ANTIMICROBIAL RESISTANCE AND PRESENCE OF INTEGRONS IN *SALMONELLA* ISOLATED FROM ANIMALS AND HUMANS IN THE UNITED STATES OF AMERICA AND UGANDA

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Program:
Food Safety

May 2010

Fargo, North Dakota

North Dakota State University
Graduate School

Title

Antimicrobial Resistance and Presence of Integrons in *Salmonella* Isolated From Animals and Humans in the United States of America & Uganda

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

ABSTRACT

Mahero, Michael Wandanje, M.S., Great Plains Institute of Food Safety, School of Food Systems, College of Agriculture, Food Systems, and Natural Resources, North Dakota State University, May 2010. Antimicrobial Resistance and Presence of Integrons in *Salmonella* Isolated from Animals and Humans in the United States of America and Uganda. Major Professor: Dr. Margaret Khaitsa.

Salmonella has been cited as one of the leading causes of food borne illness world wide and in the United States (US), as well as an indicator organism for studying antimicrobial resistance (AMR) trends. The objective of this study was to characterise AMR patterns of *Salmonella* isolates from animals and humans in North Dakota, US, and Kampala, Uganda, and determine the association between the observed AMR and presence of class 1 integrons. *Salmonella* isolates were collected from the Veterinary Diagnostic Laboratory (VDL) at North Dakota State University and the North Dakota Department of Health respectively from 2003-2008. Samples were also retrieved from archives present at the Microbiology Department, Faculty of Veterinary Medicine at Makerere University in Kampala, Uganda. AMR profiles were determined using a panel of 15 antimicrobials as per the manufacturer's instructions (Sensitire, Trek Diagnostics System, Westlake, Ohio). Screening for the class 1 integrons was done using PCR with primers specific for the *int1*. Out of 359 *Salmonella* isolates tested, 24.79% were resistant to ≥5 antimicrobials while 36.2% were resistant to at least 2. Pan susceptible isolates were mostly (65.05%) from human isolates. The most common multidrug resistant (MDR) phenotype among the tested isolates was the classic ACSSuT penta-resistance at 29.06% (50/172). The highest resistance frequency was seen against Tetracycline (39.6%) and Streptomycin (34.7 %), while 5.2% (17) of the isolates were resistant to Nalidixic acid and 56 (15.7%) to Ceftiofur. A total of 20.7% (57/276) of the ND samples tested positive for presence of class 1

integrons. Class 1 integron was significantly associated ($p< 0.05$) with AMR to Ampicillin, Kanamycin, Tetracycline, Streptomycin and Sulfisoxazole. Of all Ugandan *Salmonella* isolates tested, 94.4% (68/72) were resistant to ≥2 antimicrobials. The highest resistance was observed against Sulfisoxazole and Trimethoprim-Sulphamethoxazole, and 45.8% of human and 46.2% of cattle isolates tested positive for presence of class 1 integrons. Presence of class 1 integron was significantly associated ($p< 0.05$) with AMR to Tetracycline and Amoxicillin. DNA sequencing of the class 1 integron variable regions identified several resistance genes including *aadA1, dfrA7,* and *dfrA5* gene. The data indicated high AMR among antimicrobials widely used in veterinary and human medicine. Also, AMR was observed against drugs whose veterinary use is restricted, implying possible horizontal transmission. A good proportion (47.9% in Uganda and 29.85% in ND) of the *Salmonella* isolates from clinical cases of salmonellosis were MDR (resistant to ≥2) isolates bearing class 1 integron.

ACKNOWLEDGEMENTS

My heartfelt gratitude goes to my advisor Dr. Margaret Khaitsa for her excellent guidance and support during my academic pursuits. Her patience and confidence in me has spurred me to great heights. Indeed an exceptional mentor she has been. I would like to thank Dr. Neil Dyer, Dr. Penelope Gibbs and Dr. Charles Stoltenow for serving on my committee; their invaluable advice and support has gone a long way in shaping my research work into what it is today. I would like to extend my gratitude to Dr Denis Byarugaba and the Faculty of Veterinary Medicine in Makerere University, Uganda, for their support and input in this research. I would also like to thank Dawn Doetkott for her constant encouragement, technical support and valuable advice during my research. I would like to thank Susan Olet for her assistance with the statistics and fellow lab mates in Dr Khaitsa's lab for their unwavering support, great camaraderie and constructive critique throughout my training. A special note goes to all graduate students within the department for the cordial relationships and vibrant support during my stay here. The staff, in particular the front office team, has offered constant logistical support during my stay; thank you. I would like to appreciate the navigator family for making me feel home away from home and my friends for their constant support and encouragement. Lastly, I would like to appreciate my parents Dr. and Mrs. Mahero and my entire family for their unwavering support and kindness throughout the years.

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LIST OF SYMBOLS/ABBREVIATIONS

1. bp-Base pairs
2. ^{0}C- Degrees centigrade
3. CI-Confidence intervals
4. CLSI- Clinical and Laboratory Standards Institute
5. DNA-Deoxyribonucleic Acid
6. *g*-gravity
7. Int-F- Integrase forward primer
8. Int-R- Integrase backward primer
9. MDR-Multi Drug Resistance
10. min- Minute
11. MIC-Minimum Inhibitory Concentration
12. mls- Millilitre
13. OR-Odds Ratio
14. PCR-Polymerase chain reaction
15. P-value-Probability value
16. Pmol/L-Picomoles/litre
17. Spp-Species
18. Taq-*Thermophilus aquaticus*
19. µg-Micrograms
20. µl-Microlitre
21. USDA-United States Department of Agriculture
22. WHO-World Health Organisation

INTRODUCTION

Antimicrobials have been considered the avenue of remedy for bacterial infections in both animals and humans for the last 70 years. Indeed, if judiciously taken these medications may destroy or disable the bacterial pathogens that cause infections. However, there has been a significant increase in drug-resistant bacteria leading to failure in the treatment of infectious diseases (Alliance of Prudent Use of Antimicrobials) (http://www.tufts.edu/med/apua/Ecology/faairExecSum_6-02.html). Antimicrobial resistance (AMR) is a natural consequence of infectious agents' adaptation to exposure to antimicrobials used in medicine, food animals, food processing, crop production and the environment (2, 16, 86, 98). There has been a decline in effectiveness of existing antimicrobial agents and thus infections have become more difficult and expensive to treat and epidemics have become harder to control (47, 65, 76). It has often been reported that a number of the newly emerging resistant bacteria in animals are transmitted to humans through meat and other foods of animal origin (2, 65, 80).

In the United States of America (US), the major pathogens that have been associated with food borne outbreaks are comprised of viruses, bacteria, parasites, toxins, metals and prions (54). Of these various agents, 7 major food pathogens (*Campylobacter jejuni, Clostridium perfrigens, Escherichia coli, Listeria monocytogenes, Salmonella, Staphlococcus aureus* and *Toxoplasma gondii*) are known to cause 3.3-12.3 million cases of food borne illness and up to 3900 deaths, with an estimated total cost of $6.5-$ 34.9 billion (1995 US$) annually (15). *Salmonella* has been reported as one of the leading causes of food borne illness in the US and worldwide (54, 93). It is responsible for

approximately 1.4 million illnesses, 17,000 hospitalisations and 590 deaths in the US each year (54). According to Food Net (Food borne Diseases Active Surveillance Network), *Salmonella* prevalence has consistently remained high in comparison to the other food borne pathogens despite various intervention measures (17, 53). Unfortunately the burden of disease, associated mortality and epidemiology in sub-Saharan Africa is unknown although outbreaks with high case fatality rates are reported to the World Health Organisation (98).

Previous studies have demonstrated the presence of antimicrobial resistance (AMR) in *Salmonella* and other bacteria of family Enterobacteriaceace. Often, this resistance is encoded by integrons that occur on plasmids or that are integrated into the bacterial chromosome (34). Although this AMR genetic mechanism has repeatedly been demonstrated (31, 59) few epidemiological studies (23) have been conducted to determine how much of the phenotypic resistance is attributed to these genetic structures. Additionally, it is evident that there are other genetic mechanisms that contribute to this resistance (59) which also need to be characterized. Moreover, few studies have been done in sub Saharan Africa to investigate the role of integrons in AMR acquisition by food borne pathogens.

Therefore the goal of this study was to characterize *Salmonella* isolates from the US (North Dakota) and Uganda (Kampala) based on AMR, presence of integrons and genetic sequencing of the integron gene cassettes. Comparison of data from a developed and developing country will help understand the unique risk factors and some of the molecular mechanisms driving the emergence of AMR in the two different environments.

Hypotheses

(i) AMR is equally widespread in *Salmonella* isolated from animals and humans in the US and Uganda.

(ii) Integron mediated resistant genes (in particular class 1 integron) contribute to the majority of the observed AMR phenotype in *Salmonella* isolates in North Dakota and Uganda.

Objectives

(i) To characterise AMR patterns of *Salmonella* isolates from clinical cases of animals and humans in North Dakota, US and from Kampala, Uganda.

(ii) To assess the presence of class 1 integrons and resistance gene cassettes in the *Salmonella* isolates and their association with the observed AMR patterns.

LITERATURE REVIEW

Antimicrobial Use and Misuse

It is with greater emphasis that the "prudent use of antimicrobials" is being addressed by both the human and veterinary medical care establishments (Alliance for the Prudent Use of Antimicrobials [http://www.tufts.edu/med/apua/]). This is because the use of antimicrobials in clinical and veterinary medicine for therapy and prophylactic purposes has been recognized as a driving force for the selection of resistant bacteria. The major conduit of this resistance transmission is the acquisition of resistance genes through mutations in chromosomal loci or horizontal transfer of mobile genetic elements (9, 101). Some of these mobile elements such as plasmids have been known to not only increase bacterial resistance to antimicrobials but also their virulence and extend their host range (1).

Both the amount of antimicrobials used and how they are used contribute to the development of resistance. For instance the use of broad-spectrum antimicrobials rather than narrow-spectrum drugs is known to favor the emergence of resistance by broadly eliminating competing susceptible flora; additionally antimicrobials are frequently prescribed in the treatment of viral infections or at wrong doses for incorrect periods of time. As a result treatment failure in human cases of salmonellosis cannot be ruled out, especially with the emergence of resistance to extended spectrum cephalosporins associated with flouroquinolone resistance (32).

In veterinary practice, antimicrobials may be used for a number of reasons: therapeutic, prophylactic and growth promotion; this greatly influences the prevalence of resistance in animal bacteria and poses some risk for the emergence of antibiotic resistance

in human pathogens (31, 58, 63). In developing countries use of antimicrobials for growth promotion is on a limited scale despite the numerous antimicrobials used for treatment and prevention (57). Many of these drugs are available to the general public with little being done to monitor their use which greatly contributes to the emergence of antibiotic resistance (45, 65, 66). Currently, the possibility of horizontal transmission of resistant organisms from animals to humans (28, 60, 70, 88) has been recognized by several international-animal and human health organizations, thereby underscoring the need to limit these routes of exposure (94, 97, 99).

Antibiotic Resistance- Modes of Acquisition

Antimicrobial resistance is a natural consequence of exposure of infectious agents to antimicrobial compounds during use in agriculture, human and animal medicine. Therefore the increased usage of these compounds worldwide is a major contributor of the observed increased resistance (16, 86). Unfortunately this has resulted in more expensive and less successful treatment of parasitic, viral and bacterial diseases (65).

Bacteria can display one of three fundamental phenotypes: Susceptibility, intrinsic resistance or acquired resistance. Intrinsic resistance is usually due to natural physiological or biochemical constitution of the bacteria. This may involve the action of efflux pumps, presence of inactivating enzymes or the barrier function of the outer membrane of Gram negative bacteria (22, 72). Acquired resistance is as a result of a mutation of either a regulatory or structural gene (41). This resistance in bacteria is due to re-assortment of resistance genes, either from one DNA molecule to another (genetically) or from one bacterial cell to another (physically) (8). The transfer of DNA sequences from one cell to

another is essentially through conjugation, transduction and transformation, while molecular transfer is by classical recombination, transposition, (many drug resistance transposons have been described in both Gram-positive and Gram-negative bacteria), site-specific recombination, which achieves directed insertion of a resistance gene(s) (9, 40).

Salmonella and Antibiotic Resistance to Different Classes of Antimicrobials

Antimicrobial resistance in *Salmonella* is an issue of great concern not only in human but also in animal medicine (107). Its ubiquitous nature in the environment, ability to cause disease in a variety of food producing animals and potential to lead to life threatening invasive infections in humans, that require the use of antimicrobials (91), makes *Salmonella* an important indicator bacteria whose AMR patterns need to be continuously tracked and monitored. Consequently the value of antimicrobial susceptibility testing as the foundation for clinical treatment decisions cannot be overstated (1). The observed resistance in *Salmonella* to various antimicrobials is mediated through a host of different mechanisms, ranging from production of modifying enzymes to the action of efflux pumps.

Resistance to ***tetracyclines*** is normally coded for by a series of *tet* genes, *tet (A), tet (B), tet (C), tet (D), tet (G),* which all code for a membrane–associated efflux protein. Despite the frequent presence of tetracycline resistance, few studies have identified the tet genes responsible. The distribution of these genes varies depending on the isolates sampled; all the same *tet A* and *tet B* are the most frequently detected *tet* genes among the different serovars (55). Resistance to ***phenicols*** is mediated through enzymatic inactivation by type A or B acetyltransferases (*Cat*) as well as export of Chloramphenicol by specific

efflux proteins. Two different Cat A proteins, encoded by *catA1* and *catA2* have been described (55).

Resistance to ***aminoglycosides*** is normally as a result of modifying enzymes which attach certain groups to the aminoglycoside molecule hence curtailing its antibacterial activity. Three different classes of aminoglycoside-modyfying enzymes have been described hitherto, namely, O-adenyltransferases, N-acetyltransferase and O-phosphotransferases. There are about 20 aminoglycoside-O-transferase genes (*aad).* Among these only those whose products act at position 3" [*aadA, (3")*] and 2" [*aadB,(2")*] have so far been identified in *Salmonella.* The *aaAd* genes mediate resistance to Streptomycin and Spectinomycin whereas *aadB* genes confer resistance to Gentamycin,, Kanamycin and Tobramycin (55).

Trimethoprim resistance frequently occurs in Enterobacteriaceae and is primarily due to replacement of a Trimethoprim sensitive dihydrfolate reductase by one that is resistant to the antibiotic. Thus far about 30 different resistance genes have been identified and are grouped in two major categories, *dfr A* and *dfrB*. While *dfrB* genes are yet to be identified in *Salmonella*, there are a total of 13 different *dfrA* genes which are mainly borne in mobile genetic elements within the bacteria (55). Similarly, resistance to the ***quinolone*** Nalidixic acid (MIC $\geq$ 32 μg/ml) correlates with mutations causing decreased susceptibility to Ciprofloxacin (MIC $\geq$ 0.125 μg/ml) (61). Resistance against flouroquinolone has been reported to be due to a mutation in the quinolone determining region of gyrA leading to amino acid changes Ser83Ala and Asp87Asn.While high level flouroquinolone resistance has been attributed to the action of an efflux pump system mediated by AcrAB-TolC (7).

Resistance to ***sulphonamides*** among the *Salmonella* isolates is largely mediated by

three different sulphonamide resistance genes *sul1* (represents part of the 3'CS of class1 integrons), *sul2* and *sul 3* all of which code for sulphonamide resistant dihydropteroate synthases (55). Conversely resistance to ***penicillins*** and ***cephalosporins*** is mainly as a result of B-lactamase enzymes which inactivate the antimicrobials. There are about 350-400 different enzymes in this group from both Gram negative and Gram positive bacteria (12). Elevated MICs 8($\geq$ 8 μg/ml) to Ceftiofur are usually indicative of the presence of the AmpC gene and decreased susceptibility (MIC $\geq$ 2 μg/ml) to Ceftriaxone.

Integrons and Antibiotic Resistance

The spread of mobile genetic elements especially transposons plasmids and integrons has greatly contributed to the rapid spread of antibiotic resistance among several bacterial genera of human and veterinary importance (41). According to Bennet (8) "An integron is defined as a genetic element that possesses a site, *attI*, at which additional DNA, in the form of gene cassettes, can be integrated by site-specific recombination, and which encodes an enzyme, integrase, that mediates these site-specific recombination events" (Figure 1). The gene cassettes are mobile, free, non-replicating DNA molecules that are usually part of a plasmid or bacterial chromosome (8, 34). The gene cassette contains one gene and a 59 base element that function as a specific recombination site. The genes carried on gene cassettes usually lack promoters and are expressed from a promoter on the integron (25, 40). Four classes (class 1-4) of integrons have been described. The structure of the integron includes a 5'-conserved segment and variable region. The 5'-conserved segment consists of the *int1* gene (integrase) and a promoter region expressing the inserted gene(s). For class 1 integrons, the 3' conserved segment contains the defective quaternary

ammonium resistance gene *qac*ED1 and the *sul1* gene, which encodes resistance to sulfonamides (25, 34). The variable region, located between the two conserved segments, is the site for the insertion of antibiotic resistance gene cassettes. Class 1 integrons are the most widespread and have also been identified on transposable elements such as mercury resistance transposon Tn*21* (37). Class 2 and 3 have been associated with transposon Tn *7* are rare and are usually borne on plasmids *(56)*. While the class 4 integron is a chromosomal super-integron located on the genome of *Vibrio choerae* (43). These super integrons are the likely source of resistance gene cassettes that could be donated to other bacteria hence conferring antibiotic resistance.

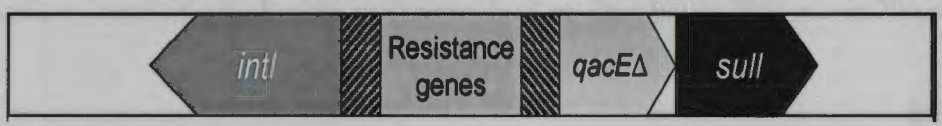


Figure 1. Schematic of class 1 integron. The structure includes the integrase gene *int1* which codes for the enzyme intergrase, a recombination site (*att1*) , and an array of resistance gene cassettes and a short recognition site for recombination (*attC).* (Adapted from Foley and Lyne(29))

In a study by Molla et al., (59) up to 53.1% of *Salmonella* isolates from slaughter animals and food products of animal origin in Ethiopia were positive for class 1 integron although none was positive for class 2 integron. In the US, Gold stein et al., (34) reported that up to 63.1 % of *Salmonella* isolates from avian species that were tested, had class 1 integrons while 8.3% of them had multi drug resistance. Miko et al., (56) reported 65 % of multi-drug *Salmonella* isolates bearing class 1 integrons and only 10% had class 2 integrons. A higher prevalence of up to 74% of class 1 integrons in *Salmonella* isolates from a veterinary teaching hospital in Colorado state University (CSU) have been observed (26). It is therefore evident that these integrons especially class 1, play an important role in

the observed antimicrobial resistance, although this is yet to be effectively quantified epidemiologically.

Food borne Illness and Antibiotic Resistance

According to Mead et al., (54) more than 200 known diseases are transmitted through food. Causes range from bacteria and parasites to prions and toxins, with symptoms ranging from mild gastroenteritis to severe neurological, hepatic and renal syndromes. Some of the major food borne pathogens include *Campylobacter jejuni, Escherichia coli* 0157:H7, *Listeria monocytogenes, Salmonella, Norwalk*-like viruses and *Cyclospora cayetanensis,* some of which were not recognised as food borne illness just 20 years ago (54).

Apart from the emergence of new food borne pathogens, many of the already existing ones such as some *Salmonella* serovars, are displaying resistance to major antimicrobials like third generation cephalosporin and flouroquinolones used in the treatment of patients. This situation is compounded by the lack of adequate information about how this resistance is acquired and hence making it hard to control (54, 58, 70).

Salmonella

Genera from the family Enterobacteriaceae are known to inhabit the intestinal tracts of humans and animals. These include both nonpathogenic species (the commensals) and pathogenic species (34, 44). Due to the close association of these species in the intestinal tract and frequent exposure to various antimicrobials, there is potential for the dissemination of antibiotic resistance genes. In vitro studies have shown that *E. coli* is capable of transferring resistance to other bacterial species, such as *Salmonella* spp., which

are disseminated through the human food chain (11, 54, 76). This mechanism of transfer is widespread and hence has been proposed as a leading cause behind the dissemination of resistance in the past 5 decades (24). With the growing problem of antimicrobial resistance, a detailed understanding of the family Enterobacteriaceae is therefore imperative for development of effective mitigation measures.

Salmonella is one of the genera within this family that has received great focus in the recent past due to its ubiquitous nature, capacity to cause invasive disease, increasing resistance seen among its serovars and its persistent high levels in the population despite several mitigation efforts. These factors also make it a good indicator of the AMR status in other genera. Salmonellae are divided into 2 main species namely, *Salmonella enterica* and *Salmonella bongori*. Majority of the serotypes (over 99%) are grouped into the species *Salmonella enterica* making up a total of more than 2,500 serotypes of *Salmonella* which can be differentiated on the basis of cell wall "O" and flagella "H" antigens. A smaller number of these serotypes are significantly associated with animal and human disease including Typhimurium, Enteritidis, Newport, Heidelberg, and Montevideo (17, 46, 47, 90).

The disease that is produced by *Salmonella* infection is called salmonellosis. Manifestation of the disease syndrome may vary from a mild self-limiting gastroenteritis, to more invasive bacteraemia and typhoid fever. Typhoid fever, which is caused by *Salmonella enterica* serovar Typhi, is specific to humans and is therefore only associated with human transmission, while the non typhoidal form of the disease is zoonotic and often self limiting (developing in 6-72 hours and lasts about 2-7days). However, this non typhoidal form may also assume an invasive nature which usually requires the use of

antimicrobials for treatment. Hence, with the emergence of antibiotic resistance and the prevalence of immuno-compromised individuals, especially in developing countries, the situation is bound to get increasingly complex (10, 29, 46, 65).

Approximately 95% of cases of human salmonellosis are associated with the consumption of contaminated products such as meat, poultry, eggs, milk, seafood, and fresh produce (23, 29, 33). An observation that has further been supported by demonstration of a clear overlap in serovars that cause disease in humans and those commonly isolated from different food sources (18). Additionally, the greater consumption of poultry, pork and beef over the past century has increased the level of exposure among many Americans to Salmonellae, many of which also bear MDR factors (14, 105). Multiple drug resistance (MDR) among the pathogenic *Salmonella* serotypes is being detected with greater frequency, including third-generation cephalosporins and flouroquinolones, which are recommended for the treatment of severe infections (29, 46, 51, 90). In the US, several reports exist of AMR and MDR in *Salmonella* isolates from humans and animals. For instance in North Dakota a study by Oloya et al., (68) observed that most of the animal salmonellosis cases were reported in cattle (64.7%) with greater multi-drug resistance (MDR) being seen in animals as compared to human isolates. A similar study in the area reported a MDR prevalence of 75.7% among *Salmonella* isolates from steers reared in a feedlot (47), while lower AMR levels in *Salmonella* isolates (37.8%) were reported by Dargatz et al., (23) where only 11.7% of the isolates displayed MDR patterns.

Much of the multi-drug resistance seen among these *Salmonella* isolates has been attributed to the emergence and spread of the multi drug resistant *S.typhimurium* DT 104 over the last ten years (77, 104). The genetic determinants of this strain are embedded in a

43kb island Salmonellae Genomic island (SG1) which is made up of integrons with various genes that code resistance to antimicrobials such as Tetracycline (*Tet G*), Chloramphenical (*Flo*) and Streptomycin *(aadA2).* It has been noted that, regardless of origin (food animal or human), these MDR-DT104 strains have had the same gene cassettes (5, 41, 42, 79, 91, 108) and while removal of antibiotic pressure should normally help reverse or slow development of resistance, this genomic island mediates persistence of these genes even in the absence of the prevailing antibiotic pressure (7, 39-41). Also of concern is the increasing trend of additional resistance among these DT104 isolates to Trimethoprim and Ciprofloxacin which have been reported in the UK (77, 80). Fortunately no DT104 R-type ACSSuT isolates in the United States have shown resistance to Trimethoprim or Ciprofloxacin.

In developing countries such as Uganda, a few comparative reports exist of AMR and MDR in *Salmonella* and other Gram negative bacteria. Bachou et al., (6) noted that non typhoidal *Salmonella* species were the most common cause of bacteraemia in severely malnourished children in Uganda. Most of these Salmonellae were resistant to Chloramphenicol, Ampicillin and Co-trimoxazole. Similarly Anguzu et al.,(3) observed high resistance amongst Gram negative bacteria to Ampicillin Chloramphenicol and Amoxcyllin while Karuiki et al., (46) reported MDR in 44.3% of non typhodial *Salmonella* (NTS) isolates obtained from cases of invasive non typhoidal infections in Kenya. In Zaire and Rwanda multi-drug resistant NTS have been cited as a major cause of bacteraemic illness among children (19, 36). Several studies have linked some of the observed resistance in *Salmonella* serovars, in both animals and humans, to the use of antimicrobials in the treatment and production of food animals with great public health repercussions (31,

58, 63). Therefore, a better understanding of the antimicrobial resistance patterns in *Salmonella* in developing countries and its associated epidemiology cannot be overemphasised.

MATERIALS AND METHODS

Study Design

The design was a retrospective study in which *Salmonella* isolates included were collected either as part of diagnostic procedures for large animal patients or as part of an active hospital surveillance program and were obtained from the Veterinary Diagnostic Laboratory (VDL) at North Dakota State University (NDSU) and the North Dakota Department of Health (NDDoH), respectively. Uganda was chosen as a developing nation that had preexisting research partnership between NDSU Department of Veterinary and Microbiological Sciences (VMS) and Makerere University (MAK) Kampala Uganda. Additional *Salmonella* isolates were obtained from the Microbiology Department at the Faculty of Veterinary Medicine, MAK, Kampala Uganda.

Ethical Approval

Approval to carry out this project was granted by the NDSU Institutional Review Board and Institutional Biosafety Committee. Pursuant to this was the training of all involved personnel in ethical policies that govern research on human cases. In Uganda, similar approval was obtained from the Uganda Council of Science and Technology, which oversees all ethical matters regarding research in Uganda.

Salmonella Isolates

This study used *Salmonella* isolates obtained from clinical cases of bovine and human salmonellosis that were presented at the VDL and NDDoH respectively during the 2003-2008 period. All isolates were cultured and characterised according to methods

optimised for *Salmonella* detection (47, 68). Additionally, samples were retrieved from archives present at the Microbiology Department, Faculty of Veterinary Medicine at MAK.

Antimicrobial Susceptibility Testing

Antimicrobial resistance of each *Salmonella* isolate was determined using a panel of 15 antimicrobials as per the manufacturer's instructions (Sensitire, Trek Diagnostics System, Westlake, Ohio). Each CMV1AGMF (Sensititre Gram Negative NARMS) plate that was used for resistance screening contained a full range of minimum inhibitory concentrations (MIC). The panel consists of 96-well microtitre plate containing 15 different NARMS antimicrobials over a wide range of concentrations. The inoculation of the panels was done in accordance with the manufacturer's instructions (Trek Diagnostics). Sensititre panels were read using the Sensititre Automated Reading and Incubation System (ARIS; TREK Diagnostic Systems) after which results were transferred to the Sensititre for Windows (SWIN) software for interpretation.

The antimicrobials tested were Amikacin, Amoxicillin/clavulanic acid, Ampicillin, Ceftiofur, Ceftriaxone, Chloramphenicol, Ciprofloxacin, Gentamicin, Kanamycin, Nalidixic acid, Streptomycin, Sulfizoxazole, Tetracycline, and Trimethoprim /sulfamethoxizole (Table 1). Antimicrobial minimal inhibitory concentrations (MICs) for *Salmonella* were determined according to manufacturer instructions using the Sensititre® semi-automated antimicrobial susceptibility system (Trek Diagnostic Systems, Westlake, Ohio). Antimicrobial resistance was determined using Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) standards, when available. For antimicrobial agents without CLSI approved standards, National Antimicrobial Resistance Monitoring System

(NARMS) interpretive criteria as established by the NARMS working group were used.

Table 2 lists antimicrobials tested and their breakpoints for *Salmonella.*

Table 1: Concentrations of antimicrobials in NARMS broth micro dilution panel CMV1AGNF

CLSI Subclass	**Antimicrobials**	**Concentration (µg/ml)**
Aminoglycosides	Amikacin	0.5-6.4
	Gentamicin	0.25-16
	Kanamycin	8-64
	Streptomycin	32-64
Aminopenicillins	Ampicillin	1-32
β-Lactamase inhibitor combinations	Amoxicillin-Clavulanic acid	1/0.5-32/16
Cephalosporins (3rd generation)	Ceftiofur	0.12-8
	Ceftriaxone	0.25-64
Cephamycins	Cefoxitin	0.5-32
Folate pathway inhibitors	Trimethoprim-Sulfamethoxazole	0.12/2.38-4/76
Phenicols	Chloramphenicol	2-32
Floroquinolones	Ciprofloxacin	0.015-4
Quinolones	Nalidixic acid	0.5-32
Sulfonamides	Sulfisoxazole	16-256
Tetracyclines	Tetracycline	4-32

Table 2: Antimicrobials contained in National Antimicrobial Resistance Monitoring System (NARMS) panel and their resistance breakpoints as described by the Clinical and

Class	Agent	Susceptible	Intermediate	Resistant
Aminoglycosides	Amikacin	≤ 16	32	≥ 64
	Gentamicin	≤4	8	≥16
	Kanamycin	≤16	32	≥64
	Streptomycin	≤32	NA	≥64
Aminopenicillins	Ampicillin	≤8	16	≥32
Blactam/Blactamase Inhibitor 2combinations	Amoxicillin /Clavulanic acid	≤8/4	16/8	32/16
Cephalosporins	Ceftiofur	≤2	4	≥8
	Ceftriaxone	≤8	16-32	≥64
	Cephalothin	≤8	16	≥32
Cephamycin	Cefoxitin	≤256	16	≥32
Folatepathwayinhibitors	Sulfamethoxazole /Sulfisoxazole	≤2/38	NA	≥512
	Trimethoprim /Sulfamethoxazole	≤8	NA	≥4/76
Phenicols	Chloramphenicols	≤8	16	≥32
Quinolones	Ciprofloxacin	≤1	2	≥4
	Nalidixic acid	≤16	NA	≥32
Tetracyclines	Tetracycline	≤4	8	≥16

Laboratory Standards Institute (CLSI).
Source National Committee for Clinical Laboratory Standards (62).

Class 1 Integron Detection

Class 1 integron detection was accomplished by characterization of the molecular structure of the *Salmonella* isolates using PCR primers specific for class 1 integrase (Int-F and Int-R), a 280 bp amplicon (34). The protocol used was previously described by Miko et al., (56). Briefly, the amplifications were performed in 23 µl 5X of Taq PCR (Polymerase Chain Reaction) Master Mix (Promega), 10 pmol/L each primer, and 2 µl template DNA. In order to extract the DNA Proteinase K was added to the samples and heated at 94°C for 5 min followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 30 s at 72°C. PCR products were analyzed by gel electrophoresis with 1.5% agarose gels. All PCRs included both positive and negative controls.

Class 2 Integron Detection

Some isolates from Uganda were tested for the presence of class 2 integrases by single PCR reaction using primers specific for the class 2 integrase gene, a 233 bp amplicon (34). The protocol used was similar to that employed by Miko et al., (56).

DNA Purification and Sequencing

A representative sample of 24 isolates of *Salmonella* was selected according to the size (gene profile) each isolate contained and the host; at least two isolates per host from each size was picked. The single reaction PCR was followed to amplify the conserved sequence as previously described by Nde et al., (63). The amplification products were purified using The Wizard® SV Gel and PCR Clean-Up System according to manufacturer's instruction. Briefly, to each unit volume of PCR products, an equal amount of membrane binding solution (Promega) was added. A SV minicolumn with a 2ml

collection tube was set for each PCR sample. The DNA sample was added to the column and centrifuged for 60s at 16000x*g* and the flow through discarded. The columns were washed with 0.7 mls of membrane wash solution (Promega) and centrifuged for another 60s. The flow-through was discarded and the column was replaced into the collection tube and a further 0.5mls of the membrane wash solution added. The columns were then centrifuged for an additional 5 min at 16000x*g*. There after each column was placed in a clean 1.5 ml microcentrifuge tube. DNA elution was completed by adding 50µl of nuclease free water (Promega) to the center of the Promega column membrane and centrifuged for 1 min. The column was removed and the elute was stored at 40^0C until use. Purified DNA was sent to Macrogen USA for sequencing. The sequences were compared with the data in the Gen Bank (http://www.ncbi.nlm.nih.gov/BLAST).

Data Analysis

Descriptive statistics of antimicrobial resistance profiles and class 1 integrons detected within the *Salmonella* isolates were computed using Epi Info version 3.3.2 software (Epi Info ^{TM}, U.S. Centers for Disease Control and Prevention (CDC), Atlanta, GA). A Chi-square test was carried out to assess the level of association between the observed AMR and the presence of Integrase 1 gene in the *Salmonella* isolates. This was determined by computing the odds ratio and 95% confidence intervals as previously described by Khaitsa et al., (48). In this analysis the antimicrobial resistance was coded as absent (0) or present (1), with the intermediate resistance being considered as resistant. Multi-drug resistance by CLSI antimicrobial subclass was defined as resistance to two or more subclasses.

A measure of effect of the presence of class 1 integron in *Salmonella* isolates on resistance to specific antimicrobials was determined by computing the *attributable risk, risk ratio and attributable fraction.* A p-value of 0.05 was used to determine the level of significance in the calculated results. In cases where any of the expected out comes had a value less than 5 (hence nullifying the assumptions of the Chi square test) Fisher's exact test was used to verify the level of significance in the observed difference.

RESULTS

Antimicrobial Resistance (AMR)

Overall, *Salmonella* isolates exhibited the highest antimicrobial resistance towards Tetracycline (39.60%), Streptomycin (34.70 %), Sulfisoxazole (33.10%), Ampicillin (32.60%) and Chloramphenicol (31.40%) (Figure 2). This antimicrobial resistance pattern was similar to that observed in *Salmonella* isolates from cattle where the highest resistance frequency was seen against Tetracycline (61.0%, 102/170), Streptomycin (54.80%, 94/171), Ampicillin (53.20%, 91/171), Sulfisoxazole (51.40%, 88/171) and Chloramphenicol (47.40%, 81/171). Among *Salmonella* isolates from humans, high antimicrobial resistance (AMR) frequencies were reported against Tetracycline 19.40%, 37/186), Chloramphenicol (16.70%, 31/186), Streptomycin (16.40%, 32/186), Sulfisoxazole (16.10%, 30/186) and Ampicillin (13.44% 25/186) (Figures 3, 4).

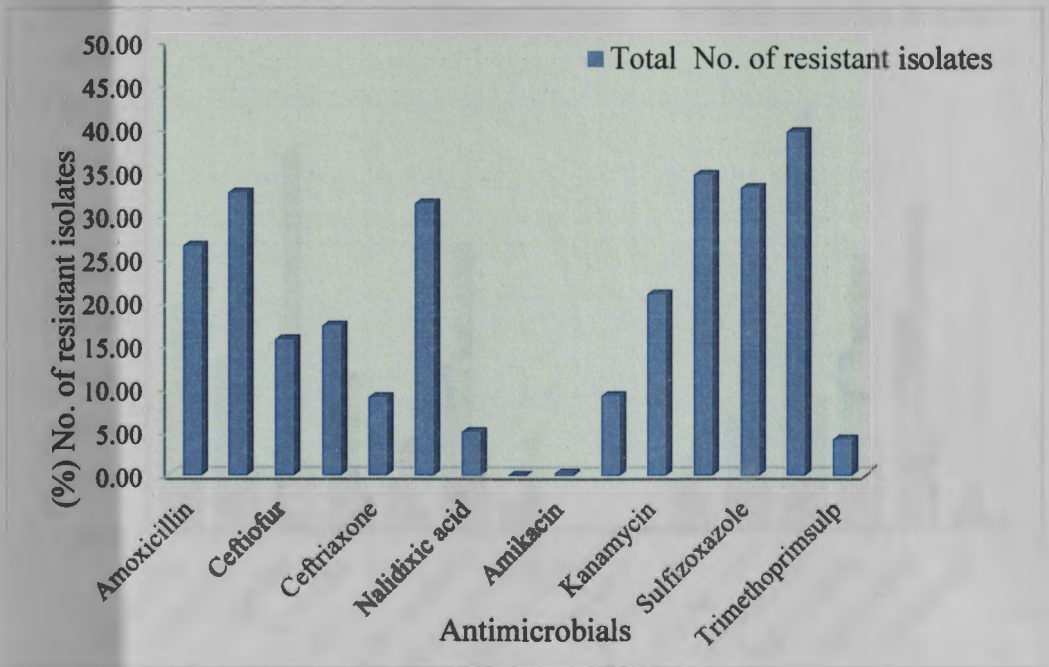


Figure 2: Overall resistance profile of North Dakota *Salmonella* isolates against National Antimicrobial Resistance Monitoring Systems panel of antimicrobials.

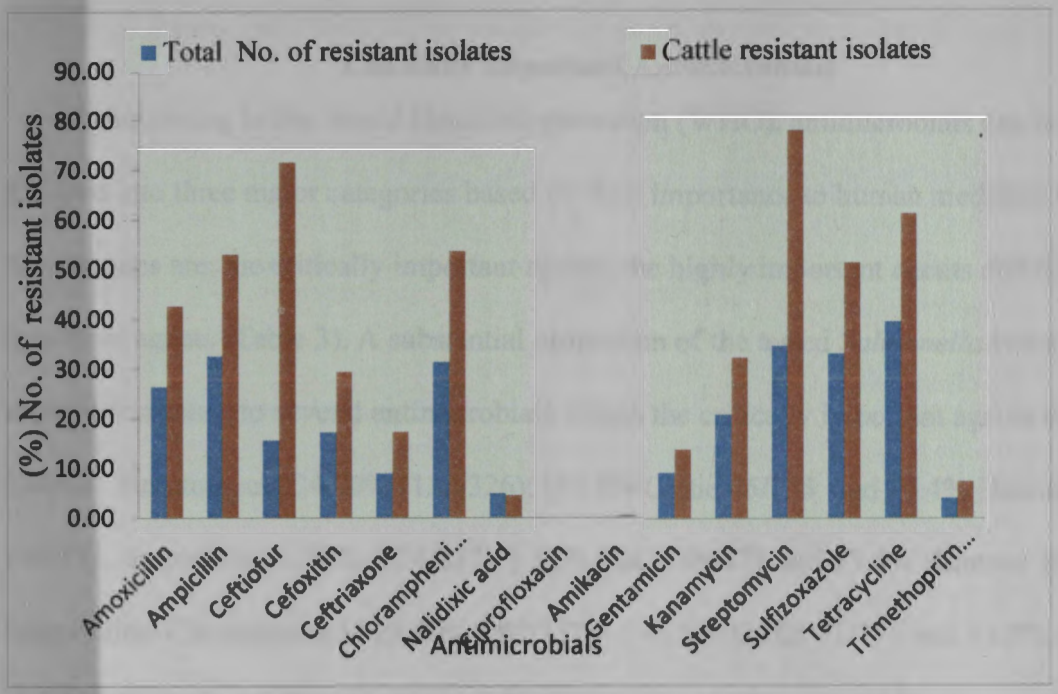


Figure 3: Comparison of overall resistance profile and antimicrobial resistance patterns in cattle *Salmonella* isolates from North Dakota.

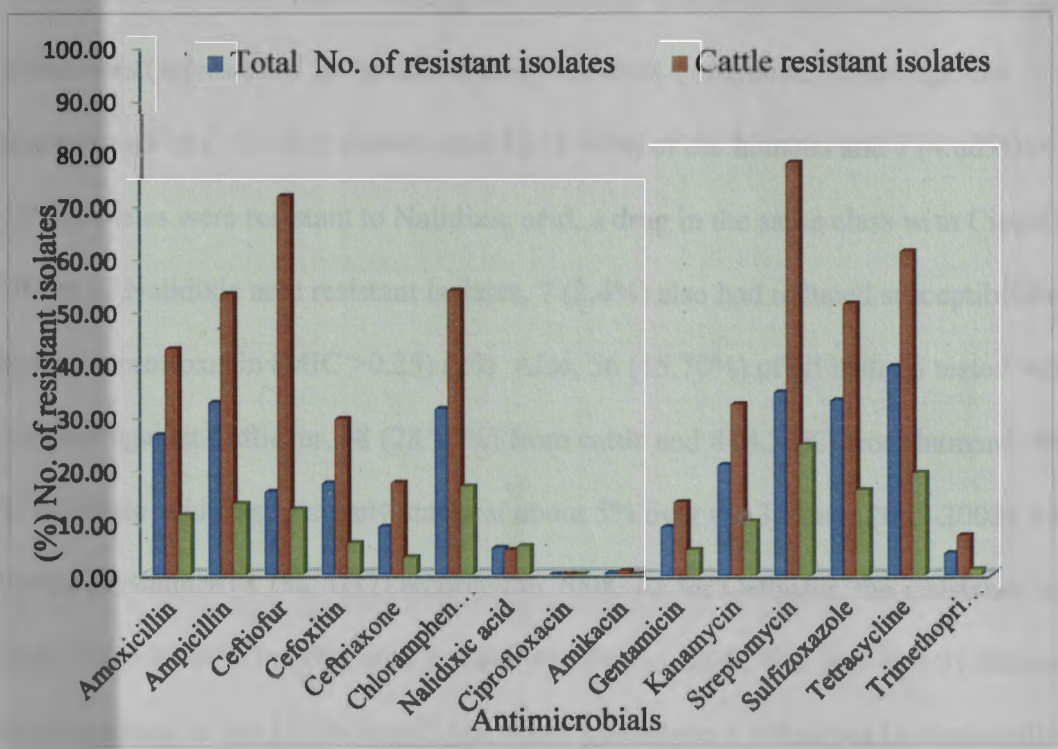


Figure 4: Resistance profile of cattle and human *Salmonella* isolates from North Dakota

Clinically Important Antimicrobials

According to the World Health Organisation (WHO), antimicrobials can be grouped into three major categories based on their importance to human medicine (96). The three groups are; the critically important agents, the highly important agents and the important agents (Table 3). A substantial proportion of the tested *Salmonella* isolates showed resistance to several antimicrobials within the critically important agents as follows: Streptomycin 34.70% (113/326); [54.8% Cattle 85/155 and 16.4% Human 28/171], Ampicillin 31.90% (114/357); [52% Cattle 89/171 and 13.4% Humans 25/186], Amoxicillin-Clavulanic acid 25.80% (92/357) ; [41.5% Cattle 71/171 and 11.3% Humans 21/186] ,Ceftiofur 15.70% (56/357) ; [28.10% Cattle 48/171 and 4.30% Humans 8/186]; Ceftriaxone 9.50 % (34/357) [16.4% Cattle 28/171 and 3.20% Humans 6/186] (Figure 5).

The resistance pattern among the clinically important antimicrobial subclasses-quinolones (represented by Nalidixic acid) and third-generation cephalosporins (represented by Ceftiofur) showed that 10 (5.40%) of the humans and 7 (4.60%) of the cattle isolates were resistant to Nalidixic acid, a drug in the same class with Ciprofloxacin. Of the 17 Nalidixic acid resistant isolates, 7 (2.4%) also had reduced susceptibilities against Ciprofloxacin (MIC >0.25) (20). Also, 56 (15.70%) of all isolates tested were resistant against Ceftiofur, 48 (28.10%) from cattle and 8 (4.30%) from humans. Resistance to Nalidixic acid was relatively stable at about 5% over the 3 years (2005-2008) with the lowest prevalence (3.1%, 1/32) recorded in 2008. As for Ceftiofur, the resistance increased from 0% to 21.90% in 2008 with a peak (33.80%) in 2006. This was closely mirrored by the prevalence of the MDR-AmpC (ACSSuT phenotype + resistance to Amoxicillin and

Ceftiofur) phenotype which, like Ceftiofur resistance, first appeared in 2004 and peaked in 2006 (Figure 6).

Table 3: World Health Organization categorisation of antimicrobials of critical importance to human beings

Critical Importance	***CLSI Subclass**	**Antimicrobial Agent**	**Categorization of Antimicrobials**
I	Aminoglycosides	Amikacin	Critically important
		Gentamicin	Critically important
		Streptomycin	Critically important
	Aminopenicillins	Ampicillin	Critically important
	β-Lactamase inhibitor combinations	Amoxicillin-Clavulanic acid	Critically important
	Cephalosporins (3rd generation)	Ceftriaxone	Critically important
	Ketolides	Telithromycin	Critically important
	Macrolides	Azithromycin	Critically important
		Erythromycin	Critically important
	Quinolones	Ciprofloxacin	Critically important

Table 3 (continued)

Critical Importance	***CLSI Subclass**	**Antimicrobial Agent**	**Categorization of Antimicrobials**
II	Aminoglycosides	Kanamycin	Highly important
	Cephalosporin (1st generation)	Cephalothin	Highly important
	Cephamycins	Cefoxitin	Highly important
	Folate pathway inhibitors	Trimethoprim-Sulfamethoxazole	Highly important
	Phenicols	Chloramphenicol	Highly important
	Sulfonamides	Sulfamethoxazole	Highly important
		Sulfisoxazole	Highly important
	Tetracyclines	Tetracycline	Highly important
III	Lincosamides	Clindamycin	

***CLSI**-Clinical and Laboratory Standards Institute
Source WHO 2003 (95).

Among the *Salmonella* isolates from Uganda high resistance was seen against Sulfisoxazole (86.10%), Trimethoprim (76.40%), Chloramphenicol (73.60%), Streptomycin (66.70%), Ampicillin (66.70%) and Tetracycline (56.90%) (Figure 7). Some

of these drugs fall under the WHO described group of critically important drugs in human medicine (Figure 8). The highest resistance was observed against Sulfisoxazole in cattle (83.3%, 8/12) and humans (91.2%, 52/57) followed by Trimethoprim in humans (85.7%, 48/12) and Nalidixic acid (72.73%, 8/11) in cattle. Relatively high resistance to Ciprofloxacin (a drug of choice for treatment of salmonellosis in humans) was seen in cattle-27.30% (3/11) and 14.29% (8/576) human isolates (Figure 7, 9).

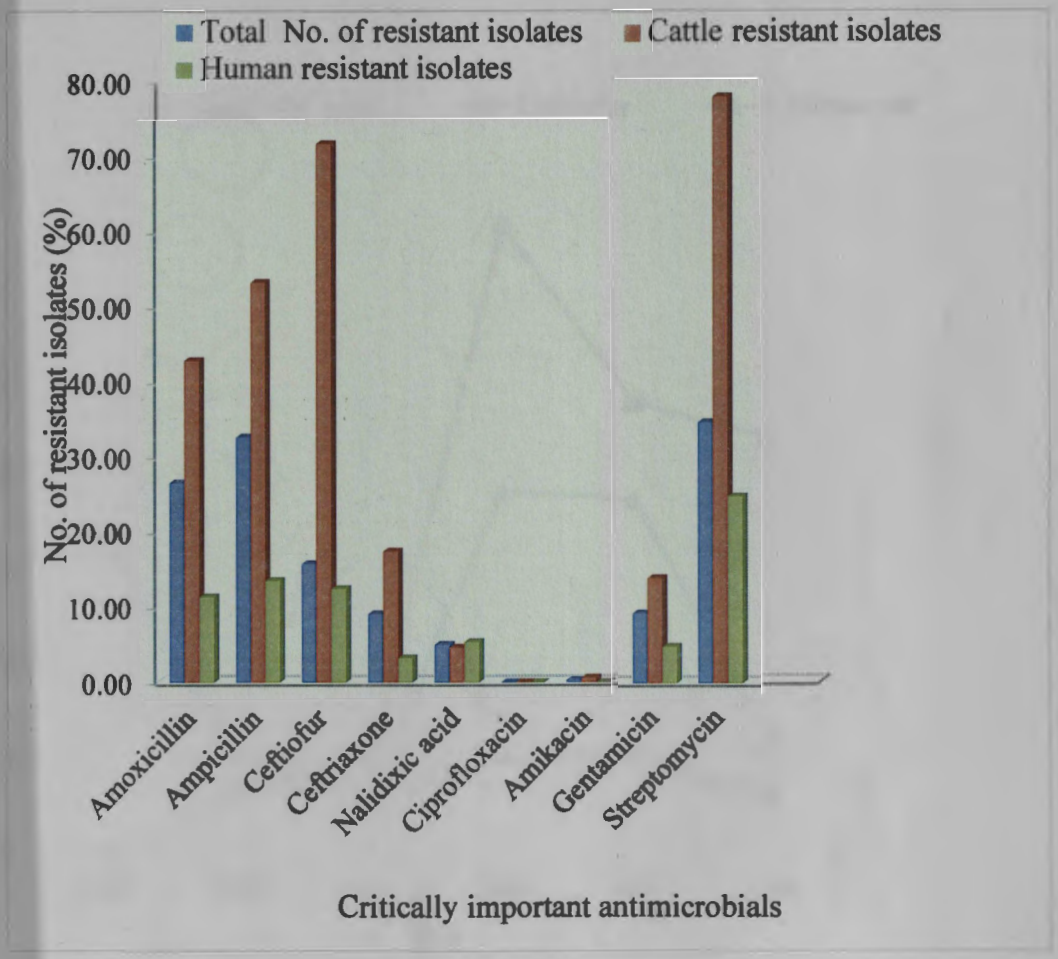


Figure 5: Resistance profile of cattle and human North Dakota *Salmonella* isolates against critically important antimicrobials.

The lowest resistance was recorded against Amikacin and Ceftriaxone 16.70% (2/12) among cattle isolates and Amikacin 0% (0/56) among human isolates (Figure 9).

Multi drug Resistance (MDR)

Overall, out of 359 *Salmonella* isolates tested 24.79% (89/359) were resistant to ≥5 antimicrobials while 36.20% (130/359) were resistant to at least 2. For cattle and human isolates 52.60% (91/173) and 20.97% (39/186), respectively, had resistance to ≥2 antimicrobials while 42.20% (73/173) and 8.60% (16/186), respectively, were resistant to ≥5 antimicrobials.

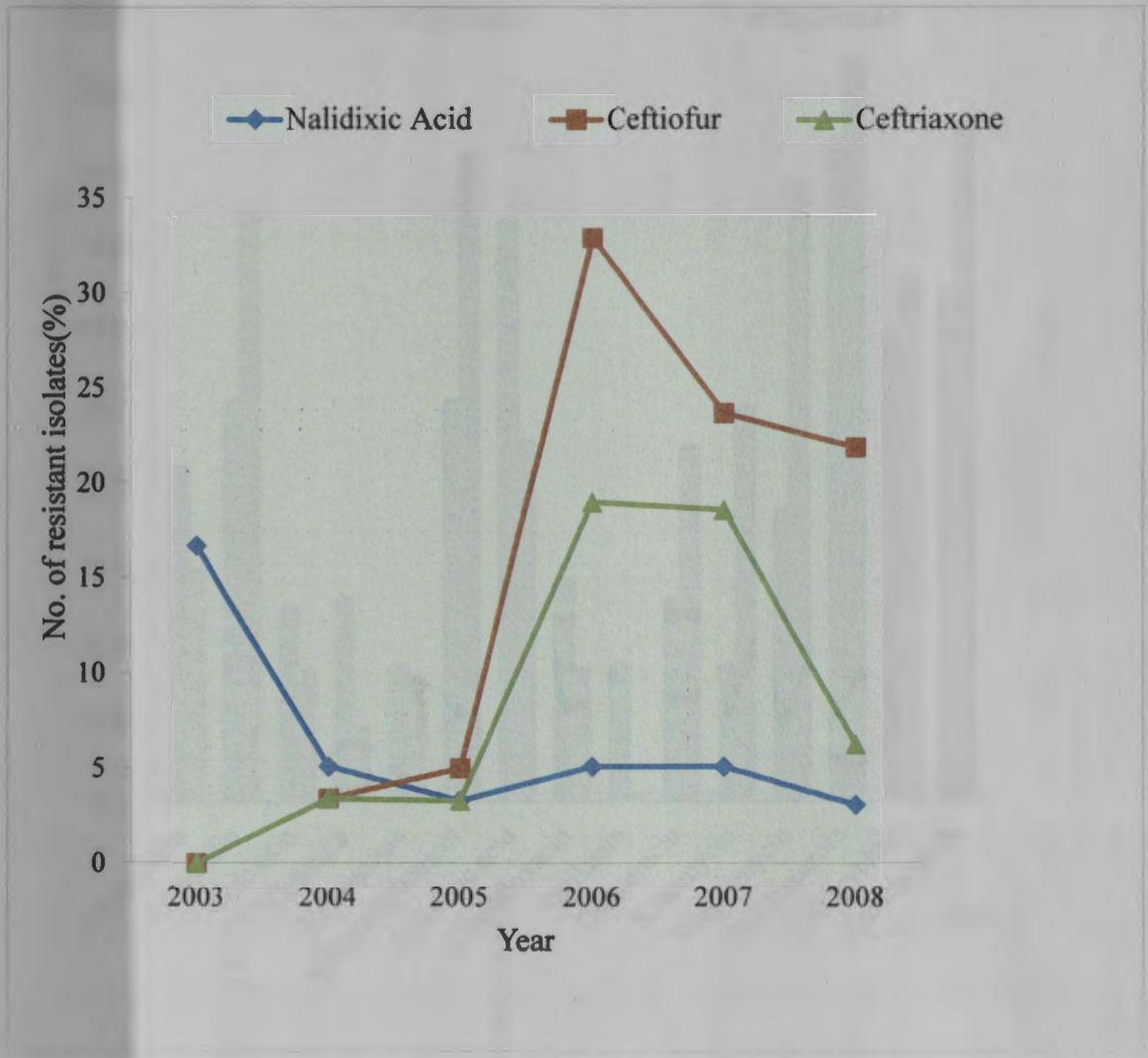


Figure 6: Comparison of resistance trends of cattle and human *Salmonella* isolates from ND 2003-2008.

Pan susceptible isolates were 28.20% (66/173) in cattle and 65.05% (121/186) in humans (Figure 10). The most common multiple drug resistance phenotype among the *Salmonella* isolates was the classic ACSSuT (Ampicillin, Chloramphenicol, Streptomycin, Sulfisoxazole, Tetracycline) penta-resistance at 29.06% (50/172), followed by the MDR-AmpC phenotype with a total of 18.02% (31/172).

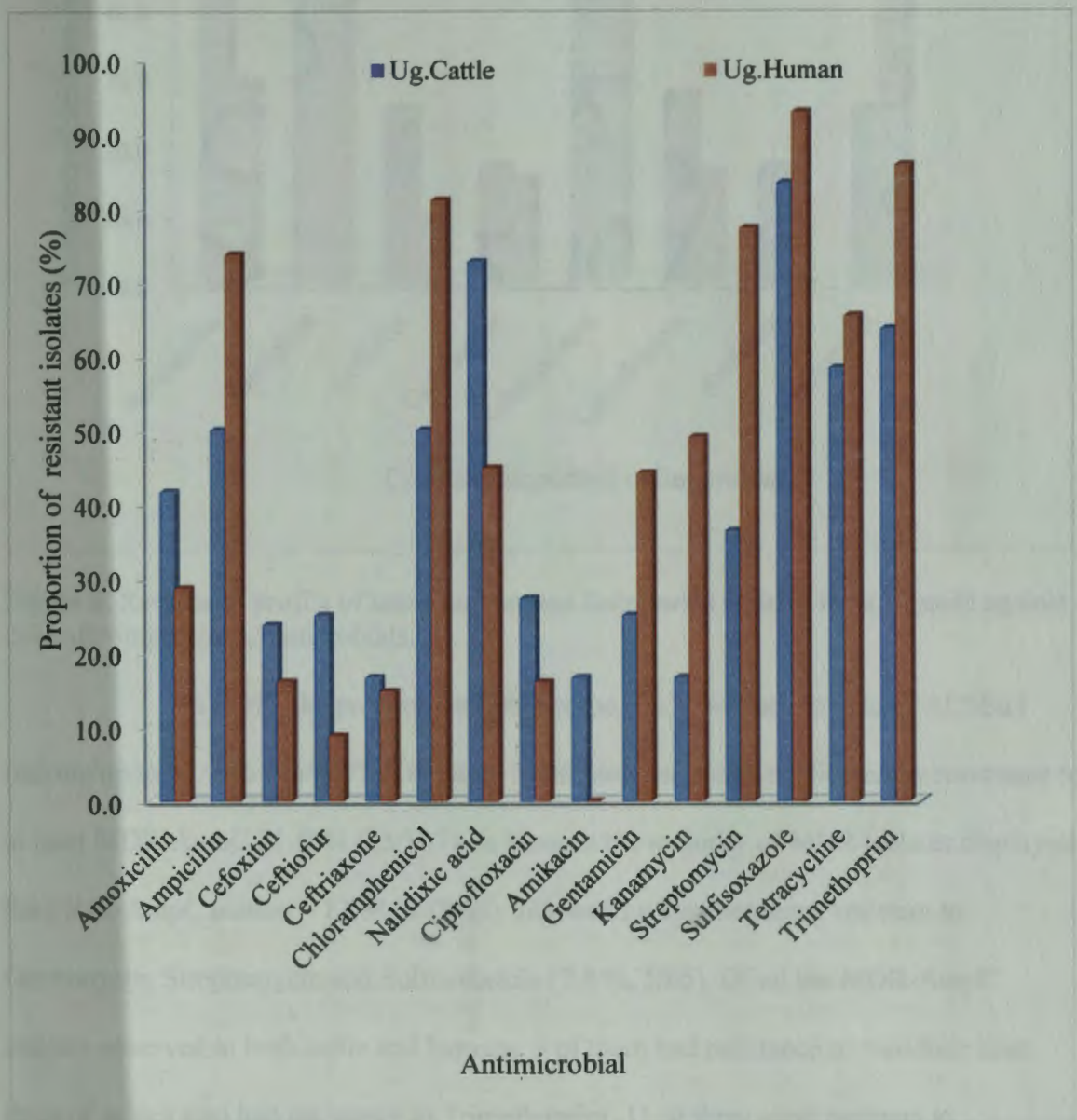


Figure 7: Resistance profile of cattle and human *Salmonella* isolates from Uganda.

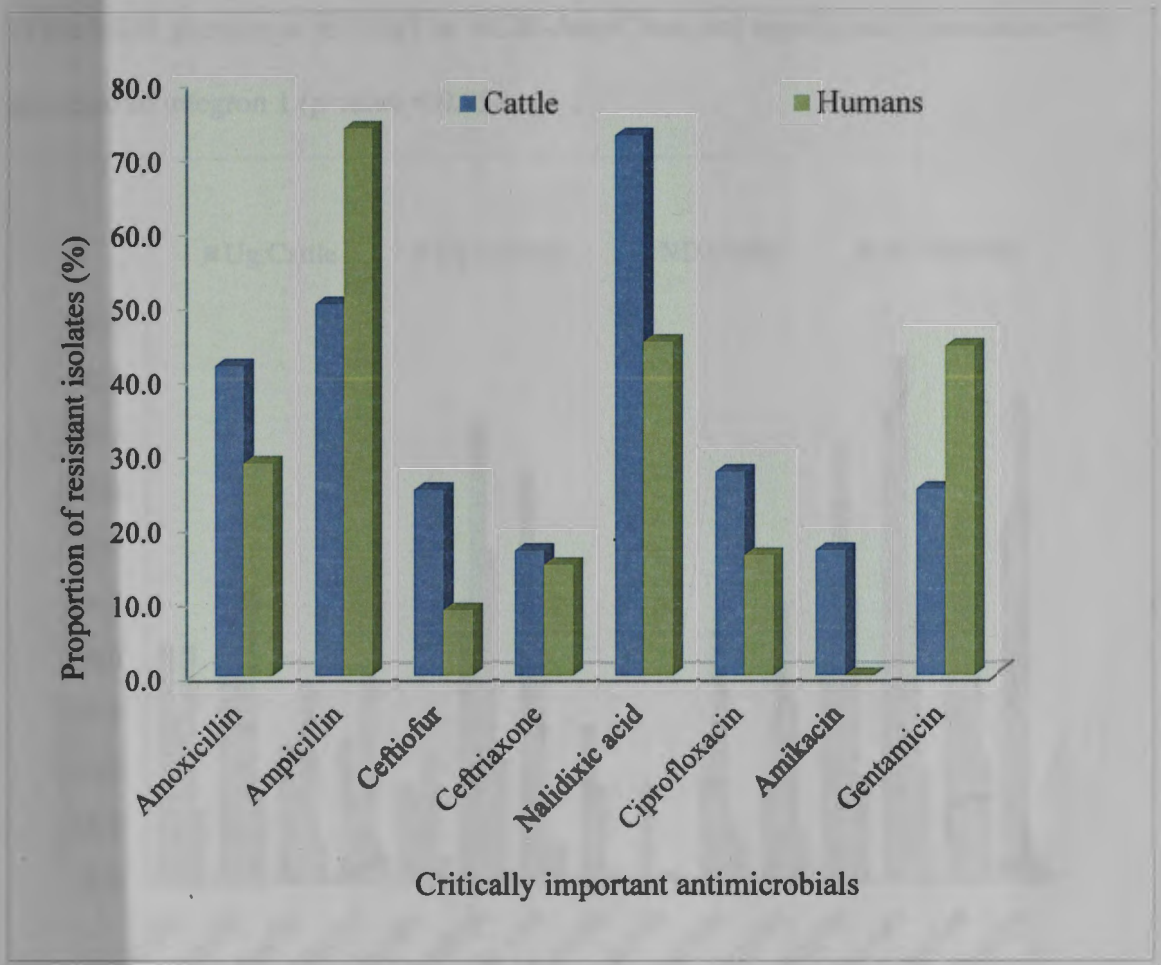


Figure 8: Resistance profile of cattle and human *Salmonella* isolates from Uganda against critically important antimicrobials.

In cattle, the predominant phenotype was resistance to at least ACSSuT making up to 42.99% (46/107) of the total MDR isolates in cattle followed by resistance to at least MDR-AmpC 21.50% (23/107). In humans the majority of MDR isolates displayed the MDR-AmpC pattern – 12.31 % (8/65) followed by the phenotype resistant to Gentamycin, Streptomycin and Sulfisoxazole (7.8 %, 5/65). Of all the MDR-AmpC isolates observed in both cattle and humans, 5 of them had resistance to Nalidixic acid three of which also had resistance to Trimethoprim; 11 of them were resistant to Trimethoprim only (a drug also used for the treatment of invasive salmonellosis). Presence

of the MDR phenotype ACSSuT or MDR-AmpC was not significantly associated with presence of integron 1 (p value < 0.05).

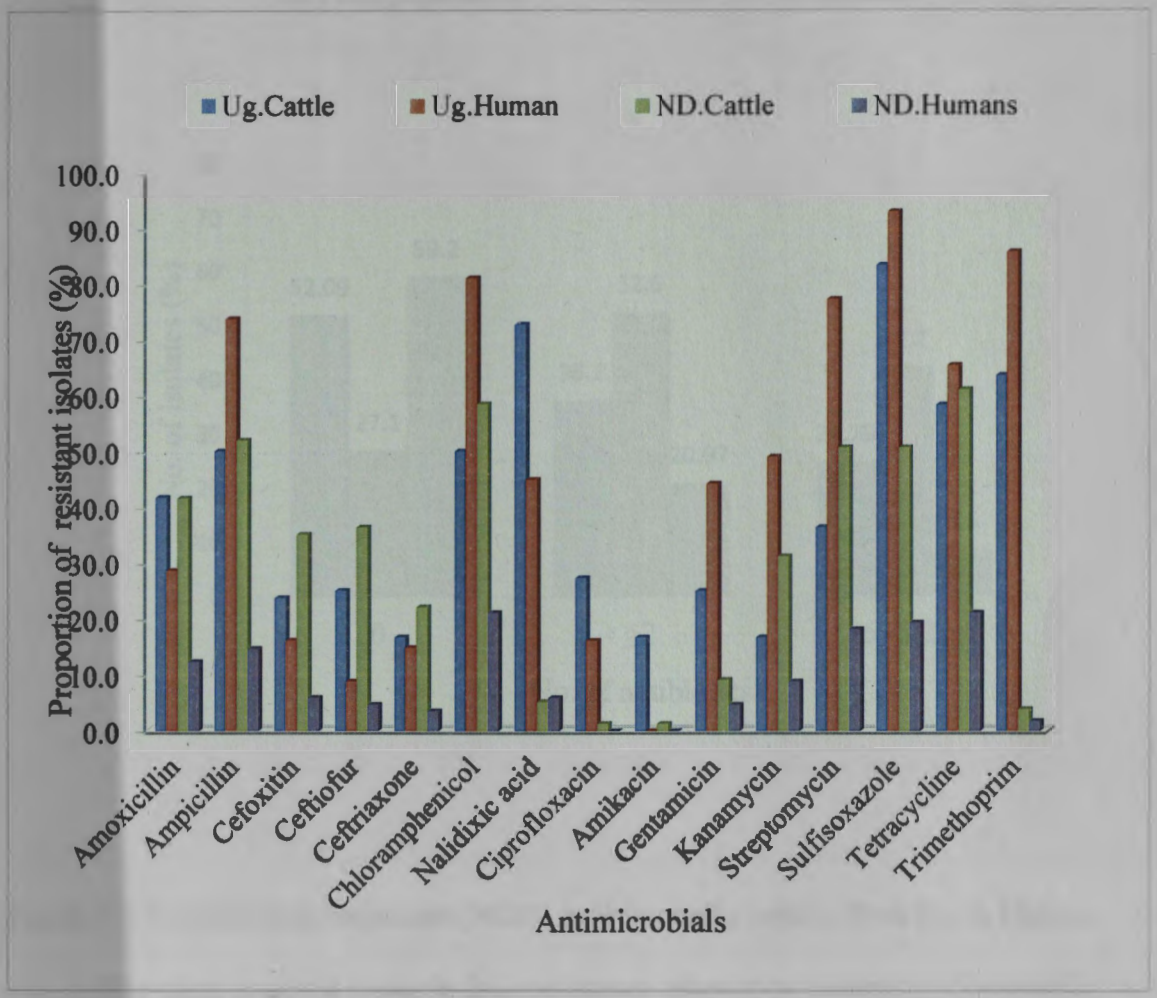


Figure 9: Comparison of antimicrobial resistance profiles from cattle and human *Salmonella* isolates from North Dakota and Uganda.

Out of all the multidrug resistant isolates (resistant to 2 or more antimicrobials) only 2 (1.16%) were resistant to Nalidixic acid while 54 (31.40%) were resistant to Ceftiofur. Of the 72 *Salmonella* isolates from Ugandan that were tested, 94.4% (68/72) were resistant to ≥2 antimicrobials while 74.6% were resistant to ≥5. All isolates were resistant to at least 1 antimicrobial (Figure 11).

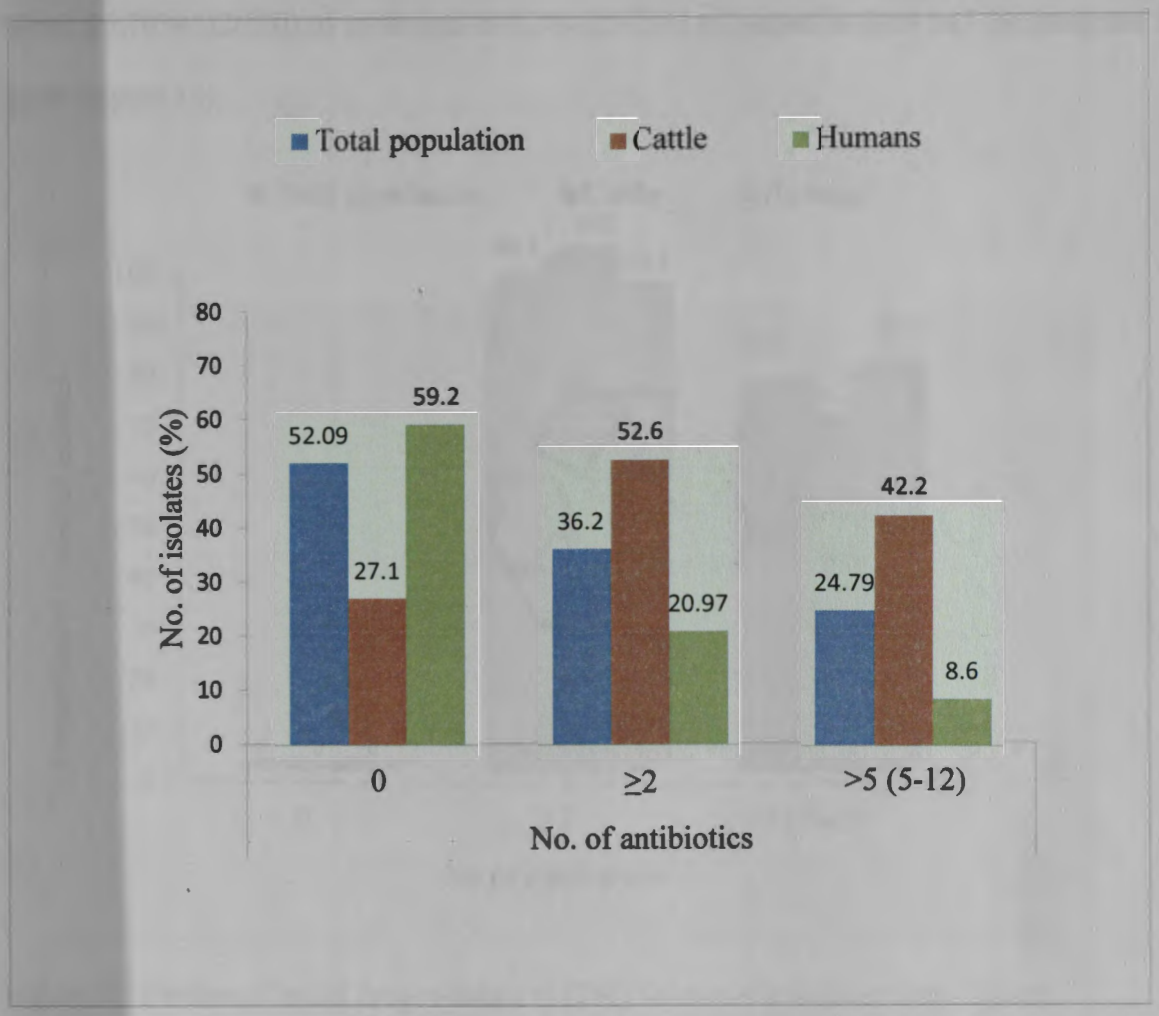


Figure 10: % Multi drug resistance (MDR) in *Salmonella* isolates from North Dakota.

The most common multiple drug resistance phenotype among the *Salmonella* isolates was resistance to ACSSuT and Trimethoprim (Ampicillin, Chloramphenicol, Streptomycin, Sulfisoxazole, Tetracycline) at 27.78% (20/72), followed by the ACSSuT-Trimethoprim and Nalidixic acid phenotype with a total of 25% (18/72).

Prevalence of Class 1 and 2 Integrons

A total of 20.70% (57/276) of the *Salmonella* isolates from North Dakota were positive for presence of the integrase 1 gene – indicative of class 1 integron presence. Of

these, 26.70% (32/120) of cattle and 16.02% (25/156) of human isolates had the integrase 1 gene (Figure 12).

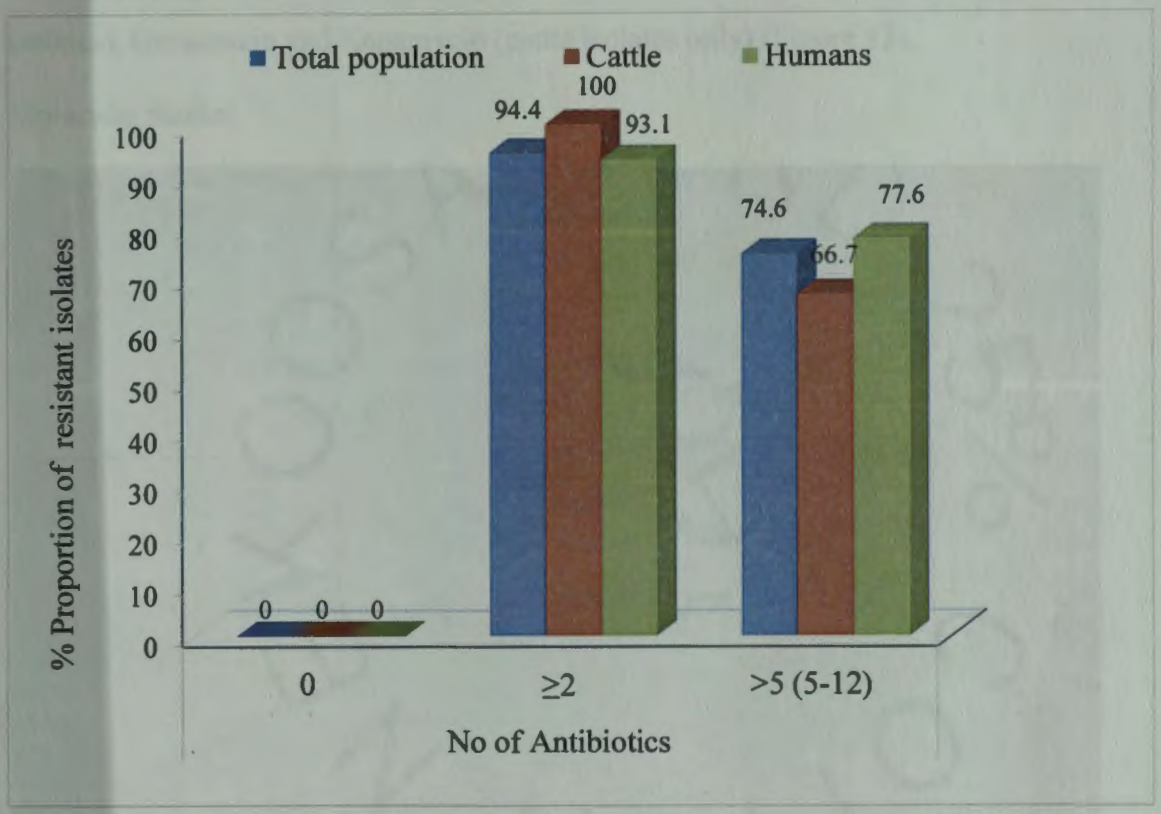


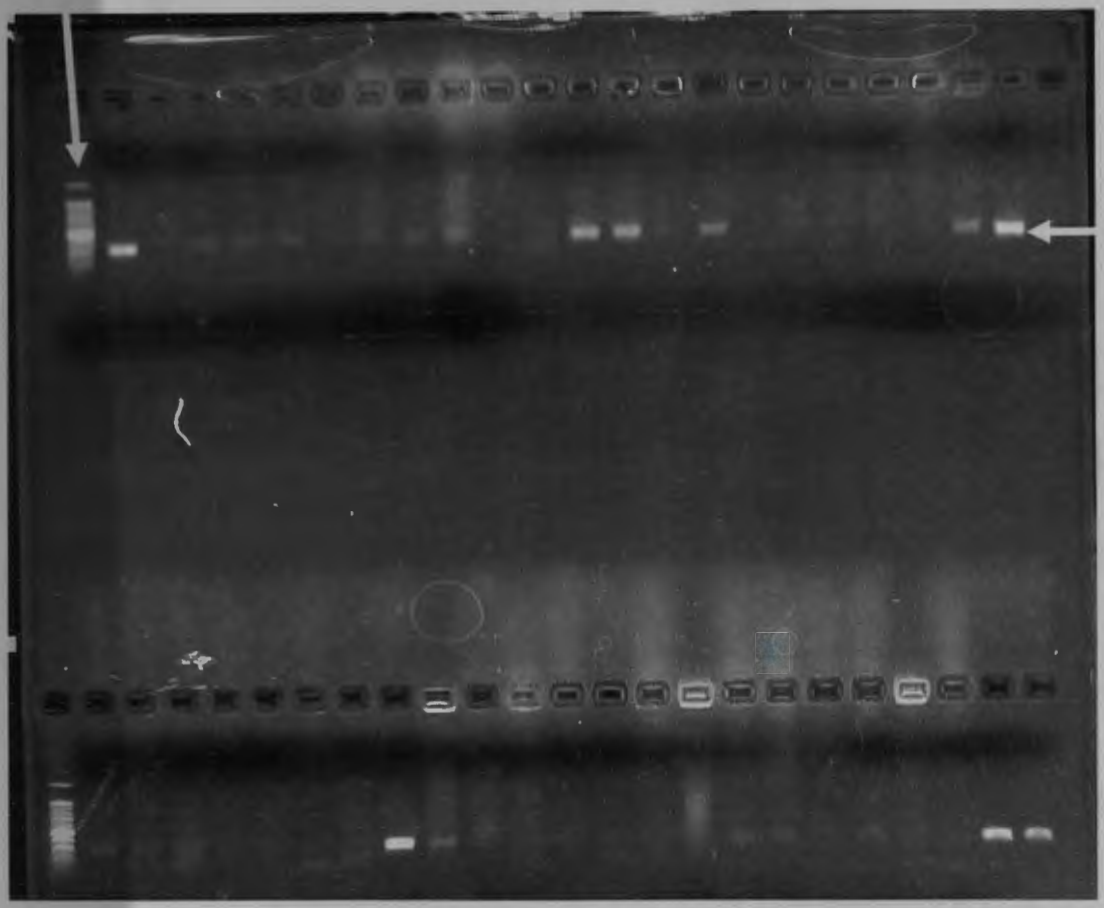
Figure 11: Percent of multi drug resistant (MDR) *Salmonella* isolates from Uganda.

Presence of class 1 integron in the *Salmonella* isolates was significantly associated with antimicrobial resistance to: Ampicillin (OR 2.78; CI 1.50, 5.14; p-value Fishers exact <0.001); Kanamycin (OR 2.56; CI 1.31, 5.01; p-value Fishers exact < 0.001); Tetracycline (OR 2.12; CI 1.16, 3.90; p-value Fishers exact 0.02) , Streptomycin (OR 2.58; CI 1.34, 4.94; p-value Fishers exact < 0.02) and Sulfisoxazole (OR 3.132; CI 1.69, 5.82; p-value < 0.001) (Table 4).

Of the samples from Uganda, a total of 45.80% (33/72) tested positive for presence of integrase 1 gene. Of these, 45.80% (27/59) of human and 46.20% (6/13) of cattle

isolates, respectively, tested positive for presence of this gene. All integron 1 isolates displayed resistance against all antibiotics apart from Amikacin (both human and cattle isolates), Gentamicin and Kanamycin (cattle isolates only) (Figure 13).

Molecular marker



280 bp *In*
1 gene

Figure 12: Image of gel with class 1 integrase amplified using *Int1* primers. The lanes contained *Salmonella* isolates tested.

Out of a subset of 30 isolates from Uganda 3 (10%) of them tested positive for integron 2 (10%, 3/30). There were higher proportions (47.9%, 34/72) of integron positive multi drug resistant *Salmonella* isolates from the *Salmonella* samples in Uganda compared to those from ND (29.85%, 40/134). Presence of class 1 integron was significantly

associated with AMR to Tetracycline (OR 5.94, CI 1.85, 19.09; p-value < 0.001) and Amoxicillin (OR 4.41; CI 1.442, 13.497, p-value < 0.01) (Table 5).

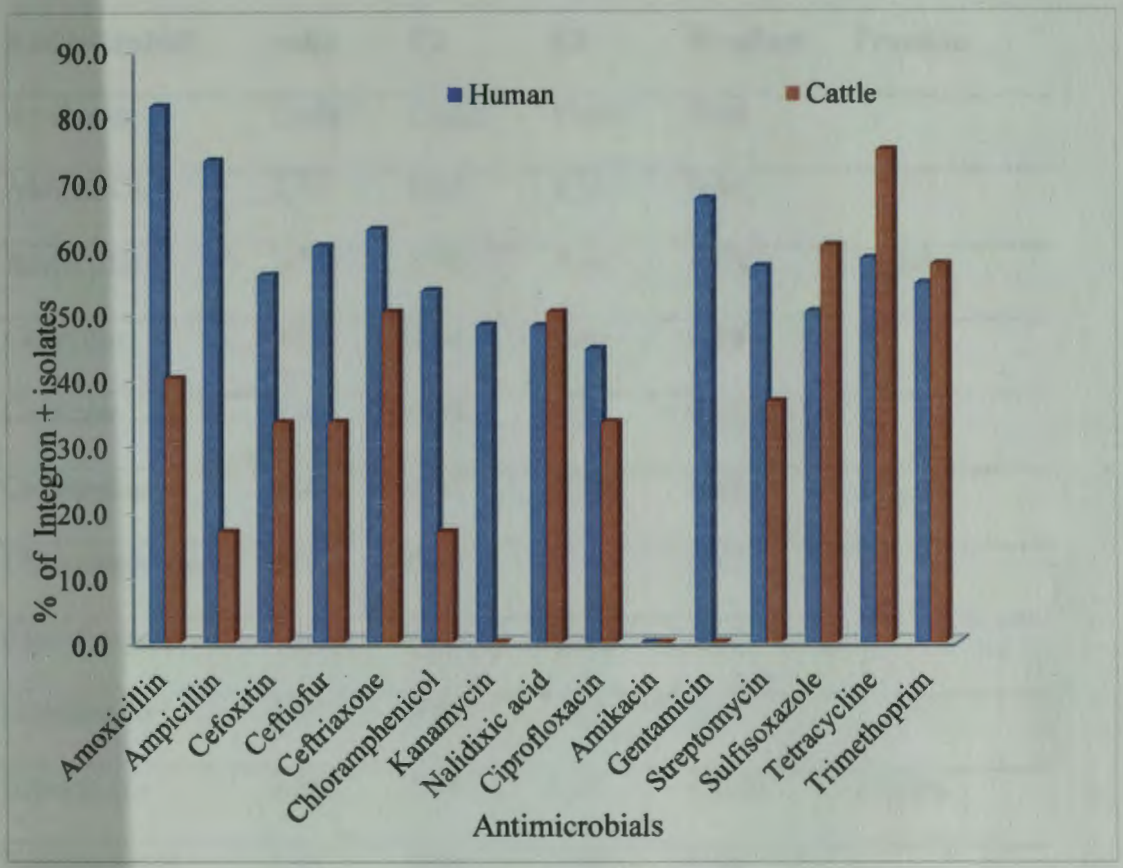


Figure 13: Proportion of integron positive resistant *Salmonella* isolates from humans and cattle in Uganda.

Association of Class 1 Integron to the Observed Antimicrobial Resistance

Of all the *Salmonella* isolates with resistance to >5 antimicrobials, 32.35% (22/68) had the integrase 1 gene. Of these 17 (30.90%) were from cattle and 5 (38.50%) from humans. In order to quantify the level of contribution of the presence of class 1 integron in the *Salmonella* isolates, to the observed resistance towards the different antimicrobials, an attributable fraction (AF) was computed.

Table 4: Association of antimicrobial resistance and presence of class 1 integron among *Salmonella* isolates from North Dakota

Antimicrobial	Odds ratio	Lower CI	Upper CI	P-values	Attributable Fraction
[redacted]n	Undef[1]	Undef	Undef	0.04	
AMOX/CLA	1.75	0.92	3.33	0.04	
Ampicillin	2.78	1.50	5.14	< 0.01	33.84%
Cefoxitin	0.87	0.14	1.20	0.14	
Ceftiofur	0.90	0.39	2.08	0.41	
Ceftriaxone	1.08	0.41	2.78	0.43	
Chloramphenicol	1.02	0.53	1.96	0.47	
Ciprofloxacin	Undef	Undef	Undef	0.01	
Gentamicin	2.21	0.89	5.48	0.27	
Kanamycin	2.56	1.31	5.01	< 0.01	17.13%
Nalidixic acid	1.55	0.04	4.96	0.48	
Streptomycin	2.36	1.34	4.94	0.02	33.07%
Sulfisoxazole	3.13	1.69,	5.82	< 0.01	37.26%
Tetracycline	2.12	1.16	3.90	< 0.01	29.92%
Trimethoprim	1.39	0.27	7.11	0.34	

[1] Undefined

Table 5: Association of antimicrobial resistance and presence of class 1 integron among [redacted]*ella* isolates from Uganda

Antimicrobial	Odds ratio	Lower CI	Upper CI	P-Values	Attributable Fraction
Amikacin	4.14	0.4893	22.58	0.2185	
AMOX/CLA	4.41	1.44	13.50	0.0048	36.31%
Ampicillin	1.766	0.62	5.03	0.013	
Cefoxitin	1.12	0.32	22.46	0.52	
Ceftiofur	1.10	0.25	4.82	0.61	
Ceftriaxone	2.0	0.58	7.88	0.26	
Chloramphenicol	1.38	0.46	4.16	0.27	
Ciprofloxacin	0.74	0.21	2.62	0.42	
Gentamicin	2.10	0.69	6.03	0.09	
Kanamycin	0.84	0.32	2.44	0.47	
Nalidixic acid	0.93	0.12	7.09	0.62	
Streptomycin	2.56	0.91	8.39	0.06	
Sulfisoxazole	5.50	0.61	49.80	0.11	
Tetracycline	5.94	1.85	19.09	< 0.01	69.23
Trimethoprim	3.60	0.88	14.75	0.40	

For *Salmonella* isolates from North Dakota presence of class 1 integron was a significantly associated with resistance to several of antimicrobials with the following values of AF: Ampicillin 33.84%; Sulfizoxazole 37.26%; Streptomycin 33.07%; Kanamycin 17.13%; Tetracycline 29.92%. Among the isolates from Uganda 36.31% of resistance towards

Amoxicillin and 65.20% of Tetracycline was attributed to presence of class 1 integron (Table 5).

DNA Sequencing

After amplification of the variable region in selected class 1 integron positive *Salmonella* isolates using primers specific for the 5'CS and 3'CS several amplicons of various sizes were detected among the isolates. The most frequently encountered profile had an amplicon that had 1000 bp followed by a 750 bp amplicon and lastly a 2000 bp amplicon (detected among the isolates from Uganda). Only 63.33% (57/90) of integrase 1(*Int 1*) positive *Salmonella* isolates from North Dakota and 67.39% (31/46) of those from Uganda contained the integron conserved sequence in their integration site.

In order to determine the content of the variable regions cradled within these integrons, the detected amplicons were subjected to DNA sequencing. Among the North Dakota isolates 2 gene cassette profiles were detected (1000 bp and 750 bp). Sequencing of the 1000 bp amplicon identified mainly the *aadA* family of genes including; *aadA1* which confer resistance to Streptomycin and Spectinomycin; acetyltransferase *(aac(6')-Ib-cr)* which confers resistance to Amikacin, Tobramycin and Kanamycin. While the 750 bp mainly contained the *dfrA1* gene.

Among the isolates from Uganda all 3 gene cassette profiles were detected (Figure 14). In one isolate two different gene cassette profiles were identified. The identified gene cassettes were *aadA1* which confer resistance to Streptomycin and Spectinomycin; dihydrofolate reductase *dfrA7, dfrA5, dfrA1* which confer resistance to Trimethoprim and aminoglycoside acetyltransferase *(aac(6')-Ib-cr)* which confers resistance to Amikacin, Tobramycin and Kanamycin, the most common profile had a combination of more than one

of these genes. Additionally, one isolate depicted some similarity (91%) to the *Salmonella enterica* subsp. *enterica* serovar Typhimurium plasmid pSLT-BT that was identified in Malawi and Kenya. This isolate was implicated in an epidemic of multiple drug resistant *Salmonella* Typhimurium causing invasive disease in sub-Saharan Africa (49). This isolate had several resistant genes including *aadA1* and *dfrA1* gene.

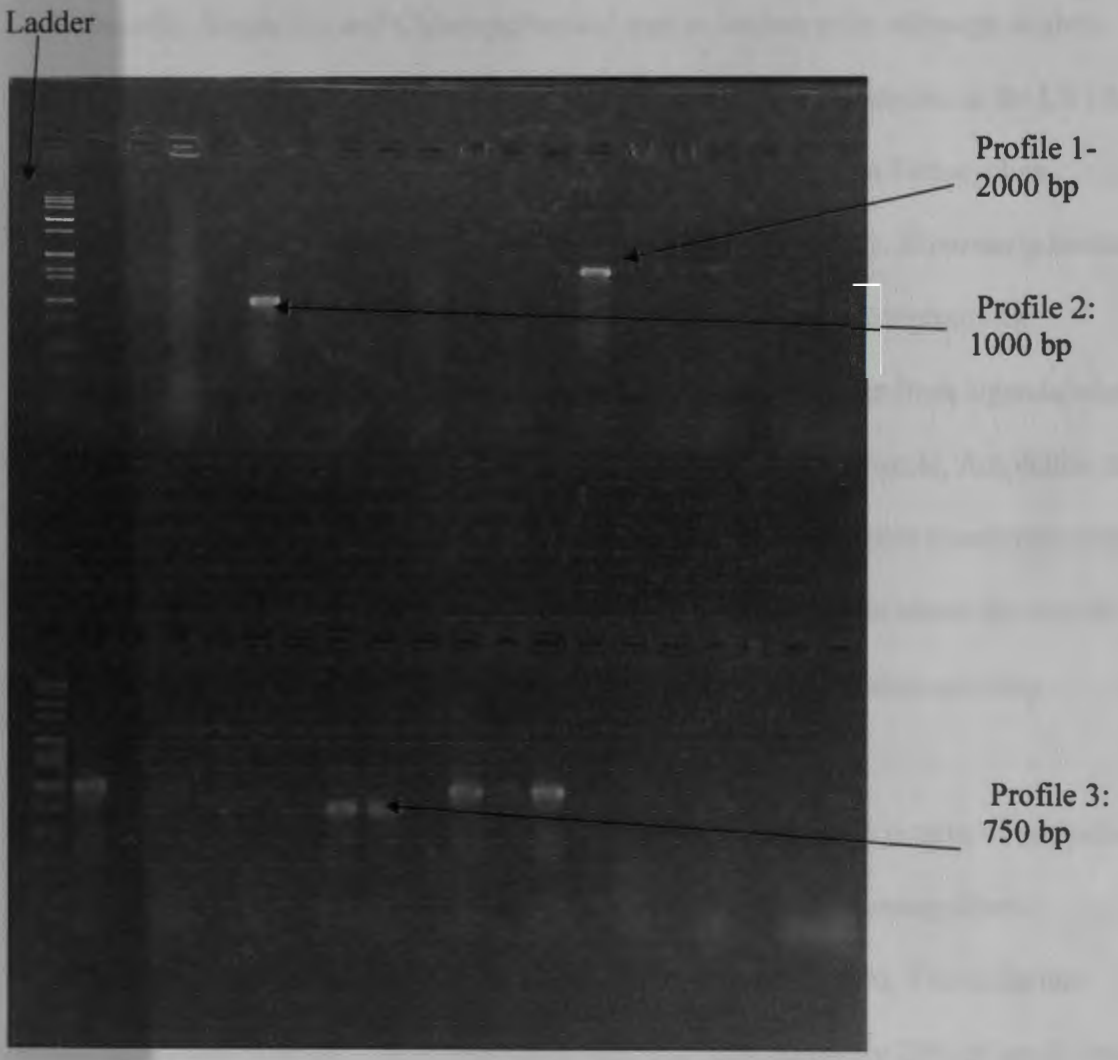


Figure 14: 1.5% Agarose gel showing different sized integron 1 gene cassette amplicons. Amplification of isolates was done using their conserved sequences (CS). MW: Hi-Lo TM DNA Ladder was used.

DISCUSSION

This study reported considerable resistance against several antimicrobials among human and cattle clinical *Salmonella* isolates, from both the US and Uganda. The data supports previous reports (6, 28, 85, 90) that antimicrobial resistance in *Salmonella* is both a human and veterinary problem. The high resistance against Tetracycline, Streptomycin, Sulfisoxazole, Ampicillin and Chloramphenicol was in tandem with, although slightly lower than, reports from four other state veterinary diagnostic laboratories in the US (AZ, NC, MO, and TN) where resistance was most often observed against Tetracycline, Streptomycin, Sulfisoxazole, Ampicillin and Chloramphenicol (107). *Salmonella* isolates from ND showed a slightly lower resistance towards Tetracycline, Streptomycin Sulfisoxazole, Ampicillin and Chloramphenicol compared to isolates from Uganda where greatest resistance was towards Tetracycline Streptomycin, Sulfisoxazole, Ampicillin and Chloramphenicol. This difference could be attributed to the easy access to antimicrobials by the general public in Uganda as compared to the US. This is made worse by over the counter prescription of most of these drugs, leading to poor prescription and drug adherence as well (45, 66, 67).

In the US the observed resistance could be a reflection of the pattern of out-patient antibacterial use in the US, which is characterized by a high use of tetracyclines, macrolides (Azithromycin) and fluoroquinolones (Levofloxacin) (35). This is further backed by reports from the USDA (4) which state that approximately 25% of small feedlot cattle operations and 70% of large feedlot operations use antimicrobials in their feed. Within these set-ups approximately 31% of cattle on small feedlot operations and 57% of cattle on large feedlot operations received antimicrobials via feed. Of the antimicrobials

used, Tetracycline and its derivatives were among the most frequently used in-feed antimicrobials on feedlot operations (4). The use of these antimicrobials in food producing animals has previously been linked to the emergence of resistant strains among human *Salmonella* isolates (1).

Another interesting observation was the similarity of the reported antimicrobial resistance profile among *Salmonella* isolates from cattle in this study, to that reported in foods such as ground meat where high resistance has been observed against Tetracycline (80%), Streptomycin (73%) and Sulfamethoxazole (69%) (92). This represents a possible route of spread of antimicrobial resistance from the cattle population to the human population (28, 68) through contamination of food products such as meat and poultry, a route that has already been identified as a potent conduit for transmission of these pathogenic isolates from animals to humans (102). However, the *Salmonella* isolates from cattle were resistant to three different kinds of cephalosporins as opposed to what had previously been cited among *Salmonella* isolates from feedlot steers in the area where all isolates were susceptible to cephalosporins (47); this difference is possibly due to the fact that the *Salmonella* isolates used in this study were from sick cattle while the feedlot cattle were apparently healthy. It has been postulated that exposure to antimicrobial agents could lead to the selection of these resistant isolates which then proliferate within the host and cause disease (33).

Third-generation cephalosporins (such as Ceftriaxone) and fluoroquinolones (such as Ciprofloxacin) are choice drugs for the treatment of invasive forms of *Salmonella* infections in humans in the US. In this study, we reported the presence of *Salmonella* strains that were resistant to these important classes of antimicrobials. Up to 17 (4.7%)

isolates were resistant to Nalidixic acid while 56 (15.70%) of all isolates tested were resistant to Ceftiofur. A total of 7 (2.4%) isolates also had reduced susceptibilities against ciprofloxacin (MIC $\geq$ 0.25 μg/ml) (20). This emergence of isolates resistant to Nalidixic acid with reduced susceptibilities to Ciprofloxacin is of great concern given the possibility of treatment failures as has previously been reported (78). The use of the flouroquinolone Enrofloxacin in food animals could also be influencing the emergence of such multidrug resistant isolates with decreased flouroquinolone susceptibility. Moreover, it has been previously noted that the introduction of this drug in veterinary therapeutics of food animals, was followed by an increase in resistance among *Salmonella* and *Campylobacter* isolates against quinolones and flouroquionolones (77).

The antimicrobial resistance patterns observed also revealed unique associations, such as, the higher resistance to Nalidixic acid among humans as compared to the cattle. This could be due to the use of flouroquinolones for the treatment of invasive salmonellosis in adults (cross resistance). Additionally, use of Nalidixic acid in poultry medicine was linked to the emergence and transmission of these resistant genes from poultry products to humans (74). Generally there was a slight decrease in the prevalence of Nalidixic acid resistant bacteria from about 15% in 2003 to 5% in 2008 among the *Salmonella* isolates from North Dakota. This could be attributed to the withdrawal of flouroquinolones from poultry production during this time period due to a ban on its use by the FDA in 2005; in fact, some production units had ceased using it as early as 2000 (71).

Another interesting observation was the high resistance of *Salmonella* isolates from cattle in the US to beta -lactam antimicrobials such as Ampicillin and Ceftiofur. While this could be attributed to the occurrence of multiple drug resistant isolates, the specific use of

some of these drugs in animal medicine, such as Ceftiofur (FDA approved for the treatment of bovine respiratory diseases) (84) could explain the greater resistance observed among the cattle isolates. This could also explain the considerable resistance observed against Ceftriaxone a drug in the same class with Ceftiofur (possibly due to cross resistance) which is not used in animal medicine but is indicated for treatment of invasive salmonellosis in children (29).

Similarly the higher resistance observed against Kanamycin in the cattle isolates could be due to cross resistance as a result of Neomycin use in cattle for the control of *E. coli* associated morbidity and mortality (21). Conversely, most of the resistance seen against Chloramphenicol in humans was not associated with resistance to any other antimicrobial. This was different from the scenario in cattle where Chloramphenicol resistance was usually associated with presence of the classic penta-resistance phenotype-ACSSuT (Ampicillin, Chloramphenicol, Streptomycin, Sulfisoxazole and Tetracycline). This finding could be due to the fact that use of Chloramphenicol in food animals was prohibited by the FDA because of its tendency to cause blood dyscrasia and its potential to induce aplastic anaemia in humans (81), whereas its use in human medicine still continues, for infections where other antimicrobials are not effective or contraindicated (81). This is a clear indication of how sustained use of a drug could result in selection of resistance genes among commensal and pathogenic bacteria.

Our results indicated a difference in the antimicrobial susceptibility of Salmonellae in different hosts (cattle and humans), and from different geographical regions. In general, isolates from cattle displayed a higher resistance than those from humans in North Dakota. The use of antimicrobials in food animals could be a major contributing factor to the higher

resistance seen among the cattle isolates (33, 75). This could be attributed to the selective pressure that results in the proliferation and dissemination of drug resistant strains (73, 89). However the lack of drug use information among the subjects from whom the isolates were obtained, limits the ability to make a definite inference linking the resistance observed against particular antimicrobials and antimicrobial use

For *Salmonella* isolates from Uganda resistance frequencies were equally high in both cattle and human isolates against most of the drugs tested, apart from Amikacin, Ciprofloxacin and the cephalosporins. This high resistance could possibly be due to the unregulated use of drugs in the in both veterinary and human medicine and the relative ease of access to antimicrobials in the country as previously noted. For example Penicillins and Trimethoprim-Sulphamethoxazole (Co-trimoxazole) are the cheapest drugs available in Uganda. This would explain the high resistance seen against these drugs (45). Conversely, the high cost of extended spectrum antimicrobials such as the cephalsporins and Amikacin would suggest lower use in both humans and cattle and explain the low resistance observed (16, 45). Also, when compared to the North Dakota isolates, the isolates from Uganda showed much higher resistance against a wider range of antimicrobials, possibly for the same reason (easy access to antimicrobials) compounded by the over–the-counter purchase of antimicrobials without much diagnostic analysis to determine the best course of treatment. Reports from neighbouring Kenya indicate that Ampicillin Chloramphenicol, Gentamicin, Trimethorpim-Sulfamethoxazole, Penicillin and Tetracycline are used as the first course of treatment in tertiary referral hospitals with Amikacin, Cefuroxime, Ciprofloxacin and Nalidixic acid as the second line of treatment. This treatment regime would probably be similar in other developing countries in Africa or Asia (27, 42).

Therefore it is possible that the availability of these drugs within these countries would probably be similar.

Moreover, the AMR levels reported in the Ugandan isolates were similar to what had been reported in a neighbouring country, Kenya, within the same region. Oundo et al., (46, 69) reported MDR in greater than 50% of non typhoidal *Salmonella* (NTS) isolates obtained from cases of invasive NTS infections in Kenya. This supports the hypothesis that similar health systems and policies in developing countries could explain the rising antimicrobial resistance reported within these nations (67). Some of the risk factors that are associated with this rising level of resistance include: antimicrobial misuse and abuse. use of poor quality antimicrobials, use of narrow repertoire of antimicrobials on most patients, inadequate sanitation in health care institutions and large proportion of immunocompromised individuals. These results underscore the need to re-evaluate the current treatment regimen for salmonellosis in Uganda given the reported resistance against Ciprofloxacin.

Among the ND isolates the multi-drug resistant ACSSuT phenotype was the predominant phenotype as previously reported (104). This phenotype has been linked to the emergence and spread of the multi drug resistant *S.* Typhimurium DT-104, whose origin has been linked to sea gulls. This strain has been credited with significantly contributing to the increase in resistance in the past ten years among *Salmonella* isolates and has been known to spread through food animals (mainly cattle, pigs, poultry) to humans (77); it has also been identified in other domestic and wild animals (13).

The majority (75%, 21/28) of the MDR-AmpC (ACSSuT phenotype + resistance to Amoxicillin and Ceftiofur) isolates were recovered from cattle, which is in agreement with

previous reports (22) of its recovery only from diseased cattle. This finding has significant implications both in human and animal medicine. Infection of cattle with such isolates would lead to complicated outcomes, and persistence of MDR-AmpC isolates within the cattle population consequently spilling over to the human population through diseased cattle or contaminated beef products as has previously been noted (21). In this study, 11(39%) of the MDR-AmpC isolates were also resistant to Trimethoprim-Sulfamethoxazole, 3 (11%) were also resistant to Nalidixic acid while 2 (7%) were resistant to Nalidixic acid only, in addition to the MDR-AmpC complex. It is important to note that resistance against Nalidixic acid is a marker for the emergence of flouroquinolone resistance or reduced susceptibilities.

Among the Ugandan isolates the most common resistance observed was resistance to the ACSSuT pentad + Trimethoprim/Sulfamethoxazole followed by resistance to ACSSuT+ Nalidixic acid. Resistance to the ACSSuT group could be linked to the emergence and spread of the notorious DT104 *S.typhimurium* strain globally (77) while the high frequency of Trimethoprim/Sulfamethoxazole and Nalidixic acid reported could be due to the low associated cost and introduction of oral forms of quinolones, respectively (67). Fluoroquinolones with Trimethoprim/Sulfamethoxazole are used for the treatment of invasive salmonellosis in humans (1); resistance to these drugs would therefore narrow the spectrum of effective antimicrobials available for treatment or control of *Salmonella* infections. Moreover, other studies (29) have also reported MDR against third-generation cephalosporins and flouroquinolones which are recommended for the treatment of severe infections.

Prevalence of class 1 integrons among the *Salmonella* isolates reported in this study (23%) was slightly lower than that identified in some previous reports (93), where up to 43% of isolates had class 1 integrons. This difference could be due to the fact that this study focused on cattle and human beings while the previous study examined a wide range of domestic animals in addition to cattle. Antimicrobial use for treatment and prophylaxis varies among the different domestic animals with strong selection pressure being exerted where higher antibiotic use is prevalent. This in turn selects for integron carrying isolates that may contain and express one or more linked antimicrobial –resistance genes (92). However, other studies (100, 103) have reported similar prevalence of class 1 integrons in *Salmonella* to that reported in this study. Also, *Salmonella* isolates from Uganda had a higher proportion of integrase 1 gene compared to those from ND. This could be due to greater antimicrobial selection pressure among the isolates from Uganda. Despite the paucity of data on class 1 integrons in *Salmonella* isolates, a few reports (30, 50, 59) have indicated equally high prevalence of these integrons in Salmonellae isolated from both humans and cattle.

Not all MDR isolates had presence of integrons. Up to 51.4%(37/72) and 70% (251/359) of multi drug resistant *Salmonella* isolates from Uganda and ND, respectively, did not have class 1 integrons further confirming the presence of other mechanisms that mediate the observed resistance. This was supported by attributable fractions (AF) that were used to quantify the association of presence of integrons with AMR in the *Salmonella* isolates; they ranged from – 65.2 % to 17.13%, indicating that not all the AMR observed was explained by presence of integrons. Rather, that other mechanisms that mediate the observed resistance exist.

The presence of mobile elements such as transposons and integrons has been credited with the rapid dissemination of antimicrobial resistance (106). In our study we report significant associations between resistance to several antimicrobials and presence of class 1 integrons. The data indicated that presence of class 1 integron explained a sizeable proportion of the multi drug resistant profiles observed. For *Salmonella* isolates from North Dakota, presence of class 1 integron was significantly associated with resistance to some of the antimicrobials tested with the following values of AF: Ampicillin 33.84%; Kanamycin 17.13%; Sulfizoxazole 37.26 %; Tetracycline 29.92%. Among the isolates from Uganda, 36.31% of resistance towards Amoxicillin and 65.20% of Tetracycline was attributed to presence of class 1 integron. Also, three different class 1 integron profiles were observed with the most common profile being a 1.0 kb integron. Just as previously reported there was a high frequency of *dfra1* (Trimethoprim) *aadA1* genes (Streptomycin) (64). Therefore, resistance against Streptomycin and Trimethoprim among these isolates is largely mediated by presence of class 1 integron (38, 39, 52, 106). The fact that the AF values were < 100% indicates that other mechanisms of AMR exist. It could also mean that the class 1 integrons were located in extra chromosomal areas such as conjugative plasmids and hence were not detected in this study. Class 2 integrons could also be contributing to the carriage and dissemination of antimicrobial resistance genes(87). Further research could focus on quantifying AFs for other mechanisms that code for AMR in *Salmonella* isolates.

CONCLUSION

In conclusion, our data provides valuable information about emerging trends in antimicrobial resistance in the study areas, which could provide information for therapeutic selection for treatment of infections; this is especially since MDR isolates have been linked to greater hospitalisations, fewer therapeutic options and more complicated outcomes (67). This study reports the presence of class 1 integrons conferring multiple resistance phenotypes among non-typhodial Salmonellae isolated from clinical cases of salmonellosis from both cattle and humans in ND and Uganda. To our knowledge this is the first report of the presence of class 2 and the second account of class 1 integrons in clinical *Salmonella* isolates from humans and cattle in Uganda.

From the results it was evident that higher resistance was not only present against antimicrobials widely used in veterinary/ human medicine, but also against drugs whose medical use is restricted implying possible horizontal transmission mediated by molecular structures such as class 1 integrons. This further emphasises the importance of integrons in the transmission of antibiotic resistance genes in MDR Gram–negative bacteria. Therefore, the presence of integrons in these multi drug resistant strains from clinical samples in North Dakota and Uganda could result in easy dissemination of this resistance to other pathogenic and non-pathogenic bacteria with negative clinical implications.

This study also shows that in both study sites a significant number of clinical cases of salmonellosis in both humans and cattle are caused by MDR isolates. The identical gene cassettes found in many of the isolates, regardless of host or geographical location, indicates that these genes may have a common source of origin with a capacity to readily be disseminated among many bacteria. This underscores the need for international co-

operation in limiting the emergence and spread of MDR *Salmonella* isolates in light of the increased international trade and travel. However, despite the identified resistant genes, integrons and their associated gene cassettes did not always explain the presence of MDR among the tested isolates. This is clearly depicted by the presence of MDR among isolates that did not have any class 1 integron cassettes and would therefore be explained by other resistant mechanisms such as mutations, presence of efflux pumps and decreased permeability. This underscores the need for further molecular studies that would determine other mechanisms involved in explaining AMR patterns of *Salmonella* isolates from different sources and their genetic relatedness.

To the best of our knowledge this was the first report of a *Salmonella* isolate in Uganda which showed similarity (91%) to the multi drug resistant *Salmonella enterica* isolate that has been linked to an epidemic of multi-drug antibiotic resistance and invasive disease in Sub Saharan Africa. This is suggestive of a clonal spread of a virulent strain of *Salmonella*, further emphasising the need for a Pan African/global approach in its control. These results do point towards the need to re-evaluate the current treatment regime for salmonellosis in Uganda. Also containment of AMR spread could be addressed through educational interventions that target the patient and clinician prescribing the drugs, to improve drug adherence and accurate prescription. Compliance with international guidelines such as the Integrated Management of Childhood Diseases, the use of an essential drug list and improved diagnostic procedures. All these measures have been reported to be effective in controlling AMR spread.

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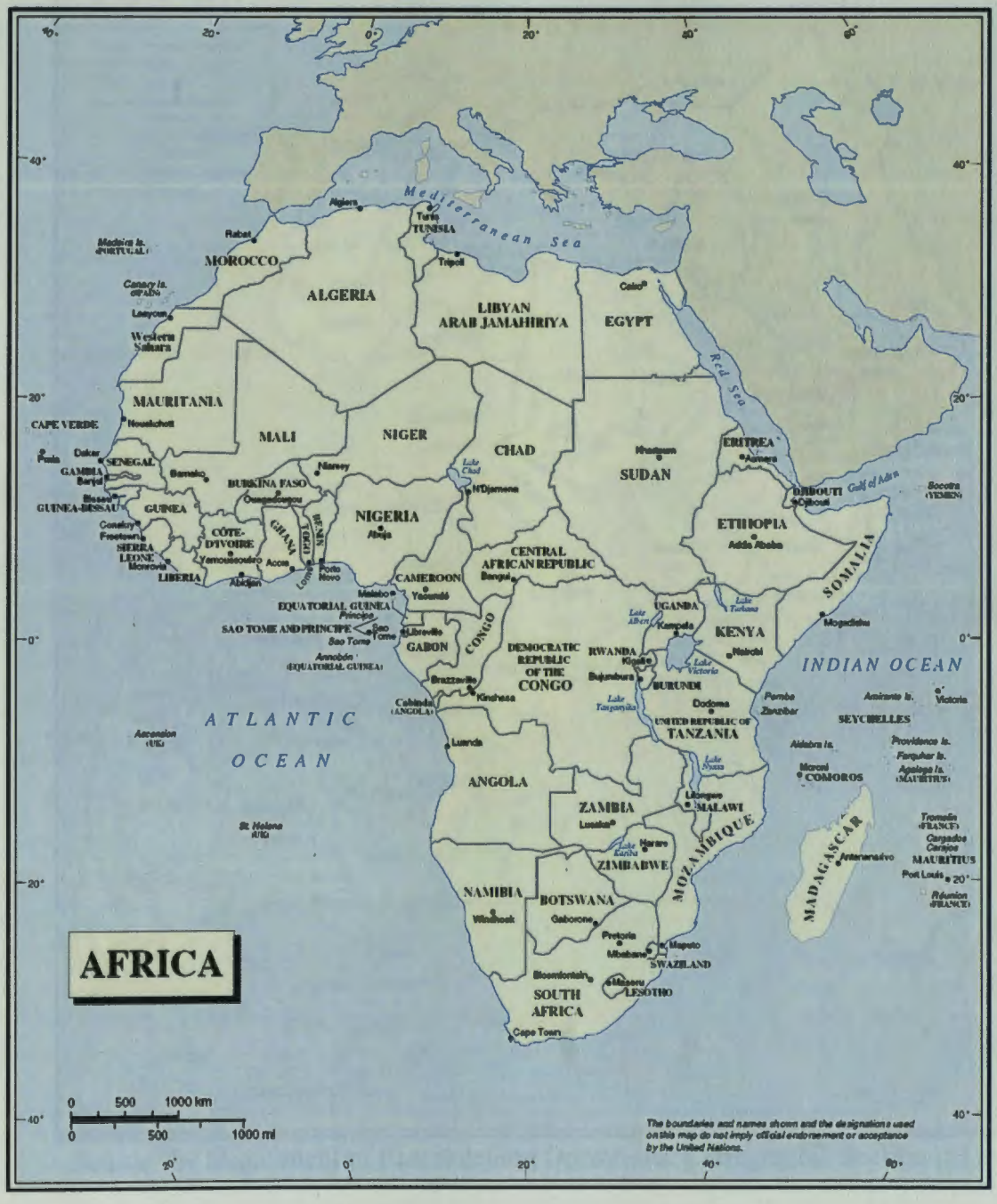
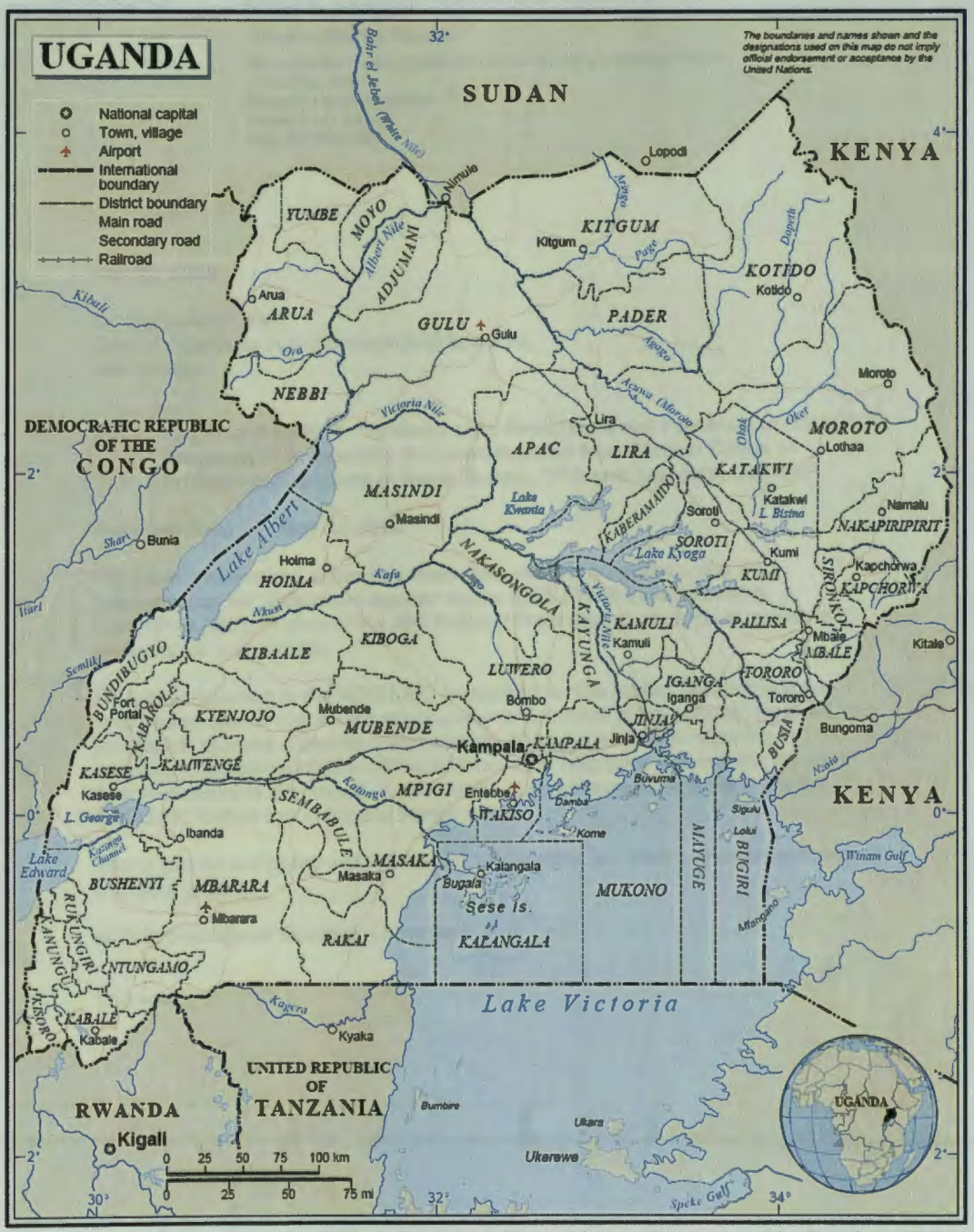
APPENDIX

Map of Africa



Source UN Department of Peacekeeping Operations. Cartographic Section.(82)

Map of Uganda



Source UN Department of Peacekeeping Operations. Cartographic Section (83)

NDSU NORTH DAKOTA STATE UNIVERSITY

701.231.8114
Fax 701.231.8098

Institutional Biosafety Committee

Office of the Vice President for Research, Creative Activities and Technology Transfer
NDSU Dept. 4000
1735 NDSU Research Park Drive
Research 1, P.O. Box 6050
Fargo, ND 58108-6050

May 22, 2009

Dr. Margaret Khaitsa
Dept of Veterinary and Microbiological Sciences
Van Es Hall

Re: IBC Project **#B0919: "Antimicrobial Resistance and Presence of Class 1 Integrons in Salmonella Serovars Isolated from Clinical Cases of Animals and Humans in North Dakota, USA and Kampala, Uganda"**

Approval Date: May 22, 2009

The project referenced above has been reviewed and accepted under the categorization for "***infectious agents***" by the NDSU Institutional Biosafety Committee (IBC). A copy of the *IBC Protocol Form* with an approval signature is enclosed for your records.

No further reporting to the NDSU IBC is required for this project unless there are unexpected events concerning exposure or containment of the agent(s) involved, or you decide to make a change in the project. Although no further reporting is necessary, an annual update will be sent to you to help track and monitor the work over the course of the project. If you decide to make changes, please notify the NDSU IBC before any change is implemented.

Thank you for complying with NDSU IBC procedures, and best wishes for success with your project.

NDSU, Institutional Biosafety Committee

Enclosure

Institutional Review Board ...for the protection of human participants in research

North Dakota State University
Sponsored Programs Administration
1735 NDSU Research Park Drive
NDSU Dept #4000
PO Box 6050
Fargo, ND 58108-6050 231-8995(ph) 231-8098(fax)

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Administration

Protocol Amendment Request Form

Use this form to request change(s) to a previously approved protocol. Examples may include, but are not limited to: addition of investigators or key personnel, revised recruitment procedures, compensation scheme, a new participant population, research setting, data collection procedures, or additional/changes to the information to be collected. These changes require review and approval by the IRB prior to implementation, except where an immediate change is necessary to eliminate a hazard to the participant. However, very minor changes such as slightly revised wording on a survey or consent form would not require IRB approval, as long as the revised document(s) will not collect identifiable or potentially harmful or sensitive information, or alter participants' understanding of the study.

Protocol Information:

Protocol #: **AG08230** Title: **Evaluation of Salmonella Occurrence in Domestic Animals and Humans in North Dakota**

Principal investigator: **Margaret L. Khaitsa** Co-investigator: **Dawn Doetkott**

Principal or co-investigator Signature, Date: Khaitsa 3/17/09

Description of proposed changes:

Provide a complete description of all proposed change(s), including justification for the change(s). Also address whether or not the change(s) will increase any risk of harm (physical, economic, psychological, or sociological) or privacy protections for subjects.

☒ Investigators or research team. *Describe change(s), including role(s) in the research; provide documentation of training, if not previously on file with the IRB.*
Addition of new personell - Michael W. Mahero (MS graduate student)l
Research Project:
Antimicrobial Resistance and Presence Of Class 1 Integrons in Salmonella Serovars Isolated from Clinical Cases Of Animals and Humans In North Dakota

Objectives:
1.To characterise AMR patterns of Salmonella isolates from clinical cases of animals and humans in North Dakota
2. Test for presence of Class 1 Integrons in Salmonella Serovars
3. Determine the association between the observed AMR and presence of class 1 integrons and
4. Assess the genotypic relatedness of the Salmonella isolates using pulsed field gel electrophoresis

Uganda National Council For Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Your Ref:.....

Our Ref:.....HS.604.. *Date:*..28/07/09.....

Mr. Michael W. Mahero
Faculty of Veterinary Medicine
Makerere University
P.O Box 7062
Kampala

Dear Mr. Mahero,

RE: RESEARCH PROJECT, "ANTIMICROBIAL RESISTANCE AND PRESENCE OF CLASS 1 INTEGRONS IN SALMONELLA SEROVARS ISOLATED FROM CLINICAL CASES OF ANIMALS AND HUMANS IN NORTH DAKOTA, USA AND KAMPALA, UGANDA"

This is to inform you that the Uganda National Council for Science and Technology (UNCST) approved the above research proposal on **June 26, 2009.** The approval will expire on **December 26, 2009.** If it is necessary to continue with the research beyond the expiry date, a request for continuation should be made in writing to the Executive Secretary, UNCST.

Any problems of a serious nature related to the execution of your research project should be brought to the attention of the UNCST, and any changes to the research protocol should not be implemented without UNCST's approval except when necessary to eliminate apparent immediate hazards to the research participant(s).

This letter also serves as proof of UNCST approval and as a reminder for you to submit to UNCST timely progress reports and a final report on completion of the research project.

Yours sincerely,

Leah Nawegulo
for: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

LOCATION/CORRESPONDENCE
Plot 3/5/7. Nasser Road

COMMUNICATION
TEL: (256) 414-250499, (256) 414 705500

THE REPUBLIC OF UGANDA

OFFICE OF THE PRESIDENT

PARLIAMENT BUILDING P.O.BOX 7168 KAMPALA TELEPHONES 25448106, 343934 343934 343926 343943, 233717, 344026 230040, FAX 256456/256141

ADM 154/212/01

July 15, 2009

The Resident District Commissioner
Kampala District

This is to introduce to you **Mahero Michael Wandanje** as a Researcher who will be carrying out a research entitled **"Antimicrobial Resistance and Presence of Class 1 Integrons in Salmonella Serovars Isolated from Clinical Cases of Animals and Humans in North Dakota, USA and Kampala, Uganda"** for a period of **03 (three)** months in your district.

He has undergone the necessary clearance to carry out the said project.

Please render him the necessary assistance

Alenga Rose
FOR: SECRETARY, OFFICE OF THE PRESIDENT

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service
Centers for Disease Control
and Prevention (CDC)
Atlanta GA 30333

To: Permittee, Public Health Service Import Permit

Subject: Approval to import etiological agents, hosts, and vectors

Your Public Health Service (PHS) import permit is attached with this letter. The PHS import permit is valid only for the material(s), locations and conditions described in your application. Please be reminded that a person may not import into the United States, nor distribute after importation, any etiological agent or any arthropod or other animal host or vector of human disease, or any exotic living arthropod or other animal capable of being a host or vector of human disease unless accompanied by a permit issued by the Director, Centers for Disease Control and Prevention (CDC).

Please be reminded that the permittee must ensure that:

1. The enclosed import permit and labels are forwarded to the shipper(s). The enclosed permit and labels may be photocopied if more are needed.
2. The shipper includes the PHS import permit with the shipping documents and the enclosed label (or copy of label) should be affixed to the outer shipping container.
3. A record of each importation (including permits and shipping documents) is maintained.
4. The shipment of etiological agents, hosts, and vectors must be packaged, labeled, and shipped in accordance with all federal and international regulations. Please note that the issuance of an import permit is not an authorization to hand carry the material.

Please also note that other permits may be required for the importation of etiological agents, hosts, and vectors. If you have questions regarding this correspondence, please contact CDC Etiologic Agent Import Permit Program at (404)718-2077 or visit our website at http://www.cdc.gov/od/eaipp/.

Robbin Weyant, PhD, CAPT, USPHS
Director, Division of Select Agents and Toxins
Coordinating Office of Terrorism Preparedness and Emergency Response
Centers for Disease Control and Prevention

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
Centers for Disease Control and Prevention
Office of Health and Safety, MS A-46
Atlanta, Georgia 30333
TEL: 404-718-2077; FAX: 404-718-2093

CDC
SAFER·HEALTHIER·PEOPLE™

Permit to Import or Transfer Etiological Agents or Vectors of Human Disease

In accordance with 42 CFR Section 71.54 of the Public Health Service Foreign Quarantine Regulations, cited on the bottom of this permit, permission is granted the permittee to import into any port under control of the United States, or to receive by transfer within the United States, the material described in item 1 below.

PHS PERMIT NO.	2009-06-107	
DATES	ISSUED: Tuesday, June 16, 2009	EXPIRES: Wednesday, June 16, 2010
1. DESCRIPTION OF MATERIAL	DNA FROM NON-TYPHOIDAL SALMONELLA SEROVARS FROM CATTLE AND HUMANS; ISOLATES OF SALMONELLA.	
2. PERMITTEE (NAME, ORGANIZATION, ADDRESS)	MARAGARET KHAITSA NORTH DAKOTA STATE UNIVERSITY 118 VAN ES HALL, 1523 CENTENNIAL BLVD. FARGO, ND 58105	TEL: 701-231-5946 FAX: 701-231-7514
3. SOURCE OF MATERIAL (NAME, ORGANIZATION, ADDRESS, COUNTRY)	DENIS K. BYARUGABA MAKERERE UNIVERSITY KAMPALA (MAK) UGANDA FAC. OF VET. MED. MAKERERE UNIV., KAMPALA. DEPT. OF MICRO. & PARASITOLOGY KAMPALA, UGANDA	
4. TYPE OF PERMIT AND INSTRUCTIONS FOR USE	☑ Multiple Importation into the US ☐ Single Transfer Within the US A. Record of each importation shall be maintained on permanent file by permittee. B. Enclosed label(s) must be forwarded to the shipper(s). C. One label shall be affixed to shipping container. Enclosed labels may be photocopied.	
5. CONDITIONS OF ISSUANCE ITEMS APPLICABLE WHEN CHECKED	☐ A. Subsequent distribution, within the U.S., of the material described in this permit is prohibited without prior authorization by the Public Health Service. ☑ B. All material is for laboratory use only - Not for use in the production of biologics for humans or animals. ☑ C. All material is free of tissues, serum and plasma of domestic and wild ruminants, swine and equines. ☑ D. Additional Requirements: ☐ File APHIS/CDC Form 2 for select agents as defined in 42 CFR 73 ☐ IATA Packaged to preclude escape. ☑ USDA permit may be required (Telephone: 301-734-3277). ☑ E. Work with the agent(s) described shall be restricted to areas and conditions meeting requirements in the CDC/NIH publication "Biosafety in Microbiological and Biomedical Laboratories." ☑ F. Packaging must conform to 49 CFR Sections 171-180. ☐ G. Select Agent. Receiving facility must be registered under 42 CFR Part 73.	
6. COPY SENT TO ☑ U.S. QUARANTINE STATION	7. Signature of issuing officer Robbin Weyant, PhD, CAPT, USPHS, Etiologic Agent Import Permit Program	

CDC 0728 (f) 13.40) REV. 2-01

42 CFR 71.54, Etiological agents, hosts, and vectors

(a) A person may not import into the United States, nor distribute after importation, any etiological agent or any arthropod or other animal host or vector of human disease, or any exotic living arthropod or other animal capable of being a host or vector of human disease unless accompanied by a permit issued by the Director.

(b) Any import coming within the provisions of the section will not be released from custody prior to receipt by the District Director of the U.S. Customs Service of a permit issued by the Director.

Note: Other permits may be required.