GENOTYPING OF MULTI-DRUG RESISTANT NON-TYPHOIDAL SALMONELLA: DETECTION OF FLUOROQUINOLONE AND OTHER MULTI-DRUG RESISTANCE DETERMINANTS

JANET AGNES MUNYALO

MASTER OF SCIENCE IN MEDICAL MICROBIOLOGY

ABSTRACT

The genus *Salmonella* belongs to the family of *Enterobacteriaceae*. Within the genus *Salmonella* there are 2 species, *Salmonella bongori* and *Samonella enterica*. There are over 2500 different serotypes or serovars that have been identified to date belonging to the above mentioned genus. Non-typhoidal *Salmonella* (NTS) is an important pathogen that causes foodborne diseases in both humans and gastrointestinal illness in animals. In humans, multi-drug resistant (MDR) NTS strains present a major health concern particularly in health care areas with inadequate resources. There is doubt that these MDR strains of *Salmonella* may not be limited to humans and zoonotic spread may be stirring. Free-range pig production around homesteads is a regular way of farming in Kenya hence increases the danger of spread of infection to human beings. Therefore pigs' serves as reservoirs of antimicrobial resistant *Salmonella* and slaughterhouse cross-contamination of pork becomes a food safety hazard. Tetracyclines, sulphonamides and trimethoprim, nitrofurans aminoglycosides, beta-lactams and the quinolones are the mostly used drugs in food-producing animals in the country. The in- feed use of co-selective agents (such as grave metal micronutrients copper and zinc) and antibiotics in swine production systems impose selective pressure and contribute to increased MDR strains. However, in Kenya and Eastern Africa there is inadequate data on the contribution of heavy metal micronutrients use in swine feed and their role on emergence of MDR *Salmonella*. There is also insufficient data on the antibiotic resistant genes in *Salmonella* isolated from swine fecal sources. This study aimed to characterize antimicrobial resistant genes in *Salmonella* isolated from swine faeces and the function of weighty metal micronutrients in the appearance of MDR *Salmonella*. A total of 171 Isolates were used in the study. Of these, 86 isolates originated from Kenya, 35 isolates from Ethiopia and 50 isolates from the United States of America (USA). Minimum Inhibitory Concentration (MIC) of copper-sulfate ($CuSO_4$) and zinc-chloride ($ZnCl_2$) was measured using agar dilution. Amplification of *pcoA* and *czcD* genes that encode for copper ions (Cu^{+2}) and zinc ions (Zn^{+2}) tolerance was done. Amplification and sequencing of quinolone resistance determining region of topoisomerases genes *gyrA* and plasmid mediated quinolone resistance gene *qnrA* were done. Amplification of class 1 integrons and Salmonella plasmid virulence (*spvA*) gene was done. Isolates from USA and Ethiopia had predetermined heavy metal tolerance (Cu^{+2} and Zn^{+2}) MICs and were used as comparative test isolates. The 86 test isolates were all subjected to MIC determination for Cu^{2+} and Zn^{2+}. 98% (N=84) of the isolates had an MIC of 4mM, 1.16% (N=1) 0.5 mM, and 1.16% (N=1) had MIC of 0.25 mM for $Zncl_2$. 98% (N=84) had MIC of 8 mM, 1.16% (N=1) 1.0 mM and 1.16% (N=1) MIC of 2.0 mM for $CuSO_4$. The zinc tolerance genes *czcD* and copper extrusion efflux gene *pcoA* were not detected in any of these isolates regardless of their MIC or MDR status. The findings demonstrated that there is no association between heavy metal resistance and antimicrobial resistance in NTS. Forty two(42) isolates out of the total 171 isolates selected with antibiotic resistance characteristic SulCip Cip and CipNa identified single and double amino-acid substitutions in *gyrA* at positions Ser83 (N = 29; →Phe, →Tyr, → Cysteine, → Gly, → Isol) and Asp87 (N = 33; →Asn, →Gly, →Ly, →Arg) and one (1) isolate carried *qnrA* gene by PCR amplification and sequencing. There was no strong relationship between NAL and CIP resistant isolates in amino acid substitutions and mutations in both positions Ser83 and Asp87. 31 out of 171 isolates were selected for integron detection with characteristic antibiotic resistance pattern SulTe by PCR amplification and 10 out of the 31 isolates were

positive for Class 1 integrons of 1.2 kb and 1.5kb size. 38 isolates were selected for *spvA* gene detection with antibiotic resistance SulCip and 3 out of the 38 isolates were found to contain *spvA* gene. This study shows the potential significance of pigs as a source of sole and many antimicrobial-resistant *Salmonella* isolates and that the use of relatively low heavy metal ion concentrations in swine feed might not be sufficient to induce increased multidrug resistance nontyphoidal *Salmonella* serotypes in swine. In addition, the study provides important information for incorporation into public health policy to control and manage *Salmonella* swine production systems in controlling antibiotic resistance transfer from swine to humans.

CHAPTER ONE

INTRODUCTION

1.1 Background

Salmonella enterica is the primary cause of food-borne diseases (11%), hospitalization (35%), death (28%) in the United States(Eaves *et al.*, 2004). NTS enterica serotypes are the most significant food-borne bacterial pathogens, with a broad host range. In Africa, multidrug-resistant NTS are the main causes of morbidity and high mortality in children under the age of 5 years, this is second in significance only to pneumococcal illness, an infection caused by the *Streptococcus pneumoniae* (Kingsley *et al.,* 2009).In Sub-Saharan countries, studies indicates that NTS are second causes of bacteraemia in small children under the age of 5 years(Kariuki *et al.*, 2006).

In Kenya, invasive nontyphoidal Salmonella (iNTS) causes harsh bacteremic sickness among human immunodeficiency virus (HIV) adults, especially among 5 years old children coinfected with malaria or HIV, this also includes the sickle cell disease or rigorous malnutrition with the occurrence of iNTS disease ranging from 166 to 568 cases per 100,000 persons per year ((Kariuki *et al.*, 2015). According to Kariuki *et al.,* (2014) over 5/24 children with alleged meningitis and/or septicemia, was caused by *S. enteritidis* and all of them were reported to have died and that septicemia caused by NTS was linked to lofty death rates in Tanzania (Vaagland *et al.,* 2004). Bacteraemia affects 1 / 6 harshly emaciated children with high death rate more especially the HIV-positive ones with increased stage of resistance to ordinary antibiotics, this was reported in Uganda (Bachou *et al.,*2006). Over 59% [94 of 160]) of NTS in South Africa were blood culture isolates with elevated levels of resistant antibiotics (Kruger *et al.,* 2004). Case-fatality levels for childhood NTS are also very

high (21–24%), even when suitable antibiotics are accessible in Malawi (Gordon *et al.,* 2008).

The frequency of invasive bacterial infections in infants in Gambia is 1009 (95% CI, 903-1124) cases per 100,000 person-years and Community-acquired invasive NTS is seen among young children of 2-29 months of age in rural the rural areas (Ikumapayi *et al.,* 2007). The presence of iNTS disease has also been reported from Mali, Mozambique where two studies were conducted and predominantly ST313 S. *typhimurium* isolates with infant incidence of 217.7 per 100 000 child-years, and South Africa were observed (Haselbeck *et al.*, 2017). It has also been reported that multidrug-resistant *S. typhimurium* is the main basis of bacteraemic sickness in infants in Rwanda and Zaire (Morpeth *et al.*, 2009). The spread of *Salmonella* illness is preferential in humans by a wide array of animal reservoir and by a wide viable division of both animals and food product (Gunell *et al.*, 2009). *Salmonella* account for an estimated 27% of all food borne illnesses caused by known bacterial agents with the majority of human salmonellosis cases related to the intake of food products that are infected (Aarestrup & Schwarz 2006).

The emergence of MDR *Salmonella* is a global burden, in topical years there has been an addition of antimicrobial resistance like resistance to nalidixic acid, among *Salmonella* spp. This resistance has also been experienced in various countries in the world (Brenner *et al.*, 2000). Nalidixic acid–resistant and ciprofloxacin-resistant *S. schwarzengrund* was reported in Taiwan (Su *et al.*, 2004) and the United States (Foley & Lynne 2007). Even though resistance to first-line antimicrobials is familiar among NTS from Kenya and other sub-Sahara African countries, resistance to ceftriaxone and fluoroquinolones is rarely reported however, according to Kariuki *et*

al., (2015), the separation and genomic description of ST313 S. *typhimurium* isolates from patients looking for treatment in Nairobi's tertiary-care and teaching hospitals showed resistance to ceftriaxone with or without combined resistance to fluoroquinolones. In Central Africa a study showed resistance to fluoroquinolones, azithromycin and third generation cephalosporins is low but emerged with reduced ciprofloxacin susceptibility, azithromycin and cefotaxime resistance at 80.7%, 4.3%, 3.0% and 2.1% respectively (Lunguya *et al.*, 2013).

1.2 Objectives

1.2.1 Main Objective

To establish antimicrobial resistance and heavy metal tolerance genes in multidrug resistant NTS isolated from swine farms in Kenya.

1.2.2 Specific Objective

i. To determine minimum inhibitory concentration (MIC) for zinc chloride and copper sulphate in multidrug resistant NTS isolated from swine farms in Kenya
ii. To determine the presence of heavy metal ion *PcoA* and *CzcD* tolerance genes in multidrug resistant NTS isolated from swine farms in Kenya
iii. To determine quinolone resistance determining regions of topoisomerase gene (QRDR) *gyrA* in flouroquinolones resistant multidrug resistant NTS isolated from swine farms in Kenya.
iv. To establish the plasmid mediated quinolone resistant gene (PMQR) *qnrA* in flouroquinolones resistant multidrug resistant NTS isolated from swine farms in Kenya.

v. To determine class 1 Integrons in multidrug resistant NTS isolated from swine farms in Kenya.

vi. To determine the presence of Salmonella plasmid virulence gene (*spvA*) in multidrug resistant NTS isolated from swine farms in Kenya

1.3 Hypothesis

Phenotypic and genotypic attributes of multidrug resistant determinants in NTS isolated from swine farms in Kenya are similar to those described in the region (such as Ethiopia) and globally (U.S.).

1.4 Problem Statement

There is increasing emergence of multidrug resistant NTS in food animal sources such as swine. Antimicrobial use in animal feed plays a significant role in horizontal transmission of antibiotic resistance in swine and other animal intensive production systems with subsequent transfer of antibiotic resistance to the human population through utilization of contaminated food animal sources. Heavy metal micronutrients usage such as zinc and copper in animal feed can co-select for antibiotic resistance pressure with development of tolerance and consequently increased multidrug resistance. In Kenya the prevalence of multidrug resistant NTS in swine production systems and mechanisms is determined in very limited studies, therefore limited data is made available on the function of heavy metal micronutrient use in animal feed in tolerance and multidrug resistant NTS. There is consequence transfer of resistance to the human population; this makes it difficult to come up with clear consistent figure as a guideline on usage of antibiotics in both human and animals.

1.5 Justification

The prevalence of multidrug resistant NTS strains in food animal sources have increased dramatically worldwide, this trend presents a great challenge to public health. Antimicrobial agents are mainly applied in human medicine to treat salmonellosis. In Agriculture antibiotics are used for therapy, metaphylasis, prophylasis and as growth promotion, this move has been controversial due to the emergence of multidrug resistant salmonella in animal intensive production systems. Studies on antibiotic resistance transfer and mechanisms of resistance mutations such as DNA gyrase have become a target for quinolone resistance in bacteria. Transfer of resistance genes such as plasmids, cross resistance and co-selection and genetic linkages have been recorded. In Kenya and Eastern Africa, however there is no sufficient data to show the work of heavy metal micronutrient use in animal feed and subsequent coming out of antibiotic resistance and resistant genes in NTS in animal production systems. This study aims to fill this gap and will be shared with public health sectors to formulate policies for controlled use and antibiotics in animal production systems.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Salmonella* Species and Disease

Salmonella are a category of strongly correlated rod-shaped, gram-stained negative bacteria with flagella characterized by specific proteins present on the bacterial and flagellar surface (www.emedicinehealth.com cited Sep 11, 2017). Members of this genus are motile by peritrichous flagella, nonflagellated variants, such as *Salmonella* serovar Pullorum and *Salmonella* serovar Gallinarum, and nonmotile strains ensuing from dysfunctional flagella occurs (Patchanee, 2008). *Salmonella* was named after Daniel E. Salmon who was a veterinary surgeon who isolate "*Bacillus choleraesuis*" from porcine intestines in 1884, the name was later altered in the year 1900 to "*Salmonella choleraesuis*" by Lignieres.

Salmonella genus is divided into 2 species namely; *Salmonella enterica* and *Salmonella bongori*, with *S. enterica* divided into 6 more subspecies (Michael *et al.*, 2017). The genus *Salmonella* is grouped into two species with multiple serotypes, this is according to the CDC system. The two spicies are *S. enterica,* and *S. bongori*, previously subspecies V (19, 21). *S. enterica* is grouped into six subspecies (4, 19), referred to by a Roman numeral and a name (I, *S. enterica* subsp. enterica; II, *S. enterica* subsp. salamae; IIIa, *S. enterica* subsp. arizonae; IIIb, *S. enterica* subsp. diarizonae; IV, *S. enterica* subsp. houtenae; and VI, *S. enterica* subsp. indica). *S. enterica* subspecies are differentiated biochemically (4, 19) and by genomic relatedness (Brenner, 2000). *Salmonella* bacteria are extensively spread in domestic and wild animals that are rampant in food animals and in pets (WHO cited January 2018). An illness of the bowel caused by *Salmonella* bacteria occurs in many wild animals, domestic animals and also in birds. This infection could easily infect humans

while all serotypes could cause diseases in humans, few are host-specific and can exist in only one or a few animal species: for example, *Salmonella enteric*a serotype dublin in cattle and *Salmonella enterica* serotype choleraesuis in pigs(WHO cited Feb. 2018).

A bacterial infection called salmonellosis can be easily transmitted into a person by taking contaminated food products and handling of pets (**www.everydayhealth.com** cited June 2015). The spread of salmonella from person-person occurs when feces of an infected person finds its way into food while cooking. (www.idph. cited January 2009). Salmonellosis ranges from *Salmonella* gastroenteritis to enteric fevers which are life-threatening febrile systemic illness that requires urgent antibiotic therapy. Crucial infections and an asymptomatic hauler state may however occur and the commonest variety of salmonellosis is a self-limited, simple gastroenteritis (Giannella, 1996). Animals are asymptomatic carriers of specific organisms and can contaminate the atmosphere to which children are exposed to **(**Sarah *et al.*, 2012).

Salmonellosis mainly presents as an acute gastroenteritis with abdominal pain, fever, vomiting, nausea, headache and diarrhoea and dehydration especially among small children and the elderly. The infection can also occur as septicaemia, and may occasionally be restricted to other body tissues. This leads to endocarditis, pneumonia, septic arthritis, cholecystitis and abscesses. Symptoms may last from 3–5 days (www2.health.vic.gov.au cited October 2015). Nontyphoidal salmonellosis is an illnesses commonly brought by all serotypes of *Salmonella* except for Typhi, Paratyphi A, Paratyphi B (tartrate negative), and Paratyphi C (Brunette *et al.*, 2017 and Coburn *et al.*, 2007). Intestinal/diarrheal disease *and* Human typhoid fever represents the most ordinary syndromes linked to *S. enterica* infection to involve the

pathogenic processes of both bacteria and the host, this is scrupulously investigated in infectious models of *Salmonella* pathogenesis (Coburn *et al.,* 2007).

Salmonella bacteria hold a number of virulence factors that contributes to its pathogenesis (microbewiki.kenyon.edu cited February 2016**).**
In addition to two T3SSs, *Salmonella* have a type I secretion system and other factors such as fimbriae, flagella and ion transporters with significant roles to establish and maintain the intracellular niche and many of these virulence factors are encoded on salmonella pathogenicity islands (SPI) on the chromosome, most notably, T3SS1 and T3SS2 are encoded on SPI1 and SPI2 respectively (Ibarra & Steele-Mortimer 2009). Invasion and early post-invasion processes are modulated by T3SS1, flagella, fimbriae and non-fimbrial adhesins; subsequently the T3SS2 and factors involved in nutrient gaining and evasion of antibacterial mechanisms are induced (Antonio, 2009). Due to its combined characteristics, *Salmonella typhi* becomes an effective pathogen with an endotoxin typical of gram negative organisms and the Vi antigen believed to boost virulence (web.uconn.edu cited September 2003).

Since antibiotics do not curtail the length of symptoms and can extend the duration of convalescent carriage, they are not habitually used to treat simple NTS gastroenteritis (emedicine.medscape.com cited July 2017).While most people infected by

Salmonella infections can recover within a week and doesn't require antibiotics, severe infections are mostly treated with either ampicillin, ceftriaxone, or ciprofloxacin and resistance to these drugs can result in increased hospitalization, invasive illnesses, and death(CDC, 2016). Due to resistance to ampicillin, chloramphenicol and trimethoprim/sulfamethoxazole in the late 1980s,

fluoroquinolones and extended spectrum cephalosporins became drugs of choice (Ramachandran *et al.*, 2017).

2.2 Antibiotic use in Swine Production

Production of conventional swine evolving regular use of antimicrobials and general incidence of antimicrobial-resistant *Salmonella* has been widely noted (Gebreyes *et al.*, 2006) and therefore in pigs, the bacterial antimicrobial resistance (AMR) is a vital public health as a result of its probable transition to human beings(Birkegard, 2017) . For a long time, *a*ntimicrobials have been used to treat diseases in animals, prevent and control diseases and also used as a growth promoters (Economou *et al.*, 2015) however, the overdose of antimicrobial agents brings some side effects on antimicrobial susceptibility (Kim, 2014).

Recently, serovars of *Salmonella* showed growth in resistance to conservative antimicrobials in animal and human therapy with grave danger to public health (Joao *et al.,* 2016). According to Gebreyes *et al.,* (2002) study on antibiotic resistance in swine in examining antimicrobial resistance of 1,257 isolates of 30 serovars of *Salmonella enterica* subsp. enterica isolated from swine, Copenhagen was extensive and became a multidrug resistant. The size of the population is significant due to its direct relationship with the antimicrobial exposure; age also becomes significant for intestinal microflora changes with the pig's age (Birkegard *et al.*, 2017).

Salmonella is closely associated with the swine production chain isolated in several production steps like animal transportation, primary production, the pre-slaughter and pre-evisceration steps (Filho, 2016). Investigations conducted to determine antibiotic resistance in all phases of pig production showed that majorly isolates from sows were pansusceptible than those from nursery or grow-finish pigs and they were

resistance to 2 or more drugs occurred in 29% of the isolates. This was considerably more likely to take place in *Salmonella* from nursery pigs than sows due to age-specific risk factor differences between production phases (Rosengren *et al.,* 2008). In South Africa, the highest level of antibiotics in animal husbandry is in swine and poultry farms operated in intensive systems. Organisms isolated from pigs presents strong resistant than those recovered from other animal sources. This is as a result of more rigorous use of antimicrobials in pigs. (Chinwe *et al.,* 2016). While in Kenya, Nyabundi, (2017) carried out a study to establish the antibiotic resistance profiles of domestic animals fecal matter and their products. The findings put the overall occurrence of Salmonella at 3.8, 3.6, 5.9 and 2.6% for pigs, chicken, eggs and cows correspondingly. The two serovars were isolated S. *typhimurium* (85%) and *S. enteritidis* (15%) to form distinct clades on the phylogenetic tree and 40% of the isolates were found resistant to one or more frequently used antibiotics like; sulphamethoxazole (200 µg), tetracycline (100 µg), cotrimaxazole (25 µg), nitrofurantoin (200 µg), nalidixic acid (30 µg), streptomycin (25 µg), gentamycin (15 µg) and ampicillin (25 µg). However, Kariuki *et al.,(*2002) investigated clonal association between NTS isolated from humans and animals and reported that many (97; 64.2%) of NTS from humans were multidrug resistant while NTS from cows, pigs, beef carcass swabs and sewers were vulnerable to tested antibiotics.

In Ghana, multidrug resistant *Salmonella typhimurium* was the prevalent isolates obtained from the studies done on pigs, the study from the 108 multiply-composite faecal samples, 72 *S. typhimurium* isolates were extracted from 72 separate composite samples representing 72 different pig farms. Of the 72 faecal isolates, 32 (52.8%) were found to be resistant to at least one antibiotic while twenty-seven isolates (71.1%) were resistant to amoxicillin and streptomycin. Resistance to tetracycline,

doxycycline, and ciprofloxacin was found in 17 (44.7%), 15 (39.5%), and 8 (21.1%) isolates, respectively (Sekyere & Adu 2015).

2.3. Transfer of Antibiotic Resistant Genes/Factors

Antimicrobial resistance is obtained through mutation or transfer of mobile genetic essentials like bacteriophages, transposons and plasmids. *Salmonella* becomes antibiotic resistant either through spontaneous mutation or via horizontal resistant gene transfers by transformation, transduction, or conjugation(Su *et al.*, 2004). Bacterial transformation is the process in which a receiver cell takes up bacterial free DNA molecules from the environment. Under in vivo circumstances, transformation only plays a partial function in the movement of resistance genes, while free DNA that originates from lysed bacteria is degraded on various ecological surroundings. Few bacteria like *S.pneumoniae* and *Bacillus* spp display an ordinary aptitude to take up DNA from its surrounding(Austin *et al.*, 2006) .Transduction is the movement of DNA from one cell to another through a replicating process of bacteriophage while bacterial conjugation ensures movement of genetic material between bacteria through direct cell-cell contact or via the sex pili.

Mobile genetic plays a major task on evolution and spread of multidrug resistance in gram-negative bacteria(Olsen & Larsson 2017). Many antibiotic resistance genes observed in gram negative microorganisms form part of a gene cassette put in an integron irrespective of the presence of antibiotic resistance and can confine gene cassettes from the surroundings and integrate them by use of site specific recombination. They are significance when included in pathogenic organisms such as *Salmonella* (Gebreyes *et al.*, 2004). The integration into transposons and plasmids, integrons takes part in the capture and distribution of resistance genes among bacteria.

Integrons contain an integrase *(int),* a 5' conserved integration site is called attachment (*attl*) and its promoter. The integrase (*int*) and integration site (*attI*) are liable of site specific integration. The 3' conserved segments carries genes to quaternary ammonium compounds (*qacΔE1*) and sulfonamides (*sul*) resistant gene cassettes with similar conserved attachment (*attC*) capable of integrating into integrons and is expressed using the promoter from the integration site.

The fact that genes yielding resistance to antibiotics are commonly used to treat human infections acquired by integron-harbouring strains potentiates and the possibilities of selection by a variety of different antimicrobials. Therefore, integron acquisition is the major cause of multiple resistance in gram-negative microorganisms(Wang *et al.*, 2013).The commonest cassettes contain genes that present resistance to a range of antimicrobial agents like, b-lactams, chloramphenicol and trimethoprim, aminoglycosides, and genes that present resistance to antiseptics and disinfectants (Wang *et al.,* 2013). A number of classes of integrons linked to antibiotic resistance have been identified and are known as the nucleotide sequence of their own integrase. So far, nine classes of integrons have been described with class 1 being the most rampant (Mazel & Davies 1999). Class 1 integrons have been reported in various countries in different *Salmonella* serovars and are situated on the so-called Salmonella genomic island 1 (SGI1) (Cirz &Romesberg 2006). SGI1 is an integrative 43 kb mobilisable chromosomal element on which antibiotic resistance genes are clustered. Flanked by two class 1 integrons, strains containing SGI1 becomes resistant to ampicillin (and amoxicillin), streptomycin, spectinomycin, chloramphenicol (and florfenicol), sulphonamides and tetracycline.

Fluoroquinolones, cephalosporins, and rifamycins antibiotics resistance comes by acquiring point mutations in genes that predetermine the drug's molecular targets or

proteins concerned in drug inactivation. Mutation is the sole way of obtaining resistance in *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* infections (Nies *et al.*, 1989).

2.4 Mechanisms of Antimicrobial Resistance

In countries like Zaire, Rwanda, Nigeria and Malawi, multidrug resistance have caused life-threatening persistent disease outburst in children (Lunguya *et al.*, 2013). In Tanzania, multidrug-resistant *S. enteritidis* was the core isolate in an epidemic of nosocomial meningitis in infants(Morpeth *et al.*, 2009), while in the Central African Republic (CAR) ,multidrug-resistant *S. typhimurium* became the main reason for community-acquired bacteraemic disease in adults and children (Cattoir *et al.*, 2007).

In developed countries, animal contact is not commonly regarded as a menace factor for NTS infection but transmission between humans becomes significant in African countries (Kariuki *et al.*, 2006). In Kenya, there is inadequate data on animal contact micronutrient use and multidrug resistance.

An increase in the antimicrobial resistance observed in *Salmonella* isolated from humans and animals associated to the rife use of antimicrobials as a significant public health issue and is recorded in many countries (Gunell *et al.*, 2009).

Several available mechanisms of antimicrobial resistance in *Salmonella* includes the production of enzymes such as *B*-lactams that inactivate antimicrobial agents, lessening of cell permeability to antimicrobials, activation of antimicrobial efflux pumps and change of the cellular target for antimicrobial agents (Gebreyes *et al.*, 2004). Miller, et al., (2004), stated that the aptitude of *Salmonella* to generate *β*-lactamases enzyme is a significant and widespread mechanism for resistance to penicillins and cephalosporins. *β*-lactamases. These are grouped into two main groups

based on resemblance in amino acid sequence (Ambler classes A through D)(Saboohi *et al.*, 2012) .

Ambler class C enzymes (Bush Group 1) are among the 9 important public health concerns. The AmpC enzyme, which is encoded by *bla*CMY is linked to resistance to a large number of *β*-lactam antimicrobials(Kiiru *et al.*, 2012). Aminoglycosides are targeted by various chemical changes that inactivate these drugs while the enzymes are termed as aminoglycoside modifying enzymes (AMEs).

The major groups include aminoglycoside, phosphotransferase(*aph*),aminoglycosidacetyltransferases (*aac*), and aminoglycoside adenyltransferases (*aad*) which function by phosphorylating, acetylating, and adenylating certain aminoglycosides, respectively (Ghaima *et al.*, 2016). Aminoglycoside phosphotransferase is an enzyme encoded by *aph*A and responsible for kanamycin resistance. The gene *aac*C encoding a functional enzyme aminoglycoside acetyltransferase is the gene encoding resistance to gentamicin, and genes *aad*A and *aad*B are respectively associated with streptomycin and gentamicin resistance (Ternhag *et al.*, 2008).

Gebreyes & Altier (2002) indicated that multidrug efflux systems are vital mechanisms of resistance in antimicrobial agents and other structurally unrelated compounds. Resistance to Zn2+ and other metals such as Co2+ and Cd2+ is conferred by genetic determinants carried by plasmid (Medardus *et al*, 2014). The CZC operon system in which the products of the czc gene clusters function as a major component of an efflux protein (CzcA), a cation funnel (CzcB), a modulator of substrate specificity (CzcC) and a protein involved in parameter of the operon (CzcD). The *CzcD* gene regulates zinc, cobalt, and cadmium efflux system, the Czc system also

mediates resistance to these heavy metal cations. Co-selection of heavy metal tolerant and antimicrobial resistant phenotypes occurs when the genes are co-located on same genetic element such as a plasmid, transposon or integron. This physical connection results in the co-selection for other genes located on the same element (Selander *et al.*, 1990).

Quinolone resistance in gram-negative pathogens is obtained by chromosomal mutations, primarily in the quinolone resistance-determining regions (QRDRs) of the target genes, *gyrA* and *gyrB*, which encode DNA *gyrase*, and *parC* and *parE*, which encode topoisomerase IV(Poirel *et al.*, 2005). Resistance is also acquired by reducing the accumulation of the antimicrobial by altered expression of porins that could lead to decreased penetration of fluoroquinolones within bacteria or increased efflux of quinolones from the bacterial cell(Nies *et al.*, 1989). Pumps are specific for one substrate or can transport a range of structurally dissimilar; they are normally associated with multiple drug resistance.

In *Salmonellae*, where DNA gyrase is the main target of quinolone action, a single point mutation in the quinolone resistance-determining region (QRDR) of *gyrA* mediates resistance to the nonfluorinated quinolone nalidixic acid and condensed susceptibility to fluoroquinolones e.g., an MIC of 0.25g/ml (Eaves *et al.*, 2004). According to Ferrari, et al., (2013), mutations in the *gyrB* and topoisomerase IV genes *parC* and *parE* are rare in *Salmonellae* (Winokur *et al.*, 2000). It was however hypothesized that those isolates with decreased susceptibility harbored a sole mutation in *gyrA*, whereas resistant isolates contained many mutations in *gyrA* and/or *gyrB* and/or *parC* and/or *parE*(Cirz & Romesberg 2006).

Resistance to fluoroquinolones in *Salmonellae* occurs as a result of mutations in the quinolone resistance—determining region (QRDR) of the DNA gyrase genes like other members of the Enterobacteriaceae (Saboohi *et al.*, 2012). The most frequently observed point mutations in *gyrA* of fluoroquinolone-resistant *Salmonellae* are the changes of the amino acids at codon 83 from serine to phenylalanine, tyrosine, or alanine and at codon 87 from aspartic acid to glycine, asparagine, or tyrosine (Nies *et al.*, 1989).Mutations at threonine 86, Asp-90, and Ala-70 in the gene encoding DNA gyrase *(gyrA*) result in quinolone resistance in *Campylobacter jejuni*, although mutations at Thr-86 are most common , high level resistance to nalidixic acid (64 to 128 ug/ml) and ciprofloxacin (16 to 64mg/ml) is associated with mutations at Thr-86-Ile(6).Resistance to fluoroquinolones is typically mediated by alterations in the target enzymes DNA gyrase (*GyrA* and *GyrB*) and topoisomerase IV *(ParE* and *ParC*) or by changes in drug entry and efflux (Poirel *et al.*, 2005) However *Qnr* (later termed *QNRA*) a plasmid mediated quinolone resistance determinant had been reported in 1998 from *Klebsiella pneumoniae* first from the United states, It has been reported since then in *Citrobacter freundii ,Escherichia coli, Enterobacter cloacae, Enterobacter sakazakii, Klebsiella pneumoniae* and *Klebsiella oxytoca* from Asia and Europe (Cattoir *et al.*, 2007). *Qnr* are pentapeptide repeat proteins that may prevent quinolones from binding to DNA topoisomerases, they confer resistance to nalidixic acid and reduced susceptibility to fluoroquinolones. Resistance to quinolone and flouroquinolone is in many cases linked to ESBL-producing organisms and integron carrying *qnr.* Studies shows the presence of *qnr* and *bla* genes on similar plasmid as one of the several possible explanations, *qnrA* gene co-resistance with blaCMY in some isolates may be on the same plasmid(Lunguya *et al.*, 2013).

The PMQR *qnrA* gene encodes a 218-amino acid protein to protect DNA-gyrase and topoisomerase IV from the quinolones' activity, this pentapeptide blocks the action of Cip, resulting in a low-level quinolone resistance with the addition of minimal inhibitory concentration of ciprofloxacin (CipMIC) (Gebreyes & Altier 2002) . Plasmid mediated quinolone resistance (PMQR) confer inferior susceptibility to fluoroquinolones and becomes background for selection of chromosome encoded resistance. The three main mechanisms involved in PMQR includes, qnr peptides protect topoisomerases, variant of aminoglycoside acetyltransferase *(aac (60)-Ib-cr),* and QepA protein modulates quinolone efflux pump (Eaves *et al.*, 2004).

The plasmid-borne *qnr* genes comprises of three families, *qnrA, qnrB,* and *qnrS,* whose nucleotide sequences vary from each other by 40% or more. The geographical distribution of *qnrA* genes is wide but that of the newer *qnr* types, *qnrB* and *qnrS* have been reported within China (Nies *et al.*, 1989). One of the main antimicrobial resistance mechanisms of *Salmonella* is conferred by lessening the ease of access of the antimicrobial to its target (Morpeth *et al.,* 2010) . Resistance to tetracycline and chloramphenicol is linked through the face of drug-efflux proteins (Jacob *et al.*, 2010). These integral-membrane proteins use energy derived from proton motive force to pump the antimicrobials out of the cytoplasm. Most of the tetracycline resistance efflux pump genes in the *tet* families is encoded by efflux pumps (Rutherford & Bird 2004), while chloramphenicol efflux pumps are encoded by *cml* and *flo* genes . The *flo* gene share 57% amino acid sequence uniqueness to *cml* gene encodes an efflux pump that confers resistance(Hill *et al.*, 1998), (Hasman *et al.*, 2006).

The sulfonamide and trimethoprim competitively inhibit the synthesis of folic acid. Resistance to sulfonamides is often required either *sul*I or *sul*II which encodes a drug-

resistant dihydropteroate synthase enzyme that have a decreased affinity for sulfonamides. Trimethoprim resistance in *Salmonella* can be conferred by *dhfr* genes encoded dihydropteroate synthase enzyme that has reduced affinity for the antimicrobials (Jacob *et al.*, 2010).

2.5 Heavy Metal Tolerance and Antibiotic Resistance

The surfacing and perseverance of MDR *Salmonella* serovars in a swine production surroundings where there is no narration of antimicrobial use recommend the existence of other risk factors such as selective pressure (Molla *et al.*, 2010).

Micronutrients like copper and zinc are incorporated in swine feed and other livestock to attain enlarged promotion as well as increasing feed effectiveness (Laupland *et al.*, 2010). Zinc and copper are important trace essentials for prokaryotic and eukaryotic cellular metabolic functions whereby Zinc becomes a cofactor of more than 300 metalloenzymes and copper is required to activate several oxidative enzymes for normal cellular metabolism(Miller *et al.*, 2005). Due to useful impact of zinc and copper in swine production, in-feed supplementation of zinc and copper in commercial production systems has become widespread (Fedorka-cray *et al.*, 1995) . On the other hand, lenience to various chemicals among bacterial pathogens, mediated by different mechanisms has also risen.

Multidrug efflux systems have become significant mechanisms of resistance against antimicrobial agents and other structurally unrelated compounds. The mechanisms of heavy metal resistance to copper in *Enterococcus faecium* isolates from pigs are linked to the carriage of a conjugative plasmid hauling copper resistance determinants like *tcrB* (Horter *et al.*, 2017) . Another efflux system linked to copper tolerance reported in gram-negative organisms is the PCO operon mediates resistance to

Cu^{+2}.Resistance to Zn^{+2} and other metals such as Co^{+2} and Cd^{+2} is conferred by genetic determinants approved by a plasmid.

In the CZC operon system the products of the *czc* gene clusters is the main component of an efflux protein(CzcA), a cation funnel (CzcB) and a modulator of substrate specificity(CzcC) (Hill *et al.*, 1998). The *CzcD* gene regulates zinc, cobalt, and cadmium efflux system, while the Czc system mediates resistance to heavy metal cations (Medardus *et al.*, 2014). Coselection of heavy-metal-tolerant and antimicrobial-resistant phenotypes occurs in many cases when the genes are collocated on genetic elements (Gebreyes & Altier 2002).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design and Sample Size

The study was a fraction of an ongoing study project "Molecular epidemiology and key issues of foodborne pathogens in Eastern Africa". Archived multidrug resistant NTS Salmonella isolates from three geographical locations were selected. These consisted of eighty six(86) from Kenya, thirty five (35) isolates from Ethiopia and fifty (50) isolates from USA/Ohio a total of one hundred and eighty four (171) isolates. The isolates were from swine fecal sources with antibiotic resistance characteristics as shown in (appendix2).

3.1.1 Purification of Archived Samples Prodecure

The isolates preserved in tryptone soya broth were revived from a freezer (-80^0C) through a series of culturing and subculturing in xylose-lysine-tergitol 4 (XLT-4) selective agar and plain media (Mueller Hinton) to obtain pure colonies. Using a wire loop a loopful of isolate was collected from the tryptone soya broth and streaked on XLT-4 media and incubated at 37^0C for 18-24 hours. Bacterial colonies with *Salmonella* characteristics, black or black centered coloration (hydrogen sulphide) with a yellow periphery were selected and subcultured on Mueller Hinton agar and the culture plate was incubated at 37^0C for 18 hours. Pure colonies were then selected and stored in freshly prepared tryptone soya broth.

3.1.2 Procedure for Determination of MIC of Copper Sulfate and Zinc Chloride in Nontyphoidal Salmonella

Eighty six (86) multidrug resistant NTS Kenya isolates with different antimicrobial resistant patterns were selected from archived *Salmonella* isolates recovered from swine fecal samples, for lenience to various concentrations of zinc chloride ($ZnCl_2$)

and copper sulfate ($CuSO_4$). The agar plate-dilution technique was applied to verify the MIC for multidrug resistant NTS to different dilutions of the heavy metals copper and zinc (Appendices 1 and 2) .The MIC was determined on Mueller-Hinton (MH)-II agar plates with the following dilution ranges: zinc chloride: 0, 0.25, 0.5, 1, 2, 4, 8 and 16mM with the pH of the medium adjusted to 5.5. Copper sulfate solutions contained the following dilution ranges: 0, 1, 2, 4, 8, 16, 20, 24, 28 and 32 mM with the pH of the medium attuned to 7.2. 20 ml of MH agar which was later dispensed on to the plates with the solutions and permitted to harden. Bacterial suspensions were attuned to 10^7CFU/ml (100µl of each inoculum at 0.5 McFarland + 900µl of sterile 0.85% NaCl solutions). Each of the 400 µl bacterial suspension was aseptically aliquoted to an equivalent to the replicator inoculum block and inoculated onto the Mueller Hinton agar plate. All test *Salmonella* isolates and control strains were tested in triplicate and the inoculated plates were incubated at 37^{o}C for 16 h. Plates were assessed for growth and MIC determined and defined as the lowest concentration to inhibit the noticeable growth of Salmonella. *Staphylococcus aureus* ATCC 29213 was chosen as the reference/control strain due to its resistance to heavy metal ions.

3.1.3 DNA Preparation

The genomic DNA was extracted by use of DNeasy Blood and Tissue kit (Qiagen, Valencia, California, USA) this followed the manufacturer's instruction as summarized in the procedure below:

1. Bacterial suspension was prepared by placing two to three colonies into 200 ul phosphate buffered saline (BPW) and the cells adjusted to 2 x 10^9 and harvested in a microcentrifuge tube by centrifuging for 10 min at 5000 x g (7500 rpm). Supernatant was discarded and the pellet re-suspended in 180 µl Buffer ATL.

2. 20 µl proteinase K was added and Mixed thoroughly by vortexing, and incubated at 56°C until the cells were completely lysed. Vortexing was done occasionally during incubation to disperse the sample using rocking incubator.

3. The mixer was Vortexed for 15 s and 200 µl Buffer AL was added to the sample, and mixed thoroughly by vortexing. Then 200 µl ethanol was added (96–100%), and mixed again thoroughly by vortexing.

4. The mixture from step 3 (including any precipitate) was pipeted into the DNeasy Mini spin column placed in a 2 ml collection tube (provided) and Centrifuged at _6000 x g(8000 rpm) for 1 min. The flow-through and collection tube were discarded

5. The DNeasy Mini spin column was placed in a new 2 ml collection tube (provided), and 500 µl Buffer AW1 added and centrifuged for 1 min at _6000 x g (8000 rpm). The flow-through and collection tube were discarded

6. The DNeasy Mini spin column was placed in a new 2 ml collection tube (provided), and 500 µl Buffer AW2 added and centrifuged for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. The flow-through and collection tube were discarded.

7. The DNeasy Mini spin column was then placed in a clean 1.5 ml or 2 ml microcentrifuge tube, and 200 µl Buffer AE pipeted directly onto the DNeasy membrane and incubation was done for 1 min at room temperature. DNA was eluted by centrifugation for 1 min at _6000 x g (8000 rpm).

3.1.4 PCR Amplification of Heavy Metal Micronutrient *pcoA* and c*zcD* (Cu2+ and Zn2+) Tolerance Genes

The Eighty six (86) multidrug resistant NTS Kenya isolates were tested for the carriage of selected tolerance genes *pcoA* for copper tolerance and *czcD* for zinc tolerance using PCR technique. *Salmonella* isolates were inoculated onto Tryptic Soy agar (TSA) plates and incubated at 37°C overnight. The PCR Primers that were used for amplification of *pcoA* gene included: Forward: (5'-CGTCTCGACGAACTTTCCTG-3') and Reverse: (5'-GGACTTCACGAAACATTCCC-3'). The thermo-cycling circumstances included hot start *Taq* polymerase 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 magnification was done in 34 cycles (34). Primers used for amplification of *czcD* gene included Forward (5' TTTAGATCTTTTACCACCATGGGCGC-3') and Reverse (5'-TTTCAGCTGAACATCATACCCTAGTTT-3'). The PCR amplification conditions included initial denaturation at 94°C for 2.5 min, annealing at 60°C for 1 min, extension at 72°C for 1 min and then amplification cycle repeated for further 28 cycles and final extension at 72°C for 5 min. 10 µl of the PCR product of each isolate tested was electrophoresed 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.

3.1.5 PCR Amplification of QRDR *gyrA* Gene Procedure

PCR was used to amplify the QRDR from *gyrA* using primers st*gyrA*1 5'-CGTTGGTGACGTAATCGGTA-3' (forward) and st*gyrA*2 5'CCGTACCGTCATAGTTATCC-3' (reverse); these primers cover the nucleotides from codon Val70 to Thr152 with an amplimer size of 251bp. The primers (reverse and forward) were constituted using PCR water to a concentration of X10. The master mix was commercially obtained in the form of PCR beads containing the following; dNTPS, *Taq* polymerase, Magnesiun chloride, the master mix was then constituted to a stock concentration of X100 by adding 100ml of PCR water. The mixture was homogenized using a PCR mixer and a working concentration of X 10 in the ratio of 1:9 was prepared for running the PCR

In a clean bench, PCR reaction was prepared in 25 volume PCR tubes as follows; 1ul Reverse primer, 1ul Forward primer, 22ul master mix and 1ul pure DNA template. Thermocycling conditions were as follows; (i) an initial denaturing step of 10 min at 95^{o}C; (ii) 30 cycles of PCR, with 1 cycle consisting of 1 min at 95^{o}C,1 min at 52^{o}C, and 30S at 72^{o}C; and (iii) a final extension step of 10 min at 72^{o}C. 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.

3.1.6 PCR Amplification of the PMQR *qnrA* Gene Procedure

Supernatant containing the DNA template was aspirated and placed in a PCR tube and the bacterial lysate discarded. The primers used to amplify *qnrA* to give 516 base pair (bp) product were 5'-ATTTCTCACGCCAGGATTTG (forward) and 5'GATCGGCAAAGGTTAGGTCA (reverse). The master mix was commercially

obtained in the form of PCR beads containing the following; dNTPS, *Taq* polymerase, Magnesium chloride. The master mix was constituted to a stock concentration of X100 by adding 100ml of PCR water. The mixture was homogenized using a PCR mixer and a working concentration of X 10 In the ratio of 1:9 was prepared for running the amplifications. In a clean bench PCR reaction was prepared in 25 volume PCR tubes as follows; 1ul Reverse primer, 1ul Forward primer, 22ul master mix and 1ul pure DNA template.

PCR conditions were; denaturation at 94^{o}C for 45s, annealing 53^{o}C for 45 s, and Elongation at 72^{o}C for 60s, cycled 32 times. 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.

3.1.7 PCR Amplification of Class 1 Integron Procedure

Variable region of class I integrons was amplified using the following PCR primers: Forward, GGC ATC CAA GCA GCA AG; Reverse, AAG CAG ACT TGA CCT GA. The master mix was commercially obtained in the form of PCR beads containing the following; dNTPS, *Taq* polymerase, Magnesiun chloride.

The master mix was constituted to a stock concentration of X100 by adding 100ml of PCR water. The mixture was homogenized using a PCR mixer and a working concentration of X 10 In the ratio of 1:9 was prepared for running the amplifications. In a clean bench PCR reaction was prepared in 25 volume PCR tubes as follows; 1ul Reverse primer, 1ul Forward primer, 22ul master mix and 1ul pure DNA template. Amplification reactions were as follows; initial denaturation for 5 min at 95^{o}C and 30

cycles of denaturation for 1 min at 95°C, primer annealing for 1 min at 54°C, and extension for 1 min at 72°C.

Ten microliters of the PCR product was stained using three microliters of bromothymol blue and 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.

3.1.8 Sequencing Procedure for gyrA, qnrA and Class 1 Integrons

PCR products from the amplification of *gyrA, qnrA* and Class 1 Integrons were processed for sequencing using QIAquick Gel Extraction Kit ((Qiagen, Valencia, California, USA) according to manufactures instructions. Aliquots of 5ul were prepared in PCR tubes for each PCR product separately. The tubes were packaged in an icebox and the parcel was send to an external facility for sequencing, Genewiz sequencing firm (Genewiz, Washington DC, United States).

3.1.9 Procedure for the Amplification of *SpvA* Gene

The primers used to amplify *SpvA* gene to give 641 base pair (bp) product were 5'-GTCAGAACCCGTAAACAGT (forward) and 5'GCACGCAGAGTACCCGCA (reverse). The master mix was commercially obtained in the form of PCR beads containing the following; dNTPS, *Taq* polymerase, Magnesiun chloride. The master mix was constituted to a stock concentration of X100 by adding 100ml of PCR water. The mixture was homogenized using a PCR mixer and a working concentration of X 10 In the ratio of 1:9 was prepared for running the amplifications. In a clean bench PCR reaction was prepared in 25 volume PCR tubes as follows; 1ul Reverse primer, 1ul Forward primer, 22ul master mix and 1ul pure DNA template. PCR conditions were; denaturatuion at 95^0C for 5min,95^0C for 1min, Annealing 540C for 1min and

final extention 72^0C for 1min for 30 cycles. 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.

CHAPTER FOUR

RESULTS

4.1 Susceptibility of MDR nontyphoidal *Salmonella* Isolates to Copper Sulphate and Zinc Chloride

For the 86 Kenyan isolates subjected to MIC determination for zinc and copper, 98% (n=84) of the isolates had an MIC of 4μg/ml, 1.2 % (n=1) MIC of 0.5 μg/ml, and 1.2 % (n=1) of the isolates had MIC of 0.25 μg/ml for Zinc. For copper, 98% (n=84) had MIC of 8 μg/ml and additionally 1.2% (n=1) of the isolates exhibited MIC of 1.0 μg/ml and 1.2% (n=1) MIC of 2.0 μg/ml . The MIC was defined as the lowest concentration that inhibits the visible growth of *Salmonella*.

Table 4.1 MIC for $CuSO_4$

MDR nontyphoidal *Salmonella* Isolates 86	F(N)	%	MIC(Mm)
	84	98	8
	1	1.2	1.0
	1	1.2	2.0
Total	**86**		

$CuSO_4$ - Copper Sulphate

F- Frequency

Table: 4.2 MIC for $ZnCl_2$

	F(N)	%	MIC(Mm)
MDR nontyphoidal *Salmonella* Isolates 86	84	98	4
	1	1.2	0.5
	1	1.2	0.25
Total	**86**		

Key

ZnCl - Zinc chloride

F -frequency

4.2 Heavy Metal Tolerance Genes *pcoA* and *czcD*

For MDR nontyphoidal *Salmonella* Kenyan isolates amplified for the detection of heavy metal tolerance genes, Zn^{2+} tolerance genes *czcD* and copper extrusion efflux gene *pcoA* were not detected in any of the isolates regardless of their heavy metal MIC or MDR status.

4.3 QRDR *gyrA* and PMQR *qnrA* Amplification

Out of the fifty (50) isolates amplified thirty seven (37) isolates contained *gyrA* gene of 251bp and one (1) isolate contained *qnrA* gene of 516 base pair (bp) product, by PCR amplification.

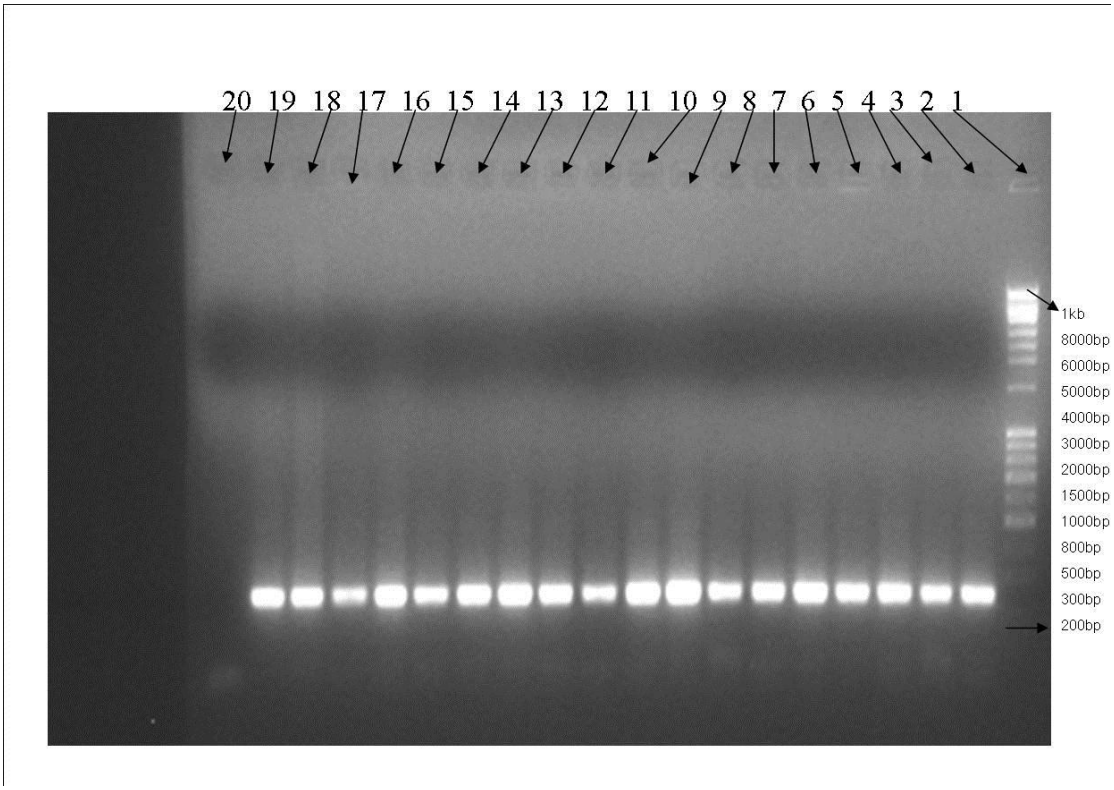

Figure 1: PCR Amplification Plate for *gyrA* gene (251bp)

Key: Cell number 1kb- DNA ladder, Cells 2 to 19 NTS DNA template, cell 20 negative control (PCR water).

4.4 *GyrA* and *QnrA* Gene Mutation Detection

Fifty isolates were screened for QRDR mutation of *gyrA* by sequencing. Forty six (46) isolates were from Kenya and Five (4) isolates from Ethiopia. Thirty seven (37) out of the 45 Kenyan isolates were ciprofloxacin resistant and eight (8) were PanSusceptible and one isolate was multidrug resistant without ciprofloxacin resistance. The thirty seven (37) Kenyan isolates produced good quality sequencing products. Of the 37 ciprofloxacin resistant good quality sequences analysed, mutation was observed .There was a change at codon Ser83 to either Phenylalanine(N=17), Glycine(N=4),Isoleucine(N=3),Cysteine (N=7). Five (5) Isolates gave no change to

codon ser83 and 2 isolates gave no amino acid codon at position83. There was observed a change in codon Asp87 to either Arginine(N=10)Lysine(N=3), Glycine(N=6),Tyrosine(N=1),Serine(1),Asparagine(N=5),Alanine(N=2) and 2 isolates did not show amino acid codon at position Asp87. Resistance to ciprofloxacin is associated with mutations at Ser83 codons substituted by phenylalanine and Asp87 codon substituted by either asparagine, glycine and tyrosine. Of the eight (8) pan susceptible isolates 2 isolates showed no mutations at both codons Ser83 and Asp87. Two isolates also gave no amino acids in both positions. Four (4) isolates showed synonymous mutations at codons Ser83 and Asp87 with codon Ser83 substituted by either Cysteine (N=1), Alanine (=2), Isoleucine (N=1) and codon Asp87 substituted by either Arginine (N=1), Alanine (N=2)(1) additionally, one isolate was multidrug resistant and ciprofloxacin susceptible and showed mutations at codon Ser83 to phenylalanine and mutation at codon Asp87 to Glycine as shown in (Table 3). Isolates from Ethiopia did not show any mutations. PMQR *qnrA* gene was detected in one Kenyan MDR nontyphpidal *Salmonella* isolate that was Ciprofloxacin resistant and with mutation in the QRDR *gyrA* gene at Asp 87 position (substituted to Asparagine) and with anonymous mutation at Ser 83position (substituted by Isoleucine).

Resistant type	Number(n)	GyrA mutation types Ser83	Asp87	qnrA (Y=1, N=0)
AmcAmpCCefCipNeoStrSulTet	1	Poor quality seq		0
AmcAmpChlCipFenNalSptStrSul Tet	1	Phenyl (1)	Gly(1)	0
AmcAMPCipGenNalSptStrSulTet	2	Gly (1) Lysine(1)		0 0
		Ser (1) a.a(1)	No	
AmcAmpCipNalStrSulTet	1	No a.a(1)	Gly(1)	0
AmcCmpCipNal	1	Pheny(1)	Asp(1)	0
CipGenNalSptStrSulTet	2	Iso(1) Gly(1)		0 0
		Pheny(1)	Asp(1)	
CipKanNalNeoNitStrTet	4	Pheny(3)	Asp(3)	0
		Ser(1) Arg(1)		0
CipNalNit	5	Pheny(4)	Asp(4)	0
		Poor quality(1)		0
SuCip	19	Iso (2)		1
		Aspar(2)		0
		Ser(2)		0
		Aspar(2)		0
		Gly(2)	Lys(2)	0
		Pheny(2)	Aspar(2)	0
		Cysteine(5)	Argi(5)	0
		Pheny(1)	N (1)	0
		N(1)		
		Tyrosine(1)		
		Ser(1)	Asp(1)	

Table 4.3: Mutations of *gyrA* and *qnrA* Gene Identified by Sequencing of MDR Nontyphoidal *Salmonella* Isolates from Swine Fecal Samples

Key: Pheny-phenylalanine, Gly-glycine, Ser-serine,a.a-amino acid, Iso-isoleucine,Arg-arginine,Aspar-aspartic acid,Asp-asparigine,Lys-lysine.

Amc-amoxicillin, Amp-ampicillin, C-chloramphenicol,Cef-cefexime,Cip-ciprifloxacin,Neo-neomycin, Str-streptomycin, Sul-sulfamethoxazole,Tet-tetracycline, Kan-kanamycin, Nit-nitrofurantoin.

4.5 Class1 Integron Profile

Class 1 integrons were detected in 9 isolates from Ethiopia and one (1) isolate from Kenya.

Forty five isolates were selected for class 1 integron detection based on antibiotic resistance pattern. Thirty one (31) isolates were from Kenya with antibiotic resistance pattern SulTe and fourteen (14) isolates from Ethiopia with antibiotic resistance pattern SulTeStr. One (1) Kenyan isolate was positive for class1 integron of 1.2 kb. For Ethiopian isolates seven(7) isolates were positive for class 1 integrons of 1.5 kb, one(1) isolates was positive for class 1 integrons of 0.7 kb and one(1) isolate was positive for class 1 integron of 1.2 kb. The ten isolates positive for class1 integrons were sequenced for detection of the presence of gene cassettes. Three gene cassettes: aadA7, dfr7 and aadA2 (aminoglycoside acetyltransferase gene) were detected from three Ethiopian isolates of class 1 integrons 1.5kb (n=2) and 1.2k b (n=1) respectively and two gene cassettes: aadA7 and dfr7 (dehydrofolate reductase gene) were detected from Kenyan isolate of 1.2 kb. The remaining six Ethiopian isolates of 1.5kb (n=5) and 0.7kb (n=1) were all negative for the presence of gene cassettes as shown in Table 4.

Isolate	Origin	Resistance pattern	Class1 integron Size (kb) gene cassette
S11589	Ethiopia	CipNalSptStrSulTet	1.5 [*aadA2*]
S11591	Ethiopia	AmpStrSulSxlTetTmp	1.5 [*dhfr* 7-*aadA7*]
S16612	Ethiopia	AmpStrSulSxlTetTmp	1.2[*aadA7*]
S11579	Ethiopia	AmcAMPCipGenNalSptStrSulTet	0.7 [Negative]
S11581	Ethiopia	AmcAmpCipGenNalSptStrSulTet	1.5[Negative]
S11583	Ethiopia	AmcAmpCipNalStrSulTet	1.5[Negative]
S11584	Ethiopia	CipGenNalSptStrSulTet	1.5[Negative]
11586	Ethiopia	CipNalSptStrSulTet	1.5[Negative]
S11588	Ethiopia	CipNalSptStrSulTet	1.5[Negative]
S16579	Kenya	SuTe	1.2 [*aadA7] [dhfr* 7]

Table 4.4: Class 1 Integron Gene Cassettes Sequences

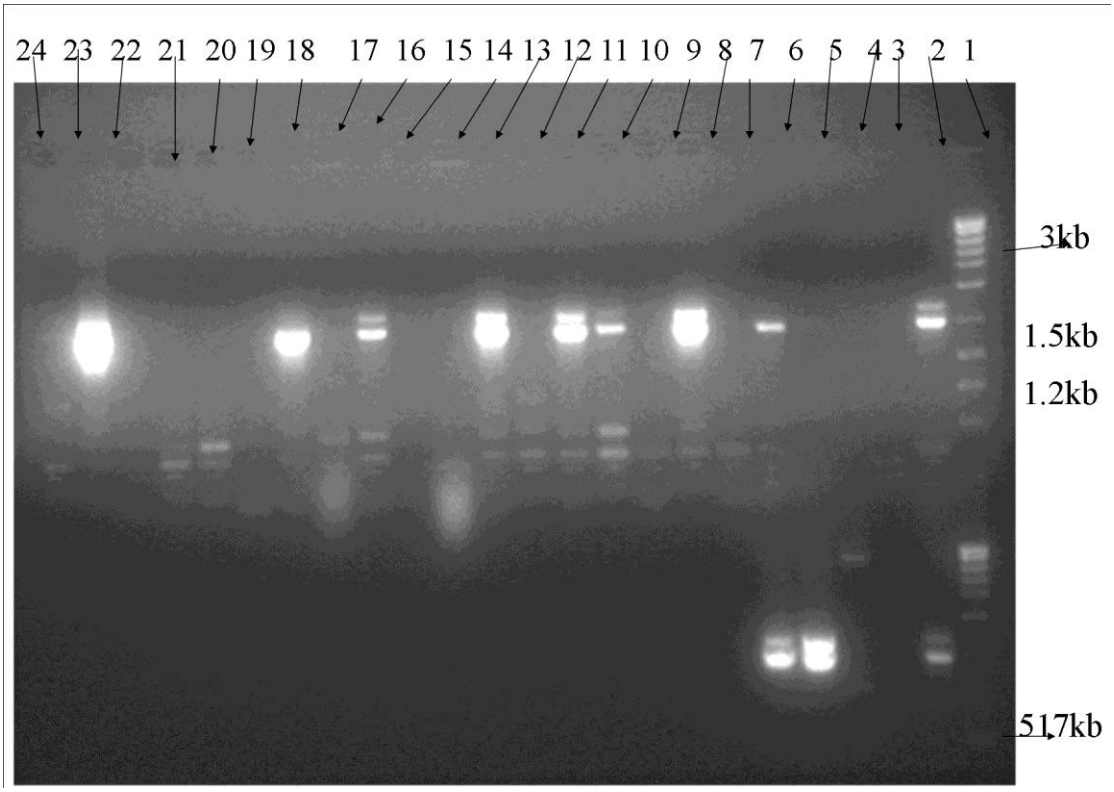

Figure 2: PCR Amplification Plate for Class 1 Integron (1.2kb, 1.5kb)

Key: cell number 1kb- DNA ladder, Cells 2 to 24 NTS DNA template.

Figure 3: Multiple Protein Sequence Alignment of *gyrA* Genes

Colour green g represents point of mutations at position Ser 83

Colour blue b represents point of mutation at position Aspar 87

4.6 *SpvA* Gene Detection

Three (3) out of thirty eighty (38) isolates tested were positive for pcr product of 641 base pair.

CHAPTER FIVE

DISCUSSION

The major finding of this study showed that multidrug resistant NTS does not share metallic ion genes (copper and zinc) of any significance compared to fully susceptible strains, and that NTS isolated from swine do carry antibiotic resistant genes that encode resistance to multiple antimicrobials. In this study the minimum inhibitory concentration (frequency) of heavy metal micronutrients copper pcoA Cu^{+2} and czcD Zn^{+2} from multidrug resistant NTS isolates from Kenya and Ethiopia was very low (3%) and there was no detection of resistance genes to heavy metals copper and zinc among all the tested multidrug resistant NTS isolates, the MIC for heavy metal micronutrients copper and zinc was found to be ($Cu^{2+} <$ 8mM, $Zn^{2+} >$ 4mM).

There is little published data on heavy metal tolerance genes in NTS from swine fecal sources in sub-Saharan Africa, however, heavy metal tolerance genes have been detected in multidrug resistant NTS isolated from clinical sources from patients with bacteremia (Kariuki *et al.*, 2015). In this study, Kariuki and his team detected heavy metal tolerance genes in multidrug resistant *Salmonella typhimurium* isolates from an outbreak involving patients, both adults and children, who firstly failed to react to ceftriaxone treatment at a referral hospital in Kenya, whereby, nine multidrug resistant *Salmonella enterica* serotype Typhimurium isolates with resistance to ampicillin, chloramphenicol, cefuroxime, ceftriaxone, aztreonam, cefepime, sulfamethoxazole-trimethoprim, and cefpodoxime were characterized and the results showed that resistance to β-lactams, including to ceftriaxone, was connected to carriage of a combination of *bla*CTX-M-15, *bla*OXA-1, and *bla*TEM-1 genes and the genes encoding resistance to heavy-metal ions that includes mercury (*mer* and *tni*

genes), tellurite (*ter* genes), arsenic (*ars* genes), and copper (*cusS* and *pcoE* genes) , were detected and were borne on the novel IncHI2 plasmid pKST313, which also carried a pair of class 1 integrons. This present study contrasts also with similar studies that showed the occurrence of a tough relationship between heavy metal resistant genes and multidrug resistant NTS isolated from swine whereby the MICs of Cu^{+2} was >20mM and for Zn^{+2} MIC (≥8 mM)(Merdadus *et al.,* 2014).

Many studies have been done on the function of heavy metal micronutrients in swine feed and the resultant increased multidrug resistant NTS in the United States(Miller *et al.*, 2005). A study by Gebreyes (Gebreyes *et al.,* 2011) suggest that the use of copper in swine feed results in higher tolerance of *Salmonella* strains to copper which in turn co-selects for antimicrobial resistance, in his study there was a significant association between the concentration of copper in feed and the MIC of isolates recovered from fecal samples for copper and heavy metal tolerance was also significantly associated with distinct multi-drug resistance types.

In addition a study in Europe shows that acquirement of Cu/Ag tolerance genes (*sil/pco* genes) might add to the appearance of particular clinically pertinent multidrug resistant NTS serotypes/clones by facilitating their endurance in diverse metal-contaminated settings, in this study done to determine the tolerance to multiple metal stressors in emerging NTS multidrug resistant serotypes and the relevant role for copper in anaerobic condition, *sil+pco* genes were detected in 37% of isolates from diverse serotypes, mainly in emergent Rissen/ ST469 and Typhimurium/ST34 European clone (100%) mostly linked to pig settings where Cu is used.(Joana Moura *et al.,*2016). In the present study absence of metal tolerance genes **(***pcoA* and *czcD* genes) for copper and zinc in multidrug resistant NTS from swine fecal sources in Kenya would probably be due to rare usage of micronutrient heavy metals copper and

zinc in animal feed most likely because in Ethiopia and Kenya micronutrients are not added in swine feed in swine production systems unlike in the developed world like the United States. Alternatively, other micronutrients may be usable in swine feed like arsenic among many others which were not tested in the study.

Salmonella plasmid virulence gene (*spvA*) was reported in multidrug resistant NTS with antibiotic resistant pattern SulCip. However there is little published data in Kenya on *spvA* antibiotic resistance genes detected in NTS isolated from swine fecal sources. However in developed countries similar studies have been done and *spvA* gene has been detected in addition to other *spv* genes in the SPV operon such as *spvBC*. For example, *S. typhimurium* isolated from apparently healthy pigs with antibiotic resistance pattern (ampicillin, kanamycin, sulfisoxazole, tetracycline and streptomycin) showed the presence of not only antibiotic resistance genes but also virulence genes such as *spvABC* (Nguyen *et al.*, 2009).

In addition a virulence plasmid was identified in a multidrug-resistant *Salmonella enteric*a serotype Typhimurium strain carrying the *spvC, rck*, and *pefA* virulence genes and two class 1 integrons linked to the Tn21 and Tn1696 transposons (Laura *et al.,* 2005). A similar study showed that Salmonella plasmid-located virulence genes *spvR, spvA, spvB, rck* and *pefA* were found on an IncFIB resistance plasmid from *S. typhimurium* isolated from pig production systems.

Class 1 integrons were detected in this present study and although Class 1 Integrons have been detected in a study done by Onsare(Onsare, 2011) from multidrug resistant NTS isolated from NTS bacteraemia patients treated at various hospitals in Nairobi, Kenya whereby In his study the most common resistance profile associated with both class I and 2 integrons included chloramphenicol-sulpbamethoxazole, ampicillin and

gentamicin and Class 1 integrons were more prevalent among the MDR strains analysed compared to class 2 integrons and contained a gene cassette in the variable region and the most common product at this variable region was approximately 739 bp. In addition a study by Kariuki (Kariuki et al., 2015) to investigate Ceftriaxone resistance *S. typhimurium* isolates from an outbreak involving patients who initially failed to respond to ceftriaxone treatment at a referral hospital in Kenya, detected a 300-kb IncHI2 plasmid which harbors genes conferring resistance to antimicrobials including aminoglycosides (aadA1),streptomycin (strA and strB), B-lactams (blaTEM-1), chloram- phenicol (catA1), trimethoprim (dhfr1), and sulfonamides (sul1 and sul2), the majority of which are within a class 1 integron. There is however little published data on class 1 I ntegrons detected from NTS isolated from swine fecal sources. One study by Kikuvi (Kikuvi *et al.*,2010) reported the occurrence of mobile genetic elements and antimicrobial resistance genes including bla_{TEM} gene allele which is usually part of transposon Tn3; *strA* gene in streptomycin-resistant isolates, *tet(A)* gene in tetracycline resistant isolates and Tn9-associated *catA1* gene in chloramphenicol resistant isolates form swine fecal sources in Kenya. A similar study in Kenya, characterizing aminoglycoside and chloramphenicol resistance in *Escherichia coli* and *Salmonella enterica* serotypes from some food animals showed that, all *Salmonella* isolates present in pigs at slaughter and on pork carcasses, were positive for *strA* gene only (Kikuvi GM 2006).

In the present study multidrug resistance was associated with the presence of *aadA2* and *dfr* gene cassettes and there are several studies that showed similar results, one of these studies showed that multidrug resistance was also strongly associated with the presence of class 1 integrons in which the *aadA2, aadA1, bla* (PSE-1), *dfrA1, dfrA5, dfrA14* or sat genes were present, as determined by nucleotide sequence determination

in *Salmonella* from food-animal sources (An.T.T.vo *et al.,* 2006). Twenty-five isolates carried class 1 integrons with an *aadA23* gene cassette or unusual class 1 integrons with a *dfrA12-orfF-aadA27* gene cassette array and both integrons were found on large conjugative plasmids in addition, Salmonella plasmid-located virulence genes *spvR, spvA, spvB, rck* and *pefA* were found on an IncFIB resistance plasmid and therefore, hybrid virulence-resistance plasmids or plasmids harbouring class 1 integrons may play a role in the maintenance and dissemination of antimicrobial resistance among *S.typhimurium* in pig production system (Lopes *et al.*,2016). Integrons are capable of trapping one or more resistance gene cassettes and furnishing a promoter for the efficient expression of antimicrobial resistance genes (Ribeiro *et al.,* 2011).

In the present study single and double substitutions in the QRDR *gyrA* and PMQR *qnrA* genes were found in the quinololone resistant NTS isolates. Forty two(42) isolates out of the total 184 isolates selected with antibiotic resistance characteristic SulCip Cip and CipNal identified single and double amino-acid substitutions in *gyrA* at positions Ser83 (N = 29; →Phe, →Tyr, → Cysteine, → Gly, → Isol) and Asp87 (N = 33; →Asn, →Gly, →Ly, →Arg) and one (1) isolate carried *qnrA* gene by PCR amplification and sequencing. However, one previous study in Kenya by (Kariuki *et al.,* 2004) did not find the common QRDR mutation among Kenyan isolates that showed increased MIC against quinolones.

This mutation has been described and reported in a study whereby a total of 27 isolates of *Salmonella enterica* serovar Typhimurium were obtained from 930 swine and 14 isolates (51.8%) were nalidixic acid resistant (MIC, 128 mg/ml) and had reduced susceptibility to various quinolones (MIC, 0.125 to 2 mg/ml) and when sequenced, 13 isolates had Asp87RTyr mutations and 1 isolate had Asp87RGly

mutation in the quinolone resistance–determining region of *gyrA*, (Lee *et al.*, 2010) however genes for *qnrA,* were not detected by PCR with specific primers unlike in this present study. In Taiwan ciprofloxacin resistant NTS strains from swine, chicken and their carcasses were detected and DNA sequence analysis revealed that mutations in the quinolone resistance–determining regions of *gyrA* (Ser83Phe, Asp87Gly or Asp87Asn), parC (Ser80Arg, or Ser80Ile or Glu84Lys), and *parE* (Ser458Pro) genes were associated with the *Salmonella* strains that demonstrated resistance to ciprofloxacin (Cheng-Chung Li *et al.*, 2009).

Mutations in the genes *gyrA*, *gyrB*, *parC* and/or *parE*, accounting for resistance to quinolones/fluoroquinolones, have normally been seen in NTS isolates (Tadesse *et al*., 2018), (Clara 2015). Different plasmid-mediated quinolone resistance (PMQR) genes, such as the gene *qnrD*, as well as *qnrA*, *qnrB* and *qnrS* variants all coding for DNA topoisomerase protecting proteins, the gene *qepA* coding for a quinolone-specific efflux pump, occurs in *Salmonella* isolates(Michael & Schwarz 2016).

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusions

The present study revealed the following;

There is no association between heavy metal tolerance genes and emergence of multidrug resistant NTS among isolates from swine fecal sources in Kenya.

This study demonstrated that multidrug resistance in NTS in swine is majorly due to antibiotic resistant genes and that among the commonly used antimicrobial agents such the Beta lactams (Ampicilin), tetracycline, sulphamethoxazole and cotrimoxazole antibiotic resistance is encoded on mobile genetic elements and in this study class 1 Integrons were detected and multidrug resistance was linked to gene cassettes *dfrA1* and *aadA2*.

Flouroquinolone resistance is majorly associated with chromosomal mutations in *gyrA* gene at positions Ser83 and Asp 87 and that plasmid mediated quinolone resistance (*qnrA*) plays a little role in mediating resistance in flouroquinolone class of antibiotics among MDR salmonella isolates from swine in Kenya.

This present study showed that Salmonella plasmid gene *(spvA*) plays a role in mediating antibiotic resistance in NTS isolated from swine fecal sources in Kenya.

In summary these observations indicate the potential importance of pigs as a source of single and multiple antimicrobial-resistant NTS isolates to commonly use and most important antimicrobials including flouroquinolones.

6.2 Recommendation

I would recommend further study to be conducted on resistance gene attributes of multidrug resistant NTS isolates from swine in Kenya including the following:

1. Determination of the presence of mutations in QRDR *gyrB parC* and *parE* which are known to mediate flouroquinolones resistance.
2. Determination of *spvBC* genes that encode for salmonella virulence and encodes for resistance.
3. Detection of PMQR *qnrB, qnrS* variants for DNA protecting topoisomerase protecting protein which have been published in studies to mediated quinolone resistance.

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