1).

Phylogenetic characterization of *16S rDNA*. Based on *16S rDNA* sequence analysis, 28 isolates and five outgroups formed two distinct clades, namely A and B (Fig. 4.2). Isolates in clade A aggregated to form three clusters namely, A_1, A_2 and A_3, in which, *Salmonella* Anatum (n = 1) and *Citrobacter* (n = 14) isolates clustered with the *E. coli* strain UMN026 (NC011751.1) to cluster A_1 at 99% similarity, eleven *Citrobacter* and *Salmonella* I 6,7:-:1,7 (n = 1) isolates showed lower sequence similarity of 64% [cluster A_2], whereas, a sequence of *Salmonella* Anatum strain 315 [JQ694223.1] and *Salmonella* I 8,20:i:- clustered at 94% to form cluster A_3. Three out-growers, namely, *Salmonella* Typhimurium strain LT2 [NC003197.1], *Enterobacter asburiae* strain LF7a [NC015968.1] and *Citrobacter rodentium* strain ICC168 [NC013716.1] clustered at 99% sequence similarity to form clade B (Fig. 4.2).

Genotyping using Pulsed-field gel electrophoresis (PFGE). PFGE DNA fingerprinting generated profiles of twenty six major genotypic clusters (A-Z) and 42 PFGE fingerprint patterns with an 80% Dice coefficient index cut-off (Fig. 4.1). The discriminatory power of Simpson's diversity index of PFGE fingerprinting was found to be 0.98 higher than that of the serotyping method ($D = 0.88$). Most isolates within a single cluster were of the same serotype, with the exception of *S*. Hadar and *S*. Karamoja, *S*. Uganda and *S*. Amager, *S*. Isangi, and *S*. I 6,7:-:- which were classified as J, L and Q clusters, respectively. Isolates *S*. Hadar and *S*. Karamoja, *S*. Uganda and *S*. Amager, *S*. Isangi and *S*. I 6,7:-:-), were found to form cluster J, L and Q-cluster, respectively, but each cluster forming different PFGE patterns, J_1 and J_2, L_1 and L_2, and Q_1 and Q_2-patterns, respectively.

Overall, M-cluster was the predominant group in this study, comprising 15 isolates of *S*. I 8,20:i:-, followed by A-cluster (4 isolates from *S*. Hadar) and W-cluster (4 isolates from *S*. I 8,20:i:-). The most common PFGE pattern in this study was M_3, which included eight isolates of *S*. I 8,20:i:-, followed by M_5, which included three isolates of *S*. I 8,20:i:- and W_1, which also included three isolates of *S*. I 8,20:i:-. Some indistinguishable isolates of *S*. I 8,20:i:- with diverse origins (from different production levels [farm and abattoir] different host species and sample types) and phenotypic characteristics (different antimicrobial resistance types [R-types]), were found, especially in the dominant groups (M_3 and M_5-patterns). These clonally indistinguishable isolates were detected on different days of isolation over a diverse geographic locations of 10 to 800 km apart (Arusha and Morogoro), for example, S.16687, S.16680, S.16682, S.16691, S.16705, S.16735, S.16811 and S.16823 in the M_3-pattern or S.16683, S.16703 and S.16750 in the M_5-pattern. Looking at the isolates in each of the main clusters, it was observed that M-cluster is composed of 15 *S*. I 8,20:i:- isolates, with different antimicrobial resistance patterns,

including R-type: Cp, AmCpTe, AmAxTe, AmAxCp, SuTeTMP, ClAxCpSuTeTMP and pansusceptible. It was also observed that A-cluster was composed of three isolates of *S*. Hadar , B-cluster (2 isolates from *S*. Manchester), J-cluster (3 isolates, 1 isolate from *S*. Hadar and 2 isolates from *S*. Karamoja), L-cluster (2 isolates, 1 isolate from each of *S*. Uganda and *S*. Amager), W-cluster (3 isolates from *S*. I 8,20:i:-), V-cluster (2 isolates from *S*. Anatum), and U-cluster (2 isolates from *S*. Colindale), all of which were susceptible to all antimicrobials used in this study. The public health important serotype *S*. Heidelberg (C-cluster) was composed of three isolates with R-type: AmAxCpSu and pansusceptible.

DISCUSSION

While other previous studies in Tanzania have also reported high prevalence of Non-typhoidal *Salmonella* (7.6 - 29%) in humans (Mtove et al., 2010; Meremo et al., 2012) and 2.3 - 37.3% in livestock (Otaru et al., 1990; Lubote et al., 2014), in this study, the *Salmonella* floor prevalence (25%) was higher than any other sources from which samples were collected. The prevalence of *Salmonella* in the cattle carcasses (3.3%) was higher than in the cattle feces (2.3%). A higher prevalence of *Salmonella* spp. on the carcasses than in the feces is a clear indication of failure to realize proper preventive measures in producing safe meat (pork, beef, mutton) and meat products for public consumption (Carrasco et al., 2012). The major source of contamination of the slaughterhouse floor and cattle carcasses could be attributed to poor handling of faecal matters during evisceration, bacterial load on the animal skin, the slaughterhouse personnel and the equipment used during the slaughter process (Teklu and Negussie, 2011). Strict preventive measures need to be instituted to limit possible contamination of the slaughterhouse floor and the dressed carcasses by foodborne pathogens (Kich et al., 2011). The individual animal-level

prevalence of *Salmonella* between the key food animals varied from 3.7% in cattle, 3.8% in poultry and 5.2% in swine, is higher than the prevalence reported by Otaru et al. (1990). The overall prevalence of Non-typhoidal *Salmonella* from this study was reported to be 4.2% similar to the prevalence (4.25%) reported by other study conducted in febrile children admitted to a referral hospital in Tanzania (Christopher et al., 2013). In another similar study conducted in apparently healthy livestock in Southern Tanzania, the overall prevalence of *Salmonella* was reported to be 2.3% (Otaru et al., 1990). In contrast to the current study, the higher *Salmonella* prevalence reported elsewhere in Tanzania were from the symptomatic children and HIV/AIDS patients admitted to referral hospitals, whereas, relatively low prevalence (2.1-2.6%) of *Salmonella* was also reported from diarrhoeic children aged less than five years admitted to major hospitals in Tanzania (Moyo et al., 2011; Oketcho et al., 2012). Previous study by Bywater et al (2004) reported persistence of *Salmonella* infections in food animals in the subclinical stage and thus they are often clinically asymptomatic carriers (Haley et al., 2015).

Some of the serovars isolated in the current study were also reported in outbreaks scenarios elsewhere, including *S*. Heidelberg and S. Hadar, (Jackson et al., 2013), *S*. Anatum (Krause et al., 2001), *S*. Virchow (Maguire et al., 2000) and *S*. Infantis (Chironna et al., 2014). In Africa, as elsewhere in the world, *S*. Heidelberg is one of the important serovars in terms of public health and is frequently involved in human and animal salmonellosis, and often exhibits MDR patterns (Evans et al., 2014; Hoffman et al., 2014). However, in the current study, of four Heidelberg isolates, one isolate was phenotypically pan-susceptible and the other three isolates were resistant to at least two antimicrobials, namely, R-type AxTe, AmAxCp and AmAxCpSu. Interestingly, *S*. Typhimurium and *S*. Enteritidis reported before in livestock and human were not recovered in this study (Allard

et al., 2013; Zheng et al., 2014). The highly dominant serovars recovered from this study included *Salmonella* I 8,20:i:- (from all the studied animal species) followed by *S*. Hadar (from swine, bovine and the slaughterhouse floor). The study conducted by Jackson et al. (2013) has reported that *S*. Enteritidis, *S*. Heidelberg and *S*. Hadar formed 80% of all outbreaks attributed to eggs and poultry. In this study, *S*. Cerro was also recovered from the dairy cattle but this study could not be certain if this serovar is adapted and persisted in the dairy cattle as reported in the United States (Kessel et al., 2007; Haley et al., 2015). The other common serovars in the current study include but not limited to *S*. Kentucky, *S*. Colindale, *S*. Uganda, *S*. Karamoja and *S*. Weltevreden as shown in Table 4.3.

Antimicrobial resistance poses a serious threat to public health and it continues to increase and is becoming one of the most devastating events ever recorded in human history (MacGowan and Macnaughton 2013). Occurrence of antimicrobial resistance in the human-animal-ecosystem interface is an evolutionary response that is highly linked to the strong selective pressure. This occurs as a result of exposure to antimicrobial agents such antibiotic and non-antibiotic agents (Kolar et al., 2001). Antimicrobial resistance in animals and environmental isolates is of public health concern because of the risk of transfer of antimicrobial resistance isolates or the resistance determinants to consumers through the food chain (Baquero et al., 2008).

Despite the dwindling rate in their effectiveness against the infectious agents, use of antimicrobials has saved countless lives (Davies and Davies, 2010). Over the years, resistance to all classes of antimicrobials has emerged, and this has led to emergence of antimicrobial resistant microbes, which are becoming a serious menace to the contemporary world (Zhang et al, 2006). The magnitude of antimicrobial resistance in

Tanzania is not well researched, although some studies have been conducted in humans than in animal subjects (Mshana et al., 2013). While this study is not intended to investigate the risk factors for occurrence and persistence of antimicrobial resistance in Tanzania (Komba et al., 2014), it is worth to enlightening on the problem of the over-the-counter sale of medicines as a source of misuse of antimicrobials. This is also considered to be among the reasons for the fading off of antimicrobials activities in treating common disease conditions (Rodriguez-Rojas et al., 2013). Previous studies have reported the clear infringement of the law with regard to stocking, distribution and dispensing of the antimicrobial agents in the country. In Tanzania, there is a fast growing unlawful practices where violation of health-related regulations is common. For example, the most important antimicrobials which are the prescription-only medicines, are stocked by drugstores which lack valid permits or are managed by staff who do not meet the qualification requirements (Goodman et al., 2007; Viberg et al., 2010).

Detection of *sul2* and *aadA1*, *blaTEM-1B*, *blaTEM-1A* from *S.* I 8,20:i:- and *S.* Manchester with susceptible phenotypes respectively, is one of the major findings in the current study (Fig. 4.1). Identification of these genes is important to understand resistance epidemiology, for verification of the carriage of antimicrobial resistance genes by *Salmonella* isolates with susceptible phenotypes, and for identification of resistant strains of *Salmonella*, when resistance genes are not phenotypically expressed (Zankari et al., 2012). Detection of other resistance genes includes *dfrA14* from *S.* I 8,20:i:-, *sul2*, *dfrA1* from *S.* I 8,20:i:-, *strB*, *sul2*, *dfrA1* from *S.* I 8,20:i:-, *strB*, *sul2*, *tet*(*A*), *dfrA14* from *S.* I 8,20:i:- and *aac(3)-Id-aadA7* from *S.* Kentucky (Fig. 4.1). As reported in this study, the *aac(3)-Id–aadA7* is an integron-borne gene, containing only a single cassette array of 1500 bp, which is shown to be transmitted by the class 1 integron-mediated MDR *S.* Kentucky.

Detection of class 1 integrons in Tanzania was also reported from *E. coli* and *S. enterica* subsp. *arizonae* from a new flock of lesser flamingoes imported from Tanzania to Hiroshima Zoological Park, Japan (Table 4.3 and 5.4). In contrast to dihydrofolate reductase (*dfrA7*) reported from the flock of lesser flamingoes, in this study, the DNA-sequencing results of the inserted gene cassette in class 1 integrons identified gene cassette harbouring aminoglycoside acetyltransferase [*aac(3)-Id*] and aminoglycoside adenyltransferase (*aadA7*) genes (Sato et al, 2009). Detection of integron-borne *aac(3)-Id-aadA7* gene is not only reported in *S.* Kentucky (Doublet et al., 2008) but also elsewhere in *S.* Newport (Doublet et al., 2004) and *Vibrio fluvialis* (Ahmed et al., 2004).

The frequency of the antimicrobial resistance in this study were shown to vary with *Salmonella* serovars. Although *S.* I 8,20:i:- was shown to be a highly occurring serovar, it was also found to be the most resistant serovar in the current study, with 57% (12 of 21) of its strains resistant to at least one antimicrobial. Also, *S.* I 8,20:i:- (33%), is reported in the current study to be highly MDR with R-types including TeSuTMP and ClAxCpTeSuTMP. The strains of *S.* I 8,20:i:- were shown to persist in different sample types from which *Salmonella* spp. were isolated (Table 4.3). None of the previous studies conducted in Tanzania and elsewhere in East Africa to date, have reported the serovar *S.* I 8,20:i:- alone or in large numbers as it was depicted in the current study. While we can not confirm if *S.* I 8,20:i:- is adapted to all food animals, we can provide enough evidence that serovar I 8,20:i:- was highly persisted in food animals for the period when this study was on implementation. With the thorough literature review done so far, none has reported recovery of the *S.* I 8,20:i:- from mastitic milk or from any other source. Although it is not uncommon to uncover the co-existence of *Salmonella* with other mastitis causing organisms, this study reported, for the first time the isolation of the *S.* I 8,20:i:- from a

mastitic dairy cow and from other source in Tanzania. In spite of the fact that the *S*. I 8,20:i:- can exist or co-exist in the mastitic milk, the current study could not conclusively confirm if the isolation of the serovar I 8,20:i:- from the mastitic milk is a determinant for the condition (Junaidu et al., 2011).

Pulsed field-gel electrophoresis (PFGE) as an epidemiologic tool provides very insightful phylogenetic relationship inference for foodborne pathogens such as *Salmonella* at serotype and strain level (Hauser et al., 2011). Comparison of the discriminatory power of serotyping and PFGE fingerprinting, using Simpson's diversity index was found to be 0.88 and 0.98, respectively. The serotyping method is inferior to differentiate between related and un-related strains (D = 0.88) compared to PFGE fingerprinting (D = 0.98). The discriminatory power of PFGE fingerprinting reported in this study is consistent with a study conducted in 128 *S*. Enteritidis by Campioni et al. (2012), which found a D of 0.98, indicating that PFGE fingerprinting method is an appropriate technique for *Salmonella* typing (Kuo et al., 2014).

The genotyping of isolates in the current study showed that the majority of the *Salmonella* isolates clustered by serotypes, and were clonally identical, with the exception of *S*. Hadar and *S*. Karamoja (J-cluster), *S*. Uganda and *S*. Amager (L-cluster), *S*. Isangi, and *S*.I. 6,7:-:- (Q-cluster). In addition, two clusters, L and Q-cluster composed of different serotypes, each further aggregated to form different PFGE fingerprint patterns, L_1-pattern (*S*. Uganda) and L_2-pattern (*S*. Amager, and Q_1-pattern (*S*.I. 6,7:-:-) and Q_2-pattern (*S*. Isangi) (Hauser et al., 2011). Although cluster and fingerprint patterns analysis in this study revealed clonal relationship among *Salmonella* isolates of the same serotypes, two isolates of different serotypes, *S*. Hadar and *S*. Karamoja, clustered at 100% genotypic threshold

clustering breakpoint, indicating a significant limitation of the XbaI restriction enzyme in discrimination of the isolates among and between serotypes. These two serotypes of *Salmonella* isolates in J_1-pattern (J-cluster), which are clonally indistinct using XbaI restriction enzyme could further cluster distinctly if an additional restriction enzyme such as BlnI is used (Kagambega et al. 2013). Elsewhere in West Africa, a recent study reported that *Salmonella* isolates of serovar Typhimurium var. Copenhagen, recovered from the cattle feces, clustered together with the S. Typhimurium isolates when XbaI was used, while all isolates clustered distinctly when BlnI was employed (Kagambega et al. 2013). A phylogeny based on WGS also is available (Timme et al., 2013).

Clonally identical *S.* I 8,20:i:- (n = 19) isolates aggregating to form fingerprint patterns M_3, M_5, and W_1, were found from more than one production levels (ADF3, DP1, DP2, IF, and KP) from Arusha and Morogoro. This originated from different host species: swine, bovine and poultry, suggesting horizontal transmission of *Salmonella* among the farm animals. The other reasons for detection of the clonally identical isolates from the livestock is attributed to the animal trade through animal auctions conducted to improve animal stock, as wel as the wandering behavior of the pastoral communities in the two regions, but also by pathogens cross-contamination among the mixed farm animals, poor sanitation, contaminated feed and shared animal attendants as observed from farm DP1 and DP2. Some clonally identical isolates detected from the carcass swabs in the abattoir (SH4 and SH5) aggregated with isolates from other production levels (livestock farms) to form M_3 and M_5-patterns, suggesting the need for proper hygiene and disinfection to contain spread of infectious pathogens (Aiello and Larson, 2002; Cozad and Jones, 2003). In spite of the fact that the current study could not establish the course of transmission of *Salmonella* isolates, the presence of clonally identical isolates from swine, bovine and

poultry (cluster A, C, E, J, L, M, V, and W), is an indication for the possibility of transmission of important foodborne pathogens such as *Salmonella* along the food chain and which could pose serious risk to public health (Hauser et al., 2011).

In conclusion, this study has demonstrated that PFGE fingerprinting provides more discriminatory power for *Salmonella* identification, and also, it can provide valuable information for disease surveillance and outbreak investigation. This study also highlighted the emergence and persistence of public health important MDR *Salmonella* isolates detected from different production levels. Despite a large variety of *Salmonella* serovars recovered from food animals (swine, bovine and poultry), this study clearly demonstrated the presence of epidemiologically important *Salmonella* serovars, including Heidelberg, Hadar, Infantis, Anatum, Virchow and Kentucky, which are frequently implicated in foodborne disease outbreak scenarios worldwide. The PFGE fingerprint patterns strongly indicated that the majority of *Salmonella* isolates were clonal. The occurrence of clonal MDR *Salmonella* isolates in food animals and animal products from pastoral communities indicates the significance of informal traditional sector as an important source of foodborne pathogens in the food chain and the entry of pathogens to the pastoralist communities. This is of great public health concern since unpasteurized milk and uncooked meat consumption is common in these communities. Therefore, we strongly recommend to institute control measures to improve biosecurity and hygienic practices along the entire production levels.

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Table 4.1: Types of samples and prevalence of *Salmonella* grouped by source and host species.

Source	Number of samples (%)	*Salmonella* prevalence (%)
Feces	**933 (60.6)**	**46 (4.9)**
Cattle	351 (22.8)	17 (4.8)
Swine	473 (30.7)	26 (5.5)
Poultry	6 (0.4)	3 (50)
Small ruminants	103 (6.7)	0 (0)
Swabs	**252 (16.4)**	**7 (2.8)**
Cattle carcass	181 (11.8)	6 (3.3)
Swine carcass	23 (1.5)	0 (0)
Cloacal swabs	48 (3.1)	1 (2.1)
Milk	**286 (18.6)**	**7 (2.4)**
Dairy cattle	238 (15.5)	7 (2.9)
Zebu cattle	48 (3.1)	0 (0)
Chicken eggs	**50 (3.2)**	**0 (0)**
Environment	**19 (1.2)**	**4 (21.1)**
Floor swabs	12 (0.8)	3 (25)
Sewage	7 (0.5)	1 (14.3)
Total	**1540 (100)**	**64 (4.2)**

Table 4.2: Basic biochemical tests, invA PCR, and antimicrobial susceptibility testing (AST) results for *Salmonella* isolates.

Basic biochemical tests conducted and results				*InvA* PCR	Antimicrobials tested	*Salmonella* isolates		
Urease test	TSI	LIA	Presence of H_2S gas			Resistant	Intermediate	Pansusceptible
						[n (%)]	[n (%)]	[n (%)]
-	+	+	+	+	Ampicillin (Am)	9 (14.1)	2 (3.1)	53 (82.8)
-	+	+	+	+	Chloramphenicol (Cl)	1 (1.6)	-	63 (98.4)
-	+	+	+	+	Streptomycin (S)	4 (6.2)	6 (9.4)	54 (84.4)
-	+	+	+	+	Trimethoprim (TMP)	11 (17.2)	-	53 (82.8)
-	+	+	+	+	Sulfisoxazole (Su)	14 (21.9)	-	50 (78.1)
-	+	+	+	+	Tetracycline (Te)	16 (25)	-	48 (75)
-	+	+	+	+	Amoxicillin-clavulanic acid (Ax)	10 (15.6)	3 (4.7)	51 (79.7)
-	+	+	+	+	Cephalothin (Cp)	9 (14.1)	7 (10.9)	48 (75)
-	+	+	+	+	Ceftriaxone (Ce)	-	-	64 (100)
-	+	+	+	+	Ciprofloxacin (CIP)	2 (3.1)	-	62 (96.9)
-	+	+	+	+	Kanamycin(Km)	-	1 (1.6)	63 (98.4)
-	+	+	+	+	Amikacin (An)	-	-	64 (100)
-	+	+	+	+	Gentamycin (Gm)	-	-	64 (100)
-	+	+	+	+	Ceftiofur (XLN)	-	1 (1.6)	63 (98.4)

Abbreviations:

TSI, Triple sugar iron agar test; LIA, Lysine iron agar test; An, Amikacin; Ax, Amoxacillin-clavulanic acid; Am, Ampicillin; Cp, Cephalothin; XLN, Ceftiofur; Ce, Ceftriaxone; Cl, Chloramphenicol; CIP, Ciprofloxacin; Gm, Gentamycin; Km, Kanamycin; S, Streptomycin; Su, Sulfisoxasole; Te, Tetracycline; TMP, Trimethoprim.

Table 4.3: Phenotypic and genotypic characterization of *Salmonella* isolates (*n* = 64) from different geographical locations, animal species and sample types from March 2013 to March 2014.

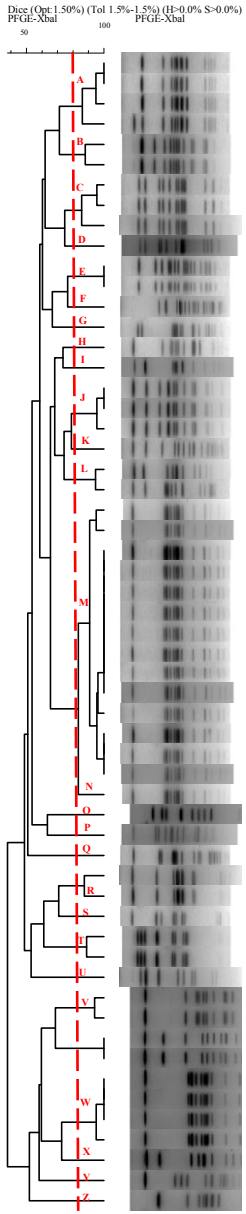
Resistance types	Serovars	Phagetype	Source (No. of isolates)	Type of sample (No. of isolates)	Year of isolation
Pansusceptible	S. Amager		Swine (1)	Feces (1)	2013
	S. Anatum		Bovine (3)	Feces (3)	2013
	S. Colindale		Bovine (2)	Feces (2)	2013
			Swine (1)	Feces (1)	2013
	S. Hadar		Bovine (2)	Feces (2)	2013
			Swine (2)	Feces (2)	2013
			Abattoir (1)	Floor swab (1)	2013
	S. Heidelberg	19	Swine (1)	Feces (1)	2013
	S. Ilala		Chicken (1)	Feces (1)	2013
	S. Infantis		Swine (1)	Feces (1)	2014
	S. Isangi		Abattoir (1)	Floor swab (1)	2013
	S. Karamoja		Bovine (1)	Carcass swab (1)	2013
			Abattoir (1)	Floor swab (1)	2013
	S. Leoben		Abattoir (1)	Sewage (1)	2013
	S. Manchester		Bovine (2)	Feces (2)	2013
	S. Roan		Bovine (1)	Feces (1)	2013
	S. Uganda		Swine (2)	Feces (2)	2013
	S. Virchow		Swine (2)	Feces (2)	2013
	S. Weltevreden		Swine (1)	Feces (1)	2013
	S. serovar 6,7:-:1,7		Bovine (1)	Feces (1)	2013
	S. serovar I 8,20:i:-		Swine (3)	Feces (3)	2013
			Bovine (6)	Milk (6)	2013
	S. serovar II 9,12:1,w:e,n,x		Chicken (1)	Feces (1)	2013
	S. serovar II 4,12,[27]:e,n,x:1,[5],7		Bovine (2)	Feces (2)	2013
Ax	S. Hadar		Swine (1)	Feces (1)	2013

Cp	S. serovar I 8,20:i:-		Bovine (1)	Milk (1)	2013
Su	S. Cerro		Bovine (1)	Feces (1)	2013
AxTe	S. Heidelberg	19	Swine (1)	Feces (1)	2013
STe	S. Anatum		Swine (1)	Feces (1)	2013
	S. Ahuza		Bovine (1)	Carcass swab (1)	2013
	S. Colindale		Bovine (1)	Feces (1)	2013
AmAxCp	S. Heidelberg	Atypical	Bovine (1)	Carcass swab (1)	2013
	S. serovar I 6,7:-:-		Bovine (1)	Carcass swab (1)	2013
	S. serovar I 8,20:i:-		Bovine (1)	Carcass swab (1)	2013
AmAxTe	S. serovar I 8,20:i:-		Bovine (1)	Carcass swab (1)	2013
AmCpTe	S. serovar I:8,20:i:-		Swine (1)	Feces (1)	2014
SuTeTMP	S. serovar I:8,20:i:-		Chicken (1)	Feces (1)	2013
			Swine (6)	Feces (6)	2013
AmAxCpSu	S. Heidelberg	19	Swine (1)	Feces (1)	2013
AmSSuTe	S. Hadar		Swine (1)	Feces (1)	2013
CipSuTeTMP	S. Kentucky*		Bovine (2)	Feces (2)	2013
ClAxCpSuTeTMP	S. serovar I 8,20:i:-		Chicken (1)	Cloacal swab (1)	2014

Abbreviations: **S.* Kentucky isolates carried *aac(3)-Id-aadA7* gene cassettes of size 1500 bp.; Ax, amoxacillin-clavulanic acid; Am, ampicillin; Cl, chloramphenicol; CIP, Ciprofloxacin; An, amikacin; Gm, gentamycin; Km, kanamycin; S, streptomycin; Su, sulfisoxasole; TMP, trimethoprim; Te, tetracycline; XLN, ceftiofur; Ce, ceftriaxone; Cp, cephalothin.

Table 4.4: Sequencing of resistant cassettes and *16S rDNA* and the resistance patterns of *Salmonella* and *Citrobacter* isolates

Sample ID	Animal host	Type of sample	Sampling date	Bacterial spp	R-Type	Accession number	Gene sequenced
S.16746	Swine	Feces	1.18.2014	*Citrobacter*	Pansusceptible	KM986871	*16S rDNA*
S.16704	Swine	Feces	1.25.2014	*Citrobacter*	Pansusceptible	KM986865	*16S rDNA*
S.16741	Swine	Feces	1.25.2014	*Citrobacter*	Pansusceptible	KM986851	*16S rDNA*
S.16744	Swine	Feces	1.25.2014	*Citrobacter*	S	KM986852	*16S rDNA*
S.16699	Swine	Feces	1.26.2014	*Citrobacter*	AmCpSSuTeTMP	KM823525	dfrA7
S.16700	Swine	Feces	1.4.2014	*Citrobacter*	AmCpTe	KM986863	*16S rDNA*
S.16708	Swine	Feces	1.4.2014	*Citrobacter*	Pansusceptible	KM986872	*16S rDNA*
S.16756	Bovine	Carcass swab	10.1.2013	*Citrobacter*	Pansusceptible	KM986870	*16S rDNA*
S.16806	Bovine	Carcass swab	10.16.2013	*Citrobacter*	TMP	KM986855	*16S rDNA*
S.16694	Bovine	Carcass swab	10.16.2013	*Citrobacter*	AmAxCp	KM986848	*16S rDNA*
S.16702	Bovine	Carcass swab	10.16.2013	*Citrobacter*	AxCp	KM986849	*16S rDNA*
S.16693	Bovine	Carcass swab	10.18.2013	*Citrobacter*	Pansusceptible	KM986854	*16S rDNA*
S.16715	Bovine	Carcass swab	10.18.2013	*Citrobacter*	Pansusceptible	KM986856	*16S rDNA*
S.16743	Bovine	Carcass swab	10.18.2013	*Citrobacter*	Pansusceptible	KM986857	*16S rDNA*
S.16790	Bovine	Carcass swab	10.18.2013	*Citrobacter*	AmAxCp	KM986859	*16S rDNA*
S.16791	Bovine	Carcass swab	10.18.2013	*Citrobacter*	Ax	KM986858	*16S rDNA*
S.16748	Bovine	Feces	10.18.2013	*Citrobacter*	AxCpSuTMP	KM823524	dfrA15
S.16696	Swine	Feces	12.28.2013	*Citrobacter*	Cp	KM986861	*16S rDNA*
S.16739	Swine	Feces	12.28.2013	*Citrobacter*	TeSSu	KM986850	*16S rDNA*
S.16679	Swine	Feces	4.10.2013	*Citrobacter*	AmAxCpS	KM986847	*16S rDNA*
S.16712	Bovine	Feces	4.3.2013	*Citrobacter*	AmAxCp	KM986853	*16S rDNA*
S.16711 [2]	Swine	Feces	5.14.2013	*Salmonella* I 8,20:i:-	TeSuTMP	KM986846	*16S rDNA*
S.16697	Swine	Feces	5.8.2013	*Citrobacter*	Pansusceptible	KM986862	*16S rDNA*
S.16788	Poultry	Chicken egg	6.27.2013	*Citrobacter*	SSu	KM986866	*16S rDNA*
S.16701	Swine	Feces	6.6.2013	*Salmonella* Anatum	STe	KM986864	*16S rDNA*
S.16774	Swine	Feces	8.27.2013	*Citrobacter*	AxCpTe	KM986868	*16S rDNA*
S.16819	Swine	Feces	8.27.2013	*Citrobacter*	AmAxCpSuTeTMP	KM823521	dfrA1
S.16720	Swine	Feces	8.3.2013	*Citrobacter*	Pansusceptible	KM986860	*16S rDNA*
S.16716 [1]	Bovine	Feces	8.4.2013	*Salmonella* I 6,7:-:1,7	Pansusceptible	KM986845	*16S rDNA*
S.16485	Bovine	Feces	8.4.2013	*Salmonella* Kentucky	CipSuTeTMP	KM823522	aac(3)-Id-aadA7
S.16486	Bovine	Feces	8.4.2013	*Salmonella* Kentucky	CipSuTeTMP	KM823523	aac(3)-Id-aadA7
S.16775	Bovine	Milk	9.23.2013	*Citrobacter*	Cp	KM986869	*16S rDNA*
S.16778	Bovine	Milk	9.4.2013	*Citrobacter*	Pansusceptible	KM986867	*16S rDNA*



ID	Serotype	Farm	Source	Sample	Location	Date	R-type	MLST type	Plasmid type	R-genes	PFGE patterns	No. of isolates
S.16681	Hadar	SF	Swine	Feces	Morogoro	040313	Pansusceptible	Unknown ST	NKP	No R-genes	A_1	2
S.16768	Hadar	SF	Bovine	Feces	Morogoro	040313	Pansusceptible	Unknown ST	NKP	No R-genes	A_2	1
S.16731	Hadar	SF	Swine	Feces	Morogoro	040313	Pansusceptible	Unknown ST	NKP	No R-genes	A_3	1
S.16763	Hadar	SF	Bovine	Feces	Morogoro	040313	Pansusceptible	Unknown ST	NKP	No R-genes	B_1	1
S.16765	Manchester	SH2	Bovine	Feces	Morogoro	062013	Pansusceptible	Unknown ST	IncFII	No R-genes	B_2	1
S.16783	Manchester	SH2	Bovine	Feces	Morogoro	062013	Pansusceptible	Unknown ST	IncI1	aadA1, blaTEM-1B,blaTEM-1A	C_1	1
S.16742	Heidelberg	MI	Swine	Feces	Morogoro	042713	AmAxCpSu	Unknown ST	NKP	No R-genes	C_2	1
S.16764	Heidelberg	MI	Swine	Feces	Morogoro	042713	Pansusceptible	Unknown ST	ColpVC	No R-genes	C_3	1
S.16808	Heidelberg	SH4	Bovine	Carcass swab	Morogoro	101613	AmAxCp	Not done	ND	Not done	D	1
S.14045	Typhimuri.		Swine	Feces	NC, USA	010509	AmAxClSSuTe	Not done	ND	Not done	E	2
S.16753	Virchow	SF	Swine	Feces	Morogoro	040313	Pansusceptible	Unknown ST	NKP	No R-genes	F	1
S.16767	Virchow	SF	Swine	Feces	Morogoro	040313	Pansusceptible	Unknown ST	NKP	No R-genes	G	1
S.16812	Hadar	MI	Swine	Feces	Morogoro	042713	AmSSuTe	Not done	ND	Not done	H	1
S.16773	Leoben	SH3	Abattoir	Septage	Morogoro	090913	Pansusceptible	Not done	ND	Not done	I	1
S.16770	Colindale	SF	Swine	Feces	Morogoro	040313	Pansusceptible	Not done	ND	Not done	J_1	2
S.10074	Enteritidis	EW	Swine	Floor swab	NC, USA	111109	AmClSSuTe	Not done	ND	Not done	J_2	1
S.16772	Hadar	SH1	Abattoir	Floor swab	Morogoro	061213	Pansusceptible	Unknown ST	No plasmid	No R-genes	K	1
S.16786	Karamoja	SH2	Bovine	Carcass swab	Morogoro	062013	Pansusceptible	Unknown ST	NKP	No R-genes	L_1	1
S.16771	Karamoja	SH1	Abattoir	Floor swab	Morogoro	061213	Pansusceptible	Not done	ND	Not done	L_2	1
S.16726	Anatum	MF	Bovine	Feces	Morogoro	052113	Pansusceptible	Unknown ST	NKP	No R-genes	M_1	1
S.16688	Uganda	DP1	Swine	Feces	Morogoro	051413	Pansusceptible	Not done	ND	Not done	M_2	1
S.16755	Amager	SF	Swine	Feces	Morogoro	040313	Pansusceptible	Not done	ND	Not done	M_3	8
S.16706	I 8,20:i:-	DP1	Swine	Feces	Morogoro	051413	SuTeTMP	Unknown ST	No plasmid	strB,sul2,dfrA	M_4	1
S.16826	I 8,20:i:-	SH4	Bovine	Carcass swab	Morogoro	101613	AmAxCp	Not done	ND	Not done	M_5	3
S.16687	I 8,20:i:-	DP1	Swine	Feces	Morogoro	051413	SuTeTMP	Failed run	No plasmid	sul2,dfrA	M_6	1
S.16680	I 8,20:i:-	DP2	Swine	Feces	Morogoro	060613	SuTeTMP	Unknown ST	ColRNAI	strB, sul2, tet(A), dfrA14	N	1
S.16682	I 8,20:i:-	DP2	Swine	Feces	Morogoro	060613	Pansusceptible	Unknown ST	No plasmid	No R-genes	O	1
S.16691	I 8,20:i:-	DP2	Swine	Feces	Morogoro	060613	SuTeTMP	Unknown ST	ColRNAI	No R-genes	P	1
S.16705	I 8,20:i:-	DP2	Bovine	Milk	Morogoro	060613	Pansusceptible	Not done	ND	Not done	Q_1	1
S.16735	I 8,20:i:-	DP2	Swine	Feces	Morogoro	060613	Pansusceptible	Unknown ST	NKP	No R-genes	Q_2	1
S.16811	I 8,20:i:-	IF	Poultry	Cloacal swab	Morogoro	012614	ClAxCpSuTeTMP	Not done	ND	Not done	R	1
S.16823	I 8,20:i:-	SH5	Bovine	Carcass swab	Morogoro	101813	AmAxTe	Not done	ND	Not done	S_1	1
S.16814	I 8,20:i:-	KP	Swine	Feces	Morogoro	012514	AmCpTe	Not done	ND	Not done	S_2	1
S.16683	I 8,20:i:-	DP1	Swine	Feces	Morogoro	051413	Pansusceptible	Unknown ST	No plasmid	sul2	T	1
S.16750	I 8,20:i:-	DP2	Poultry	Feces	Morogoro	060613	SuTeTMP	Unknown ST	NKP	Not done	U_1	1
S.16703	I 8,20:i:-	ADF3	Bovine	Milk	Arusha	101013	Cp	Unknown ST	NKP	No R-genes	U_2	1
S.16690	I 8,20:i:-	DP1	Swine	Feces	Morogoro	051413	SuTeTMP	Unknown ST	ColRNAI.	dfrA14	V_1	2
S.16722	Roan	MF	Bovine	Feces	Morogoro	052113	Pansusceptible	Unknown ST	NKP	No R-genes	W_1	3
S.16575	- (A-I), -G, .		Swine	Feces	Kenya	112013	Pansusceptible	Not done	ND	Not done	W_2	1
S.16692	Ilala	DP2	Poultry	Feces	Morogoro	060613	Pansusceptible	Not done	ND	Not done	X	1
S.16785	I 6,7:-:-	SH4	Bovine	Carcass swab	Morogoro	101613	AmAxCp	Unknown ST	NKP	No R-genes	Y	1
S.16760	Isangi	SH2	Abattoir	Floor swab	Morogoro	062013	Pansusceptible	Failed run	No plasmid	No R-genes	Z	1
S.16766	DNCS	DP2	Poultry	Feces	Morogoro	060613	Pansusceptible	Unknown ST	NKP	No R-genes		
S.16759	DNCS	SH2	Bovine	Feces	Morogoro	062013	Pansusceptible	Not done	ND	Not done		
S.16815	DNCS	SH2	Bovine	Feces	Morogoro	062013	Pansusceptible	Failed run	NKP	No R-genes		
S.16825	Ahuza	SH5	Bovine	Carcass swab	Morogoro	101813	AmAxCp	Unknown ST	NKP	No R-genes		
S.16709	Colindale	BS1	Bovine	Feces	Morogoro	041013	Pansusceptible	Unknown ST	NKP	No R-genes		
S.16769	Colindale	BS1	Bovine	Feces	Morogoro	041013	Pansusceptible	Unknown ST	NKP	No R-genes		
S.16685	Anatum	MF	Bovine	Feces	Morogoro	052113	Pansusceptible	Not done	ND	Not done		
S.16713	Anatum	MF	Bovine	Feces	Morogoro	052113	Pansusceptible	Unknown ST	NKP	No R-genes		
S.16717	I 8,20:i:-	ADF3	Bovine	Milk	Arusha	101013	Pansusceptible	Unknown ST	NKP	No R-genes		
S.16718	I 8,20:i:-	ADF3	Bovine	Milk	Arusha	101013	Pansusceptible	Unknown ST	NKP	No R-genes		
S.16727	I 8,20:i:-	ADF3	Bovine	Milk	Arusha	101013	Pansusceptible	Unknown ST	NKP	No R-genes		
S.16695	I 8,20:i:-	ADF3	Bovine	Milk	Arusha	101013	Pansusceptible	Unknown ST	NKP	No R-genes		
S.16719	Uganda	DP1	Swine	Feces	Morogoro	051413	Pansusceptible	Unknown ST	NKP	No R-genes		
S.16781	Weltevreden	DP1	Swine	Feces	Morogoro	051413	Pansusceptible	Unknown ST	IncFII	No R-genes		
S.16810	I 8,20:i:-	ADF4	Bovine	Milk	Arusha	101513	Pansusceptible	Not done	ND	Not done		

Abbreviations: ND: not done; DNCS: do not conform serotypes; NKP: no known plasmid

Figure 4. 1: Dendrogram representing genetic relatedness among *Salmonella* isolates based on PFGE fingerprints. Percentage (%) similarity was determined by Dice coefficient and UPGMA clustering

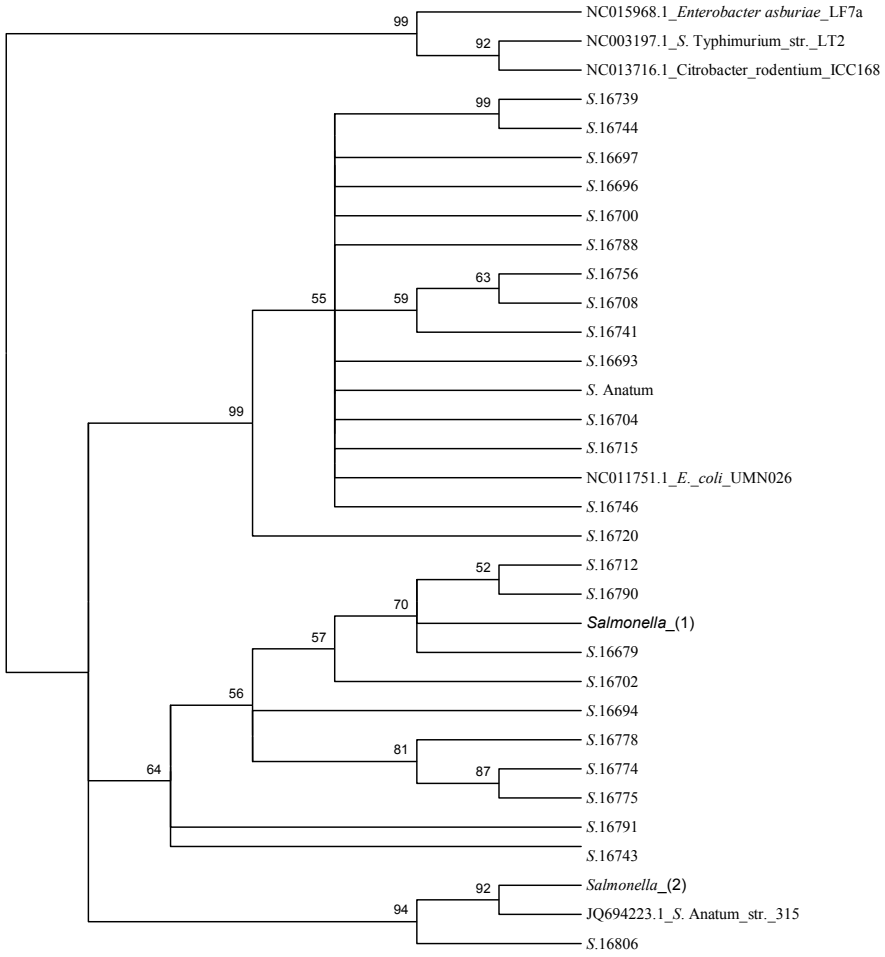


Figure 4.2: Neighbor-Joining tree based on analysis of partial *16S rDNA* sequences of bacterial isolates from different animal hosts. *S.* Typhimurium strain LT2 [NC003197.1], *Enterobacter asburiae* strain LF7a [NC015968.1], *Citrobacter rodentium* strain ICC168 [NC013716.1], *S.* Anatum strain 315 [JQ694223.1] and *Escherichia coli* strain UMN026 [NC011751.1] were chosen to provide the outgroup sequences.

CHAPTER FIVE

5.0 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

While other previous studies have also found high prevalence of *Salmonella* isolates from the floors of the animal houses and the abattoirs (Hald *et al*., 2003; Molla *et al*., 2010), the current study has reported a high prevalence of *Salmonella* isolates ranging from 13.9 to 25% in the swine barn floors and abattoirs. Additionally, in this study, the prevalence of *Salmonella* isolates from the meat carcasses from cattle that are the principal meat and milk producer in Tanzania was found to be 3.3% higher than the 2.3% of the cattle faeces. Occurrence of high prevalence of *Salmonella* isolates in the meat carcasses indicates failure to follow proper preventive measures in producing safe meat for public consumption (Arguello *et al*., 2012; Carrasco *et al*., 2012). Some of the possible sources of contamination of cattle carcasses are through personnel, poor handling of faecal matter during evisceration and equipment used during the slaughter process (Hald *et al*., 2003; Arguello *et al*., 2012).

In this study, the pulsed field-gel electrophoresis (PFGE) DNA fingerprints have shown aggregates of clonally identical multi-drug resistant (MDR) *Salmonella* isolates from more than one farm (DP1, DP2, IF), originating from different animal hosts (swine, bovine and poultry). Occurrence of clonally identical isolates indicates the possibility of cross-transmission of *Salmonella* isolates between the farm animals due to intermixing of the animals as it was evident in farm DP1 and DP2. One of the clonally identical isolates (S.16823) detected from the carcass swabs from the abattoir (Fig. 4.2) aggregated with

other MDR *Salmonella* isolates from the farms DP1, DP2 and IF. Other reason for detection of the clonally identical isolates from the farm animals is attributed to poor sanitation, contaminated grazing lands and shared animal attendants as observed in farm DP1 and DP2. Isolation of clonally identical foodborne pathogens such as MDR *Salmonella* along the food production could bring a serious public health risk if they infect humans (Hauser *et al*., 2011; Hauser *et al*., 2012).

Besides the high prevalence of *Salmonella* isolates, occurrence of the antimicrobial resistance in the foodborne pathogens is becoming a serious public health threat. These antimicrobial resistant *Salmonella* isolates have public health implications if they find their way into humans and food animals. Thus, humans can be infected and colonized with the antimicrobial resistant pathogens, and this may limit therapeutic options in case of a disease occurrence (Nweneka *et al*., 2009; Rodriguez-Rojas *et al*., 2013). In this study, antimicrobial resistance was very common among the United States isolates, with 90% of *Salmonella* isolates that originated from feed samples, 92.3% of isolates from drag swabs and 98.02% of isolates from swine faecal samples, showing resistance to one or more of the antimicrobials. In addition, thirty eight percent of the *Salmonella* isolates from food animals and animal products from Tanzania were showing resistance to one or more of the antimicrobials.

The rapid spread of antimicrobial resistance between the susceptible and resistant bacteria in the microbial population is mediated by mobile genetic elements such as plasmids, transposons, integrons and resistance cassettes (Dantas and Sommer, 2014). Besides the spread by mobile genetic elements, antimicrobial resistance in foodborne pathogens is also disseminated through horizontal transfer by transformation, transduction, or conjugation of

individual antimicrobial resistance genes (Carattoli, 2003). In this study, three mobile genetic elements such as plasmid replicons, which are responsible for replication of antimicrobial resistance genes, class 1 integrons and resistance gene cassettes, including *aac(3)-Id-aadA7*, *dfrA1-orfC*, *dfrA7* and *dfrA15*, were detected from MDR *Salmonella* isolates. Detection of class 1 integrons in Tanzania was also reported from *Salmonella enterica* subsp. *arizonae* from the flock of lesser flamingoes imported from Tanzania to Japan (Sato *et al*., 2009). Identification of the individual resistance genes and resistance gene cassettes are also important to understand the antimicrobial resistance epidemiology (Zankari *et al*., 2012). Thus, in the current study, a number of the individual resistance genes and resistance gene cassettes such as *strB*, *sul2*, *tet*(A), *dfrA1*, *dfrA14*; *aadA1*, *blaTEM-1B* and *blaTEM-1A*, were also detected from food animals and animal products in Tanzania.

Previous studies have reported persistence of MDR *Salmonella* isolates in production environment regardless of the use of antibiotics in the farms. Besides the direct selective pressure of antimicrobial resistance, co-selection due to other structurally related or unrelated chemical agents has also been a concern for the rising trend in multidrug resistance. However, there have been very limited studies conducted in this area. The current study attempts to fill the knowledge gap, mainly focusing on the use of heavy metal micronutrients and biocide. Previous studies have identified genetic elements among *Salmonella* strains that render some strains resistant to heavy metal micronutrients, including copper and zinc (Choudhury and Srivastava, 2001; Aarestrup and Hasman, 2004). Such resistant strains were shown to carry genes associated with multiple antimicrobial resistance factors (Ciraj *et al*., 1999; Hasman and Aarestrup, 2002). This study has reported a wide range of the levels of copper and zinc used in swine feed and

concentrations which are much higher than those recommended by the National Research Council (NRC).

It was previously reported that as the part of feed ingredients are absorbed within the gastrointestinal tract, the rest of elements such as heavy metal micronutrients tend to be more concentrated in the feces (Jacob *et al*., 2010). The recent NRC report indicated that even though high levels of dietary copper and zinc have been shown to improve animal performance, a large percentage of consumed minerals such as copper and zinc is excreted in the faeces and ends up as environmental contaminants (NRC, 2012). It is worth noting that in the current study, the concentrations of both micronutrients (zinc and copper) were also found to be higher in the faecal samples than in the feed as previously reported (Poulsen and Larsen, 1995; Jondreville *et al*., 2003).

A few studies have reported different tolerance levels of important foodborne pathogens such as *Salmonella* to different concentrations of Zn^{2+} and Cu^{2+} (Hasman and Aarestrup, 2002, Karbasizaed *et al*., 2003). This study has reported up to 8 mM as the highest level of tolerance of *Salmonella* isolates to Zn^{2+}, which is higher than the level reported elsewhere (Aarestrup and Hasman, 2004). Furthermore, the highest level of tolerance of *Salmonella* isolates to Cu^{2+} reported in this study was 24 mM, which is smaller than the maximum tolerance reported by the previous study conducted in food animals (Aarestrup and Hasman, 2004). The increased tolerance level of *Salmonella* isolates to Zn^{2+} and Cu^{2+} is attributable to the use of the respective heavy metal micronutrients in swine feed and also expression of the tolerance genes in foodborne pathogens such as *Salmonella* (Nies, 1999).

While there have been very limited studies conducted to date, investigating the association between heavy metal tolerance and specific antimicrobial resistance patterns, a study in 1984 in drinking water reported the overall association between copper and zinc tolerance and antimicrobial resistance (Calomiris *et al*., 1984). In this study, the polymerase chain reaction (PCR) results on the level of tolerance gene carriage by *Salmonella* isolates were consistent with the increase in the level of the heavy metal minimum inhibitory concentration (MIC) of *Salmonella* isolates. This observation is attributable to the fact that antibiotic resistant and heavy metal-tolerant genes can be functionally associated with some genetic elements, which can cause a positive co-selection (Baker-Austin *et al.*, 2006). It is also reported elsewhere that carriage of the heavy metal tolerance genes (*pcoA* or *czcD*) is not constantly associated with copper or zinc tolerance of *Salmonella* isolates because of the presence of other mechanisms that may result in copper or zinc tolerance besides *pcoA* and *czcD*, respectively, that also encode copper and zinc tolerance in *Salmonella* (Kim *et al*., 2002; Shepherd *et al*., 2013).

Control of *Salmonella* as a zoonotic foodborne pathogens in terms of disinfection of animal contact surfaces such as barn and abattoir floors, are very important to limit the risk of contaminated animal products from reaching the consumers and public (Fraise, 2002; Gantzhorn *et al*., 2014). Over the years, biocides were broadly used to prevent microbial growth and thus, playing an important role in preventing the spread of pathogenic bacteria (Gantzhorn *et al*., 2015). Biocidal substances such as glutaraldehydes and quaternary ammonium compounds (QAC) are commercially available for disinfection purposes to inhibit bacterial growth and colonization (Maillard, 2007). However, the use

of biocides is implicated to contribute to the emergence of biocide-tolerant foodborne bacterial pathogens (Russell, 2004; Carson *et al*., 2008).

Different types of biocides were long been used for disinfection purposes as a measure to control the risk of contaminated food products from reaching the consumers and public at large (Gantzhorn *et al*., 2014). Thus, disinfection is well regarded as a crucial step in achieving a defined, desired hygiene status in food production and processing areas. Public health important foodborne pathogens, such as *Salmonella* serovars, may remain in food animal houses, including swine barn floors, abattoir lairages, floors and associated environments even after cleaning and disinfection, and this may pose a risk of transfer of foodborne pathogens and contamination food animal products such as animal carcasses.

In a study conducted in France, approximately, 38% of the pre-disinfected poultry farms tested positive for *Salmonella* before placing day-old chickens (Rose *et al*., 2000). In the current study, the prevalence of *Salmonella* isolates detected from swine barn floors before disinfection was two times higher (13.9%) than after disinfection (6.7%). Previous study conducted in Denmark also reported a similar observation in which the prevalence of *Salmonella* isolates before cleaning was found to be 17.7% and was then reduced to 5.2% after disinfection of abattoir with the biocide (Gantzhorn *et al.*, 2014). Besides the reduction of *Salmonella* isolates by 51.8% in the swine barns, the use of biocides consequentially lead to a significant increase on the prevalence of MDR *Salmonella*, which was found to be 8.2%. While biocides play an important role in limiting the potential sources of infection, there is a rising concern about the increasing use of biocides over the selection pressure they could create to aggravate the rising trend of antimicrobial resistance in foodborne pathogens as well as the potential for cross-resistance to clinically

important antibiotics (Poole, 2002; Russell, 2003). In the current study, the proportion of *Salmonella* isolates that showed high tolerance to different concentrations of the biocides used was found to be 97% and 96.6% for Biosentry® and Synergize®, respectively. In addition, almost all the *Salmonella* isolates with multi-drug resistance phenotypes were biocide-tolerant *Salmonella* isolates.

While integrons are known mobile genetic elements associated with dissemination of antimicrobial resistance (Stokes and Hall, 1989), they also harbour tolerance genes, *qacE1* and *qacEΔ1* on their 3'-conserved segment (CS) end, which encode resistance to quaternary ammonium compounds (QAC) (Hsu *et al*., 2006; Chuanchuen *et al*., 2007). In this study, nearly 25% of *Salmonella* isolates carried the *qacEΔ1* and about 15% of the *qacEΔ1* genes were disseminated among the *Salmonella* isolates by Class 1 integrons. Therefore, it is concluded that all of the *intI1*-positive isolates carried *qacEΔ1* genes in their 3' -CS end, confirming that the *qacEΔ1* gene is linked to the integrons. Also, in the current study, the comparisons of abundances of Class 1 integrons and biocide resistance genes (*qacEΔ1*) in *Salmonella* isolates showed a significant correlation, suggesting that the multi-drug resistance efflux pumps, such as the *qacEΔ1* in foodborne pathogens are common transporters for extrusion of a wide range of structurally dissimilar toxic compounds from the cytoplasm of the bacterial cells (Wan and Chou, 2015).

5.2 Conclusion

Non-typhoidal *Salmonella* (NTS) are recognized as one of the major bacterial foodborne pathogens. They are of great importance in the sub-Saharan Africa, as they are a cause of concerns for some salmonelloses in humans, including, the high case fatality of extra-

intestinal non-typhoidal salmonellosis with meningitis in children and adults and cases of nosocomial outbreak of neonatal *Salmonella* Enteritidis meningitis in children. Of significant importance as far as public health is concerned, is the potential of resistant foodborne pathogens of food animal origin, when they infect and colonize humans. The occurrence of antimicrobial resistance is mainly due to the selective pressure of various factors, including the overuse and misuse of antimicrobials in humans, food animals, and agriculture. Therefore, this study, investigated the role of heavy metal micronutrient and biocide interventions in the emergence and persistence of heavy metal and biocide-tolerant *Salmonella* and also their co-selective association with multi-drug resistant (MDR) *Salmonella* isolates. The study further investigated the molecular epidemiology and antimicrobial resistance of *Salmonella* isolates from food animals and animal products.

Investigation of the role of various chemical interventions in the United States swine production systems, including heavy metal micronutrients (Zn and Cu) and biocides (Biosentry® and Synergize®) in the emergence of heavy-metal and biocide-tolerant *Salmonella* and their co-selective association with MDR *Salmonella* revealed that: (a) The level of carriage of tolerance genes, *pcoA* or *czcD*, by *Salmonella* isolates were consistent with the increase in the level of the Cu^{2+} or Zn^{2+} MIC of *Salmonella* isolates. This observation indicates that antibiotic resistance and heavy metal tolerance genes can be functionally associated with some genetic elements, which can cause a positive co-selection for antimicrobial resistance and heavy metal tolerance genes in the foodborne pathogens. (b) Presence of strong statistical associations between the level of Class 1 integrons (*intI1*) and disinfectant resistance genes (*qacEΔ1*) in *Salmonella* and the level of MDR *Salmonella*, from which they were detected, indicates the involvement of *qacEΔ1* as

one of the common transporters for extrusion of a wide range of structurally dissimilar toxic compounds from the cytoplasm of the bacterial cells, such as *Salmonella* strains.

Investigation to determine the molecular epidemiology and antimicrobial resistance of *Salmonella* isolates from food animals and animal products revealed that: (a) The pulsed field-gel electrophoresis (PFGE) DNA fingerprints of *Salmonella enterica* subspecies *enterica* serovar 8,20:i:- [S.I 8,20:i:-], which apparently, did not conform any known *Salmonella* serovars, have shown aggregates of clonally identical *Salmonella* isolates from more than one farm (DP1, DP2, IF), abattoir (SH5) and from different animal hosts (swine, bovine and poultry). This *Salmonella* isolate S.I 8,20:i:- was the highly occurring *Salmonella* isolates from food animals and animal products during the period of sampling. (b) The current study has also revealed involvement of plasmid replicons (*incFII*, *Colpvc* and *ColRNAI*), integrons (Class 1 integrons), resistance genes and cassettes (*strB*, *sul2*, *tet*(A), *blaTEM-1B*, *blaTEM-1A*, *aadA1*, *dfrA1*, *dfrA7*, *dfrA14*, *dfrA15*, *dfrA1-orfC* and *aac(3)-Id-aadA7*) in dissemination of antimicrobial resistance in *Salmonella* in food animals and food products in Tanzania. The occurrence of clonal transmission of *Salmonella* isolates and the presence of different antimicrobial resistance determinants in food animals and animal products, and the fact that *Salmonella* can invade different hosts without any greater resistance, indicates that *Salmonella* are of great potential as they can pose the public health threat.

5.3 Recommendations

Study recommends that:

a) The commercial swine owners in the United States should adhere to the levels of micronutrients depicted by the United States - National Research Council (NRC) and the laws should be enforced.

b) There should be a coordinated regular surveillances and monitoring on the use and sale of antimicrobials and the laws and bylaws should be enforced.

c) The governments in sub-Saharan Africa should devise a national surveillance programmes for antimicrobial resistance in humans and agriculture.

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APPENDICES

Appendix 1: Journal Article in Applied and Environmental Microbiology

In-Feed Use of Heavy Metal Micronutrients in U.S. Swine Production Systems and Its Role in Persistence of Multidrug-Resistant Salmonellae

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The study aimed to characterize the role of heavy metal micronutrients in swine feed in emergence of heavy-metal-tolerant and multidrug-resistant *Salmonella* organisms. We conducted a longitudinal study in 36 swine barns over a 2-year period. The feed and fecal levels of Cu^{2+} and Zn^{2+} were measured. *Salmonella* was isolated at early and late finishing. MICs of copper sulfate and zinc chloride were measured using agar dilution. Antimicrobial susceptibility was tested using the Kirby-Bauer method, and 283 isolates were serotyped. We amplified *pcoA* and *czcD* genes that encode Cu^{2+} and Zn^{2+} tolerance, respectively. Of the 283 isolates, 113 (48%) showed Cu^{2+} tolerance at 24 mM and 164 (58%) showed Zn^{2+} tolerance at 8 mM. In multivariate analysis, serotype and source of isolates were significantly associated with Cu^{2+} tolerance ($P < 0.001$). Fecal isolates were more likely to be Cu^{2+} tolerant than those of feed origin (odds ratio [OR], 27.0; 95% confidence interval [CI], 2.8 to 250; $P = 0.0042$) or environmental origin (OR, 5.8), implying the significance of gastrointestinal selective pressure. *Salmonella enterica* serotypes Typhimurium and Heidelberg, highly significant for public health, had higher odds of having >20 mM MICs of Cu^{2+} than did "other" serotypes. More than 60% of *Salmonella* isolates with resistance type (R-type) AmStTeKm (32 of 53) carried *pcoA*; only 5% with R-type AmClStSuTe carried this gene. *czcD* gene carriage was significantly associated with a higher Zn^{2+} MIC ($P < 0.05$). The odds of having a high Zn^{2+} MIC (≥8 mM) were 14.66 times higher in isolates with R-type AmClStSuTe than in those with R-type AmStTeKm ($P < 0.05$). The findings demonstrate strong association between heavy metal tolerance and antimicrobial resistance, particularly among *Salmonella* serotypes important in public health.

Nontyphoidal *Salmonella enterica* serotypes are among the most important food-borne bacterial pathogens, with a broad host range, including food animals and humans. *Salmonella enterica* remains one of the leading causes of food-borne illness (11%), hospitalization (35%), and death (28%) in the United States (1). In addition, most strains of the commonly occurring serovars, such as Typhimurium, have been shown to exhibit multidrug resistance, resistance to two or more antimicrobials (2–4). Previous studies on antimicrobial resistance have shown the emergence of multidrug-resistant (MDR) *Salmonella* in swine production systems even when there was no history of using antimicrobials, either as therapeutics or as growth promoters (5–7). The emergence and persistence of MDR *Salmonella* serovars in a swine production environment where there is no history of antimicrobial use suggest the presence of other risk factors such as selective pressure, including the use of heavy metal micronutrients in intensive swine production units.

Micronutrients such as copper and zinc, among many others, are included in swine feed and other livestock to achieve growth promotion and increase feed efficiency (8, 9). Zinc and copper are essential trace elements for prokaryotic and eukaryotic cellular metabolic functions. Zinc is a cofactor of more than 300 metalloenzymes, including alkaline phosphatases, whereas copper is needed for activation of several oxidative enzymes required for normal cellular metabolism (10, 11). Due to the proven and anticipated beneficial effects of zinc and copper in swine production, in-feed supplementation of zinc and copper in commercial production systems has been very common (12, 13).

On the other hand, tolerance to various chemicals among bacterial pathogens, mediated by different mechanisms, has also been on the rise. Multidrug efflux systems have been shown to be important mechanisms of resistance against antimicrobial agents and other structurally unrelated compounds, including heavy metals and biocides. The mechanisms of heavy metal resistance to copper in *Enterococcus faecium* isolates from pigs have been associated with the carriage of a conjugative plasmid carrying copper resistance determinants such as *tcrB* (14–16). Another efflux system that has also been associated with copper tolerance reported in Gram-negative organisms is the PCO operon, which mediates resistance to Cu^{2+}.

Resistance to Zn^{2+} and other metals such as Co^{2+} and Cd^{2+} is conferred by genetic determinants often carried by a plasmid such as pMOL30. In the CZC operon system, the products of the *czc* gene clusters function as a main component of an efflux protein

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(CzcA), a cation funnel (CzcB), a modulator of substrate specificity (CzcC) (17–20), and a protein involved in regulation of the operon (CzcD). The CzcD gene is involved in the regulation of a zinc, cobalt, and cadmium efflux system, the Czc system, which mediates resistance to these heavy metal cations (19, 21).

Coselection of heavy-metal-tolerant and antimicrobial-resistant phenotypes often occurs when the genes are colocated on genetic elements such as a plasmid, transposon, or integron (22–24). The efflux pump in bacteria plays a major role in development of resistance to several classes of antimicrobial agents. The presence and significance of resistance factors against micronutrients among *Salmonella* strains in the United States are poorly understood, and their association with antimicrobial resistance in the production environment has not been investigated. The present study was conducted to characterize the role of heavy metal micronutrient interventions (such as those by copper and zinc) in the emergence of heavy-metal-tolerant *Salmonella* and also its coselective association with multidrug-resistant *Salmonella*. Further, we investigated the association with carriage of specific efflux gene markers.

MATERIALS AND METHODS

Study design and sample collections. This study was part of a large longitudinal group-randomized controlled study designed to investigate the association of heavy metal micronutrients in swine feed with the occurrence and persistence of multidrug-resistant (MDR) *Salmonella*. Briefly, three vertically integrated commercial swine production systems (systems 1, 2, and 3) selected based on their history of *Salmonella* occurrence were included. From each system, three farms were selected (total of 9 farms). At each farm, four barns were randomly selected for further follow-up in this study, and all barns used standardized disinfection systems to limit introduction of additional potential confounding effects. We visited each farm at two stages (early finishing and late finishing) in four replicates (repeated visits to the same barns during the study period of October 2007 to November 2009). Each replicate visit consisted of sampling assigned barn floors before and after disinfection, pigs at early and late finishing stages, and pooled feed samples (25). Sampling was done from all the 36 barns for a period of more than 2 years. A total of 48 fresh fecal samples (25 g) were aseptically collected from each barn in four replicates at the early finishing stage (6 to 9 weeks of age) (n = 6,842) and at late finishing stages (26 to 28 weeks of age) of production (n = 6,093) from individual pigs. Some samples were lost/missed at different stages of the study (48 samples/barn × 36 barns × 4 replicates = 6,912 samples). Approximately 100 g of pooled feed samples (1 sample per barn collected from all 36 barns at 2 stages and 4 replicates with 13 losses to follow-up) was aseptically collected from 36 barns (n = 275) over a period of 2 years. Each pooled feed sample per barn was aseptically collected from the feeder bin in sterile Whirl-Pak bags and shipped to the laboratory on the same day as collection. For each farm, a survey assessment including questions about basic production, herd health management, biosecurity, and in-feed use of heavy metals (copper and zinc) was done.

***Salmonella* isolation and identification.** Salmonellae were isolated and identified according to conventional methods as described previously (26, 27). Briefly, a 10-g portion of each fecal and feed sample was preenriched in 90 ml of buffered peptone water (BPW; Becton, Dickinson, Sparks, MD), and 90 ml of BPW was added to each Whirl-Pak bag containing individual drag swabs and incubated at 37°C overnight. The remaining portions of fecal and feed samples were stored at −20°C. After overnight incubation, 100 μl of the preenriched suspension was added into 9.9 ml of Rappaport-Vassiliadis (RV) enrichment broth (Becton, Dickinson, Sparks, MD) and incubated at 42°C for 24 h. A 10-μl portion of the suspension was inoculated onto xylose-lactose-Tergitol 4 (XLT-4) agar (Becton, Dickinson, Sparks, MD) plates and incubated at 37°C for 24 h, and incubation was extended to 48 h in cases where colonies were doubtful. Three presumptive *Salmonella* colonies were selected from each positive plate for biochemical testing. Each selected presumptive *Salmonella* colony was then inoculated onto triple sugar iron (TSI) agar slants (Becton, Dickinson, Sparks, MD), lysine iron agar (LIA) slants (Becton, Dickinson, Sparks, MD), and urea broth (Becton, Dickinson, Sparks, MD) and incubated at 37°C for 24 h. All biochemically confirmed *Salmonella* isolates were then stored at −80°C until further testing.

Phenotyping. *Salmonella* isolates recovered from swine feed (n = 30), swine barn floors (n = 1,628), and swine feces (n = 4,504) were serogrouped using commercially available polyvalent O and group-specific antisera (Mira Vista, Copenhagen, Denmark) according to the recommendations of the manufacturer. Of all *Salmonella* isolates biochemically confirmed (n = 6,162), 283 *Salmonella* isolates were systematically selected based on origin and phenotypic characteristics (serogrouping and antimicrobial resistance profiles) and submitted to the National Veterinary Services Laboratories (USDA-NVSL, Ames, IA) for serotyping. *Salmonella* isolates were tested for antimicrobial susceptibility to a panel of 12 antimicrobials using the Kirby-Bauer disc diffusion method according to the guidelines of the CLSI (28). The antimicrobials used and their respective disc potencies were as follows: ampicillin (Am; 10 μg/ml), amoxicillin-clavulanic acid (Ax; 30 μg/ml), amikacin (An; 30 μg/ml), ceftriaxone (Ce; 30 μg/ml), cephalothin (Ch; 30 μg/ml), chloramphenicol (Cl; 30 μg/ml), ciprofloxacin (CIP; 5 μg/ml), gentamicin (Gm; 10 μg/ml), kanamycin (Km; 30 μg/ml), streptomycin (St; 10 μg/ml), sulfisoxazole (Su; 250 μg/ml), and tetracycline (Te; 30 μg/ml). We used *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853 as control strains. *Salmonella* isolates showing resistance to three or more antimicrobials were classified as multidrug resistant (MDR), and those isolates showing intermediate resistance profiles were considered susceptible.

Copper and zinc level determinations in swine feed and fecal samples. Pooled feed and fecal samples were shipped to the Ohio State University soil analytic laboratory for copper and zinc level determinations. The concentrations of copper and zinc in pooled feed and fecal samples were measured by inductively coupled plasma emission spectroscopy (PerkinElmer Optima 2000) using a method adapted from the work of Luo et al. (29). Quality control samples included a negative control (blank), positive control (two matrix spikes), and one standard reference material per batch of 20 samples. The methods have been modified from those of Lindsey et al. (30), Ollers et al. (31), Zhu et al. (32), and USEPA 3051b (33).

MIC of copper sulfate and zinc chloride. Three hundred forty-nine *Salmonella* isolates, from 283 samples, recovered from floor swabs (n = 179 samples), feces (n = 94 samples), and feed samples (n = 10 samples) with different antimicrobial resistance patterns were systematically selected for tests of tolerance to different concentrations of zinc chloride ($ZnCl_2$) and copper sulfate ($CuSO_4$). The agar plate-dilution method was used to determine the MIC against *Salmonella* as described before (34). The susceptibilities were determined on Mueller-Hinton II (MH-II) agar plates with a dilution range for zinc chloride of 0, 0.25, 0.5, 1, 2, 4, 8, and 16 mM, with the pH of the medium adjusted to 5.5. Copper sulfate solutions contained the dilution range of 0, 1, 2, 4, 8, 16, 20, 24, 28, and 32 mM with the pH of the medium adjusted to 7.2. Briefly, 25 ml of MH agar was aseptically dispensed and allowed to solidify. Bacterial suspensions were adjusted to 10^7 CFU/ml (100 μl of each inoculum at a 0.5 McFarland standard plus 900 μl of sterile 0.85% NaCl solutions). Each of the 400-μl suspension was aseptically aliquoted to a corresponding well of the replicator inoculum block. All test *Salmonella* isolates and control strains were tested in triplicate. The inoculated plates were incubated at 37°C for 16 to 20 h. Plates were assessed for growth, and the MIC was determined. The MIC was defined as the lowest concentration that inhibits the visible growth of *Salmonella*. *Enterococcus faecium* A17 sv 1 HHA 210, *S. aureus* C10682, *S. aureus* ATCC 29213, and *S. aureus* SO385 were used as reference strains. The reference strains were generously provided by Henrik Hasman (Technical University of Denmark).

Identification of heavy metal micronutrient (Cu^{2+} and Zn^{2+}) tolerance genes. The 283 *Salmonella* isolates were tested for the carriage of selected tolerance genes (*pcoA* for copper tolerance and *czcD* for zinc tolerance) using PCR. Briefly, *Salmonella* isolates were inoculated onto tryptic soy agar (TSA) plates and incubated at 37°C overnight. The genomic DNA was extracted using the Qiagen DNeasy tissue kit according to the manufacturer's instructions (Qiagen Ambion, Austin, TX, USA). Primers used for amplification of the *pcoA* gene included Forward (5′-C GTCTCGACGAACTTTCCTG-3′) and Reverse (5′-GGACTTCACGAA ACATTCCC-3′). The thermocycling conditions included Hot Start *Taq* activation at 95°C for 5 min, denaturation at 95°C for 1.5 min, annealing at 57°C for 1.5 min, and extension at 72°C for 2 min, and amplification was done in 34 cycles (35). Primers used for amplification of the *czcD* gene included Forward (5′-TTTAGATCTTTTACCACCATGGGCGC-3′) and Reverse (5′-TTTCAGCTGAACATCATACCCTAGTTT-3′) (36). The PCR amplification conditions were initial denaturation at 94°C for 2.5 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, and then the amplification cycle was repeated for a further 28 cycles and final extension was done at 72°C for 5 min. Ten microliters of the PCR product of each isolate tested was electrophoresed on a 1% agarose gel stained with 5 μl of 10-mg/ml ethidium bromide for 1 h at 120 V using 0.5× Tris-borate-EDTA (TBE) as running buffer. A 1-kb Plus DNA ladder was used as a molecular size marker.

Data analysis. Data were first summarized descriptively: levels of *Salmonella* prevalence in different samples, proportions of isolates that were resistant or were carrying heavy metal tolerance genes, and copper and zinc MICs were calculated. To explore the role of heavy metals and heavy metal tolerance in *Salmonella* in swine production, the data were analyzed with SAS v.9.3 (SAS Institute Inc., Cary, NC) using a generalized linear mixed model approach (PROC GLIMMIX), with binary distribution and logit link. The clustered data structure was accounted for by testing and including production company, farm, and barn as random effects in the models. Only one isolate per sample was included in the statistical modeling. Isolates were categorized based on their heavy metal (Zn and Cu) MIC values into high- or low-MIC groups (Zn MIC equal to 8 = high; Cu MIC equal to 24 mM = high), as well as on carriage of the zinc (*czcD*) or copper (*pcoA*) tolerance gene (yes/no). These characteristics were used as outcomes in the statistical analysis, and separate models were run for Cu and Zn.

Isolates were categorized into three groups based on their serotype: Typhimurium, Heidelberg, and "other." Based on their resistance patterns (R-type), isolates were also categorized into four groups: AmClStSuTe, AmStTeKm, resistant to any other combination of drugs, or pansusceptible. Associations between the above-mentioned outcomes and feed and fecal levels of the metals, source of the isolates (fecal, feed, or floor swab), different serotypes, and resistance patterns were assessed. Initially, all factors were individually modeled against the outcome in a univariate screening, and all that were associated with the outcome with P of <0.2 were included in a full model. Nonsignificant ($P > 0.05$) variables were dropped one at a time until all variables remaining in the model were significant. Of the 283 isolates selected for further characterization, 100% of serovar Heidelberg and Typhimurium isolates were multidrug resistant; thus, no further statistical modeling was performed on multidrug resistance.

RESULTS

Antimicrobial resistance of *Salmonella*. *Salmonella* isolates were detected in 17.2% (1,180/6,842) and 7.1% (431/6,093) of the pigs at early and late finishing stages, respectively. Interestingly, in each of the three production companies different *Salmonella* serovars appeared to dominate: Typhimurium was found most frequently in production system 1 (61.8% of all isolates), Heidelberg was found most often in production system 2 (50.3%), and "other" serovars were isolated most commonly in production system 3 (56.5%). Antimicrobial resistance was very common among the isolates, with 90% (27 of 30) of *Salmonella* isolates that originated from feed samples, 92.3% (1,503 of 1,628) of isolates from drag swabs, and 98.02% (4,415 of 4,504) of isolates from swine fecal samples showing resistance to one or more of the antimicrobials tested. Almost two-thirds, 60% (18 of 30), of the *Salmonella* isolates recovered from feed samples, 51.4% (836 of 1,628) of the isolates from barn floor drag swabs, and 76.1% (3,428 of 4,504) of the isolates from swine fecal samples were multidrug resistant (MDR). Table 1 summarizes the phenotypic properties of *Salmonella* serotypes ($n = 283$) further characterized using phenotypic and genotypic approaches.

Fecal and feed levels of heavy metal micronutrients (Zn^{2+} and Cu^{2+}). The level of zinc in the pooled feed samples varied between 77 mg/kg and 2,000 mg/kg of feed with a median of 139.8 mg/kg. The levels of Zn^{2+} in fecal samples were found to be significantly higher than the in-feed levels, ranging between 536.5 mg/kg and 12,557.2 mg/kg with a median of 941.1 mg/kg. Copper levels were found to range between 3.2 mg/kg and 365.2 mg/kg in pooled feed samples, with a median of 31.5 mg/kg. Copper levels in fecal samples were also higher, ranging from 71.2 mg/kg to 2,397 mg/kg with a median of 137.6 mg/kg. Table 2 depicts the levels of copper and zinc in feed and fecal samples. Overall, the Zn^{2+} levels in feed or fecal samples were not associated with the occurrence of high tolerance of Zn^{2+} (≥8 mM). On the other hand, copper tolerance was significantly associated with the Cu^{2+} levels found in fecal matter but not in feed.

Analysis of copper (Cu^{2+}) tolerance. Source of the isolates (fecal, feed, or floor swab sample), serotype, the resistance pattern that the isolates exhibited, and the copper level in fecal matter were associated with Cu^{2+} MICs in the univariate screening ($P < 0.2$). Forty-seven percent (134 of 283) of the isolates showed tolerance to Cu^{2+} at 24 mM, while the remaining 53% showed tolerance ranging from 4 mM to 20 mM. Carriage of the *pcoA* gene ($P = 0.8473$), copper level in the swine feed ($P = 0.6501$), or stage of sampling was not significantly associated with copper tolerance of *Salmonella* isolates. The carriage of the *pcoA* gene versus Cu MIC in *Salmonella* isolates recovered from fecal matter, feed, and barn floors is shown in Tables 1 and 3. When using multivariate analysis, two variables, serotype ($P = 0.0006$) and source of the isolates ($P < 0.0001$), remained significant in the model (Table 4). Resistance pattern became nonsignificant, and its effect appeared to be explained by the serotype when the two were included in the model simultaneously.

The odds of *Salmonella* isolates having high Cu^{2+} MICs (≥20 mM) were 5.8 times higher if the isolates originated from fecal samples rather than from floor swabs (95% confidence interval [CI] for an odds ratio [OR] of 3.1 to 11.1, $P = 0.0002$), indicating the significance of selective pressure in the gut ecosystem. Isolates of fecal origin were also significantly more likely to be tolerant to Cu^{2+} (MIC, ≥20 mM) than were those originating from feed samples (OR, 27.0; 95% CI, 2.8 to 250; $P = 0.0042$) (Table 4).

We found that serotype Heidelberg had 5.6-times-higher odds (95% CI for OR, 2.3 to 13.5; $P = 0.0002$) of having a MIC higher than 20 mM MIC than did "other" serotypes. Similarly, Typhimurium isolates were 1.3 times as likely to have high Cu^{2+} MICs (>20 mM) as were the "other" serotypes, but the difference was not statistically significant (95% CI for OR, 0.7 to 2.4; $P = 0.4741$). Also, serotype Heidelberg was 4.4 (95% CI for OR, 1.8 to 10.5; $P = 0.0009$) times as likely to have a MIC higher than 20 mM as was serovar Typhimurium (results not shown).

TABLE 1 Summary of phenotypic properties of *Salmonella* isolates ($n = 283$)

Serotype (no. of strains)	Resistance pattern (R-type), no. of strains	Source	Zn				Cu			
			MIC (mM)	Presence of *czcD* (no. of strains)[a]	Level in sample (mg/kg)		MIC	Presence of *pcoA* (no. of strains)[a]	Level in sample (mg/kg)	
					Feed	Fecal			Feed	Fecal
Schwarzengrund (3)	AmStTeAxChKm (1)	Swab	8	0	196	1,436	24	0	14	260
	Pansusceptible (2)	Swab	8	0	196	1,436	24	0	14	260
Mbandaka (7)	AmClStSuTeAx (1); AmSuChCe (1); StSuTe (2)	Swab	8	0	196–527.3	1,436–4,167.2	20–24	0	14–194.7	260–1,209.8
	AmStTe (1); AmClStSuTeAxCh (1)	Fecal	4–8	0	109.0–144	674–9,027.2	20–24	0 (1), + (1)	157.7–173	1,142–1,704.2
	SuTe (1)	Feed	8	0	1,668	10,530	16	1	268	1,852
Anatum (9)	AmTeAxChCeXNL (1); AmTeAxChXNL (1); AmStTeAxCh/XNL (3)	Swab	8	0	140.1–171.3	774–1,383.1	20–24	0	11.9–97	137.8–742
	Pansusceptible (2)	Swab	4–8	0	171.3–201	1,017–1,691	20	0	34	406
	AmStTeAxCh (2)	Fecal	8	0	96	787	24	0	146	1,496
Derby (17)	StSuTe (4)	Swab	4–8	0	132.7–201	939.9–11,631	20	0	9.4–34	112.9–406
	AmStSuTeAxChCe/XNL (6); AmClStSuTe (1); AmStSuTe/Km (2)	Fecal	4–8	0	108.8–2,000	865.9–11,185	20–24	0	8.3–316	110.6–1,911
	AmStSuTe (1); AmStSuTeAxCh (1)	Swab	4–8	0	1,389.1–1,393.2	8,714.8–10,146.2	20	1	249.3–268.3	1,645.5–1,875.3
	Pansusceptible (1)	Fecal	4	0	91.4–166.3	639.2–1,229.4	20–24	0 (6), + (1)	6.2–173	95–143.9
	Te (1)	Feed	8	0	147.6	719.7	16	0	146.2	919.2
Heidelberg (72)	AmStTeKm (1)	Feed	4	0	127.2	736.1	20	1	20.9	86.7
	AmStTeKm (12); AmStTeKmGm (2); AmStSuTeKmGm/Ax (2)	Swab	4–8	0	93.4–7,383.7	710.8–8,494.7	20–24	0 (7), + (9)	18.7–1,384.3	106.6–1,564.3
	AmStTeKm/AxChkmXNL (31); AmStSuTeKm/Gm (10); AmClStSuTeKm (1)	Fecal	4–8	0	79–2,000	675.3–11,631	20–24	0 (17), + (25)	135.4–308	137.3–2,130.7
	AmStTeAxChKmXNL (5); AmStTeAxChKmGm (4)	Fecal	4–8	0	92.9–1,549.1	675.3–8,952	20–24	0 (4), + (5)	203.2–291.1	12,176–1,839.1
	AmStTeAxChKmAnXNL (1); AmStTeAxChKm (2)	Fecal	8	0	178–191	1,059–1,197	24	0	14–87	160–172
	AmStTeKm (1)	Feed	4	0	1,330.3	948.6	20	0	229.5	1,412.7
Typhimurium (122)	AmClStSuTe (28); AmStSuTeKm/Ax (4); AmClStSuTeAx/Ch (3)	Swab	4–8	0 (2), + (12)	109.5–183.1	541–970	16–24	0 (10), + (4)	7.8–173	81.9–1,142
	AmClStSuTe/Ch (2); AmClStSuTeKm/Gm (2)	Swab	4–8	0 (5), + (14)	97.5–1,685	541–11,365	16–24	0 (18), + (1)	7.3–271	89.3.1–2,001
	AmClStSuTe (39); AmClStSuTeAx (9); AmStSuTeKm (4)	Fecal	4–8	0 (7), + (45)	81.3–1,762.2	787–11,365	16–24	0 (47), + (5)	9.9–262	90.7–2,001
	AmStTeKm (8); AmClStSuTeKm (3)	Fecal	4–8	0 (11)	89.1–1,728.2	692.6–7,852.4	20–24	0 (2), + (9)	7.5–365.2	152.3–1,703
	AmClStSuTeAxCh (6); AmClStSuTeAxChKmXNL (2)	Fecal	4–8	0 (1), + (7)	140–161.1	674–1,383.1	20–24	0 (7), + (1)	7.6–173	119.1–1,142
	AmClStSuTeAxChXNL (4); AmClStSuTeAxChKmGm (1); AmClStSuTeCh (1)	Fecal	4–8	0 (4), + (1)	144–160.9	674–1,063.6	20–24	0	14.4–173	123.8–1,142
	AmClStSuTeAxKmGm (1); ClStSuTe (2); AmClStSuTeChKmGm (2)	Fecal	4–8	0 (3), + (2)	99.1–1,347.5	541–8,952.0	4–24	0 (3), + (2)	3.2–203.2	101.8–1,543.4
	AmClStSuTe (1)	Feed	8	1	116.2	761.3	20	0	19.4	87.8
Worthington (8)	AmTeAxChCeXNL (2)	Fecal	8	0	115.2–134.4	1,066.7–1,463.5	24	2	9.3–31.4	126.8–177
	AmStTeAxChKmGm (1)	Swab	8	0	81.9	732.1	24	1	133	935.7
	AmStTeAxChKmXNL (1)	Fecal	8	0	1,645	12,325	24	1	302	2,397
	AmStTe (1)	Feed	8	0	1,685	11,365	24	1	271	2,001
	AmTeAxChCe (1); AmTeAxChCeXNL (1)	Swab	8	0	119.6	734.6	20	1	7.3	89.3
	AmStTeAxChKmGmXNL (1)	Swab	8	0	81.9	732.1	24	0	133	935.7
Rissen (1)	AmStTeKm (1)	Swab	8	0	129.6	1,254.7	20	1	15.7	138.7
Enteritidis (1)	AmClStSuTe (1)	Swab	8	0	140	774	16	0	97	742

Infantis (9)	AmClStSuTeAxChKmXNL (2); AmClStSuTe (1)	Fecal	4–8	0 (2), + (1)	86.4–146.9	1,383.1	16–20	0	11.9–16.2	135.6
	AmClStSuTeAxChKmGm (2); AmClStSuTeAxChKmGmXNL (1); AmAxChXNL (1)	Fecal	4–8	0	111–169.1	674–1,266	16–20	0	17–173	149.2–1,142
	AmClStSuTeChKmGmXNL (1); AmAxChXNL (1)	Swab	8	0	97.5–160.9	990.5–1,063.6	20	0	11.7–15.2	123.8–144.4
Agona (5)	StSuTeKmGm (2); AmStSuTeAxChKmGmXNL (1)	Fecal	4	0	105.1–177.3	991-.9–1,007.7	16–20	0	8.5–14.9	133.9–140.5
	AmStSuTeAxChKmGmXNL (1)	Swab	8	0	173.6	883.2	20	1	10.1	133.9
	Te (1)	Feed	8	0	1,375	6,895.0	16	0	279.2	1,391.4
Senftenberg (7)	AmClStSuTeAxChGmKmXNL (4); AmClStSuTeGm (1); StSuTeKmGm (1)	Fecal	4–8	0	178–1,636.2	657.8–8,364.9	20–24	0 (1), + (4)	14–291.1	172–1,643.1
	Pansusceptible (1)	Swab	8	0	191	1,059	24	1	87	160
Amsterdam (1)	Pansusceptible (1)	Swab	8	0	198	1,048	20	0	21	161
London (1)	Te (1)	Feed	4	0	1,558	12,120	20	1	262	2,144
Ohio (2)	Pansusceptible (1)	Swab	8	0	191	1,059	24	0	87	160
	AmClStSuTeKmGm (1)	Fecal	4	0	155.2	1,043.7	24	1	I0.9	107.8
Inverness (1)	Pansusceptible (1)	Feed	8	0	131	924	20	0	4.8	99
Muenchen (3)	AmStTeKm (1)	Fecal	8	0	147	1,691	24	0	138	406
	Pansusceptible (2); AmStTeKm (1)	Fecal	8	0	192	1,183	20	0	15	120
Rough_Ob:e,n,x (1)	Pansusceptible (1)	Fecal	8	0	192	1,183	20	0	15	120
Serogroup E (5)	AmStSuTeChCeGm (1); AmStTeKmGm (1); AmClStSuTeChKmGm (1); ClStSuTe (1)	Fecal	4–8	0	139.5–1,393.2	855.2–1,215.9	16–24	0 (2), + (2)	23.1–249.3	85.5–1,875.3
	Te (1)	Feed	8	0	119.9	731.4	16	0	14.1	102.0
Serogroup A-I+ (3)	AmStTeKmGm (1); AmStTe (1)	Swab	8	0	81.9–1,552.1	732.1–8,629.2	16	+ (2)	133–229.2	935.7–1,498.1
	AmStSuTeKmGm (1)	Fecal	8	0	111	675.4	16	1	17	78.2
Serogroup B (5)	AmClStSuTeChKmGm (2); AmClStSuTe (1); AmStTeKmGm (1)	Fecal	4–8	0	116.2–1,393.2	761.3–10,146.2	16	+ (4)	8.8–249.3	87.8–1,875.3
	StSuTe (1)	Swab	8	0	1,389.1	8,714.8	20	1	268.3	1,645.5
Serogroup A-S+ (2)	AmClStSuTe (1); AmStTeKmGm (1)	Fecal	8	0	119.6–155.2	734.6–1,142.1	16	+ (2)	7.3–10.3	89.3–127.8

[a] 0, negative; +, positive.

TABLE 2 Zinc and copper levels in swine feed and fecal samples[a]

Heavy metal	Type of sample	Level (mg/kg)				Dietary Zn and Cu requirement (mg/kg)
		Median	Minimum	Maximum	95% CI of $\bar{x}$	
Zinc	Feed	139.8	79	7,383.7	563.14 ± 79.57	50–100
	Feces	941.1	541	12,325	3,070.17 ± 393.47	
Copper	Feed	31.5	3.2	1,384.3	123.33 ± 13.64	3–6
	Feces	137.6	71.2	2,397	806 ± 73.89	

[a] Dietary requirements are based on the guidelines of the National Research Council, 2012 (8).

The copper extrusion efflux gene, *pcoA*, was detected in 35% (99/283) of the *Salmonella* isolates (Table 3). More than 60% of *Salmonella* isolates with R-type AmStTeKm (32 of 53) carried the *pcoA* gene, while 5% of those with R-type AmClStSuTe (4 of 84) carried *pcoA*. The *pcoA* gene was detected in all three categories of serotypes regardless of their MDR status (Table 3). Of the detected *pcoA* genes, 40% were found in serovar Heidelberg and 22% were found in serovar Typhimurium. A lower proportion of these genes was also detected in the following serotypes: Senftenberg, Worthington, Derby, Ohio, Mbandaka, London, Agona, and Rissen. This finding implies the common occurrence of the gene in *Salmonella* (Table 3).

Analysis of zinc (Zn^{2+}) tolerance. In the univariate screening, carriage of the *czcD* gene, serotype, resistance pattern, and source of the isolates were all significantly associated with high Zn^{2+} MICs. However, neither Zn^{2+} levels in the feed ($P = 0.9613$) nor those in the feces ($P = 0.8043$) were significantly associated with high Zn^{2+} MIC levels. Sixty percent (171 of 283) of the isolates showed zinc tolerance at 8 mM, and the remaining 40% (112 of 283) showed tolerance at 4 mM. The carriage of the *czcD* gene versus Zn MIC in *Salmonella* isolates recovered from fecal samples, feed, and barn floors is shown in Tables 1 and 3. In the final model, carriage of the *czcD* gene, serotype, and source of the isolates remained significantly associated with high Zn^{2+} tolerance (Table 5). Isolates carrying the *czcD* gene had 10.6-times-higher odds of having a Zn^{2+} MIC of 8 mM or more than did those not carrying the gene (95% CI for OR, 4.0 to 27.8; $P < 0.0001$), adjusting for the serotype. "Other" serotypes had 4.5 (95% CI for OR, 2.2 to 9.5; $P < 0.0001$)- and 2.4 (95% CI for OR, 1.0 to 5.6; $P = 0.0504$)-times-higher odds than serotype Typhimurium of having a high Zn^{2+} MIC, whereas Heidelberg isolates were only approximately half as likely to have a high Zn^{2+} MIC as were Typhimurium isolates (OR = 0.52; 95% CI for OR, 0.211 to 1.289), but the difference was not statistically significant ($P = 0.1578$) (Table 5). In the model with serotypes, resistance pattern became nonsignificant and its effect appeared to be explained by the serotype. In contrast to the findings with copper tolerance, isolates from floor swabs had 6.5-times-higher odds (95% CI for OR, 3.2 to 12.9; $P < 0.0001$) of having high tolerance for Zn^{2+} than did fecal isolates. Also, isolates from feed samples had 3.0-times-higher odds of having a Zn^{2+} MIC of >8 mM than did fecal isolates, even though the difference was not significant (95% CI for OR, 0.7 to 13.5; $P = 0.1546$) (Table 5).

Assessment of association between tolerance and gene carriage showed that isolates with a high Zn^{2+} MIC also were more likely to carry the *czcD* gene (Table 3). The odds of *czcD* gene carriage were 5.2 times higher for isolates with high Zn^{2+} MICs than for those with low Zn^{2+} MICs (OR = 5.2; 95% CI, 2.4 to 11.5; $P < 0.0001$). However, 9.8% of the *Salmonella* isolates (11/112) with low Zn^{2+} MICs also carried the *czcD* gene, suggesting that carriage of this gene is not always associated with a Zn^{2+}-tolerant phenotype. Of the high-zinc-tolerant isolates, 52% belonged to serovar Typhimurium and 12% belonged to serovar Heidelberg. No *Salmonella* serovar Heidelberg carried the *czcD* gene, indicating a different mechanism for tolerance, whereas 69% of the Typhimurium isolates carried the *czcD* gene. Interestingly, none of the pansusceptible *Salmonella* isolates (12 of 283, 4.2%) were found to carry the *czcD* gene even though 66.7% (8/12) were tolerant to Zn^{2+} at 8 mM (Table 3). This indicates the presence of other mechanisms apart from the *czcD* gene. While the finding is just in contrast to the carriage of the *pcoA* gene encoding copper tolerance, it shows the occurrence of a strong association between distinct heavy metal tolerance and antimicrobial resistance (R-types).

The Zn^{2+} tolerance gene, *czcD*, was detected in 30% (85 of 283) of the *Salmonella* isolates. None of the *Salmonella* isolates with R-type AmStTeKm carried the *czcD* gene, while 84% of those with R-type AmClStSuTe (71 of 84) carried *czcD*. The *czcD* gene was almost exclusively detected in serotype Typhimurium (84 of 85); none of the Heidelberg isolates carried the gene and only one of the isolates belonging to the "other" serotypes carried the gene.

DISCUSSION

The magnitude of multidrug resistance in *Salmonella* and other pathogens at the human-animal and ecosystem interface has been a major concern globally. As we previously reported (25), in addition to isolation of *Salmonella* from feces and barn floor swabs, in the current study, *Salmonella* was also detected in 3.6% (10/275) of the commercially processed swine feed samples. Besides the direct selective pressure of antimicrobial resistance, coselection due to other structurally related or unrelated chemical agents has also been a concern for the rising trend in multidrug resistance. However, there have been very limited studies conducted in this area. The current study attempts to fill the knowledge gap, mainly focusing on the use of heavy metal micronutrients. Previous studies have identified genetic elements among *Salmonella* strains that render some strains resistant to heavy metal micronutrients, including copper (37–39) and zinc (39, 40). Such resistant strains were shown to carry genes associated with multiple antimicrobial resistance factors (34, 38).

Heavy metal micronutrients such as Zn^{2+} at relatively low concentrations are essential for microorganisms since they provide vital cofactors for metalloproteins and enzymes (2, 41). The use of copper in swine feed has also been repeatedly shown to have a positive effect in production performance (42, 43), especially when dietary Cu^{2+} is supplemented above the National Research Council (NRC) (8) requirement of 5 mg Cu^{2+}/kg (44). Copper

TABLE 3 Heavy metal micronutrient (Zn^{2+} and Cu^{2+}) phenotypes, genotypes (*czcD* and *pcoA*), and association with resistance types (R-types)

Heavy metal micronutrient	MIC (mM)	Heavy metal tolerance gene[a]	R-type, *n* (%)										
			AmCfiSûTe/Ax/Ch	AmŠTeKm/Gm	AmŠûTeKm/Gm	AmŠTeAxChKm/Gm	AmŠTeAxChKmXNL	StŠuTe	Te	Pansusceptible	Others	Total
Zinc	4	*czcD* (+)	11 (9.1)									11 (3.8)
		czcD (−)	4 (3.5)						1 (0.9)	4 (3.5)	38 (34)	101 (35.6)
	8	*czcD* (+)	67 (39.2)	42 (37.5)	4 (3.5)	3 (2.7)	5 (27)				7 (4.1)	74 (31.1)
		czcD (−)	10 (8.3)	18 (14.9)	11 (6.4)	2 (1.2)	1 (0.6)	7 (4.1)	3 (1.8)	8 (4.7)	37 (21.6)	97 (34.2)
Copper	1	*pcoA* (+)										
		pcoA (−)										
	2	*pcoA* (+)										
		pcoA (−)										
	4	*pcoA* (+)									1 (0.5)	1 (0.3)
		pcoA (−)										
	8	*pcoA* (+)										
		pcoA (−)										
	16	*pcoA* (+)	1 (0.5)	3 (1.5)					3 (3.0)		94 (47.5)	98 (35)
		pcoA (−)	3 (3.0)						1 (1.4)		94 (47.5)	100 (35.3)
	20	*pcoA* (+)		10 (13.9)	2 (2.6)		1 (1.4)	2 (2.8)			12 (16.7)	28 (9.9)
		pcoA (−)	5 (6.9)	6 (8.3)	1 (1.3)			5 (6.9)		7 (9.7)	20 (2.8)	44 (15.5)
	24	*pcoA* (+)	2 (1.9)	25 (24.8)	11 (10.9)	4 (3.9)	4 (3.9)			1 (1.0)	9 (8.9)	56 (19.8)
		pcoA (−)	3 (2.9)	16 (15.8)	19 (18.8)	2 (1.9)	1 (1.0)			4 (3.9)		45 (15.9)

[a] +, detected; −, not detected.

TABLE 4 Multivariable model with copper tolerance (Cu MIC = 24 mM) as outcome

Serotype/sample	Estimate	SE	OR (95% confidence interval)	*P* value
Serotype				0.0006
Typhimurium	0.2373	0.3311	1.27 (0.66–2.43)	0.4741
Heidelberg	1.7212	0.4486	5.59 (2.31–13.52)	0.0002
Other	Reference			
Sample type				<0.0001
Fecal	1.7633	0.3266	5.83 (3.07–11.09)	<0.0001
Feed	−1.5257	1.1477	0.22 (0.02–2.08)	0.1849
Floor swab	Reference			

and zinc are considered two of the most widely researched alternatives to growth promotion antibiotics to enhance swine performance and maintain health (45). In the current study, we found a wide range in the levels of copper and zinc used in swine feed and concentrations often much higher than the NRC recommendations, as depicted in Table 2. In addition, it is worth noting that the concentrations of both micronutrients were higher in the fecal samples than in the feed. This is expected, and it was previously reported that as the various feed ingredients are absorbed within the gastrointestinal tract, a few elements such as heavy metal micronutrients tend to be more concentrated in the feces, and as a result, a large percentage of the consumed dietary copper and zinc ends up in feces (8), indicating a relatively low retention in the intestine and significant excretion of these minerals in feces. Previous reports (46) also showed that this phenomenon is particularly more significant in swine and poultry feces and reported that the concentration of zinc in swine feces is 10 to 100 times higher than that in dairy manure and that liquid swine manure had six times as much copper as did liquid dairy manure. In addition, the recent NRC report (8) indicated that even though high levels of dietary copper and zinc have been shown to improve animal performance, a large percentage of consumed minerals such as copper and zinc (approximately 90 to 95%) is excreted in the feces and ends up as environmental contaminants. In this study, we observed that the level of micronutrients, particularly zinc, in the

TABLE 5 Multivariable model with zinc tolerance (Zn MIC = 8 mM) as outcome

Serotype, sample type, or carriage	Estimate	SE	OR (95% confidence interval)	*P* value
Serotype				0.0003
Heidelberg	−0.6507	0.4594	0.52 (0.211–1.289)	0.1578
Others	0.8609	0.4379	2.37 (0.999–5.602)	0.0504
Typhimurium	Reference			
Sample type				<0.0001
Feed	1.095	0.7672	2.99 (0.66–13.54)	0.1546
Floor swabs	1.8727	0.3493	6.51 (3.27–12.94)	<0.0001
Fecal	Reference			
czcD carriage				<0.0001
Yes	2.3576	0.4912	10.57 (4.02–27.79)	<0.0001
No	Reference			

swine feed is much higher than the NRC daily requirements. While we cannot confirm it, the high level of use could potentially ameliorate the coselective pressure and its association with antimicrobial resistance. This area may need further investigation.

A few studies have reported different tolerance levels of important food-borne pathogens such as *Salmonella* and *E. coli* to different concentrations of Zn^{2+} and Cu^{2+} (34, 47). This study has reported up to 8 mM as the highest level of tolerance of *Salmonella* isolates to Zn^{2+}, which is higher than the level reported by Aarestrup and Hasman (39). The highest level of tolerance of *Salmonella* isolates to Cu^{2+} reported in our study was 24 mM. This is smaller than the maximum tolerance reported by the same study (39). The increased tolerance level of *Salmonella* isolates to Zn^{2+} and Cu^{2+} is attributable to the use of the respective micronutrients in swine feed. Zinc is known to inhibit some of the bacterial populations in the intestinal tract and thereby improve the health or feed conversion ratio of the food animals (39, 48, 49).

Carriage of the *pcoA* gene or level of copper in swine feed was not significantly associated with copper tolerance of *Salmonella* isolates. This may have different implications. One clear reason might be the presence of various other mechanisms that may result in copper tolerance besides *pcoA* and associated operon systems. Other genes, including *cuiD* and *scsC*, and other mechanisms have also been reported to encode copper tolerance in *Salmonella* (50, 51).

The PCR results on the level of *czcD* gene carriage by *Salmonella* isolates were consistent with the increase in the level of the Zn^{2+} MIC of *Salmonella* isolates. The PCR results on the level of *pcoA* gene carriage by *Salmonella* isolates were also consistent with the increase in the level of the Cu^{2+} MIC of *Salmonella* isolates. The two multidrug-resistant R-types AmClStSuTe and AmStTeKm were observed to be the highest-occurrence resistance types in the *Salmonella* isolates in this study; however, their occurrence varied by the type of heavy metal tolerance gene carriage. It should be noted that *Salmonella enterica* serovar Typhimurium strains of phage type DT104, often containing the R-type AmClStSuTe, are some of the major strains reported worldwide and are commonly isolated from humans and food animals (52, 53). While there have been very limited studies conducted to date investigating the association between heavy metal tolerance and specific antimicrobial resistance patterns, a study in 1984 in drinking water reported the overall association between copper and zinc tolerance and antimicrobial resistance (54). Heavy metal tolerance was more common among isolates from the fecal samples than among those of environmental origin (drag swabs).

In summary, the findings in this study clearly demonstrated the presence of a strong association between decreased susceptibility to heavy metals and antimicrobial resistance among *Salmonella* serovars isolated from swine, swine feed, and barn floors. The detection of decreased susceptibility to heavy metal micronutrients (copper and zinc) and associated genetic determinants among various *Salmonella* strains has implications for the control of multidrug-resistant *Salmonella* strains, which are of public health and veterinary medicine significance. However, further studies investigating the role of coselection and mechanisms of genetic linkage could shed further light on the relationship and its significance.

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Appendix 2: Approval for Animal Use Protocol for Research in Tanzania

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Our Ref: SUA/FVM/R.1/10 Your Ref: Date: 1st February 2013

Dr. Julius John,
Faculty of Veterinary Medicine,
Sokoine University of Agriculture,
P.O. Box 3016.
Chuo Kikuu - Morogoro.

Dear Dr. Julius John

RE: APPROVAL FOR ANIMAL USE PROTOCOL FOR THE RESEARCH ENTITLED MULTIDRUG RESISTANT *SALMONELLA* ISOLATES FROM FOOD ANIMALS AND ANIMAL PRODUCTS IN PASTORAL ZONES OF TANZANIA: PREVALENCE AND MOLECULAR CHARACTERISATION

Kindly refer the above heading.

I am delighted to inform you that your animal use protocol submitted for review has been reviewed by the Faculty Research and Publication Committee using the criteria for Humane cares and use of Animal Procedures. The approval is valid for three years; 4th February 2013 to 3rd February 2016 subject to satisfactory annual reports. The committee advised observation and adherence to the approved protocol and recommend use of professional veterinarian in handling of animals. Should you become compelled to make necessary changes during the implementation of your research please make sure that you submit an amendment for review and approval.

The committee congratulates your team for this innovative research and looks forward to its successful implementation of the research.

Yours sincerely,

Professor Robinson Mdegela, PhD chair
Faculty Research and Publication Committee

C.C: Dean, FVM