Norwegian University of Life Sciences
Faculty of Veterinary Medicine

Philosophiae Doctor (PhD)
Thesis 2018:83

Non-Typhoidal *Salmonella* in Layer Hen Farms in Uganda; Occurrence, Biodiversity and Antimicrobial Resistance

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Odoch Terence Amoki

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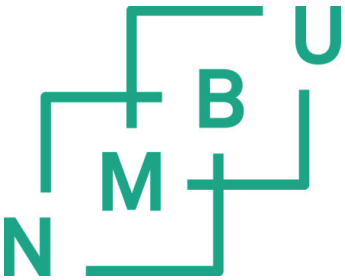
Adamstuen (2018)



Thesis number 2018:83

ISSN 1894-6402

ISBN 978-82-575-1767-0

Series of dissertations at the

Norwegian University of Life Sciences

Thesis number 2018:83

ISSN 1894-6402

ISBN 978-82-575-1767-0

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1 ACKNOWLEDGEMENT

This PhD study was undertaken at the Department of Food Safety and Infection Biology of the Norwegian University of Life Sciences (NMBU), from 2015 to 2018. My deep appreciation for the financial support that was provided by the Norwegian Programme for Capacity Development for Higher Education (NORHED), under a project (UGA-13/0031), Capacity Building in Zoonotic disease Management using Integrated Approach to Ecosystems Health at Human-Livestock-Wildlife interface in Eastern and Southern Africa (CAPAZOMANINTECO) to the College of Veterinary Medicine Animal Resources and Biosecurity (CoVAB), Makerere University, Kampala, Uganda.

I would like to express my sincere gratitude to the main supervisor of this PhD project, Professor Yngvild Wasteson whose magnanimous and unwavering efforts provided direction that led to the successful completion of this PhD study. It will not be possible to forget the role of Professor Yngvild. Also, I am highly indebted to Associate Professor Trine L'Abée-Lund my co-supervisor at NMBU who physically inducted me to some of the laboratory procedures, introduced me to the laboratory team, and kept her door opened for consultations without the need for appointments. Trine in addition, provided significant intellectual guidance and material support that effectively facilitated the timely completion of my PhD studies. Similarly, I must extend special thanks to Professor Eystein Skjerve, the CAPAZOMANINTECO coordinator at NMBU, not only for timely provision of all the logistical and welfare support, but also for technical and scientific contributions to this PhD project. Associate Professor Clovice Kankya, the overall coordinator, CAPAZOMANINTECO, spent sleepless nights working with partners developing the proposal that eventually secured the funding for this project. I sincerely owe him a lot, he kick-started my PhD journey with full support that included but not limited to approving my application and proposal, logistical, academic and intellectual backup. Clovice, as a co-supervisor, remained passionate and committed throughout. In addition, he provided for my family demands during my long stays away from home. My appreciation and thanks to Associate Professor Nasinyama for his encouragement and support particularly at the initial phases that propelled me to love working with foodborne pathogens.

I would like to express my sincere thanks to the researchers and technicians at the Department of Food Safety and Infection Biology, NMBU and affiliated institutions. The warm welcome I was accorded made me feel at home. Dr Toril Lindbäck, the most dedicated laboratory based researcher

I have ever met in my life, rescued me in many instances in the laboratory. Kristin O'Sullivan was always ready to help, ensured the laboratory supplies are in place all the time, and was my immediate full time laboratory mentor. Associate Professor Marina Aspholm, very cool and nice, ensured that I benefited from infection biology and antimicrobial resistance training program at University of Bergen. Helge Christoffer Høgberg Hansen, a fantastic dedicated fellow who deeply embedded himself in my project fully and accomplished a lot of laboratory tasks that were very challenging. Professor Henning Sørum for the positive and negative controls of the resistance genes used in this project and scientific advice. Thanks to Dr Bjarne Bergsjø for serotyping and Dr Camilla Sekse for helping with Bionumerics analysis of pulsed-field gel electrophoresis (PFGE) profiles.

Thanks to our collaborators from the University of Zurich; Professor Paul Togerson, Professor Reinhard Furrer, Dr Sonja Hartnack and Gilles Kratzer for building my capacity in the use of R-statistics and helping with additive Bayesian network modelling.

I need not underscore the assistance and cooperation I received from Olga Anna Osinska, Aud Kari Fauske, Marte Monshaugen, Gaute Skogtun, when working with them in the laboratories.

And to all the PhDs, Postdocs, researchers and staff at the Department of Food safety and Infection Biology, NMBU; Hildegunn Iversen, Kristina Borch-Pedersen, Ingun Lund Witsø, Anne Kijewski, Kristian Høy Horsberg. My special thanks to you. You were all nice and I enjoyed all the moments we had together, sharing ideas and some of you directly helping me.

To my fellow PhD students on CAPAZOMANINTECO project, Luke Nyakarahuka, Doreen Sitali, Mumba Chisoni, Nuor Madut, Ceasor Sobe. It was nice interacting, encouraging and supporting each other.

To all the academic, technical and support staff affiliated to the Department of Biosecurity, Ecosystems and Veterinary Public Health (BEP), of Makerere University where I work, thank you for your helping hands. In addition my special appreciation to all the district officials, research assistants and farmers, you accepted to work and supported this study at different levels.

I am highly indebted to my dear wife Janet Martha Odoch, my children (Pius, Paul, Patra, Peninah, Pauline) and all other children under my care, for your patience and endurance during the long periods of my absence from home as I pursued this PhD studies.

All these happened because of God the almighty. I will always keep praying to God for divine intervention in our daily lives.

2 ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AMR	Antimicrobial resistance
ASM	American Society of Microbiology
BEP	Department of Biosecurity Ecosystems and Veterinary Public Health of Makerere University
BPW	Buffered peptone water
CAPAZOMANINTECO	Capacity building in zoonotic disease management using integrated approach to ecosystems health at human-livestock-wildlife interface in Eastern and Southern Africa
CDC	Centre for Disease Control and Prevention
CDDEP	Centre for Disease Dynamics, Economics and Policy
CoVAB	College of Veterinary Medicine Animal Resources and Biosecurity
CRISPRs	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
ECDC	European Centre for Disease Prevention and Control
EMA	European Medicines Agency
ESBL	Extended spectrum beta lactamase
EU	European Union
EUCAST	European Union Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization of the United Nations
HE	Hektoen agar
HGT	Horizontal gene transfer
HIV	Human immunodeficiency virus
iNTS	Invasive non-typhoidal *Salmonella*
ISO	International Organization for Standardization
LMIC	Low and middle income countries

MDR	Multidrug resistant
MLST	Multilocus sequence typing
mRNA	messenger ribonucleic acid
MRSA	Methicillin resistant *Staphylococcus aureus*
MSRV	Modified semisolid Rappaport Vassiliadis
NAADS	National Agricultural Advisory Services
NDA	National Drug Authority
NFDA	National Food and Drug Authority
NMBU	Norwegian University of Life Sciences
NORHED	Norwegian programme for capacity development for higher education
NTS	Non-typhoidal *Salmonella*
iNTS	Invasive non-typhoidal *Salmonella*
NVI	Norwegian Veterinary Institute
OH	One Health
OIE	World Organization of Animal Health
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction restriction fragment length polymorphism
PFGE	Pulsed-field gel electrophoresis
PT	Pulsotype
QRDR	Quinolone resistant determining region
RAPD-PCR	Random amplified polymorphic deoxyribonucleic acid polymerase chain reactions
rep-PCR	Repetitive element polymerase chain reaction
RNA	Ribonucleic acid
SS	Salmonella-Shigella
UBOS	Uganda Bureau of Statistics

WGS	Whole genome sequencing
XLD	Xylose Lysine Deoxycholate
WHO	World Health Organization

3 SUMMARY

Non-typhoidal *Salmonella* (NTS) is an important global foodborne pathogen mainly acquired in humans from animal sources through consumption of contaminated animal products, contacts with animals or their environment, contaminated water and vegetables. One of the main sources of NTS is poultry and poultry products. In addition to causing illnesses in humans and animals, NTS is increasingly implicated in the spread of antimicrobial resistance (AMR) between humans and animals. Occurrence of AMR in NTS to commonly used antimicrobials complicates management of infections and limits the choice of drugs. In Uganda, the occurrence of AMR in NTS from livestock (poultry inclusive) is poorly known and yet animal agriculture, which is an important economic activity in many households, could be contributing to the spread of drug resistant NTS. The aim of this study was to determine occurrence, diversity and antimicrobial resistance of non-typhoidal *Salmonella* in layer hen farms from selected districts of Uganda.

In this thesis, a cross sectional study was designed to collect data from the districts of Wakiso, Masaka and Lira in Uganda. Data was collected in 2015 and 2016 from randomly selected poultry farms after determination of the sample size. Questionnaires were directly administered to collect data on farm management practices, disease prevention and control as well as demographic data of farmers and farm managers. In addition, faecal samples were collected from poultry houses for isolation and identification of NTS isolates. All the isolates were then later tested for antimicrobial susceptibility testing using Kirby disk diffusion methods and minimum inhibitory concentrations (MIC) by micro brsoth dilutions. Molecular subtyping of the isolates was performed by pulsed-field gel electrophoresis (PFGE) and screening for resistance genes and integron genes was done by polymerase chain reaction amplifications. Sequencing was done to detect specific mutations involved in quinolone resistance.

A total of 237 farms were involved in this study. Farm prevalence of NTS was estimated at 20.7 % of the study farms (95 % confidence interval (CI): 15.6 – 25.6 %). Altogether, 11 NTS serovars were identified (Newport, Hadar, Aberdeen, Heidelberg, Bolton, Mbandaka, Uganda, Typhimurium, Enteritidis, Kampala, Zanzibar), these were further typed by PFGE into 15 pulsotypes and 10 nontypable isolates. Large farms and the presence of other animal species on the farm were associated with NTS prevalence on farms. However, having a separate house for birds newly brought to the farms was found to be protective (OR = 0,4; 95 % CI: 0.2–0.8).

A high level of phenotypic AMR in NTS was estimated (57.7 % of the isolates), the highest level observed was resistance to ciprofloxacin (50.0 %) followed by resistance to sulfonamides (26.9 %) and resistance to sulfamethoxazole/trimethoprim (7.7 %). Resistance was significantly associated with sampled districts ($p = 0.034$). Multidrug-resistance (MDR) was detected in 15.4 % of the isolates. Resistance to ampicillin was linked to farm size with large farms being more likely to display ampicillin resistance. Resistance towards trimethoprim was linked positively to resistance towards sulfonamides, sulfamethoxazole/trimethoprim and ciprofloxacin. Resistance towards sulfonamides was also linked positively to resistance to ciprofloxacin. There was also a positive association between resistance to chloramphenicol and ampicillin, with all isolates being either both susceptible or resistant. Seven AMR genes (*blaTEM-1, cmlA, tetA, qnrS, sul1, dhfrI/dhfrVII*) conferring resistance to major classes of antimicrobials (beta lactams, chloramphenicol, tetracyclines, quinolones, sulfonamides, trimethoprim, respectively) commonly used in human and veterinary medicine were identified. Six (11 %) of the phenotypically resistant isolates carried genes that encode for class 1 integrons.

In this study high levels of reduced susceptibility (37.7 % of the 85 samples tested) to ciprofloxacin in NTS was detected and this was significantly associated with the districts where they were isolated ($p= 0.014$) and presence of *qnrS* genes ($p<0.01$). However, none of the isolates had the classical mutations at *gyrA* (S83F and D87N)/*gyrA* (S83F and D87G) and *parC* (T57S and S80R) normally associated with quinolone resistance in *Salmonella.*

The high prevalence of NTS estimated in this study and determination of some factors associated with NTS in layer hen farms in Uganda is important information that should inform local, national and international efforts to control NTS. In addition, the determination of AMR patterns, linkages, and genes to important antimicrobials used in human and veterinary medicine calls for immediate and concerted efforts to strengthen strategies to combat AMR. It is recommended that more longitudinal studies involving wider geographic coverage to further understanding of occurrence of AMR and underlying mechanisms in NTS isolates from livestock is performed. This is particularly urgent in Uganda and other developing countries where data is still very limited.

4 NORSK SAMMENDRAG

Non-tyfoid *Salmonella* (NTS) er på globalt nivå viktige matbårne patogener. Mennesker smittes ofte gjennom konsum av forurensede animale produkter, vann og frukt/grønt, eller ved direkte kontakt med dyr og dyrenes miljø. En av de viktigste kildene for smitte med NTS er fjørfe og fjørfeprodukter. I tillegg til at disse bakteriene forårsaker sykdom hos mennesker og dyr, settes NTS også i økende grad i forbindelse med spredning av antimikrobiell resistens (AMR) mellom dyr og mennesker. Opptreden av resistens overfor vanlig brukte antimikrobielle midler i NTS kompliserer håndtering av sykdom og begrenser valg av medikamenter. Det er lite kunnskap om forekomsten av AMR i NTS isolert fra husdyr (inkludert fjørfe) i Uganda. Det er imidlertid mulighet for at husdyrproduksjon, som er en viktig inntektskilde for mange familier og husholdninger, kan bidra til spredning av resistente NTS. Formålet med denne studien var å kartlegge forekomst, biodiversitet og resistens overfor antimikrobielle midler i NTS i fjørfebesetninger i utvalgte distrikter i Uganda.

Som utgangspunkt for denne avhandlingen ble det designet en tverrsnittstudie for å samle inn data fra fjørfebesetninger i distriktene Wakiso, Masaka og Lira i Uganda. Innsamlingen ble gjort i 2015 og 2016, basert på besøk i et tilfeldig utvalg av fjørfebesetninger. Antall besetninger ble beregnet i forkant av besetningsbesøkene. Spørreskjema ble brukt for å samle inn data om driftsmessige forhold og kontroll og forebygging av sykdom, samt demografiske data om dyreeiere og gårdsbestyrere. Fecesprøver ble samlet inn fra fjørfehusene for isolering av NTS isolater. Alle isolater identifisert som NTS ble testet for følsomhet overfor antimikrobielle midler ved bruk av Kirby disk diffusjonstest og måling av minste hemmende konsentrasjon (MIC, minimum inhibitory concentrations). Subtyping av isolatene ble utført ved bruk av pulsfelt-gelelektroforese (PFGE) og analyse for påvisning av aktuelle resistensgener og integrase-gener ble gjort ved PCR. Videre ble det foretatt sekvensering av PCR-amplifiserte elementer for å detektere spesifikke mutasjoner involvert i resistens overfor kinoloner.

Totalt ble 237 besetninger inkludert i studien. På besetningsnivå ble det estimert en NTS-prevalens på 20,7 % (95 % konfidensintervall (CI): 15,6 – 25,6 %). Tilsammen ble det identifisert 11 NTS serovarianter (Newport, Hadar, Aberdeen, Heidelberg, Bolton, Mbandaka, Uganda, Typhimurium, Enteritidis, Kampala, Zanzibar). Isolatene kunne videre deles inn i 15 pulsotyper ved bruk av PFGE, med unntak av 10 isolater som ikke var mulige å subtype. Store besetninger og samtidig tilstedeværelse av andre dyrearter viste seg å være assosiert med forekomst av NTS på

den enkelte gård. På den annen side ble det påvist en beskyttende effekt av å ha separate hus for nye fjørfe ved introduksjon til besetningene (OR = 0,4; 95 % CI: 0,2–0,8).

Det kunne påvises fenotypisk uttrykk av resistens overfor ett eller flere antimikrobielle midler hos en høy andel av isolatene (estimert til 57,7 %). Høyest andel av resistens ble påvist overfor ciprofloksacin (50,0 %), sulfonamider (26,9 %) samt trimetoprim–sulfametoksazol (7,7 %). Resistens var signifikant assosiert med distrikt (p = 0,034). Multiresistens ble påvist i 15,4 % av isolatene. Det kunne påvises en sammenheng mellom resistens overfor ampicillin og besetningsstørrelse, ved at det var større sannsynlighet for å påvise isolater med ampicillinresistens i besetninger med et høyt antall dyr. Det ble påvist en positiv assosiasjon mellom uttrykk av resistens overfor trimetoprim og resistens overfor sulfonamider, trimetoprim–sulfametoksazol og ciprofloksacin. Resistens overfor sulfonamider var videre positivt assosiert med resistens overfor ciprofloksacin. Det kunne også dokumenteres en korrelasjon mellom resistens mot kloramfenikol og ampicillin; enten var alle isolater følsomme eller resistente overfor begge midler.

Til sammen ble det identifisert syv resistensdeterminanter (*blaTEM-1, cmlA, tetA, qnrS, sul1, dhfrI/ dhfrVII*) kjent for å kode for viktige klasser av antimikrobielle midler (henholdsvis betalaktamer, kloramfenikol, tetracykliner, kinoloner, sulfonamider, trimetoprim) som er vanlig i bruk i human- og/eller veterinærmedisin. I totalt seks (11 %) av de fenotypisk resistente isolatene ble det påvist gener som koder for klasse 1 integroner.

I denne studien ble det påvist et høyt nivå av redusert følsomhet overfor ciprofloksacin blant de innsamlede NTS isolatene (37,7 % av totalt 85 isolater). Redusert følsomhet var signifikant assosiert med opprinnelsesdistrikt (p=0,014) og tilstedeværelse av *qnrS*-genet (p<0,01). Det kunne imidlertid ikke påvises noen av de klassiske mutasjonene i GyrA (S83F og D87N)/GyrA (S83F og D87G) og ParC (T57S og S80R) som er kjent å være assosiert med kinolonresistens hos *Salmonella*.

Den høye forekomsten av NTS som er estimert i denne studien sammen med identifisering av enkelte faktorer av betydning for forekomst av NTS i fjørfebesetninger i Uganda er viktig kunnskap som kan brukes på lokalt, nasjonalt og internasjonalt nivå i felles arbeid for å kontrollere NTS. I tillegg viser beskrivelsen av resistensmønstre og resistensgener overfor viktige antimikrobielle midler som brukes i human- og veterinærmedisin nødvendigheten av en umiddelbar og samlet anstrengelse for å styrke AMR bekjempelsesstrategier. Det anbefales at det bør gjennomføres flere longitudinelle studier som involverer større geografiske områder for å

komme fram til en bedre forståelse av AMR og underliggende resistensmekanismer hos NTS fra husdyr. Det er spesielt presserende at slik arbeid utføres i Uganda og andre utviklingsland der kunnskap om slike forhold er meget begrensede.

5 LIST OF PAPERS

Paper I

Terence Odoch, Yngvild Wasteson, Trine L'Abée-Lund, Adrian Muwonge, Clovice Kankya, Luke Nyakarahuka, Sarah Tegule and Eystein Skjerve. Prevalence, antimicrobial susceptibility and risk factors associated with non-typhoidal *Salmonella* on Ugandan layer hen farms. BMC Veterinary Research (2017) 13:365. DOI 10.1186/s12917-017-1291-1.

Paper II

Terence Odoch, Camilla Sekse, Trine M. L'Abee-Lund, Helge Christoffer Høgberg Hansen, Clovice Kankya and Yngvild Wasteson. Diversity and Antimicrobial Resistance Genotypes in Non-Typhoidal *Salmonella* Isolates from Poultry Farms in Uganda. International Journal of Environmental Research and Public Health 2018, 15, 324; doi:10.3390/ijerph15020324.

Paper III

Sonja Hartnack, Terence Odoch, Gilles Kratzer, Reinhard Furrer, Yngvild Wasteson, Trine M. L'Abee-Lund and Skjerve, Eystein. Additive Bayesian networks for antimicrobial resistance and potential risk factors in non-typhoidal *Salmonella* isolates from layer hens in Uganda (Manuscript)

Paper IV

Terence Odoch, Helge Christoffer Høgberg Hansen, Trine M. L'Abee-Lund, and Yngvild Wasteson. Emergence in poultry of non-typhoidal *Salmonella* with reduced susceptibility to ciprofloxacin but without classical mutations (Manuscript)

6 INTRODUCTION

6.1 Poultry production, poverty alleviation and food security in Uganda

Globally, the poultry industry is the fastest growing livestock subsector especially in developing countries. In Uganda, it provides an important source of income and nutrition for a country with an estimated poverty level at 21.4 % of the population as estimated by Uganda Bureau of Statistics (UBOS) recent report (Uganda Bureau of Statistics, 2018). Peri-urban, small-scale poultry production has been expanding tremendously in Uganda, providing income and significantly contributing to poverty reduction in many households in Uganda. The concern about reducing poverty in Uganda has been at the centre of the government of Uganda programmes in the last decades. Because Uganda's economy largely depends on agriculture, agriculture remains one of the key pillars to fight poverty and reduce food insecurity. Livestock agriculture plays an important role not only in rural livelihood but also in urban and peri-urban agriculture (Herrero et al., 2013). The poultry population in Uganda was estimated to have increased from about 38.6 millions in 2009 to 46.3 millions by 2016 (Figure 1). Indigenous poultry population were at 39.2 millions (87.7 %) while exotic (imported poultry breeds) poultry were at 5.5 millions constituting 12.3 % of poultry population in Uganda in 2014. Total egg production was estimated to have increased from 761.3 millions in 2010 to 856.8 millions in 2014 (Uganda Bureaus of Statistics, 2015) . Small-scale producers mainly characterize the poultry industry in Uganda, with chicken being the main poultry kept. The small-scale producers keep chicken for egg production, sale of live chicken and domestic consumption. estimated that indigenous breeds of chicken was found in almost 99.2 % of chicken keeping households, with about 50 % of all households in Uganda owning chicken in 2008. Chicken keeping households typically owning an average of six chickens. Other poultry kept were ducks owned by 4.3 % of household and turkeys owned by 1.3 % of households. There were also some limited number of households keeping guinea fowls, geese and pigeons (Uganda Bureaus of Statistics, 2015).

The need to improve and increase poultry production has led to intensification and widespread use of antimicrobials, this coupled with poor biosecurity practices, present significant public health risks. One of the most important global foodborne pathogens, non-typhoidal *Salmonella* (NTS) is mainly transmitted from poultry and livestock products and is increasingly being implicated in the spread of antimicrobial resistance.

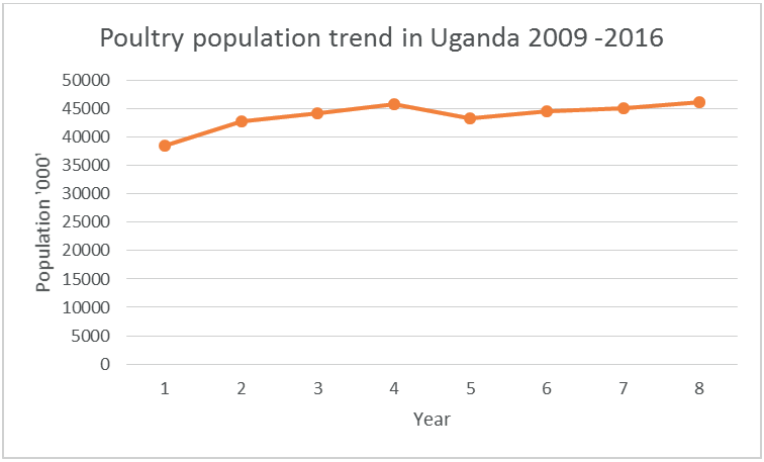

Figure 1. Poultry population in Uganda 2009 – 2016 (Adapted from Uganda Bureau of Statistics)

6.2 Non-typhoidal *Salmonella* (NTS)

6.2.1 Nomenclature and classification of *Salmonella*

Salmonella belong to the family *Enterobacteriaceae,* which mainly inhabit intestinal tracts of humans and animals. The genus *Salmonella* consists of Gram-negative, non-spore forming, rod shaped, facultative anaerobic and predominantly motile bacteria. *Salmonella* organisms are ubiquitous and they can survive several weeks in dry environments and months in water. Currently, the generally accepted nomenclature classifies *Salmonella* into two species, *S. enterica* and *S. bongori*. This is based on the differences in their 16 rRNA sequence analysis (Popoff, Bockemuhl, & Gheesling, 2003). The species *S. enterica* is divided into six subspecies (Table 1) based on their genomic relatedness and biochemical properties (Reeves, Evins, Heiba, Plikaytis, & Farmer, 1989).

Table 1. *Salmonella* nomenclature

***Salmonella* species**	***Salmonella* subspecies**	**Habitat**
Salmonella enterica	*enterica* (I)	Warm blooded animals
	salamae (II)	Cold blooded animals, environment
	arizonae (IIIa)	Cold blooded animals, environment
	diarizonae (IIIb)	Cold blooded animals, environment (also occur naturally in some sheep)
	hountenae (IV)	Cold blooded animals, environment
	indica (VI)	Cold blooded animals, environment
Salmonella bongori	*bongori* (V)	Cold blooded animals, environment

According to Ryan, O'Dwyer, and Adley (2017) the terms serotypes and serovars are synonomous, however World Health Organization (WHO)/Pasteur Institute use serovar and Center for Disease Control and Prevention (CDC) and American Society of Microbiology (ASM) use serotype although they are now moving to use serovar for international consistency. Most serovars causing infections in humans and animals belong to the subspecies *enterica. Salmonella* is further classified by serovars basing on two antigenic determinants: the somatic (O) antigen, and flagellar (H: phases 1 and 2) antigen as described according to White-Kauffmann-Le Minor. The Vi capsular antigen is mostly used for *S.* Typhi and sometimes *S.* Dublin, *S.* Paratyphi C. (Dieckmann & Malorny, 2011).

For *Salmonella enterica* subspecies *enterica*, the serovars are for historic reasons named according to the disease associated with the infection and the geographic area where the first isolation took place or typical habitats. For other subspecies of *S. enterica* and in S. *bongori* serovars antigenic formulae are assigned using Kauffmann-White-Le Minor scheme (Grimont & Weill, 2007). For

the first mention in a publication, the name is written in full eg. *Salmonella enterica* subsp. *enterica ser.* Typhi, then subsequent use the name can be condensed to *Salmonella* with name of the serovar eg. *Salmonella* Typhi. The name of the serovar is not written in italics and the first letter of the Roman alphabet in capital. A serovar is named after meeting the full antigenic definition of a serovar (Ryan et al., 2017). By 2010, 2659 serovars of *Salmonella* were identified. *Salmonella* serovars and their antigenic formulae are listed in the White-Kauffmann-Le Minor scheme. The World Health Organization's Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute in Paris, France is mandated to annually update the White-Kauffmann-Le Minor scheme and publish the report in the journal Research in Microbiology.

Salmonella serovars are also grouped into host-specific, host-restricted and generalists (Uzzau et al., 2000). Host-specific serovars like Typhi, Paratyphi, Gallinarium, and Pullorum only cause disease in one host species (Baumler, Tsolis, Ficht, & Adams, 1998; Uzzau et al., 2000). The host-restricted serovars are predominantly associated with one species, eg *S.* Dublin in cattle and *S.* Cholerasuis in pigs, although they can spread and cause infections in other species. The generalist serovars like *S.* Typhimurium and *S.* Enteritidis tend to have a broad host range, although some subtypes with a narrow host range have been described (Gyles, 2004). The adaptation of the different serovars with respect to their clinical syndromes is shown in Figure 2.

Salmonella serovars are broadly divided into typhoidal and non-typhoidal *Salmonella* (NTS) serovars based on clinical manifestations. Typhoidal serovars; Typhi, Sendai and Paratyphi A, B, C are specific human restricted serovars causing typhoid fever, also called enteric fever, an invasive life threatening febrile illness (Crump, Luby, & Mintz, 2004). Typhoidal *Salmonella* infections mainly cause systemic diseases. The rest of the serovars are NTS and are predominantly found in animal reservoirs (Haeusler & Curtis, 2013; Hohmann, 2001).

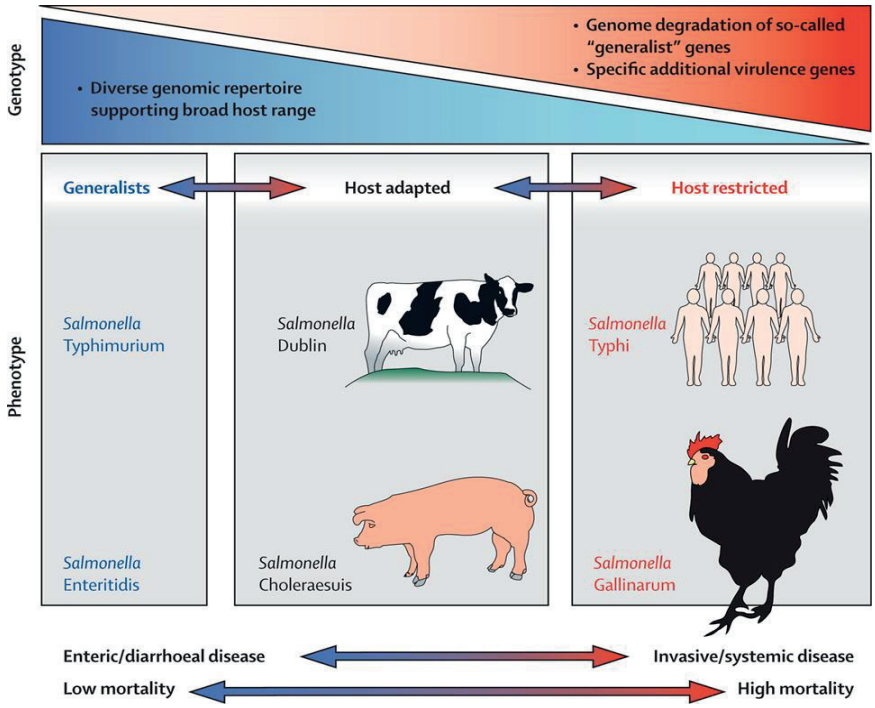

Figure 2. Features of host adaptation in salmonellae and effects on clinical syndrome in the host (Feasey, Dougan, Kingsley, Heyderman, & Gordon, 2012)

6.2.2 Isolation and characterization of NTS

6.2.2.1 Isolation of Salmonella

Isolation and identification procedures yield *Salmonella* isolates that can be used for further characterization like serotyping, antimicrobial susceptibility testing and other typing methods. Culture techniques are generally laborious and time demanding. A number of selective media are available for isolation of *Salmonella*. These include low-selective media (MacConkey agar, deoxycholate agar), intermediate-selective media (Salmonella-Shigella [SS] agar, Hektoen [HE] agar), and highly selective media (selenite agar with brilliant green).

Standard culture and isolation procedures for *Salmonella* have been developed and continuously improved by International Organization of Standardization (ISO), international organizations like WHO and some laboratories have optimized methods that are used. For NTS, a new standard (ISO, 2017), ISO 6579-1:2017, Microbiology of the food chain -- Horizontal method for the detection,

enumeration and serotyping of *Salmonella* -- Part 1: Detection of *Salmonella* spp., has been released recently and incorporates ISO 6579:2002/Amd 1:2007 (ISO, 2007), which had been widely used. This protocol can be used for isolation of NTS from food products for human consumption, animal feed, environmental samples, and samples from primary production stage such as feces and dusts.

NTS can be identified biochemically by an array of tests. This can be done manually, or by use of kits designed for identification of *Enterobacteriacae* (e. g. API20E, BioMerieux, Marcy l'Etoile, France). Although the API20E kit is quite simple and easy to use, it remains a bit expensive compared to other culture methods.

6.2.2.2 *Typing techniques*

Typing methods are not only important for epidemiological investigations and source tracing but also for understanding zoonotic potential and disease outcomes. Typing methods, phenotypic and genotypic, should be able to type all the isolates in a study (high typeability) and discriminate isolates (discriminatory power) appropriately (van Belkum et al., 2007). The methods should also have high reproducibility. Ideal methods for typing of NTS should be rapid, robust, portable, and sensitive. A number of studies have used and compared different methods (Reche et al., 2003; Tatavarthy, 2005; Wattiau, Boland, & Bertrand, 2011). Typing methods is an area under constant development and it is normally useful to apply more than one method depending on the surrounding circumstances.

6.2.2.3 *Phenotypic typing*

Traditional phenotypically based approaches include biochemical profiling (biotyping), phage typing, serotyping, and antimicrobial resistance profile (Herikstad, Motarjemi, & Tauxe, 2002; Olsen, Brown, Skov, & Christensen, 1993). Although phenotypically based techniques remain the methods of choice in many situations, genotypic methods are becoming increasingly applied.

Serotyping techniques for *Salmonella* are based on a well-established Kauffmann-White-Le Minor scheme. It identifies somatic (O), flagellar (H) and sometimes capsular (Vi) antigens through reactions with specific antisera. The O antigen determines the serogroup and the H antigen determines the serovars (Nataro, Bopp, Fields, Kaper, & Strockbine, 2011). These antigens vary with 64 O and 114 H variants identified (Grimont & Weill, 2007; McQuiston, Fields, Tauxe, & Logsdon, 2008; Popoff, Bockemuhl, & Brenner, 1998). By 2010, the number of *Salmonella*

serovars stood at 2659. Serotyping by antibody microarrays, which reduces analysis time, has been developed. However, it awaits to be validated on large scale before further development (Cai, Lu, Muckle, Prescott, & Chen, 2005). A major disadvantage of serotyping methods is that it cannot discriminate isolates in epidemiological investigations.

Biochemical profiling (biotyping) is useful particularly isolate discrimination in *Salmonella* (Barker & Old, 1989). It is reaction to a series of biochemical tests including production of H_2S, glucose and lactose fermentation, and lysine decarboxylation. A scheme for biotyping has been fully described (Duguid, Anderson, Alfredsson, Barker, & Old, 1975). However, it is time consuming, laborious and expensive (Tatavarthy, 2005).

Phage typing (PT) works on the ability of a given phage to lyse the investigated strain based on the host's specificity of the bacteriophages (Anderson & Williams, 1956). For NTS, the most important schemes exist for *S*. Enteritidis, *S*. Typhimurium, and *S*. Virchow. Several PT schemes of clinical and epidemiological importance have been developed for other *Salmonella* serovars as well (Castro et al., 1992). *Salmonella* PT is simple to implement, economical and less expensive. However, it requires highly skilled staff (Threlfall, 2000). A major disadvantage is that not all serovars are typeable by PT due to limited number of available phages.

Antimicrobial resistance profiling (antibiogram) is a result from testing susceptibility to different antimicrobials. It is usually cheap and no complicated equipment required (Barco, Barrucci, Olsen, & Ricci, 2013), but because of its limited discrimination power it has to be complemented with other subtyping techniques. The discriminative power depends on the antimicrobial tested, the stability, diversity and the relative prevalence of the detectable acquired resistance (van Belkum et al., 2007). Phenotypic susceptibility profiles like those obtained by Kirby-Bauer methods provide very useful data for epidemiological investigations. Patterns such as ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracyclines) for *S*. Typhimurium DT 104 and ACSSuTTm (ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracyclines, and trimethoprim) for *S*. Typhi have become very useful markers that are used globally.

6.2.2.4 *Genotypic typing methods*

A number of molecular approaches have been developed to replace or complement traditional phenotypic methods, which are often slow, laborious and can be imprecise (Wattiau et al., 2011). Genotypic methods offer many advantages like increased discriminatory power, better standardization and better reproducibility (Castro et al., 1992; Herrera-Leon et al., 2007;

Wiedmann, 2002). A recent review by Shi, Singh, Ranieri, Wiedmann, and Switt (2015) puts the currently available methods in to 3 categories; (i) molecular typing methods; pulsed-field gel electrophoresis (PFGE,) ribotyping, repetitive element sequence-based polymerase chain reaction (rep-PCR), (ii) methods based on serovar-specific genomic markers, and (iii) direct methods that target genes encoding antigens.

The DNA subtyping banding based methods target mainly bacterial chromosomes. The banding patterns are generated after restriction digestion of DNA or from PCR amplified DNA fragments (Hartmann & West, 1997; Nair, Schreiber, Thong, Pang, & Altwegg, 2000; Ribot et al., 2006). The limitations for banding based methods is the prediction of highly homologous patterns and prediction of polyphyletic serovars (Shi et al., 2015). Among the genotypic methods, PFGE is still considered the gold standard. It is discriminatory and therefore quite useful for outbreaks investigations and source attribution. However, PFGE is time consuming and labour intensive. Other banding pattern-based methods are; ribotyping, random amplified polymorphic DNA-PCR (RAPD-PCR), PCR (rep-PCR), PCR-restriction fragment length polymorphism (PCR-RFLP), and amplified fragment length polymorphism (AFLP) (Shi et al., 2015).

DNA sequencing-based molecular typing method like multilocus sequence typing (MLST) classifies *Salmonella* according to allelic profiles of selected housekeeping genes (Achtman et al., 2012; Enright & Spratt, 1999). Clustered regularly interspaced short palindromic repeats (CRISPRs) typing is another sequenced base method. It has been optimised for use in other species, but it's application in *Salmonella* is still limited (Gomgnimbou et al., 2012).

Direct methods are based on PCR, sequencing, or probes that target genes encoding the somatic (O) and flagellar antigens (Braun et al., 2012). Whole genome sequencing (WGS) has gathered success and the next generation is likely to become the method of choice as it is becoming cheaper, can detect single nucleotide differences and allows distinction of strains of high clonality (Salipante et al., 2015). WGS data requires competence in bioinformatics and infrastructure to store and analyse data. It has been used to predict *Salmonella* serovars (Arrach et al., 2008; Malorny, Bunge, Guerra, Prietz, & Helmuth, 2007).

6.2.3 Non-typhoidal *Salmonella*: A global foodborne pathogen

NTS is one of the leading causes of global food-borne disease outbreaks and illnesses, posing huge challenges to public health systems around the world (Painter et al., 2013; Routh et al., 2015; Scallan et al., 2011). The true global burden of NTS in humans remains uncertain. An earlier study

estimated annual cases of NTS to range from 200 millions to 1.3 billions cases annually (Coburn, Grassl, & Finlay, 2007). NTS mainly cause gastroenteritis which has been estimated to cause 93.8 million enteric infections and 155,000 diarrheal deaths annually with 80.3 millions of the cases foodborne (Majowicz et al., 2010). An estimate of NTS in the US put the annual figure at 1 million cases (Scallan et al., 2011), while a study in the European Union (EU) estimated annual cases of salmonellosis to range between 16 and 11,800 per 100,000 persons (Havelaar, Ivarsson, Lofdahl, & Nauta, 2013). Enteric NTS infections are estimated to account for 4.8 million disability-adjusted life years (Lozano et al., 2012).

The burden of invasive non-typhoidal *Salmonella* (iNTS) is equally high especially in Africa. A recent estimate of of iNTS puts the global figure at 3.4 million cases with 681,316 deaths annually. In Africa, iNTS is estimated at 227 cases per 100,000 persons per year compared to the global average of 49 cases per 100,000 persons per year. Out of the iNTS cases, 63.7 % occurred in children under five years of age globally and 68.3 % occurred in children under 5 years of age in Africa (Ao et al., 2015). In Sub Sahara Africa it is estimated that iNTS causes 2,000 – 7,500 cases per 100,000 HIV infected adults (Gilks, 1998; M.A. Gordon et al., 2008; Graham, Molyneux, et al., 2000; Reddy, Shaw, & Crump, 2010; van Oosterhout et al., 2005).

NTS serovars are the second most prevalent foodborne pathogen worldwide after *Campylobacter*. Most foodborne disease outbreaks and infections caused by NTS are associated with consumption of foods of animal origin like eggs, chicken, beef, pork, dairy products, but also vegetables and fruits (Bayer et al., 2014; Callejon et al., 2015; Dechet et al., 2014; Fashae, Ogunsola, Aarestrup, & Hendriksen, 2010; Foley SL, 2008; Painter et al., 2013; Scallan et al., 2011). However, NTS infections can also be contracted through direct contacts with infected individuals, animals, pets, reptiles or amphibians (Hohmann, 2001; Mermin et al., 2004).

Global estimates have consistently demonstrated that NTS cause more deaths than typhoidal *Salmonella* (Ao et al., 2015; Crump et al., 2004; Lozano et al., 2012). While NTS epidemiology is well understood in developed countries, this is not the case especially in developing countries in Africa. The application of more advanced technologies should support more studies in Africa to help better understand NTS reservoirs and transmission dynamics. Emergence of antimicrobial resistance (AMR) is likely to influence the incidence of iNTS and related deaths in Africa.

6.2.4 Non-typhoidal *Salmonella* in humans

The subspecies *Salmonella enterica* subspecies *enterica* serovars are responsible for approximately 99 % of *Salmonella* infections in humans and warm blooded animals, leaving the other five subspecies of *S. enterica* and *S. bongori* mainly to be found in environment and cold blooded animals (Brenner, Villar, Angulo, Tauxe, & Swaminathan, 2000). Only about 30 serovars, all belonging to *Salmonella enterica* subspecies *enterica* account for over 90 % of diseases caused by *Salmonella* in humans and animals. Most NTS illnesses are caused by *S. enterica* subspecies *enterica* serovars Enteritidis, Typhimurium, Newport, Heidelberg and Javiana.

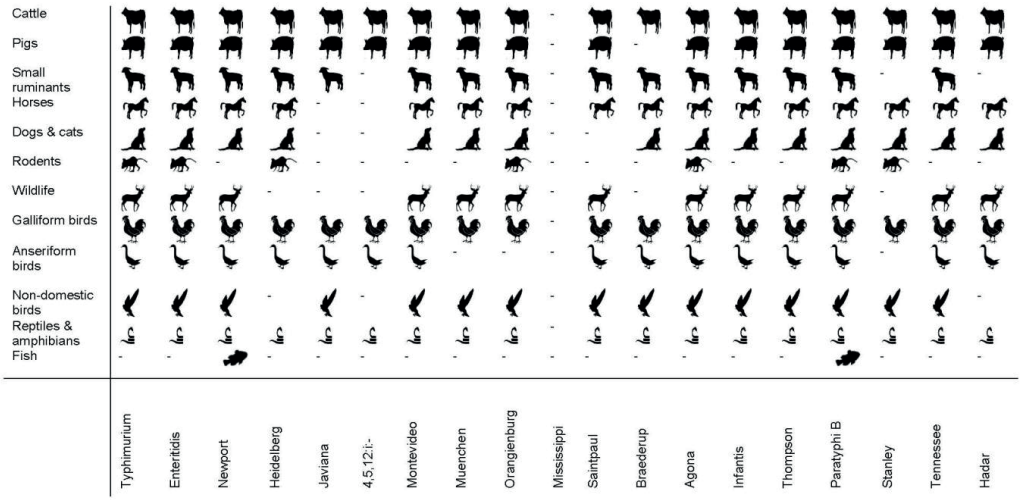

Figure 3. Distribution of the 20 common human *Salmonella* serovars among animals based on US data (Hoelzer, Switt, & Wiedmann, 2011)

The disease caused by NTS in humans can be mild, normally characterized by self-limiting gastroenteritis lasting three to seven days with the main symptoms being diarrhoea, abdominal pain, and vomiting. Such gastroenteritis can be outbreak-related or occur as sporadic cases. A lot of sporadic cases go unnoticed and/or unreported. In the developed world, most NTS infections cause mainly self-limited gastroenteritis apart from individuals with specific risk factors (Laupland et al., 2010), while in developing countries, a serious aspect of the disease is iNTS, that leads to

the development of bloodstream infections and bacteraemia, especially in immunocompromised individuals with HIV infections (Gruenewald, Blum, & Chan, 1994; Larsen et al., 2011), young, elderly persons and malnourished (Feasey et al., 2012). The iNTS is the most common bacteria isolated from bloodstream in patients with fever. In Africa iNTS is mainly caused by *S.* Typhimurium and *S*. Enteritidis although there are some reports of cases caused by other serovars (Berkley et al., 2005; Beyene et al., 2011; M.A. Gordon et al., 2008; MacLennan & Levine, 2013; Reddy et al., 2010; Tennant et al., 2010; Wadula et al., 2006).

In Africa it is estimated that iNTS is responsible for up to 39 % community acquired bloodstream infections (Uche, MacLennan, & Saul, 2017). The main clinical presentation of iNTS in Africa is febrile systemic illnesses resembling enteric fever, with diarrhoea often absent (M. A. Gordon et al., 2002; Graham, Walsh, Molyneux, Phiri, & Molyneux, 2000; Peters et al., 2004).

The self-limiting mild NTS disease in humans normally does not require antimicrobial treatment. However, infections caused by the more severe iNTS require antimicrobial drugs. In the past *Salmonella* organisms were quite sensitive to a number of antimicrobials like ampicillin, amoxicillin, trimethoprim-sulphamethoxazole, and chloramphenicol. However, the development of multidrug resistant strains of *Salmonella* especially in Africa has complicated management of NTS disease (M.A. Gordon et al., 2008; Kingsley et al., 2009). Therefore, because of AMR, empirical management of NTS diseases require the use of 3rd generation cephalosporins (eg. ceftriaxone) and fluoroquinolones (eg. ciprofloxacin) which are quite expensive for the ill financed health systems in most developing countries.

There is enough scientific evidence from recent studies to show that vaccines against NTS can be developed successfully. For typhoid fever, a conjugate vaccine has been developed and approved by WHO for use in children above 6 months of age (Burki, 2018). The feasible candidate vaccines for NTS are mainly targeting O antigens, flagellin proteins and other membrane proteins (Haselbeck et al., 2017). These include live attenuated vaccines, sub-unit based and recombinant antigen based. Currently monovalent and bivalent vaccines for *S.* Typhimurium and *S.* Enteritidis are under development. Larger efforts are meant to target multivalent vaccines targeting 5-6 serovars (Tennant, MacLennan, Simon, Martin, & Khan, 2016). Development of NTS vaccine is however being complicated by enormous numbers of serovars of NTS, and immune-compromised nature of susceptible patients.

6.2.5 Non-typhoidal *Salmonella* in livestock and poultry production

There is some overlap among the common NTS serovars causing diseases in humans and animals. A study by Centers for Disease Control and Prevention (CDC), US found that, out of the ten top serovars causing human illnesses, eight are also among the top in one of the food animals (Foley SL, 2008). Thus, livestock provides the primary sources and reservoirs for NTS. The disease is most common in poultry, swine and reptiles that are important sources for human infections, but other animal sources are also important in the transmission of NTS to humans (Hoelzer et al., 2011). Serovar distributions vary greatly among animal populations even in the same geographical region.

The zoonotic NTS normally colonize the intestinal tract of a variety of animal species, but in most cases they remain carriers (Brackelsberg, Nolan, & Brown, 1997; Kumar, Saxena, & Gupta, 1973). Normally animals get exposed through the fecal-oral route, i.e. by intake of polluted feed and water, or consumption of pasture contaminated with feces from other carrier animals. Animals that become clinically infected with NTS, particularly young animals, will manifest symptoms like pyrexia, lethargy, depression, anorexia, dehydration and diarrhoea that can progress to dysentery. Such animals exhibit higher prevalence of shedding compared to asymptomatic carriers. However, asymptomatic carriers can intermittently shed NTS over a variable period. When the disease become endemic, NTS organisms survive in farm environments, which become important reservoirs of infections (Cummings et al., 2009; Giles, Hopper, & Wray, 1989).

In poultry, NTS has been reported in most galliform birds (chicken, turkey, quails, pheasants) and high prevalence rates have been seen in many commercially raised poultry (Hoelzer et al., 2011). Symptoms vary by age groups and serovars (Kabir, 2010), but exposure to the generalist serovars mainly result in asymptomatic carriers. However, some cases of mortality have been reported in chickens. These generalist serovars, like *S.* Enteritidis, cause public health threats and significant economic losses. The host restricted serovars, *S.* Pullorum and *S.* Gallinarium cause severe illnesses with high mortality and economic losses to commercial farms. *S.* Pullorum cause pullorum disease mainly in young birds causing septicaemia, anorexia, diarrhoea, dehydration, and high mortality. In adults, it causes decreased fertility and egg production. *S.* Gallinarium cause fowl typhoid in young and adults with symptoms similar to *S.* Pullorum infections (Hoelzer et al., 2011). NTS in poultry is mainly transmitted horizontally through direct and indirect contacts, but sometimes also vertical transmission through eggs.

6.2.6 Prevention and control of non-typhoidal salmonellosis

As more than 90 % of salmonellosis are foodborne, control and prevention of NTS dissemination should start primarily at primary food production enterprises and continue at all stages along the food chain. Therefore, ensuring sanitation along all stages from production to processing and handling at home is key to minimize transmission along the food chain not only of NTS but also other foodborne infections (Eng et al., 2015). Unfortunately, crucial elements like safe water and food hygiene is lacking in many developing and underdeveloped countries (Clasen, Schmidt, Rabie, Roberts, & Cairncross, 2007). At the farm level, promotion of biosecurity and good farming practices is important to reduce risks of transmission from farm environments of NTS to other animals and humans. In poultry farms, supplies of day old chickens and breeding stock should be from reputable companies known and certified as NTS free. The same should also apply in case of feeds supplies. Culling of affected flock should be done in a manner that does not expose humans to NTS. That means following the recommended hygiene and biosecurity practices in poultry production.

Several studies have shown high infections and occupational risks among veterinarians, farm workers and persons in constant contact with animals (Hendriksen, Orsel, Wagenaar, Miko, & van Duijkeren, 2004; Radke, McFall, & Radostits, 2002). Personnel working in farms should avoid occupational hazards by ensuring personal protection at all times, including washing of hands after handling animals. Children should be taught how to handle animals, pets and chicken in a proper way like have their hands washed immediately after touching of animals and birds, and always washing hands before eating anything. A reduction of malaria and HIV is known to be associated with reduction of iNTS in humans. Persons handling foods should be free of salmonellosis.

Cross contamination in processing units and in the kitchen should be avoided. Uncooked meat should be separated from cooked foods and ready to eat products. Hands should be washed before and between handling foods. Consumption of raw or undercooked animal products like eggs, meat, and milk should be avoided. Farm and industry workers need education and sensitization on basic hygiene and food safety practices. Regional and national integrated surveillance and monitoring for foodborne diseases including NTS should be enhanced in farms, environment and communities. This especially should be initiated in developing countries where such programs do not exist to ensure early detection and to prevent spread of NTS infections particularly.

Vaccines for some specific serovars of NTS are available for veterinary use, but not yet in use in many developing countries and farmers are still reluctant to their use because of costs. Currently, vaccines for typhoid fever has been developed, though not widely in use yet (Burki, 2018). NTS vaccines for humans are still under development (Tennant et al., 2016)

6.3 Antimicrobial resistance

Several definitions of AMR exist (Guardabassi & Courvalin, 2006; WHO, 2017). WHO (2017) defined AMR as the ability of a microorganism (like bacteria, viruses, and some parasites) to stop an antimicrobial (such as antibiotics, antivirals and antimalarials) from working against it. As a result, standard treatments become ineffective; infections persist and may spread to others. AMR is not absolutely a new problem, it has been known for long that development of AMR is a natural process (Benveniste & Davies, 1973; Bhullar et al., 2012; Mukerji et al., 2017; Rosenblatt-Farrell, 2009). Resistance bacteria was realized immediately after the first antibiotic, penicillin, was discovered (Abraham & Chain, 1988) . Microbes are becoming increasingly resistant to available drugs, and apparently, AMR is affecting all infections that were once susceptible to certain antimicrobials. The accelerated development and rapid spread of AMR necessitates urgent attention. Several pathogens that have developed resistance are listed across the world. Even more threatening are organisms developing multidrug resistance (Dalhoff, 2012; Doyle, 2015; Koirala, 2011). These multidrug resistant organisms cause infections that can only be managed by few antimicrobials. If this development continues, the world can reach a phase in our history, the post antibiotic era, where the available drugs will not work anymore and death after surgeries, caesarean sections, and infections will be widespread. Injudicious use of antimicrobials especially broad spectrum ones, put pressure on organisms including commensals that are normal flora in humans, animals and environment (Lammie & Hughes, 2016). Therapeutic and non-therapeutic antimicrobial use in animal production is known to accelerate the emergence of antimicrobial resistant strains. There exist reports of AMR in humans that have been traced back to antimicrobial resistant microbes in livestock (Paphitou, 2013).

AMR data on bacteria from humans are more available compared to data on bacteria from animals. Although, sometimes controversial, the use of antimicrobials in animals is attributed to AMR resistance in human pathogens. The widespread transfers of AMR genes that occur from

commensals to human pathogens support the argument that animal reservoirs are sources of AMR genes in human pathogens.

6.3.1 Global public health threat of antimicrobial resistance

AMR is duly recognized as a global health challenge (FAO, 2016; OIE, 2016; WHO, 2015; WHO, OIE, & FAO, 2016). AMR is a threat to health security, food security, and economy and is affecting multiple sectors. The crisis of AMR is growing worldwide although the full impact is unknown globally, however its spread is likely to undo all the benefits long associated with antimicrobial therapy (O'Neill, 2016). AMR is rapidly increasing while the tools for combatting it are decreasing. New AMR mechanisms are rising and spreading globally, resistance is developing to drugs used to manage globally important diseases like human immunodeficiency virus (HIV), tuberculosis (TB), malaria, and different bacterial infections. AMR is a big threat to the realization of sustainable development goals especially for developing countries and its impact affect all tenets of society. It poses a complex challenge that will require coordinated actions. Currently there is no global system in place to monitor and track AMR. With globalized food systems and travels, the food supply chain is an integral part of the spread of AMR.

A recent report estimates death due to AMR at 700,000 annually and this is expected to increase to 10 million annually by 2050, if no action is taken today (O'Neill, 2016). Models based on available data (Figure 4) shows that the biggest impact, latitude and burden of AMR will most likely be in low to middle income countries (LMIC). The estimated death to be attributed to AMR by 2050 in different regions of the world is shown in Figure 4 (O'Neill, 2016). In the US, AMR is estimated to cause more than 23,000 annual deaths and more than 2 million illnesses (CDC, 2013) and in the EU AMR will cause 25,000 deaths per year and 2.5 million extra hospital days (ECDC/EMEA, 2009). In India, AMR is already causing 58,000 deaths annually in babies acquiring resistant infections from their mothers (Laxminarayan et al., 2013). There is lack of reliable data on the state of AMR in most developing countries, especially in Africa as surveillance for AMR is only done in some limited countries.

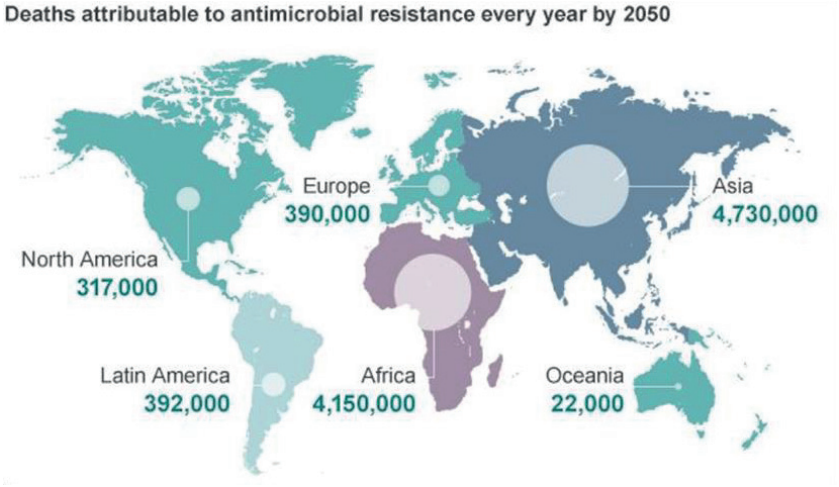

Figure 4. Estimated global distribution of human death due to antimicrobial resistance (O'Neill, 2016)

A recent publication on the state of the world's antibiotics by Center for Disease Dynamics, Economics & Policy, Washington, US provided an analysis of the global patterns and trends on AMR (CDDEP, 2015). According to the CDDEP report, resistance to first line and last resort antibiotics is rising, moreover for LMIC, new drugs are not widely available and affordable. WHO (2014) estimated global antimicrobial resistance and listed *E. coli*, *Klebsiella pneumoniae,* and *Staphylococcus aureus* as the top three organisms of greatest concerns in hospital and community acquired infections. It also listed *Streptococcus pneumoniae*, NTS, *Shigella* spp. and *Neisseria gonorrhea* as the top organisms of high concerns in community acquired infections. Two studies in the African countries of Tanzania and Mozambique have shown increased mortality as a result of drug resistant infections (Kayange, Kamugisha, Mwizamholya, Jeremiah, & Mshana, 2010; Roca et al., 2008). Current reports indicate that AMR is increasing globally, particularly methicillin-resistant *S. aureus* (MRSA), extended-spectrum beta-lactamase producers, and carbapenem-resistant *Enterobacteriaceae.*

AMR is now commonly seen in NTS from various sources. A recent review and meta-analysis of health outcomes of NTS showed that *S*. Enteritidis, *S*. Typhimurium, *S*. Newport, and *S*. Heidelberg are the most reported MDR pathogens in high income countries (Parisi et al., 2018). Since the discovery in 1990s of the spread of MDR *S*. Typhimurium phage type 104 (DT104) world wide

(Molbak et al., 1999), MDR in NTS is increasingly reported (L. H. Su, Chiu, Chu, & Ou, 2004). *S.* Typhimurium DT104 is known to exhibit resistance to five antimicrobial agents: ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (ACSSuT). For example, in the European union a study in human isolates of NTS collected between 2000 – 2004 and tested for resistance towards 10 antimicrobials reported prevalence increasing from 57 to 66 %, but MDR decreasing from 18 to 15 % (Meakins et al., 2008), while a study in Kuwait and United Arab Emirates found MDR rates at 9.8 and 4.1 %, respectively (Rotimi et al., 2008). In Africa, emergence of two clades of *S.* Typhimurium ST313 with a temporal relationship to acquired AMR, especially to first line antibiotics and association with HIV, has been reported (Okoro et al., 2012). AMR development is a natural process arising from selection and genetic mutations, however the pace of development of AMR is influenced a lot by human activities, fueled by high burden of infectious diseases in LMIC, easy access to antimicrobials across the counter without prescriptions leading to misuse and overuse particularly in humans and animals. Other factors like increased global trade and travels that has enhanced interconnectedness of countries, this means organisms can move very rapidly from one part of the globe to another. Particularly when travelers move from countries where resistant strains exist and where strong mechanisms to contain them resistance don't exist. In addition, few LMIC have systematic antimicrobial surveillance system.

Recognizing the global challenges posed by AMR, WHO/FAO/OIE agreed to embark on joint efforts to control AMR under a tripartite agreement, and approved a global action plan on AMR adopting a One Health approach (WHO, 2015). WHO has put in place some efforts in Africa to improve laboratory based surveillance (WHO, 2013) and some countries are beginning to establish structures for surveillance of AMR in selected pathogens.

6.3.2 Types of antimicrobial resistance in bacteria

6.3.2.1 Intrinsic antimicrobial resistance

Intrinsic resistance is a result of innate ability by bacteria to resist antimicrobial agents. It is natural inherent structural and functional characteristics of bacteria that make them insensitive, never susceptible or non-responsive to certain antimicrobial substance or class (Fernández & Hancock, 2012). Intrinsic resistance can be due to absence of the target site on the bacteria for the drug, inaccessibility or impermeability of the drug into the bacterial cell, extrusion of the drug by chromosomally encoded active exporters, and innate production of enzymes that inactivate the drug

(Gupta, Limbago, Patel, & Kallen, 2011; Munita & Arias, 2016; Shaikh, Fatima, Shakil, Rizvi, & Kamal, 2015). For example, the outer membrane of Gram negative bacteria prevent the entry of penicillins and most beta lactams and larger molecular size antibiotics like bacitracin, vancomycin cannot pass through the porins of Gram negative bacteria (Nikaido, 1992, 1994). Intrinsic resistance is normally species or genus specific and is not horizontally transferable to other bacteria, only vertically to offspring. Intrinsic resistance is not affected by use or misuse of antimicrobials (Capita & Alonso-Calleja, 2013).

6.3.2.2 Acquired antimicrobial resistance

Acquired antimicrobial resistance happen when the microorganisms become resistant to antimicrobials to which they were once sensitive or susceptible. Since the discovery of antibiotics, bacteria have developed wide mechanisms of acquired resistance. This can happen as a result of genetic changes like in mutations or acquisition of genes through horizontal gene transfer (HGT) involving mobile genetic elements. HGT can occur by conjugation, transformation, and transduction (Figure 5)

Conjugation is the transfer of mobile DNA elements between two bacterial cells, it requires cell to cell contact between the donor and the recipient of the mobile DNA structure. Conjugation process results in the dissemination of genes encoding for AMR and probably plays the biggest role in the spread of AMR (Rowe-Magnus & Mazel, 1999). The conjugative spread of AMR genes is mainly mediated by mobile genetic elements, especially plasmids (Carattoli, 2013; Guardabassi & Courvalin, 2006; L.H. Su, Chu, Cloeckaert, & Chiu, 2008).

Transformation represents the uptake of naked or extracellular donor DNA from the surrounding environment. The bacteria should be in a competent state before transformation occurs. Transformation requires homology between DNA of donor and recipient, therefore can only occur between closely related bacteria. The DNA from the donor is incorporated in the chromosome of the recipient cell (Guardabassi & Courvalin, 2006).

Transduction is the acquisition of DNA from bacteriophages. There are examples where the entire mobile genetic elements are mobilized through transduction (Willi, Sandmeier, Kulik, & Meyer, 1997).

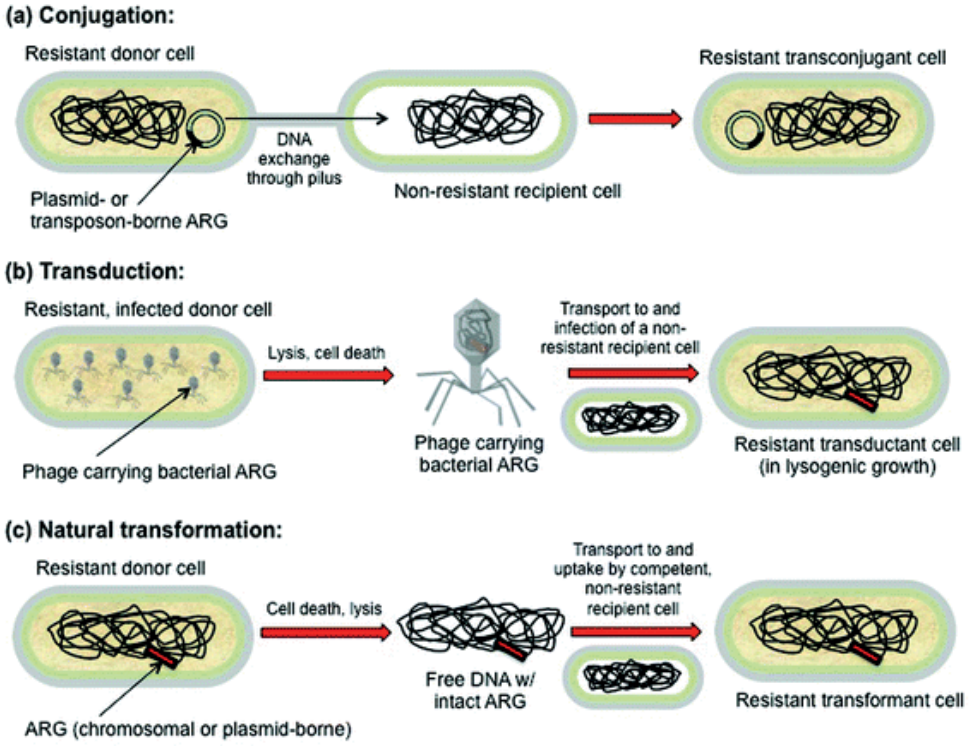

Figure 5. Illustrations of major aspects of horizontal gene transfer by means of (a) conjugation, (b) transduction, and (c) natural transformation **(Dodd, 2012).** Reproduced by permission of the Royal Society of Chemistry.

Acquired resistance can also be transferred vertically through spontaneous and induced gene mutations. Bacteria have acquired different mechanisms of resistance that are not exclusive and an interplay of many mechanisms can result in high level resistance (Arzanlou, Chai, & Venter, 2017). In isolates from animal sources, resistance has been reported to all the major classes of antimicrobials following introduction; beta lactams (CIPARS, 2006; Jacoby & Munoz-Price, 2005; Olesen, Hasman, & Aarestrup, 2004), macrolides (Roberts, 2004; Topp, Renaud, Sumarah, & Sabourin, 2016; Zemlickova, Jakubu, Marejkova, Urbaskova, & Pracovni Skupina Monitorovani, 2014), aminoglycosides (Byarugaba, Kisame, & Olet, 2011; Donabedian et al., 2003; Jaimee & Halami, 2016), quinolones and fluoroquinolones (Acar & Goldstein, 1997; Miro et al., 2004;

Planta, 2007; Robicsek, Jacoby, & Hooper, 2006), tetracyclines (Roberts, 2005; Thaker, Spanogiannopoulos, & Wright, 2010), chloramphenicols (Bissonnette, Champetier, Buisson, & Roy, 1991; Schwarz, Kehrenberg, Doublet, & Cloeckaert, 2004; G.D. Wright, 2005), sulfonamides (Huovinen, 2001; Maka, Mackiw, Sciezynska, Modzelewska, & Popowska, 2015; Skold, 2001). Acquired resistance normally affects strains of the same species or genus (Miro et al., 2004; Robicsek et al., 2006).

6.3.3 Mobile genetic elements and acquired antimicrobial resistance

Mobile DNA is any segment of DNA that is capable of translocating from one part of the genome to another (van Hoek et al., 2011). The mobile genetic elements that are involved in HGT include mainly mobilizable and conjugative elements (plasmids), integrated and conjugative elements (conjugative transposons,) insertion sequences, bacteriophage through a process of transduction, mobile introns, and integrons. Mobile genetic elements capture antimicrobial resistance genes and play an important role in transmission of antimicrobial resistance genes (ARG) from environment to pathogens of humans and animals. They are mainly acquired through transformation and conjugation.

Plasmids are extra-chromosomal circular structures of DNA that replicate independently of the cells' chromosome. Plasmids contain genes not essential for the host bacterial survival, but may contain genes for AMR and virulence factors. Plasmids that harbor genes for conjugation are called conjugative plasmids and plasmids that contain an origin of transfer are called mobilizable plasmids (van Hoek et al., 2011). Plasmids therefore contribute to the dissemination and persistence of AMR genes posing enormous challenges (Carattoli, 2013). Some plasmids have a broad host range and hence can be transferred to several species of bacteria and some have a narrow host range and transfer limited to same species or genus.

Integrative and conjugative elements, also called conjugative transposons, are pieces of DNA that can migrate through the genome of an organism (Saedler & Gierl, 1996). They are integrated into a host genome and are passively propagated during chromosomal replication and cell division (Johnson & Grossman, 2015).

Integrons are bacterial genetic elements that allow the shuffling of smaller mobile elements called gene cassettes (Canal et al., 2016). They are not truly mobile elements, as the mobility is limited to the gene cassettes. At least six classes of integrons have been determined according to their *intI* gene. Classes 1, 2 and 3 are the most studied and are largely implicated in the dissemination of

antibiotic resistance (Ploy, Lambert, Couty, & Denis, 2000) .

6.3.4 Mechanisms of antimicrobial resistance

Bacteria have developed a number of antimicrobial resistance mechanisms. These mechanisms have been elaborated and explained for most pathogenic organisms in humans and animals (Blair, Webber, Baylay, Ogbolu, & Piddock, 2015; Founou, Founou, & Essack, 2016; Sirijan & Nitaya, 2016). The main mechanisms are decreased influx and increased efflux of antimicrobials, target modifications, modification of the antimicrobials and biofilm formation. Some of these targets for antibiotics and mechanisms of resistance are summarized by G. D. Wright (2010) shown in Figure 6.

Decreased influx is achieved through decreased cellular expression of porins and mutations in porin genes, hence making the cell wall less permeable to antimicrobials. The outer membrane porins (OMP) are used in the uptake of antimicrobials like tetracycline, beta-lactams and chloramphenicol. Increased efflux through an active multidrug resistant efflux pumps as a result of over expression or mutation in the genes, occurs through a large family of protein pumps that eject antibiotics from inside the cell. These multidrug transporters are found in almost all bacteria (Schwarz, Cloeckaert, & Roberts, 2006). Five super families of efflux pumps have been described; ATP-binding cassette (ABC) family, the small multidrug resistance family, the major facilitator super family, the resistance-nodulation-division (RND) family, and the multidrug and toxic compound extrusion family (Sun & Yan, 2014).

Target modifications mainly occur through mutational and chemical changes, protection of the target sites, the replacement of sensitive targets by functionally analogous but insensitive ones, and overproduction of sensitive targets (Mascaretti, 2003). A typical example for mutation alteration is seen in fluoroquinolone resistance in bacteria, where mutation occurs in genes for DNA gyrase (topoisomerase II and topoisomerase IV) at a particular site referred to as quinolone resistance determining region (QRDR), so the cellular targets are hence protected from the actions of the antimicrobial (Blair et al., 2015).

Modification of the antibiotics after it has gained entry in to the bacteria can be through enzymatic degradation or modification so that it can no longer bind to the target (Blair et al., 2015). Modifications of enzymes as observed in aminoglycoside resistance, for example as the case in acetyltransferases. These are encoded by a number of genes that have been elucidated (van Hoek et al., 2011). In enzymatic modifications, bacteria produce enzymes that modify the drugs

chemically. This can be through attachment of acetyl, adenyl or phosphate groups on specific sites of the antimicrobial molecules. This method is commonly seen in phenicols, aminoglycosides and also macrolides (Ramirez & Tolmasky, 2010; Schwarz et al., 2004). In case of enzymatic inactivation, bacteria produce enzymes that bind directly to the antimicrobial molecule and disintegrate it. An example of this is seen in beta-lactamases both in Gram positive and Gram negative bacteria, reported in penicillins, cephalosporins, monobactams, and/or carbapenems (Bush & Jacoby, 2010).

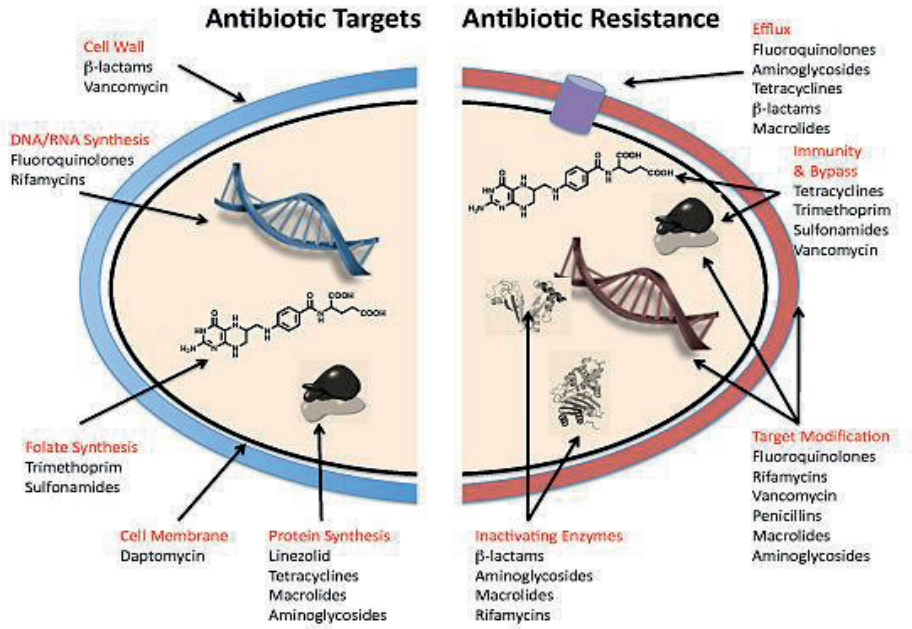

Figure 6. Antimicrobial target and antimicrobial resistance mechanisms (G. D. Wright, 2010)

6.3.5 Transmission of antimicrobial resistance in the food chain

The use of antimicrobials to treat humans and animals can lead to selection, spread and dissemination of antimicrobial resistant microorganisms between humans and animals. Transfer of these resistant microorganisms therefore is possible either by direct contact, inhalation of dust and aerosols that contain bacteria, or via the food chain (Schwarz, Kehrenberg, & Walsh, 2001). Transmission of antimicrobial resistant bacteria in the food chain is effected by ingestion of resistant microorganisms from contaminated food. Once in the gut these microorganisms can transfer the resistance genes to other gut microbiota by means of mobile genetic elements including phages, transposons and plasmids (Hudson et al., 2017). Vertical and horizontal gene transfer can occur concomitantly. The processes involved are emergence, invasion and occupation (Baquero, Lanza, Canton, & Coque, 2015).

AMR bacteria can enter the food chain following use of antimicrobials in agriculture. Consequently, this can lead to selection of AMR organisms that can ultimately get transmitted to

humans anywhere along the food chain. Contamination with AMR bacteria and genes can happen through many ways. Antimicrobial resistant bacteria and genes are found in the soil, water, human and animal faeces. They can enter animal products by faecal contamination during slaughter and processing and plant products can be contaminated by use of contaminated irrigation water. Food may also be contaminated by the environment and through cross contamination by food handlers (Verraes et al., 2013). During processing of some products, microorganisms are intentionally added and these may contain AMR genes. The potential transmission routes of AMR in the food chain are illustrated in figure 7.

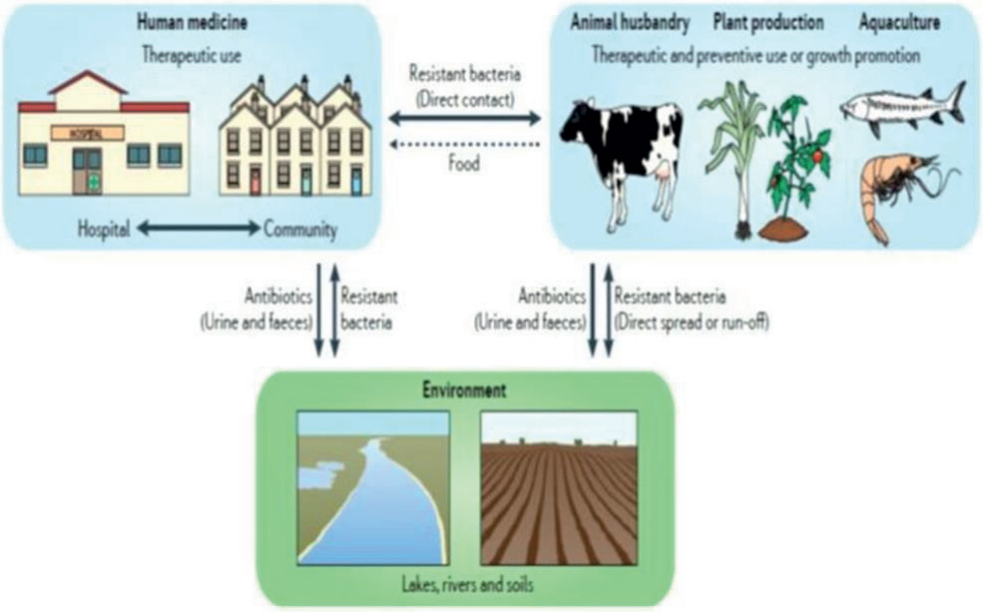

Figure 7. Transmission of different transmission routes of antibiotic resistance in the food chain. Adapted by permission from MacMillan Publishers Ltd: [Nature Reviews Microbiology], (Andersson and Hughes, 2014), Copyright (2014)

6.4 Knowledge gaps

Foodborne diseases cause high morbidity and mortality worldwide, but detailed extent and economic burden remains poorly understood, especially in LMIC. Data on major foodborne illnesses is scarce in LMIC, and yet experts believe these countries carry heavy burdens of these diseases (Grace, 2015; Kaferstein, 2003).

In high income countries NTS is known to cause mainly gastroenteritis while in Africa, NTS is a major cause of bacteraemia in children, immunocompromised persons, HIV patients and the elderly (Feasey et al., 2012). Although the primary sources of NTS are animals and animal products, the magnitude of transmission to humans is still unknown. The occurrence of NTS in animal reservoirs is poorly understood in developing countries. In addition, most studies on risk factors associated with the occurrence of NTS are conducted in USA and Europe, the few studies from Africa were mainly from West Africa and North Africa. Consequently, not so much is known about factors associated with NTS in Sub Saharan Africa, including Uganda.

In developed countries, many programmes have been established for routine monitoring and surveillance for NTS and important foodborne diseases to support strategies for control of foodborne diseases with data that are scientifically generated. On the contrary, most developing countries including Uganda do not have systematic surveillance for foodborne pathogens (Ao et al., 2015). Consequently, in developing countries, the extent of the burden of NTS and the transmission dynamics is not well estimated, a fact that compromises control efforts and in effect food safety. Even where some isolation is done, normally *Salmonella* is identified only to species level and on rare instances serotyped, so largely, further characterization and typing is not undertaken, leaving large data gaps that would help with understanding the epidemiology of NTS. So in essence, very few epidemiologic and microbiological studies have been undertaken in African countries to understand NTS in livestock.

In addition to causing morbidity and mortality, increasing reports of AMR in NTS is of great concern particularly the occurrence MDR NTS and their potential roles in the transmission of AMR genes (Feasey et al., 2012; Lynne, Rhodes-Clark, Bliven, Zhao, & Foley, 2008; Yang et al., 2016). AMR in NTS is a real public health challenge and needs to be better understood and effectively managed with emphasis on primary production units. Its occurrence anywhere can affect any part of the world. Most studies on AMR in NTS in Africa focused mainly on human isolates with very few on livestock. The few studies on livestock, like those done by Adesiyun et al. (2014), Aimey et al. (2013), Cardinale, Tall, Gueye, Cisse, and Salvat (2004) and (Raufu et al., 2014) were mainly from West Africa and North Africa. Because of limited research and surveillance, there is paucity of data from East Africa including Uganda on NTS. The significant data gaps in developing countries hinder development of effective control systems and risk-mitigation strategies at multiple levels.

It is noteworthy that similar antimicrobial compounds are used in treatment of human illnesses and poultry production in Uganda, but limited documentation exists of the susceptibility to antimicrobials and carriage of AMR genes in zoonotic pathogens like NTS. As a result sound epidemiological and microbiological data is lacking. In addition there is lack of knowledge on the prudent use of antimicrobials. Consequently efforts to control NTS and AMR can be limited by complex interplay of multiple factors.

The need to generate data on AMR and foodborne pathogens from developing countries and Sub Saharan Africa is critically urgent, especially from primary production units. There is limited

epidemiologic research and lack of appropriate laboratory infrastructure to undertake routine culture, isolation and antimicrobial susceptibility testing.

AMR in *Salmonella* are known not only for the decreased susceptibility to different antimicrobials, but also that the organisms become more virulent, causing prolonged sufferings of the affected (Travers & Barza, 2002). There is therefore dire need, especially in Uganda to improve understanding of AMR considering that indiscriminate and injudicious use of antibiotics in animals and humans could be aggravating development and spread of antimicrobial resistance. There is need to have more accurate data from all parts of the world if local, national and international control strategies to combat AMR and foodborne diseases are to be effective.

7 AIM AND OBJECTIVES

The main aim of this study was to determine occurrence, diversity and antimicrobial resistance of non-typhoidal *Salmonella* in layer hen farms from selected districts of Uganda.

Specific objectives were;

i. To estimate prevalence and identify modifiable risk factors for non-typhoidal *Salmonella* in layer chicken farms (Paper I).
ii. To determine the diversity of non-typhoidal *Salmonella* isolates from commercial layer farms (Paper II).
iii. To evaluate antibiotic resistance in non-typhoidal *Salmonella* isolates from commercial layer chicken farms (Paper I and II).
iv. To determine association of risk factors to antimicrobials resistances and if antimicrobial resistances are linked to each other (Paper III).
v. To characterize resistance genes and integrons from phenotypically resistant non-typhoidal *Salmonella* isolates from layer chicken farms (Paper II).
vi. To analyze for mutations in the quinolone resistance-determining region (QRDR) of the DNA gyrase (*gyrA, gyrB*) and topoisomerase IV (*parC, parE*) in ciprofloxacin resistant isolates (Paper IV).

8 MATERIALS AND METHODS

8.1 Study area

The study was carried out between June 2015 and August 2016 in the districts of Wakiso, Masaka and Lira in Uganda (Figure 8). Masaka district is located between latitudes $00^0$25'S and $00^0$15' N and longitudes N34^0 00'E and N35^0 00'E. It is estimated to have a human population of 314,000 (Uganda Bureau of Statistics, 2017a). The district receives two rainy seasons, the first and the heaviest falling between March – May, while the second rainy season is normally between September – December. The average rainfall is between 1100 mm-1200 mm falling in average of 100 – 110 days annually. Masaka district has 44.9 % of its population engaged in livestock farming (Uganda Bureau of Statistics, 2017b).

Wakiso district is located between latitudes $00^0$10'N and $01^0$00' N and longitudes $32^0$01'E and $32^0$52'E. It is estimated to have a human population of 2,391,500 (Uganda Bureau of Statistics, 2017a). The district has bimodal rainfall with peak April – May and October – November. The average rainfall is 1320 mm but areas around the lake Victoria receive 1750 and 2000 mm. Wakiso district has 25.0 % of its population engaged in livestock farming (Uganda Bureau of Statistics, 2017b).

Lira district is located between latitudes 1° 21'N and 2° 42"N and Longitudes 32^0 51"E and 34^0 15"E. The estimated human population is 439,200 (Uganda Bureau of Statistics, 2017a). The rainfall is bimodal with peak April – May and August – October. The average rainfall is between 1200 mm-1600 mm. Lira district has 61.4 % of its population engaged in livestock farming (Uganda Bureau of Statistics, 2017b).

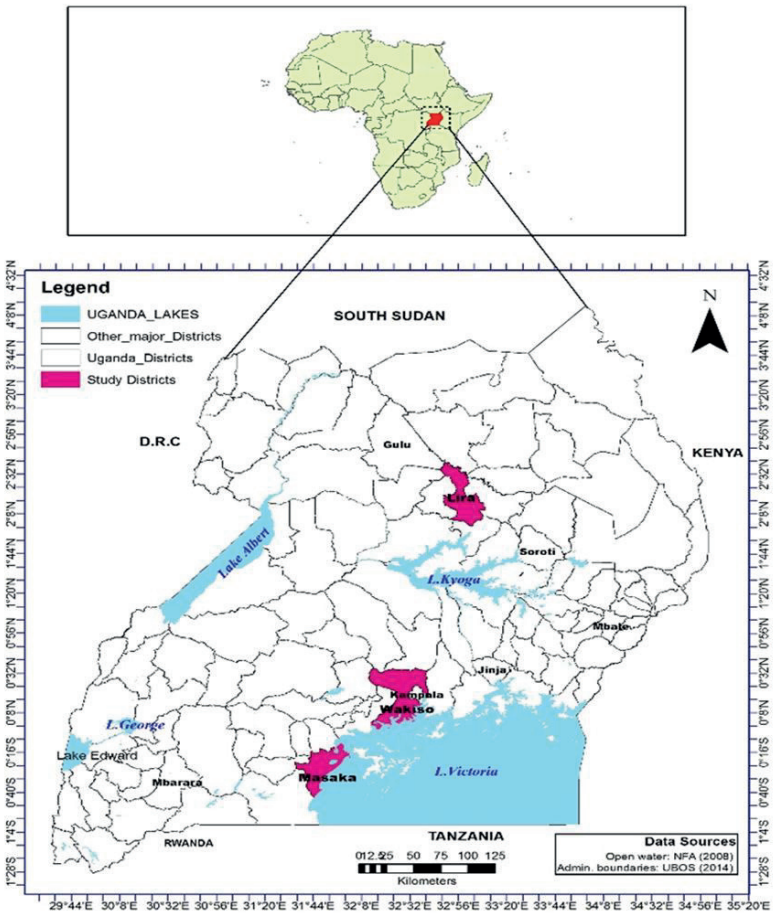

Figure 8. Showing the study districts in Uganda

8.2 Study design and sample size determination

This study was a cross sectional survey with farms randomly selected from a list of farms from Veterinary authorities in the districts. The sampling frame was generated from a list of farmers participating in National Agricultural Advisory Services (NAADS) program in the three selected districts of Uganda. Because most poultry farmers in Uganda are smallholders, a minimum number of 50 chicken was considered for a farm to participate. A sample size calculator, FreeCalc sample size calculation for imperfect tests (www.epitools.ausvet.com.au, accessed on 3rd, June, 2015) was

used. The required sample size for demonstrating disease freedom was calculated as previously described (A. Cameron, 1999; A. R. Cameron & Baldock, 1998). The calculator had as input the population size, sensitivity (60 %), specificity (100 %), design prevalence (5 %), and the desired type 1 and type 2 errors were all assumed at 0.05. Because of small populations, the modified hypergeometric option was used. Farms were selected by use of computer generated random numbers. The selected farmers were asked for their consent and those who were not willing to participate were replaced by random selection of others from the same list.

8.3 Questionnaire administration

All questionnaires were directly administered onsite by the researcher and research assistants (Figure 9). The questionnaire was pre-tested before administration. The questionnaires collected information on general farm management practices and characteristics, disease prevention, control and management as well as demographic data of the farmers and managers (Annex II). The questionnaire had mainly close-ended questions (80 %) and was designed in English, but where a respondent was not competent in English, then it was translated in to the local language of the area.

Figure 9. Administration of questionnaire, Masaka district, Uganda

8.4 Collection of fecal samples

Collection of fecal samples followed a standardized scheme designed to take care of wide variation in poultry housing and farm sizes. Each house was divided into sectors of about 5 m by 5 m (25

m^2), and one sample was collected from each sector. This approach was adapted from previous studies (Snow et al., 2010; Van Hoorebeke et al., 2009). The samples from each house were pooled together and transported to the laboratory in a cool box with icepacks within less than 8 hours. All flocks were sampled once (Figure 10).

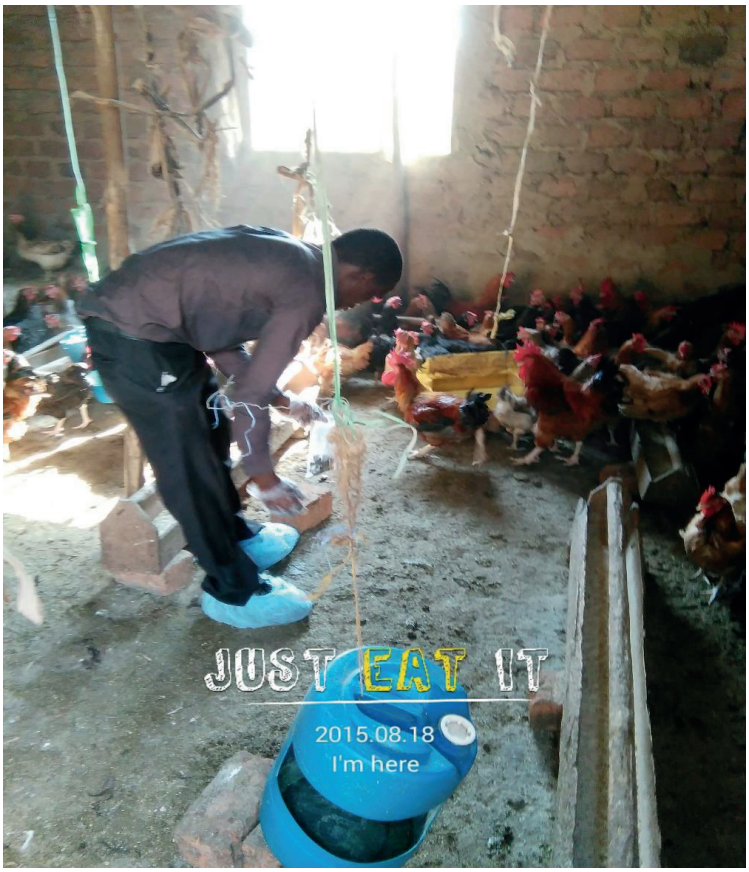

Figure 10. A research assistant collecting fecal samples from a poultry house in Wakiso, Uganda

8.5 Laboratory methods

8.5.1 Bacterial culture, Isolation and Identification of NTS serovars

Culture and isolation of NTS followed standard procedures according to ISO 6579:2002/Amd 1:2007 Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples

from the primary production (ISO, 2007). Briefly, pooled samples were homogenized, 25 g weighed and added to 225 ml of Buffered Peptone Water (BPW) for pre-enrichment and incubated for 20 h at 37 °C. The culture obtained was subjected to selective
Modified Semisolid Rappaport Vassiliadis (MSRV) agar plates and incubated at 41.5 °C for 24–48 h. One colony from each culture indicative of NTS was further plated on selective Xylose Lysine Deoxycholate (XLD) agar and incubated at 37 °C for 24 h. Presumptive NTS colonies
were stored at −20 °C in Mueller-Hinton agar. The samples were later transported to Norway where they were sub-cultured on blood agar plates and stored at 4 °C. Biochemical confirmatory tests were done by using the API-20E (BioMerieux, Marcy l'Etoile, France) identification system. All isolates were serotyped according to the Kauffman–White–Le–Minor technique (Grimont & Weill, 2007) at the Norwegian Veterinary Institute.

8.5.2 Antimicrobial susceptibility testing

Kirby-Bauer disk diffusion method

Antibiotic susceptibility testing of all identified isolates was performed on 13 antibiotics (NEO-SENSITABS™, Rosco, Denmark) using the standard Kirby-Bauer disk diffusion methods on Muller-Hinton agar. *Escherichia coli* ATCC 25922 was used as quality control and was provided by the bacteriology laboratory at NMBU. The 13 antibiotics tested were gentamycin (GEN10 µg), sulfonamide (SULFA240 µg), trimethoprim-sulfamethoxazole (SxT 25 µg), ciprofloxacin (CIPR1 µg), cefotaxime (CTX 30 µg), meropenem (MPR 10 µg), chloramphenicol (CLR30 µg), ceftazidime (CAZ30 µg), ampicillin (AMP10 µg), amoxycillin clavulanic acid (AMC30 µg), trimethoprim (TRIM5 µg), tetracycline (TET30 µg), and enrofloxacin (ENROF10 µg). Their selection was based on the common antibiotics used in Uganda and those recommended by World Health Organization (WHO). Results were interpreted according to Clinical and Laboratory Standards Institute(CLSI, 2015), except for ciprofloxacin (CIPR1µg) which was interpreted using CLSI (CLSI, 2013).

Minimum inhibitory concentrations (MIC) determination

In order to detect reduced susceptibility to ciprofloxacin, minimum inhibitory concentrations (MIC) for all the isolates had to be determined. This was done using a commercially available dehydrated panel (SensiTitre® TREK EUVSEC, TREK diagnostics Ltd). The intervals assayed

ranged from 0.015 – 8 µg/mL and isolates with MIC >0.06 µg/mL were considered to have reduced susceptibility to ciprofloxacin as interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org).

8.5.3 Pulsed-Field Gel Electrophoresis (PFGE) and BioNumerics Analysis

The PulseNet standardized protocol for PFGE for molecular subtyping of *Salmonella* was used (https://www.cdc.gov/pulsenet/pathogens/pfge.html). Overnight cultures were used to prepare DNA templates according to the PulseNet protocol. The DNA plugs were digested with the restriction enzyme, *Xba*I for 1.5 – 2 hrs at 37 ºC in a waterbath. *S*. Braenderup H9812 was used as a molecular size standard in all PFGE investigations. Restriction fragments were separately in 1% agarose gel. Electrophoresis was performed with the CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA, USA) with the following set parameters: initial switch time 2.2 s, final switch time 63.8 s, voltage-6 V, time-19 h and temperature 14 ºC. The gels were stained with ethidium bromide and the bands visualized under UV transillumination and captured by GelDoc EQ system with Quantity One®software (Version 4.2.1; Bio-Rad Laboratories, Hercules, CA, USA). PFGE banding patterns were compared using a combination of visual inspection and the BioNumerics software vers. 6.6.11 (Applied Maths, Ghent, Belgium). A dendrogram was generated using band-based dice similarity coefficient andthe unweighted pair group method using a geometric average (UPGMA) with 1.2 % position tolerance and 1.2 % optimization. A cutoff of 97 % similarity was used to define a PFGE pulsotype (PT).

8.5.4 Bacterial DNA extraction

Total DNA for PCR were extracted using the boiled lysate method (A. M. Ahmed, Hussein, & Shimamoto, 2007). This was done by taking 200 µL of an overnight culture, mixing with 800 µL of sterile distilled water and boiling for 10 minutes. The resultant solution was centrifuged at 13,000 rpm for five minutes and the supernatant was used as a DNA template. This was kept at -20 ºC for subsequent use.

8.5.5 PCR amplifications and sequencing

All the isolates that were classified as resistant according to the results of the disc diffusion test were screened by PCR for genes encoding integron class 1 and 2 and presence of 22 of the most relevant AMR genes corresponding to their phenotypic resistance pattern (Paper II). These genes are known to confer resistance to six commonly used classes of antimicrobials (beta-lactams,

tetracyclines, phenicols, fluoroquinolones, trimethoprim, and sulfonamides).

To investigate for mutations in the QRDR region, the genes *gyrA, gyrB, parC and parE* were amplified by PCR with specific PCR primers and conditions. This was done only for isolates phentypically resistant to ciprofloxacin. One PCR product from each of the AMR and integron PCRs were purified and sequenced (GATC Biotech, Cologne, Germany) and the sequence results analyzed using BLAST and compared to GenBank database (http://blast.ncbi.nlm.nih.gov/blast.cgi) to confirm the PCR results. For some (Paper IV), the sequence data was imported to BioEdit program and inspected for mutations through alignment. To check for mutations in the QRDR region, translation of the nucleotide sequence into proteins was done and then aligned with a reference sequence of the wild type *S.* Typhimurium strain LT2 in the NCBI database (Accession Number AE006468).

8.6 Data management and statistical methods

A database was created in Excel and later exported to the Statistical Package for Social Scientists (SPSS, version 20) computer program for further data analyses. An initial descriptive statistics was performed and later associations assessed with chi square statistics. This was followed by a multivariable logistic model built based upon variables with p-values < 0.20 in the initial analyses. The model was built utilizing a backward selection among the candidate variables (**p** < 0.20 from initial analyses) strategy using the Likelihood Ratio test with for comparing models [32]. The final model was assessed for fit using the Hosmer- Lemeshow test (Paper 1).

Additive Bayesian network (ABN) analysis was performed with the R package abn (Lewis & Ward, 2013). An exact search was used to first find an optimal model (Koivisto & Sood, 2004). Then to adjust from over fitting a non-parametric bootstrapping analysis was performed. This step was done many times to allow computation of the supporting matrix of the network. Then a 50 % threshold was applied to keep only well supported arcs (Paper III).

9 SUMMARIES OF RESULTS/INDIVIDUAL PAPERS

9.1 Paper 1

The aim of this paper was to estimate the prevalence, determine antimicrobial susceptibility and identify risk factors associated with NTS presence in laying hen farms in Uganda through a cross-sectional study. A total 237 farms were involved, out of these, 49 farms (20.7 %; 95 % Confidence interval (CI): 15.6–25.6 %) were positive for NTS presence. Altogether, ten NTS serovars were identified among the confirmed 78 isolates, and the predominant serovars were *S*. Newport (30.8 %), *S*. Hadar (14.1 %), *S*. Aberdeen (12.8 %), *S*. Heidelberg (12.8 %), and *S*. Bolton (12.8 %). Phenotypic AMR was detected in 45 (57.7 %) of the isolates and the highest resistance was against ciprofloxacin (50.0 %) followed by sulfonamides (26.9 %) and sulfamethoxazole/trimethoprim (7.7 %). Resistance was significantly associated with sampled districts (p = 0.034). Resistance to three or more drugs, multidrug-resistance (MDR) was detected in 12 (15.4 %) of the isolates, 9 (75 %) of these were from Wakiso district. A multivariable logistic model identified large farm size (OR = 7.0; 95 % CI: 2.5–19.8) and the presence of other animal species on the farm (OR = 5.9; 95 % CI: 2.1–16.1) as risk factors for NTS prevalence on farms. Having a separate house for birds newly brought to the farms was found to be protective (OR = 0,4; 95 % CI: 0.2–0.8). This study highlighted a high prevalence and diversity of NTS species in laying hen farms in Uganda and identified associated risk factors. In addition, it demonstrated high levels of AMR in isolates of NTS. This could be because of overuse or misuse of antimicrobials in poultry production. Also importantly, the insights provided in this study justifies a strong case for strengthening One Health practices and this will contribute to the development of NTS control strategies at local, national and international levels.

9.2 Paper 2

The aim of this study was to subtype a collection of NTS originating from poultry farms in Uganda, and to evaluate a subgroup of phenotypically resistant isolates for common antimicrobial resistance genes and associated integrons. PFGE revealed 15 pulsotypes representing 11 serovars from 75 isolates, as 10 were non-typable. Thirty one (57.4 %) of the 54 resistant isolates carried at least one of the seven genes (*blaTEM-1, cmlA, tetA, qnrS, sul1, dhfrI, dhfrVII*) identified by PCR and six (11 %) carried class 1 integrons. This study showed that a diversity of NTS-clones are present in Ugandan poultry farm settings, while at the same time similar NTS-clones occur in different farms

and areas. The presence of resistance genes to important antimicrobials used in human and veterinary medicine was demonstrated, hence the need to strengthen strategies to combat antimicrobial resistance at all levels.

9.3 Paper 3

The aim of this study was to determine if specific risk factors are associated with single AMRs and if specific AMRs are linked to each other. Data from a previous study was re-analysed using additive Bayesian networks (ABN).

Solely resistance to ampicillin was linked to farm size and management with large farms being more likely to display ampicillin resistance. In Uganda, large farms tend to use a wide spectrum of antimicrobials, consequently selection pressure can lead to propagation of antimicrobial resistant isolates. Still this needs to be interpreted with caution, as there were only four isolates with ampicillin resistance.

Resistance towards trimethoprim was linked positively to resistance towards sulfonamide, sulfamethoxazole/trimethoprim and ciprofloxacin. Resistance towards sulfonamide was also linked positively to resistance to ciprofloxacin. There was also a positive association between resistance to chloramphenicol and ampicillin, with all isolates being either both susceptible or resistant. Negatively associated were resistance to ampicillin and to sulfamethoxazole-trimethoprim. Resistance to tetracycline was not linked to any other antimicrobial resistance.

The increasing incidence of ciprofloxacin resistance has frequently been reportedly associated with resistance to other antimicrobials and multi resistant isolates. Unfortunately the cause of these associations has not been fully understood. The association of sulfonamides and trimethoprim/sulfamethoxazole can be explained by the fact these are mainly formulated in combinations and some times administered simultaneously. This can probably be regarded as an important driver for evolution of this linkage. Many of the genes coding for AMR characteristics are located on mobile genetic elements and these genes are disseminated between related and unrelated bacteria through horizontal gene transmission. However, we do not have any data on the location of the genes encoding the AMR characteristics in the bacterial isolates analysed in this study, and can therefore only speculate that one explanation for the AMR linkages observed in the ABN analysis is the physical linkage of genes on the same mobile genetic element. Although the results needs to be interpreted with caution due to a small data set, ABN analysis allowed us to

describe an association between farm size and ampicillin resistance and linkages to each other of specific AMRs.

9.4 Paper 4

The aim of this study was to determine the occurrence of reduced susceptibility to ciprofloxacin in non-typhoidal *Salmonella* (NTS) isolates and to detect for mutations in the quinolone resistance-determining region (QRDR). Reduced susceptibility to ciprofloxacin was detected in 32 (37.7%) of the 85 isolates of NTS and this was significantly associated with the district where they were isolated ($p= 0.014$) and presence of *qnrS* genes ($p<0.01$). The MIC for isolates with reduced susceptibility ranged from 0.12 – 1.00 µg/mL, an indication of low-level resistance. Fourteen (43.8 %) of the 32 isolates were identified with *qnrS* genes distributed among three serovars; Bolton (n=4), Mbandaka (n=5), and Newport (n=5). All the isolates of *S.* Mbandaka with reduced susceptibility to ciprofloxacin had the *qnrS* gene, while it was 5/9 and 4/5 for Newport and Bolton, respectively. Classical mutations at GyrA (S83F and D87N)/GyrA (S83F and D87G) and ParC (T57S and S80R) normally associated with quinolone resistance in *Salmonella* were not detected. However, 7 isolates had point mutations at codon 67 of the QRDR.

The study has shown significant presence of reduced susceptibility to ciprofloxacin in NTS isolates without the classical mutations. There is need for further investigations of other mechanisms underlying increasing resistance to fluoroquinolones in NTS and factors driving it.

10 DISCUSSIONS

10.1 Methodological considerations

This study was carried out in the three districts of Uganda known for high poultry production. It was on this basis and also because of lack of primary data that these districts were purposively selected. This was necessary especially as the study was targeting commercial poultry farmers mainly, and in addition appropriate for working within the limited resources. To balance geographic regions, one of the districts, Lira, was selected from the northern region of Uganda. Lira district in addition has high numbers of farmers keeping indigenous breeds and increasing numbers of smallholder poultry farmers. However, purposive sampling being non-random tends to bias the results of a study, this was a possibility in the study. In Uganda there is no formal register of farmers, so no complete sampling frame existed and the study relied on farmers registered in National Agricultural Advisory Services (NAADS) to provide a sampling frame for random selection. NAADS is a government of Uganda program to promote commercialization of agriculture and improve productivity. Unfortunately, many farmers are not registered in the NAADS program and they were left out, thus to some extent this limits the interpretation of the results of this study.

The study was a cross-sectional study designed to determine NTS positive farms as one of the key objectives. A cross-sectional study by design is a snapshot, depending on the status of the birds at the point in time of sample collection. Birds that are carriers of NTS normally shed the organisms in the feces intermittently, so there are chances that a number of birds shedding NTS at a time is less than the infected (Carrique-Mas & Davies, 2008). In addition, the determination of a farm as positive for NTS was based only on fecal sampling, NTS in farms can be found in feces, litter, water, feeds. However, environmental sampling of poultry houses that involves fecal droppings and dust sampling is still one of the most cost effective and sensitive ways of monitoring NTS in farms (Arnold, Carrique-Mas, McLaren, & Davies, 2011). The size of poultry houses varied tremendously in the study areas, as there is lack of standardized designs for poultry houses in Uganda. One of the large poultry farms is shown in Figure 11.

Figure 11. One of the poultry farms in Masaka, Uganda

These wide variations in poultry houses poised enormous challenges in the design of the sampling protocol. To improve representativeness of the samples a standardized sampling protocol was adapted from previous studies (Snow et al., 2010; Van Hoorebeke et al., 2009).

In assessing the occurrence of resistance genes in phenotypically resistant isolates, this study purposively selected 22 genes and could only identify a few. Antimicrobial resistomes are increasingly diverse, complex, broad and dynamic. For example currently there are more than 40 tetracycline resistance determinants identified and more than 30 gene variants encoding dihydrofolate reductase that are resistant determinants for trimethoprim have been identified (Nguyen et al., 2014; van Hoek et al., 2011; Wang et al., 2015).

In determination of diversity of NTS, the numbers of isolates were few and from limited geographical coverage of the study areas. In addition, PFGE, which is a gold standard for subtyping *Salmonella* spp. could not subtype some isolates. Therefore, to draw inference for the whole country needs to be done with caution. Use of WGS for a larger collection of NTS isolates, which is becoming more accessible, could be utilized in future studies.

10.2 Prevalence, diversity and factors associated with NTS in layer hen farms

In this study, we estimated occurrence of NTS in more than 20 % of layer hen farms. This is lower than reported in other countries in Africa (Andoh et al., 2016; Bouzidi et al., 2012; Dione, Ieven, Garin, Marcotty, & Geerts, 2009; Fagbamila et al., 2017) but also higher when compared to other studies in Africa and elsewhere (Arnold et al., 2010; Eguale, 2018). Occurrence of NTS in poultry vary a lot in countries, even within the same country variations are seen in place and time. However,

for this study occurrence of NTS were not significantly associated with the study areas. In some countries in the European Union (EU) flocks are almost free of NTS (Mølbak, Olsen, & Wegener, 2006), while very high prevalence have been reported in other countries (Tu et al., 2015). This study unveiled 11 NTS serovars circulating in poultry farms in Uganda. These were Newport, Bolton, Heidelberg, Hadar, Mbandaka, Aberdeen, Zanzibar, Typhimurium, Enteritidis, Uganda, Kampala. Some of these serovars were also reported in related studies recently undertaken in Uganda (Afema et al., 2016; Ikwap et al., 2014). The serovars identified were mostly clonally related in spite of originating from diverse geographical areas. For example, all isolates of the most prevalent serotype *S.* Newport belonged to the same pulsotype and they are therefore clonally related. This shows that similar clones are easily being transmitted from one region to another.

This study is one of the few providing insights to occurrence of NTS in Ugandan poultry. Like in other countries, prevalence and diversity of NTS in poultry farms is expected to vary within Uganda. These variations are a manifestation of a wide range of management practices, risk factors and disease control strategies. Generally in Uganda, most farm management practices are characterized by low biosecurity, birds mixing with other birds and animals under free-range systems, inadequate animal health services, and lack of systematic control measures. All these favor transmission of infections. Most of the serovars identified have been reported in previous foodborne disease outbreaks. Plausibly, the foodborne disease burden is usually high in the most vulnerable populations in developing countries (Grace, 2015). In spite of being an important global foodborne pathogen of great public health consequence, NTS in poultry farms and other livestock is poorly investigated in most developing countries. It is crucial that efforts to control NTS be put in place, the focus should start from primary production units and farm ecosystems.

As result of a univariate analysis, a number of demographic, management and production variables were associated with occurrence of NTS. However, a final logistic regression model identified large farm size, presence of other poultry species, and keeping of records to be associated with NTS, while having a separate housing for newly brought in birds was a protective factor. Large farms as a risk factor for NTS occurrence have been reported in studies from Britain (Snow et al., 2010), Trinidad and Tobago, Grenada and St. Lucia (Adesiyun et al., 2014), France and Belgium (Namata et al., 2008). Most studies to determine prevalence and risk factors for NTS have been mainly undertaken in developed countries, with quite a few studies in developing country settings. A wide range of risk factors has been reported in a number of studies (Aury et al., 2010; Cardinale

et al., 2004; Chemaly et al., 2009; Henzler, Kradel, & Sischo, 1998; Huneau-Salaun et al., 2009; Le Bouquin et al., 2010; Mollenhorst, van Woudenbergh, Bokkers, & de Boer, 2005; Sasaki et al., 2012; Schulz et al., 2011).

A number of factors affect the occurrence and transmission of NTS from their reservoirs. In most cases transmission are mainly horizontal and favoured by a number of management and environmental factors. A systematic review of observational studies on risk factors associated with NTS in laying hen farms by Denagamage, Jayarao, Patterson, Wallner-Pendleton, and Kariyawasam (2015) revealed multiple factors related to biosecurity measures, management practices and the environment. In Uganda, as the farms increase in size, keeping hygiene standards and biosecurity become challenging. These increase challenges of keeping infections under control. Moreover much of the farm operations are manual, meaning more largely unskilled laborers are required, and on the other hand some farms have limited farm labor. Consequently, comprehensive cleaning, disinfection and biosecurity practices are difficult to enforce. The current study identified presence of other animal species as another risk factor for NTS. Keeping of other animals on the same farm is quite a common feature in Ugandan farms. All these animals and poultry in most cases share water, feeds and space, thus increasing the opportunities for the spread of the bacteria due to direct or indirect contacts. Zoonotic NTS have multiple host range and can easily get transmitted in such settings (Gyles, 2004; Hoelzer et al., 2011). The other animal species may be reservoirs of NTS and thus, contribute to the maintenance of high prevalence of NTS at a farm. Having separate housing for birds newly introduced in the farm was a protective factor for NTS. New birds can introduce infections, therefore housing new birds separately provides an opportunity for close observation so that birds that are healthy are the ones ultimately allowed to mix. The sick birds can either be euthanized or they can be provided timely treatment before they are released to mix with other birds on the farms.

The food supply chain is becoming more complex, consequently the probability of contamination is higher especially where there is low biosecurity, informal marketing, poorly organized infrastructures with poor regulatory mechanisms as is the case with most LMIC (Grace, 2015; Nadimpalli et al., 2018). A surveillance program for NTS in primary production points and from farm ecosystem would be an important activity that should be initiated and supported in order to generate data for a database. NTS organisms should be isolated from different sources, serotyped

and subtyped. The high prevalence of NTS in poultry farms as demonstrated in this study warrants a program of intervention utilizing sound principles of one health.

10.3 Antimicrobial resistance in NTS isolates

This study revealed high level of phenotypic resistance in NTS isolated from poultry farms. Some of the genes responsible for the underlying resistance mechanisms were characterized. Resistance was identified to antimicrobial agents commonly used for human and animal treatment in Uganda. A number of studies with varying results have demonstrated the increasing occurrence of AMR in NTS to commonly used antibiotics in human and animal treatment (Dione et al., 2009; Dogru, Ayaz, & Gencay, 2010; Lynne et al., 2008; Yang et al., 2016). Indeed, AMR in NTS is increasingly being reported (Septimus & Kuper, 2009; Streit, Jones, Toleman, Stratchounski, & Fritsche, 2006). There is growing evidence showing that widespread use of antimicrobials is linked to development of AMR in NTS. Acquisition of resistance from NTS has been reported to be related to international travels (Crump et al., 2011). From published studies, there are wide variations in the prevalence of AMR in NTS isolates from humans and animals, with some studies reporting 80 % or more resistant isolates (Crump et al., 2011; Hu et al., 2017). Some limited studies of this kind have been done on AMR in NTS in livestock from Africa and in other LMIC (Abdel-Maksoud et al., 2015; A. M. Ahmed, Shimamoto, & Shimamoto, 2014; Andoh et al., 2016; Ben Salem et al., 2017; Tabo et al., 2013). For example, one study in Tunisia found resistance to at least one of thirteen tested antimicrobials in 33.8 % of the isolates from poultry (Ben Salem et al., 2017).

In general terms, emergence of serovars of NTS which are MDR is largely associated with widespread use of antimicrobials like sulfonamides, tetracyclines, aminoglycosides, trimethoprim in humans and livestock. In this study MDR was seen in *S*. Bolton, *S*. Mbandaka, *S*. Hadar, and *S*. Newport. Surprisingly, the highest phenotypic resistance was observed against ciprofloxacin, a fluoroquinolone that is not normally used in livestock in Uganda. The knowledge on AMR in NTS isolated from livestock farms supports the needed effort to combat AMR in the country. It is worthwhile to note that MDR strains of NTS are now reported in a wide range of other NTS serovars like Agona, Anatum, Choleraesuis, Derby, Dublin, Kentucky, Pullorum, Schwarzengrund, Senftenberg, Typhimurium, and Uganda (Chen et al., 2004; Gebreyes & Thakur, 2005; Pan et al., 2009). There is currently enough body of evidence to show that MDR NTS are amplified within livestock and animal populations where they get widely disseminated (Heurtin-Le Corre, Donnio, Perrin, Travert, & Avril, 1999; Hur, Jawale, & Lee, 2012). In addition, MDR NTS are increasingly

being isolated from different food products worldwide (Bouchrif et al., 2009), and MDR NTS strains carrying AMR genes are the most frequent cause of MDR outbreaks (Doyle, 2015).

Arguably, in Uganda AMR could be increasing due to overuse or misuse of antimicrobials in especially commercial poultry production, coupled with low biosecurity and poor farming practices. Of particular concern is the high resistance to ciprofloxacin, an important drug used in Uganda for treatment of different bacterial infections in humans. This has serious public health implications, as available options might prove expensive for the majority of Ugandans. Occurrence of AMR was significantly higher in the Wakiso district. This district surrounds Kampala, the capital city of Uganda, the hub of commercial activities involving poultry and poultry products. The potential danger is that the Wakiso district can be a focus of spread of AMR to other districts in the country. The fact that AMR is seen to the common classes of antimicrobials put farmers at high risks, as cheaper options of effective antimicrobials are scarce to none.

It was possible to obtain networks including seven potential risk factors and seven antimicrobial resistances using Additive Bayesian network (ABN) modeling. So far, very few studies like those by Ludwig et al. (2013) and Hidano et al. (2015) have used ABN for analysis on antimicrobial data. In both studies, not binary data (being resistant or not) but continuous data, assumed to be Gaussian, as zones of inhibition measured in mm were considered. In our study, due to recent adaptions in the ABN code, it was possible to directly include the dichotomized antibiotic resistance data, based on CLSI, without encountering the issue of sparse data. Still due to sparse data, inevitably present in a small data set, not all associations were estimated. Another novelty lies in the opportunity to also include multinomial data.

ABN models determined positive linkages among resistance to a number of antimicrobials, particularly ciprofloxacin resistance was linked to quite a number of other antimicrobial resistance. There is increasing reports of ciprofloxacin resistance associated with resistance to other antimicrobials in MDR isolates. Unfortunately, the cause of these associations has not been fully understood (Giufrè et al., 2012; Grude et al., 2008; Strand et al., 2014). The association of sulfonamide and trimethoprim/sulfamethoxazole can be explained by the fact these are mainly formulated in combinations and some times administered simultaneously. This can probably be regarded as an important driver for evolution of this linkage. Many of the genes coding for AMR characteristics are located on mobile genetic elements, and that these genes are disseminated between related and unrelated bacteria through horizontal gene transmission (Canal et al., 2016;

Carattoli, 2013; Johnson & Grossman, 2015). However, this study did investigate the location of the genes encoding the AMR characteristics in the bacterial isolates analyzed, and can therefore only speculate that one explanation for the AMR linkages observed in the ABN analysis is the physical linkage of genes on the same mobile genetic element. Use of antimicrobials is a main driver for development and dissemination of AMR. Although the results needs to be interpreted with caution due to a small data set, additive Bayesian network analysis allowed us to describe an association between farm size and ampicillin resistance and the linkages to each other of specific AMRs.

According to WHO, AMR is now a global health security threat. As reported earlier AMR is already resulting in the death of 700,000 persons annually and this is expected to reach 10 millions by 2050. In LMIC, AMR is fueled by high disease burdens, easy access to antimicrobials and unprecedented use. Resistant strains of microorganisms in any country of the world can easily spread to another country especially due to increased global travel, as "resistance anywhere is resistance everywhere" (Prescott, 2014). AMR is multifaceted at the interface of human, animal and plant health, food hygiene and environmental science (Butaye, van Duijkeren, Prescott, & Schwarz, 2014). AMR therefore spans across the main domains of one health and clearly epitomizes its principles. An integrated approach to effectively control AMR should adopt one-health practices.

10.4 Occurrence of AMR and integron genes in NTS

In this study, 22 AMR genes that are known to be common within the Enterobacteriaceae family were investigated for their presence in the NTS isolate collection originating from the initial study of poultry farms (Collignon, Powers, Chiller, Aidara-Kane, & Aarestrup, 2009). However, only seven genes (*sul*1, *dhfr*I, *dhfr*VII, *qnrS, tetA, cmlA*, *bla*TEM-1) were identified. These are the common genes that encode resistance to sulfonamides, trimethoprim, quinolone, tetracycline, and beta-lactam antibiotics. Although in a number of phenotypically resistant isolates, the responsible resistance genes were not identified, identification of some of the resistance genes to common classes of antibiotics is an excellent step towards understanding mechanisms underlying AMR in NTS and other *Enterobacteriacae*. The observed discordance could be due to presence of other and more unusual resistance mechanisms encoded by genes that were not probed in this study. For example, neither *sul1* nor *sul2* genes were detected in the nine phenotypically sulfonamide resistant *S*. Newport isolates, meaning that some other genes or resistance mechanisms were responsible for

the observed resistance. Resistance to sulfonamide is known to be mediated by the enzyme dihydropteroate synthetase, encoded by *sul1, sul2, sul3* and *sul4* genes (Razavi et al., 2017). In fact, a lot of AMR mechanisms are at play in poultry farms and in other livestock farms that warrant further investigations and better understanding.

Genes encoding class 1 integrons were identified in six *S*. Hadar isolates. Many investigations on the occurrence of integron genes have yielded varying results. What is reported here, though, is similar to findings in other studies (A. M. Ahmed, Nakano, & Shimamoto, 2005; Guerri, Aladuena, Echeita, & Rotger, 2004; Li, Zhou, & Miao, 2017; Peirano, Agerso, Aarestrup, dos Reis, & Rodrigues, 2006; Randall, Cooles, Osborn, Piddock, & Woodward, 2004). The role of integrons in the dissemination of multiple AMR genes is well known (Kheiri & Akhtari, 2016; Randall et al., 2004). Class 1 integron genes are associated with *sul1* genes, and are mobilizable elements that can be incorporated in transposons and transferred horizontally. Integrons therefore could be important drivers of AMR in poultry and livestock farm environment in Uganda and other developing countries.

All the tetracycline resistant isolates carried *tetA* genes, a result similar to what has been reported in previous studies undertaken in Thailand, Australia, Germany, Morocco, and Egypt (H. A. Ahmed, El-Hofy, Shafik, Abdelrahman, & Elsaid, 2016; Chuanchuen & Padungtod, 2009; Miko, Pries, Schroeter, & Helmuth, 2005; Murgia et al., 2015; Pande, Gole, McWhorter, Abraham, & Chousalkar, 2015), but in contrast to another study in Egypt (El-Sharkawy et al., 2017). Many genes responsible for tetracycline resistance have been identified and described (Roberts, 2005). Tetracycline resistance genes are associated with efflux pump mechanisms implying that these are the predominant mechanisms for tetracycline resistance in NTS in these areas. The presence of only *tetA* gene in a diverse sample of NTS isolates that shows that the gene originates from a common source. Resistance to tetracycline is not surprising, as tetracycline is used extensively because it is cheap and readily available (OIE, 2015).

The only gene encoding chloramphenicol resistance identified was *cmlA*. This finding is consistent with an earlier study (Abatcha, Zakaria, Gurmeet, & Thong, 2015). They were identified in four MDR *S*. Mbandaka isolates that were also carrying genes encoding resistance to beta-lactams ($bla_{\text{TEM-1}}$), and quinolones (*qnrS*). Use of chloramphenicol for animal treatment is banned in many countries, including Uganda, due to health hazards associated with the persistence of residues in foods (Berendsen et al., 2010). The fact that resistance to chloramphenicol is observed in NTS

isolates from poultry is an indication of some abuse of chloramphenicol in human medicine or existence of some favourable conditions allowing horizontal transfer of genes from other bacteria. The gene *bla*$_{TEM-1}$ is reported to be the most widely distributed of beta–lactamase gene worldwide (Peirano et al., 2006). Not much information is available on the occurrence of beta-lactamase encoding genes in isolates from poultry in Uganda, but similar results have been reported in studies elsewhere (Eguale et al., 2017; Giuriatti et al., 2017; Qiao et al., 2017). Carriage of *bla*$_{TEM-1}$ gene is a threat to the potency of beta-lactam antibiotics. These isolates were resistant to ampicillin, a drug still widely used in Uganda for both human and animal treatment. The PMQR *qnrS* was the most prevalent gene among the phenotypically resistant isolates and it was the only PMQR gene detected. There is emergence and rapid spread of resistance to fluoroquinolone worldwide putting to doubt the traditional understanding that resistance to fluoroquinolones is mainly through mutations and transferred vertically (Robicsek et al., 2006; Strahilevitz, Jacoby, Hooper, & Robicsek, 2009).

10.5 Reduced susceptibility to ciprofloxacin in NTS

The prevalence of reduced susceptibility to ciprofloxacin in the tested NTS isolates was almost 38% in this study. However, mutations in the quinolone resistance-determining region (QRDR) of the gyrA and parC genes were absent. Chromosomal point mutations in the quinolone resistance-determining region (QRDR) of the DNA gyrase (genes *gyrA* and *gyrB*) and DNA topoisomerase encoded by *parC* and *parE* genes is the main mechanism of resistance to flouroquinolones in Enterobacteriaceae (Correia, Poeta, Hebraud, Capelo, & Igrejas, 2017; Fabrega, Madurga, Giralt, & Vila, 2009; Redgrave, Sutton, Webber, & Piddock, 2014). The reduced susceptibility to ciprofloxacin were strongly associated with the presence of plasmid mediated quinolone resistance gene (PMQR), *qnrS* and the district of origin. PMQR gene *qnrS* was the only PMQR gene identified from among isolates that were phenotypically resistant to ciprofloxacin. This finding is in agreement with some similar studies undertaken previously (Ata, Yibar, Arslan, Mustak, & Gunaydin, 2014; Oh et al., 2016; Strahilevitz et al., 2009). The strong association between the presence of *qnrS* gene with reduced susceptibility to ciprofloxacin have been well documented. One similar study by Thong, Ngoi, Chai, and Teh (2016) detected only *qnrS1* gene, but found silent multiple mutations at sites outside the *parE* QRDR. PMQR genes are known to play an important role in fluoroquinolone resistance in Enterobacteriacae (Sato et al., 2013; Wong, Chan, Liu, & Chen, 2014). The main PMQR genes (*qnrA, qnrB, qnrC, qnrD and qnrS*) have been widely

described to date. The PMQR gene is known to confer low level resistance to ciprofloxacin, they are rapidly disseminated and therefore suspected to be responsible for the increasing resistance to fluoroquinolone (Parry et al., 2010; Robicsek et al., 2006; Sjolund-Karlsson et al., 2015; Strahilevitz et al., 2007). There have been suggestions that qnr proteins protect DNA gyrase and topoisomerase IV from quinolone inhibition and that isolates with *qnr* genes may be less likely to develop topoisomerase mutations than others (Cesaro et al., 2008). A number of studies have reported occurrence of fluoroquinolone resistant isolates without the typical mutations in the *gyrA* and *parC* genes (Cavaco, Hendriksen, & Aarestrup, 2007; Eguale et al., 2017; Gunell et al., 2009; Harrois et al., 2014; Lin, Chen, Chan, & Chen, 2015; Sato et al., 2013), and in some cases mutations have been reported outside the QRDR (Friedman, Lu, & Drlica, 2001; Jaktaji & Mohiti, 2010; Ranjbar et al., 2016).

The mechanisms associated with this reduced susceptibility to ciprofloxacin in NTS need further investigations. Since all fluoroquinolones have the same mechanism of inhibition of the topoisomerase gyrase genes, resistance to any one of them will confer resistance to all others and this can complicate their use in fighting infections, an unfortunate situation in resource limited settings.

11 CONCLUSION AND RECOMMENDATIONS

In this study NTS was prevalent in more than 20% of the farms that were sampled based on fecal sampling. Through subtyping, the diversity of NTS isolates from three districts in Uganda has been explored. Isolation and characterization of NTS from poultry and other livestock should be an ongoing program to further divulge the diversity of NTS circulating in animal populations and environment. These should go hand in hand with appropriate investigations modifiable risks factors that need to be identified and mitigated.

Efforts geared towards prevention and control of NTS organisms from entering the food chain should prioritize promotion of good agricultural practices, farm biosecurity practices, prudent and judicious use antimicrobials in Uganda and other LMIC. This needs to be supported by a good surveillance and monitoring system. Such system should urgently be put in place and supported by effective prevention- and control measures at healthcare facilities to ensure AMR, NTS and other pathogens do not get transmitted from such facilities to communities. Such systems have significantly reduced NTS prevalence and other foodborne pathogens in developed countries. Unfortunately, such systems neither exist for NTS nor other foodborne pathogens in most developing countries, including Uganda.

An estimate of the prevalence and occurrence of AMR in NTS isolates form commercial laying chicken farms in Uganda was determined in this study. This provides an important insight on the presence of resistance in NTS isolated from poultry farms. The AMR and integron genes present in NTS isolates from Ugandan poultry have been unveiled. Additionally, the presence of PMQR demonstrated in this study is of great significance, since the genes are associated with plasmids, a mobile genetic element that can be transferred horizontally to microorganisms belonging to the same species or between different species. Increasing cases of AMR isolates of NTS and their frequent carriage of transmissible AMR genes pose threats to human, animal and environmental health. Increase in resistance in NTS to sulfonamides/trimethoprim and ciprofloxacin has immediate implications for public health systems in Uganda. These are first line drugs used in managing bacterial infections in most healthcare facilities, particularly sulfonamide/trimethoprim (co-trimoxazole) which is recommended and widely used to control opportunistic infections in HIV/AIDS patients. Therefore there is a need to monitor the use of antimicrobials and occurrence of AMR genes in farm ecosystems in developing countries, in order to institute measures to contain spread of AMR. More investigations need to be undertaken to further enhance understanding of

the driving forces in farm ecosystems for the development of AMR in important foodborne pathogens like *Salmonella.*

This study has added to growing pieces of evidence of the existence of high prevalence of low-level resistance to ciprofloxacin in NTS without classical mutations. Multiple mechanisms underlie fluoroquinolone resistance in NTS and other Enterobacteriaceae. The extent to which the different resistance mechanisms contribute either singly or in combination needs further investigations, especially in developing countries where data is lacking. Capacity to perform susceptibility testing and to determine genotypic resistance in NTS should be enhanced in order to support generating more robust data on ciprofloxacin resistance in NTS. The underlying drivers of the wide spread resistance and spread of resistance to fluoroquinolones in Uganda needs to be explored and brought under control.

Transmission of AMR is complex and factors affecting spread of AMR are interrelated. Some linkages between antimicrobial resistance and risk factors were determined and also linkages of antimicrobial resistances with specific antimicrobials. ABN analysis with bigger sample sizes will be required in future studies as small sample size limited interpretation of the current results. Particular attention should be paid to the dynamics of transmission of AMR especially the roles being played by mobile genetic elements.

Poultry keeping is predicted to continue growing in developing countries including Uganda, and poultry farm environments as demonstrated in this study will remain a significant source of spread of AMR genes. As of now, like many other developing countries, the use of antimicrobials in poultry production in Uganda is indiscriminate and in a poorly regulated environment. Because of easy access across the counter, antimicrobials are often overused and misused. These practices may be aggravating spread of AMR genes in populations. Governments of Uganda should ensure that the benefits of improved public health and reduced AMR are properly factored into investment decisions about improved access to water and sanitation infrastructures.

Farmers have to be educated on prudent use of antimicrobials. Indeed, there has to be a delicate balance to ensure judicious use to achieve access, not excess. The indiscriminate and injudicious use of antimicrobials in animals and poultry production, and poor control of use of antimicrobials both in human and animal health are drivers for development and dissemination of AMR. At the country level, Uganda should have mechanisms to effectively enforce prevention of 'over- the- counter' sales of antimicrobials without prescriptions. In Uganda, all drugs are supposed

to be regulated by National Drug Authority (NDA), and currently a bill is being debated to change NDA to a National Food and Drug Authority (NFDA) to ultimately merge food and drug control under one authority. This is intended to ensure antimicrobial stewardship and effective regulations. A public awareness campaign with consistent messages developed by the authorities in Uganda in conjunction with appropriate international partners for the promotion of efforts to combat AMR and NTS should be adapted to local conditions and norms. These messages can be spread through mass media (including social media).

Evidence based on this study support the growing call for better approaches to help in combating AMR. The global efforts to improve the collection and use of surveillance data regarding the use of antibiotics in agriculture, and the emergence and spread of drug resistant microbes amongst animals should be quickly cascaded to LMIC. Adopting ‘’One Health” (OH) approaches to generate data on AMR and zoonotic infections like *Salmonella* organisms that originate from humans, animals, and environmental seems more appropriate. In addition, information education and communication materials developed in the perspective of One Health should ensure that control and prevention efforts are more effective presumably.

12 FUTURE WORK

- More longitudinal studies need to be undertaken to determine prevalence diversity and factors associated with NTS from more geographic regions in Uganda and to monitor trends.
- Better understanding of the dynamics of NTS in farm environments, along the food chain, from farm to fork and in human populations is needed.
- Further determination is required of antimicrobial resistance genes and molecular mechanisms underlying resistance in NTS from humans, animals, food and environmental sources.
- There is need for further investigations of the factors associated with the incessant circulation of resistant bacteria and their resistant genes in the environment.
- The mobility of AMR genes and the roles of mobile genetic elements in the spread of AMR in farms and environment need deeper studies with particular attention paid on plasmids profiling, and PMQR genes.
- Further investigations are required to unveil the genes and mechanisms underlying the growing resistance in NTS to fluoroquinolones. There is need to investigate plasmids and incompatibility typing and plasmid sequencing particularly in the fluoroquinolone resistant isolates.
- More investigations need to be undertaken to further enhance understanding of the driving forces in farm ecosystems for the development of AMR in important foodborne pathogens like *Salmonella.* The interrelationships of these factors and linkages with resistance patterns should be explored using a suitable analytical tool like ABN use large datasets.
- Strengthening laboratory based surveillance that should include routine blood culture, sensitivity testing, serotyping from febrile illnesses in humans and animals to support more studies on *Salmonella* epidemiology and AMR.
- Future studies should in addition utilize more advanced newer technologies like WGS, amplicon sequencing using next generation sequencing technologies.
- There is need to build competencies in bioinformatics and big data infrastructures.
- Data on the types of antimicrobials and amounts used in Uganda and other developing countries need to be collected.

- Data collection tools should be standardized and harmonized so that comparisons of results from different countries are possible.

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14 ERRATA

Title page **OCCURENCE** *replaced by* **OCCURRENCE**

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Table of Contents: **ERRATA** included in the table of contents

Page layout: **Pages 7**, and **17** were empty and deleted

Page 16, l.6: ***Uganda Bureau of Statistics*** *formatted to* **Uganda Bureau of Statistics**

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Page 29, l.12: **Resistant** *replaced by* **Resistance**

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Page 38, l.2: **to the spread** *replaced by* **selection, spread**

Page 38, l.3: **antimicrobial resistance** *replaced by* **antimicrobial resistant microorganisms**

Page 38, l.3: **of resistant microorganisms** *replaced by* **of these resistant microorganisms**

Page 38, l.5: **Antimicrobials** *replaced by* **antimicrobial resistant bacteria**

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Page 44, l.10: ***The National Population and Housing Census 2014 – Area Specific Profile Series, Kampala, Uganda, 2017*** *replaced by* **Uganda Bureau of Statistics, 2017b**

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***Kampala, Uganda*, 2017** *replaced by* **Uganda Bureau of Statistics, 2017b**

Page 44, l.18: ***Uganda Bureau of Statistics*, 2017** *replaced by* **Uganda Bureau of Statistics, 2017a**

Page 44, l.21: ***The National Population and Housing Census 2014 – Area Specific Profile Series, Kampala, Uganda, 2017*** *replaced by* **Uganda Bureau of Statistics, 2017b**

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Page 60, l.1: **However, in this** *replaced by* **However, this**

Page 60, l.10: **resulting n** *replaced by* **resulting in**

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Page 65, l.29: **drives** *replaced by* **drivers**

Page 69: In the reference Abraham, E., & Chain, E. (1988), **denicillin** *replaced by* **penicillin**

References: **Salmonella, Escherichia coli, Campylobacter, Pseudomonas aeruginosa, Shigella, Haemophilus influenza, Klebsiella, Enterobacter** *were formatted to italics* ***Salmonella, Escherichia coli, Campylobacter, Pseudomonas aeruginosa, Shigella, Haemophilus influenza, Klebsiella, Enterobacter***

Page 75: Reference **Graham, S.M. (2002)** was deleted from reference list

15 APPENDIX I: ENCLOSED PAPERS

PAPER 1

Odoch et al. BMC Veterinary Research (2017) 13:365
DOI 10.1186/s12917-017-1291-1

BMC Veterinary Research

RESEARCH ARTICLE Open Access

Prevalence, antimicrobial susceptibility and risk factors associated with non-typhoidal *Salmonella* on Ugandan layer hen farms

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Abstract

Background: Non-typhoidal *Salmonella* (NTS) are among the leading global foodborne pathogens and a significant public health threat. Their occurrence in animal reservoirs and their susceptibilities to commonly used antimicrobials are poorly understood in developing countries. The aim of this study was to estimate the prevalence, determine antimicrobial susceptibility and identify risk factors associated with NTS presence in laying hen farms in Uganda through a cross-sectional study.

Results: Pooled faecal samples were collected from 237 laying hen farms and these were analysed for NTS following standard laboratory procedures. In total, 49 farms (20.7%; 95% Confidence interval (CI): 15.6–25.6%) were positive for NTS presence. Altogether, ten *Salmonella* serotypes were identified among the confirmed 78 isolates, and the predominant serotypes were *Salmonella* Newport (30.8%), *S.* Hadar (14.1%), *S.* Aberdeen (12.8%), *S.* Heidelberg (12.8%), and *S.* Bolton (12.8%). Phenotypic antimicrobial resistance was detected in 45(57.7%) of the isolates and the highest resistance was against ciprofloxacin (50.0%) followed by sulphonamides (26.9%) and sulphamethoxazole/trimethoprim (7.7%). Resistance was significantly associated with sampled districts ($p = 0.034$). Resistance to three or more drugs, multi-drug resistance (MDR) was detected in 12 (15.4%) of the isolates, 9 (75%) of these were from Wakiso district. A multivariable logistic model identified large farm size (OR = 7.0; 95% CI: 2.5–19.8) and the presence of other animal species on the farm (OR = 5.9; 95% CI: 2.1–16.1) as risk factors for NTS prevalence on farms. Having a separate house for birds newly brought to the farms was found to be protective (OR = 0,4; 95% CI: 0.2–0.8).

Conclusion: This study has highlighted a high prevalence and diversity of NTS species in laying hen farms in Uganda and identified associated risk factors. In addition, it has demonstrated high levels of antimicrobial resistance in isolates of NTS. This could be because of overuse or misuse of antimicrobials in poultry production. Also importantly, the insights provided in this study justifies a strong case for strengthening One Health practices and this will contribute to the development of NTS control strategies at local, national and international levels.

Keywords: Non-typhoidal *Salmonella*, Antimicrobial susceptibility, Risk factor, Layer hens, Prevalence

Background

Non-typhoid *Salmonella* (NTS) is one of the leading bacterial causes of food-borne illnesses, posing huge challenges to public health systems around the world [1–3]. A recent estimate of the global burden of NTS morbidity and mortality showed that enteric NTS cause 93.8 million illnesses with 155,000 deaths annually, while invasive NTS were estimated to cause 3.4 million cases with 681,316 deaths annually [4–6]. African countries have a relatively low level of reported NTS gastroenteritis, but a much higher level of invasive non-enteric NTS infections, estimated at 227 cases per 100,000 persons per year compared to the global average of 49 cases per 100,000 persons per year [4]. This distribution of

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salmonellosis makes Africa the leading continent with invasive non-enteric NTS cases accounting for more than half of the global cases [4]. In humans, many of the gastroenteric infections caused by NTS are self-limiting, and thus, many sporadic cases go unnoticed and/or unreported. However, a serious aspect of this situation is that some of the gastroenteric infections may develop into bacteraemia, which is an emerging opportunistic infection in individuals infected with HIV, the elderly and in children [7, 8].

The reservoirs of food-borne NTS are often located in the primary food animal production. Many of these zoonotic NTS are able to colonize the intestinal tract of a variety of animal species, and in most of these cases, the animals are healthy and asymptomatic. Faecally contaminated foodstuffs like meat, eggs, dairy products and sometimes vegetables are the main sources of salmonellosis in humans [2, 3, 9, 10]. The dissemination of NTS is also a growing concern due to increasing cases of drug resistant isolates and their frequent carriage of transmissible antimicrobial resistance genes. Even more worrying is the rising occurrence of multidrug-resistant NTS, including cases reported in some African countries [5, 11]. Because of multidrug-resistance, treatment with first line drugs is often no longer an alternative, and this puts pressure on the use of second or third line drugs. Some limited studies in Africa on antimicrobial resistance in NTS isolates from animal sources have been undertaken, but with varying results [11–13].

Many prevalence and risk factor studies of NTS in layer and broiler populations have been conducted in the USA and Europe [14–17]. A systematic review of risk factors associated with laying hen farms identified multiple risk factors related to biosecurity measures, management factors and the environment [18]. In addition, developed countries have put in place monitoring and surveillance systems for antimicrobial resistance targeting important zoonotic pathogens like NTS. Unfortunately, such systematic surveillance neither exist for NTS nor other food-borne pathogens in most developing countries, including Uganda [19]. Consequently, the role of poultry as a reservoir and source of NTS in developing countries is poorly understood. Furthermore, the development of antibiotic resistance of NTS to commonly used antibiotics in agricultural production needs prompt investigation, commencing with susceptibility testing. The significant data gaps in developing countries hinder development of effective control systems and risk-mitigation strategies at multiple levels.

Since most human NTS infections originate from animal sources, prevention and control at pre-harvest level in the primary production units is an effective way to minimize NTS dissemination and transmission to humans through the food chains [20–22]. The aim of this study was to estimate the prevalence, test antimicrobial susceptibility and identify risk factors of NTS using production and management information from a sample of commercial laying chicken farms.

Methods

Study area and recruitment of farms

The study was carried out between June 2015 and August 2016 in the districts of Wakiso, Masaka and Lira in Uganda. Wakiso district (00°24′N, 32° 29′E) and Masaka district (00° 30′S, 31° 45′E) are located in the central region of Uganda, while Lira district is located in northern region of Uganda (02°20′N, 33°06′E). These districts were purposively selected because of their high commercial poultry households that make them the hub of poultry industry in Uganda. The sampling frame was generated from a list of farmers participating in National Agricultural Advisory Services (NAADS) program in the three selected districts of Uganda. In Uganda, the majority of poultry farmers are smallholders, and therefore the inclusion criterion was that for a farm to participate, it should have a minimum number of 50 chicken. A sample size calculator, FreeCalc sample size calculation for imperfect tests (www.epitools.ausvet.com.au, accessed on 3rd, June, 2015) was used. The required sample size for demonstrating disease freedom was calculated as previously described [23, 24]. The calculator had the following input; population size (Masaka = 147, Lira = 145, Wakiso = 77), sensitivity (60%), specificity (100%), design prevalence (5%), and the desired type 1 and type 2 errors were all assumed at 0.05. Because of small populations, the modified hypergeometric option was used. A total sample size of 237 (Masaka = 85, Lira = 84, Wakiso = 68) was calculated for individual districts. Computer-generated random numbers were used to select farms. After selection, farmers (respondents) were asked for their cooperation and willingness to participate, and after that a verbal consent was obtained. Those who were not willing to participate were replaced by random selection of others from the same list. Questionnaires were administered to all participating farms and faecal samples were taken for bacteriological analysis.

Sample collection

Flocks and poultry house sizes varied a lot in the study areas; and because of this, the sampling scheme was standardized. In cases where a farm had more than one poultry house, but with the same age group of birds in each house, one house was randomly selected and sampled. On the other hand, if a farm had more than one house but with different age groups, all the houses were sampled. Each house was divided into sectors of about 5 m by 5 m (25 m^2), an approach adapted from previous studies [25, 26]. One sample was collected from each

sector using sterile gloves and boot swabs. The samples from each house were pooled together, transferred to a properly labelled sterile container and put in a cool box with ice packs. The samples were transported to the laboratory within less than 8 h and processing began immediately. All flocks were sampled once.

Bacterial diagnostics and identification of *Salmonella* serotypes

Culture and isolation of *Salmonella* spp. followed standard procedures according to *ISO 6579:2002/Amd 1:2007 Annex D: Detection of Salmonella spp. in animal faeces and in environmental samples from the primary production* [27]. Briefly, pooled samples were homogenized, 25 g weighed and added to 225 ml of Buffered Peptone Water (BPW) for pre-enrichment and incubated for 20 h at 37 °C. The culture obtained was subjected to selective Modified Semisolid Rappaport Vassiliadis (MSRV) agar plates and incubated at 41.5 °C for 24–48 h. One colony from each culture indicative of NTS was further plated on selective Xylose Lysine Deoxycholate (XLD) agar and incubated at 37 °C for 24 h. Presumptive NTS colonies were stored at −20 °C in Mueller-Hinton agar. The samples were later transported to Norway where they were sub-cultured on blood agar plates and stored at 4 °C. Biochemical confirmatory tests were done by using the API-20E (BioMerieux, Marcy l'Etoile, France) identification system. All isolates were serotyped according to the Kauffman–White–Le–Minor technique [28] at the Norwegian Veterinary Institute.

Antibiotic susceptibility testing

Antibiotic susceptibility testing of all identified isolates was performed on 13 antibiotics (NEO-SENSITABS™, Rosco, Denmark) using the standard Kirby-Bauer disk diffusion methods on Muller-Hinton agar. Interpretation of sensitive (S), intermediate (I) or resistant (R) was done according to Clinical and Laboratory Standards Institute [29], except for ciprofloxacin (CIPR1μg) which was interpreted using CLSI [30]. The 13 antibiotics were selected based on the common antibiotics used in Uganda and those recommended by World Health Organization (WHO) for routine integrated antimicrobial resistance monitoring [31]. These were gentamycin (GEN10μg), sulfonamide (SULFA240μg), trimethoprim-sulfamethoxazole (SxT 25 μg), ciprofloxacin (CIPR1μg), cefotaxime (CTX 30 μg), meropenem (MPR 10 μg), chloramphenicol (CLR30μg), ceftazidime (CAZ30 μg), ampicillin (AMP10μg), amoxycillin clavulanic acid (AMC30μg), trimethoprim (TRIM5μg), tetracycline (TET 30μg), and enrofloxacin (ENROF10μg). *Escherichia coli* ATCC 25922 was used as quality control. NTS isolates showing resistance to three or more antibiotics were classified as multidrug-resistant.

Questionnaire administration

All questionnaires were directly administered onsite after pre-testing. Pre-testing of the questionnaire was done by three trained research assistants in neighbouring districts of Kampala and Mukono in central Uganda and Kole in northern Uganda. These districts have similar production and management systems to the study districts. After pre-testing, the research team reviewed the questionnaires before final administration. The final questionnaire had 80% close-ended questions and was used to collect variables to determine risk factors. The questionnaires collected information on general farm management practices and characteristics, disease prevention, control and management as well as demographic data of the farmers and managers. The questionnaire was written in English, but the research assistants would determine whether the respondent was competent in English or not. Where the respondent was not competent in English, the research assistant would translate in the local language of Luganda (in the case of Wakiso and Masaka districts) and Lwo (in the case of Lira district). The selected households were identified with the help of the local veterinary personnel and chairpersons of local council one. Local council one is the smallest administrative unit in Uganda. Data entry was done by a trained assistant at the College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, Kampala, Uganda.

Data management and analysis

After establishing the database, data were exported to the Statistical Package for Social Scientists (SPSS, version 20) computer program for further data analyses. A farm was considered positive if one of the pooled faecal samples taken tested positive for NTS. An initial descriptive statistics using tables and chi-square testing was performed to assess the association of each variable independently. This was followed by a multivariable logistic model built based upon variables with p-values < 0.20 in the initial analyses. All candidate variables were tested for collinearity with other variables using tabulation, and if collinearity was found, the most biologically relevant variable was chosen. The model was built utilizing a backward selection among the candidate variables ($p < 0.20$ from initial analyses) strategy using the Likelihood Ratio test with for comparing models [32]. The final model was assessed for fit using the Hosmer-Lemeshow test [33].

Results

Number of samples, NTS prevalence and serotypes

A total of 237 farms participated in the study (Wakiso, $n = 68$; Lira, $n = 84$; Masaka, $n = 85$). Sampling according to the standardized sampling scheme resulted in 366

pooled samples from the 237 farms. Of the 237 farms, 49 (20.7%; 95% CI = 15.6,-25.6%) were positive for NTS. Salmonella isolates were recovered from 78 of the 366 samples (21.3%).

Ten *Salmonella* serotypes were identified from the 78 isolates recovered (Table 1). Of the 49 NTS-positive farms, five farms were contaminated with two different serotypes. All these farms had three or more poultry houses and the serotypes were from different houses. The farms were from the two districts of Wakiso and Masaka representing the central part of Uganda.

Antimicrobial susceptibility of NTS

Forty five, 57.7% (95% CI: 47.4–67.9%) of the 78 isolates were resistant to at least one antibiotic in the disc diffusion test. Resistance varied significantly by district ($p = 0.034$); highest in Wakiso with 75.9% of the isolates from the district resistant to at least one of the tested antibiotics; this was followed by Lira with 52.0% and Masaka with 41.7% of the isolates resistant. The highest resistance was against ciprofloxacin (50.0% of the isolates) followed by sulphonamide (26.9%), sulfamethoxazole/trimethoprim (7.7%), trimethoprim (7.7%), and tetracycline (5.1%), then ampicillin (5.1%), chloramphenicol (5.1%), and enrofloxacin (5.1%). All the isolates were susceptible to meropenem, gentamycin, amoxicillin-clavulanic acid, ceftazidime, and cefotaxime. Multidrug-resistance was seen in 12 (15.4%) of the isolates and five multidrug-resistant phenotypes were identified (Table 2).

Factors associated with NTS

Some key demographic factors, farm management, disease prevention and control practices showing significant association with the prevalence of NTS on farms are presented in Table 3. These were included as candidate variables for the multivariable logistic model (insert Table 3).

Table 1 Distribution of NTS serotypes in layer hen farms in Uganda

Serotype	Number of farms	Percentage (%) of positive farms	Percentage (%) of farms investigated
S. Newport	13	26.5	5.5
S. Hadar	7	3.0	3.0
S. Aberdeen	10	20.4	4.2
S. Heidelberg	7	3.0	3.0
S. Bolton	6	6.0	2.5
S. Enteritidis	4	8.2	1.7
S. Mbandaka	3	6.1	1.3
S. Kampala	2	4.1	0.8
S. Typhimurium	1	2.0	0.4
S. Uganda	1	2.0	0.4

A final multivariable logistic model identified some risk factors for presence of *Salmonella* spp. (Table 4). Due to collinearity between some variables, type of poultry house and number of houses were dropped (collinear with farm size). Having a written biosecurity plan, and having a separate house for sick birds were also dropped (collinear with having a separate house for new chicken). The final multivariate logistic model found that larger farms had significantly more NTS. Similarly, presence of other livestock species like cattle, goats, sheep, pigs were significantly associated with presence of NTS (Table 4). Another variable that came out to be associated with presence of NTS was keeping of records. On the other hand, the use of separate houses for birds newly brought into the farms reduced the probability for presence of NTS. The model fit was, however, limited, as shown by the Hosmer Lemeshow test ($p < 0.05$).

Discussion

This study represents, to our knowledge, the first estimate of the prevalence of NTS in laying hen farms in Uganda. NTS prevalence was estimated at 21% of the farms with ten different serotypes identified. High phenotypic resistance to antimicrobials was found among the isolates with almost 58% of the isolates found resistant. In this study, the logistic regression model identified large farm size, presence of other poultry, and keeping of records as factors associated with occurrence of NTS in Uganda.

NTS prevalence of 21% is not surprisingly high considering how the poultry industry operates in the country, where disease control efforts are poor and deficient. In Uganda, chickens are not vaccinated against NTS. The available vaccines is for fowl typhoid targeting *Salmonella* Gallinarum and the protocol used is not ideal for identification of *S.* Gallinarum. Vaccinations of poultry in Uganda are not mandatory. Most commercial layer hen farms include fowl typhoid vaccinations in their routine vaccination schedules. A similar study in Senegal reported detection of NTS in faecal samples in 35.1% of farms [34] and in Nigeria, a related study reported isolation of NTS in

Table 2 Multidrug resistant profiles of NTS isolates from Wakiso, Lira and Masaka districts, Uganda 2017

Serotype	Resistance profile	No of isolates	District where isolates were recovered
S. Bolton	CIPR, SULFA, TET	1	Lira
S. Mbandaka	CIPR, CLR, AMP	4	Masaka (2), Wakiso (2)
S. Hadar	SULFA, TRIM, SxT	4	Wakiso
S. Hadar	CIPR, SUL, TRIM, SxT	2	Wakiso
S. Newport	CIPR, SULFA, TET, ENROF	1	Wakiso

AMP Ampicillin, *CIPR* Ciprofloxacin, *CLR* Chloramphenicol, *ENROF* Enrofloxacin, *SULFA* Sulphonamides, *SxT* Sulfamethoxazole-trimethoprim, *TET* Tetracycline, *TRIM* Trimethoprim

Table 3 Key demographic factors, disease management practices and farm characteristics associated with the prevalence of *Salmonella*, with *p*-values from simple chi-square analyses

Variable	Category	*Salmonella* positive farms (%)	*p*-value
Sex of farmer	Male ($n = 108$)	31 (28.7)	0.004
	Female ($n = 129$)	18 (14.0)	
Sex of manager	Male ($n = 85$)	27 (31.89)	< 0.001
	Female ($n = 115$)	12 (10.4)	
	Not applicable ($n = 37$)		
Age of the manager	< 20 years ($n = 5$)	4 (80.0)	0.001
	20–35 years ($n = 103$)	18 (17.5)	
	36–50 years ($n = 85$)	23 (27.1)	
	> 50 years ($n = 35$)	3 (8.6)	
	Missing ($n = 9$)		
Education level of the farmer	Primary ($n = 44$)	5 (11.4)	0.012
	Secondary ($n = 76$)	12 (15.8)	
	Tertiary ($n = 102$)	31 (30.4)	
	Missing ($n = 15$)		
Farm size (no. of birds)	Small (50–500) ($n = 162$)	19 (11.7)	< 0.001
	Medium (501–1000) ($n = 33$)	14 (42.4)	
	Large (> 1000) ($n = 38$)	14 (36.8)	
	Missing ($n = 4$)		
Number of poultry houses	One house ($n = 135$)	12 (8.9)	< 0.001
	Two houses ($n = 45$)	8 (17.8)	
	Three houses ($n = 32$)	21 (65.6)	
	> 3 houses ($n = 25$)	8 (32.0)	
Management system	Free range ($n = 47$)	2 (4.3)	< 0.001
	Semi intensive ($n = 90$)	11 (12.2)	
	Intensive ($n = 98$)	35 (35.7)	
	Others ($n = 2$)		
Use of protective clothing	Yes ($n = 136$)	35 (25.7)	0.031
	No ($n = 99$)	14 (14.1)	
	Missing ($n = 2$)		
Who does vaccination	Private ($n = 88$)	19 (21.6)	0.029
	Self/family ($n = 136$)	25 (18.4)	
	Employee ($n = 9$)	5 (55.6)	
	Missing ($n = 4$)		
Reuse of egg trays	Yes ($n = 105$)	29 (27.6)	0.034
	No ($n = 119$)	19 (16.0)	
	Missing ($n = 13$)		
Who treats the birds	Self ($n = 155$)	22 (14.2)	< 0.001
	Government/Animal Health Worker ($n = 11$)	0 (0.0)	
	Private/Animal Health worker ($n = 52$)	24 (46.2)	
	Missing ($n = 19$)		
Presence of other livestock	Present ($n = 101$)	26 (25.7)	0.097
	Not present ($n = 136$)	23 (16.9)	

Table 3 Key demographic factors, disease management practices and farm characteristics associated with the prevalence of *Salmonella*, with *p*-values from simple chi-square analyses *(Continued)*

Variable	Category	*Salmonella* positive farms (%)	*p*-value
Having a separate house for new birds	Yes ($n = 136$)	24 (17.6)	0.134
	No ($n = 97$)	25 (25.8)	
	Missing ($n = 4$)		
Disposal of dead birds	Burying ($n = 109$)	21 (19.3)	< 0.001
	Burning ($n = 17$)	12 (70.6)	
	Throw away ($n = 45$)	9 (20.0)	
	Giving to animals (dogs and pigs) ($n = 32$)	4 (12.5)	
	Drop in a pit ($n = 24$)	3 (12.5)	
	Missing ($n = 10$)		
Keeping of pets	Yes ($n = 137$)	35 (25.5)	0.025
	No ($n = 99$)	14 (14.1)	
	Missing ($n = 1$)		
If yes, species of pets	Dogs ($n = 62$)	20 (32.3)	0.006
	Cats ($n = 24$)	0 (0.0)	
	Both dogs and cats ($n = 51$)	15 (29.4)	
Keeping of records	Yes ($n = 153$)	43 (28.1)	< 0.001
	No ($n = 80$)	5 (6.2)	

12.5% of poultry droppings [35]. A more recent study in Nigeria by Fagbamilla et al. [36] estimated NTS prevalence of 43.6% in commercial poultry farms. In Algeria, a study in laying hen flocks by Bouzidi et al. [37] found that eight out 18 flocks were contaminated with NTS.

The identification of ten different serotypes can be regarded as a manifestation of the heterogeneous reservoirs and sources of NTS contamination. *S.* Newport was the most prevalent serotype, compared to the more commonly reported *S.* Enteritidis and *S.* Typhimurium in poultry isolates [37–40]. All the NTS serotypes identified in this study are zoonotic, and are known to have caused human disease outbreaks elsewhere. Consequently, this high prevalence of zoonotic NTS in the poultry reservoir constitutes a public health threat. *S.* Typhimurium, *S.* Newport, *S.* Hadar and *S.* Heidelberg have also been reported by Centers for Disease Control and Prevention (CDC) as the most threatening serotypes to public health because of their association with multidrug-resistance [41]. *S.* Mbandaka has been reported in many poultry products across the world [41–45]. A recent publication by Afema et al. [40] reported detection of mainly *S.* Kentucky, *S.* Heidelberg, *S.* Enteritidis, *S.* Typhimurium, and *S.* Virchow from poultry faeces. Also another study by Ikwap et al. [46] reported most of the serotypes of this study in isolates from piggeries in Uganda.

Studies of antibacterial susceptibility in NTS from African countries show highly variable results. An occurrence of almost 58% of isolates resistant to at least one antibiotic, is higher than what was reported in a similar study in Chad, that found overall resistance to 16 antibiotics tested at 33% [13]. However, it is lower compared to a study in Sudan that reported antibiotic resistance in NTS isolates at 98% [47] and similar to a more recent one in Ghana that reported resistance at 60.6% [48]. In Ethiopia, a study on NTS isolates from dairy cattle by Eguale et al. [49] found resistance at 30%. This high level of resistance could be associated with overuse and misuse of antibiotics in poultry farming. The significantly higher resistance level of NTS from Wakiso, which is

Table 4 Results from multivariable logistic regression showing identified factors associated with *Salmonella* spp. prevalence, with odds ratios with 95% Confidence Interval (CI) and corresponding p-values for the variables

Variable	Level	Odds Ratio	95% CI	*p*-value
Farm size (no. of birds)	Medium vs small	7.0	2.5–19.8	< 0.001
	Large vs small	5.9	2.1–16.1	0.001
Presence of other animal species	Present vs absent	5.0	2.1–16.1	< 0.001
Houses for new birds	Present vs absent	0.4	0.2–0.8	0.014
Records	Present vs absent	6.7	2.2–20.2	0.001

the immediate district surrounding the capital Kampala should be of concern. Kampala is the biggest hub of trade and movements of people, animals and animal products in the country, and resistant bacteria can potentially spread from here to all regions in the country.

Multidrug-resistance was seen in *S.* Bolton, *S.* Mbandaka, *S.* Hadar, and *S.* Newport isolates. High levels of multidrug-resistance have been reported elsewhere in Africa [11, 12, 48, 50]. The bacteria expressing resistance towards antimicrobials of which some are commonly used in humans and animals exposes a daunting challenge. Increasing development of antimicrobial resistance against commonly used drugs like ciprofloxacin, tetracyclines, sulphonamides sulfamethoxazole_trimethoprim (co-trimoxazole) in Uganda poises a great threat to public health and economy. Tetracyclines and sulphonamides are among the most widely used drugs for treatment and prophylaxis in food animals [51]. Increasing resistance toward these antimicrobials will render them less available leaving farmers with no cheaper options. In Uganda, ciprofloxacin is not licensed for use in poultry production, but is widely used for treatment of many human infections, including salmonellosis. The mechanisms behind the observed high resistance to ciprofloxacin in this study needs to be investigated.

Resistance to sulfamethoxazole-trimethoprim (co-trimoxazole) was also seen in this study. Co-trimoxazole is a drug that is used in Uganda for controlling opportunistic infections in persons living with HIV/AIDS [52, 53]. This causes concern as many of these patients will succumb to opportunistic infections [52–54] and also considering that most patients cannot afford other options of antimicrobials. All isolates were susceptible to the extended-spectrum beta-lactams (meropenem, cefotaxime, ceftazidime) that were tested. Efforts should be put in place to maintain this status. Strategies to reduce antimicrobial resistance in Ugandan farm settings should focus on improving management, biosecurity, and sensitization of key stakeholders such as farmers, farm workers, policy makers, drug dealers, animal health workers and veterinarians.

At univariate analysis, a number of demographic farm management and production variables were associated with occurrence of NTS on farms. The final logistic regression model built identified large farm size, presence of other poultry species, and keeping of records as factors associated with NTS in Uganda. The final model was also tested for the random effect of village to assess the degree of independence. While initial analyses indicated that village had some effect, the final model revealed no such effect – indicating that the factors found were stable across the study districts. A large farm was one of the risk factors associated with NTS determined by the model. This is in concordance with the fact that farm size has been significantly associated with presence of NTS in studies conducted in Britain [26], Trinidad and Tobago, Grenada, and St. Lucia [55], France [15, 56] and Belgium [57]. A previous study of presence of NTS in layer and broiler flocks in the Kampala region in Uganda by Nasinyama et al. [58] identified bird type, flock size and downtime as significant risk factors. In the Ugandan setting, bigger farms tend to attract many activities; visitors and the obtaining of feeds, feed ingredients, chickens and other supplies from multiple sources, many of which informal and unregulated. Under these situations with low biosecurity practices, keeping adequate hygiene standards can be difficult. In addition, most routine operations like mixing of feeds, feeding of birds, watering and vaccinations are manual requiring many workers, some of them coming from outside the farms. Some farms have a limited workforce and are therefore less effective in keeping high standards of routine hygienic practices. All these factors can be further complicated by farms experiencing erratic outage of power and water supplies. As a range of management factors are related to farm size, we may not have identified the most biologically important causal factors.

The current study identified presence of other animal species as another risk factor for NTS. When present on farms, other animal species will most likely share water feeds and space with the chicken and thereby increasing the opportunities for the spread of the bacteria due to direct or indirect contacts. The other animal species may be reservoirs of NTS and thus, contribute to the maintenance of high prevalence of NTS at a farm.

Surprisingly, keeping records emerged as a factor increasing the risk of infection. This could be because in Uganda record keeping is poor among smallholder farmers, and this study found a significant association of NTS with large farms. Keeping records is therefore probably correlating with large farms. It remains open if this variable should be in the model, but leaving out this variable did not affect the estimates for the other variable much. The variable is therefore not considered to be a confounder, but it may perhaps represent other factors.

Having separate housing for birds newly introduced in the farm was associated with lower levels of NTS. The lower level of NTS in farms using separate houses for new chicken is as expected and may represent other factors related to general hygiene, potentially reducing the risk of introduction and maintenance of NTS at farms. Normally housing new birds separately provides an opportunity to observe and provide timely treatment before they are released to mix with other birds on the farms.

The prevalence estimate in the current study is associated with several uncertainties. The study was a cross-sectional study including only one sampling occasion per farm. Depending on the infection levels, it is possible that in some farms a small proportion of birds shed NTS in faeces and this is normally intermittent [59].

Consequently, the prevalence and number of NTS in faecal droppings may therefore change over time and analysis of this requires a longitudinal monitoring scheme. The sampling of faecal droppings only, in order to determine the presence or absence of NTS, may be a limiting factor as NTS in farm settings can be carried in litter, feeds and water as well. However, pooled faecal and environmental sampling of poultry houses is still better than sampling individual birds for detection of NTS on farms as reported in previous studies [60, 61]. In addition, this study targeted commercial egg laying farms who are registered, and yet a lot of farmers are not registered with NAADS. However, these results provide an important insight into the occurrence of NTS in Ugandan poultry, particularly in the absence of previous similar studies. In this study, data on the sources of day old chicks were not captured and this could be included in future studies of NTS in Uganda as sources of day old chicks are potential risk factors for *Salmonella.* In addition, the sampling strategies limited identification of more than one serotype in small farms with only one house as only one sample was taken and one colony was picked for serotyping.

Conclusions

A high prevalence and high levels of antimicrobial resistant NTS in commercial laying chicken farms in Uganda was revealed in this study. Large farms and presence of other animal species at the farm were identified as risk factors for NTS. Both these risk factors are associated with biosecurity challenges. Although limited, this study should pave way for informing the establishment of proper NTS control systems based on empirical scientific evidence.

Further characterization of NTS from the poultry reservoir that is documented through the present work will be necessary in order to elucidate the transmission dynamics and dissemination of these important zoonotic bacteria. Particular emphasis needs to be given to the determination of antimicrobial resistance genes and their mobility in future studies.

Additional file

Additional file 1: Questionnaire used for study data collection. (DOCX 33 kb)

Abbreviations

CDC: Centre for Disease Control and Prevention; CI: Confidence interval; MDR: Multi-drug resistance; MSRV: Modified Semi solid Rappaport Vassiliadis (MSRV); NAADS: National Agricultural Advisory Services; NTS: Non-typhoidal *Salmonella*; UNCST: Uganda National Council of Science and Technology; WHO: World Health Organization; XLD: Xylose Lysine Deoxycholate

Acknowledgements

The authors thank all farmers, farm managers, district authorities and research assistants for their various contributions including their valuable time and knowledge. We are equally very grateful for the support from the researchers and technicians at the Department of Food Safety and Infection Biology at NMBU, particularly Toril Lindbäck and Kristin O' Sullivan. We would like to acknowledge Nasinyama George of Kampala International University for the advice and guidance at the conception of this study. Our thanks to Bergsjo Bjarne of Norwegian Veterinary Institute for serotyping the isolates.

Funding

This work was supported by the Norwegian Programme for Capacity Development in Higher Education and Research for Development (NORHED project No.UGA-13/0031) based at Makerere University, Kampala Uganda and Norwegian University of Life Sciences, Oslo.

Availability of data and materials

The dataset from which these results were generated are not publically available at this point as this study is part of an on-going PhD research at Norwegian University of Life Sciences and the university takes responsibility of storing the primary data. But this can be availed on reasonable request from the first author. The questionnaire can be accessed as additional file (see Additional file 1).

Authors' contributions

TO was the principal investigator, contributed to conception, design, data collection, analysis and drafting of the manuscript. YW contributed to conception design, supervision, editing and approval. TML supervised the lab analysis, participated in drafting and reviewing of the manuscript. AM contributed to the design and review of the manuscript. CK helped with the acquisition of funding, supervision, editing and reviewing the manuscript. LN helped with field data collection and writing. ST participated in field data collection, isolation and writing. ES contributed to acquisition of funding, design, data analysis, writing and editing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved and granted permission (number A532) by Uganda National Council of Science and Technology (UNCST). All farmers (respondents) who participated were asked for verbal consent before being interviewed. According to UNCST this is acceptable especially for the purpose of not excluding illiterate respondents and where no samples were to be taken from humans and live chicken.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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Received: 6 June 2017 Accepted: 17 November 2017
Published online: 29 November 2017

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PAPER 2

International Journal of
Environmental Research and Public Health

MDPI

Article

Diversity and Antimicrobial Resistance Genotypes in Non-Typhoidal *Salmonella* Isolates from Poultry Farms in Uganda

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Received: 14 December 2017; Accepted: 9 February 2018; Published: 13 February 2018

Abstract: Non-typhoidal *Salmonella* (NTS) are foodborne pathogens of global public health significance. The aim of this study was to subtype a collection of 85 NTS originating from poultry farms in Uganda, and to evaluate a subgroup of phenotypically resistant isolates for common antimicrobial resistance genes and associated integrons. All isolates were subtyped by pulsed-field gel electrophoresis (PFGE). Phenotypically resistant isolates (n = 54) were screened by PCR for the most relevant AMR genes corresponding to their phenotypic resistance pattern, and all 54 isolates were screened by PCR for the presence of integron class 1 and 2 encoding genes. These genes are known to commonly encode resistance to ampicillin, tetracycline, ciprofloxacin, trimethoprim, sulfonamide and chloramphenicol. PFGE revealed 15 pulsotypes representing 11 serotypes from 75 isolates, as 10 were non-typable. Thirty one (57.4%) of the 54 resistant isolates carried at least one of the seven genes ($bla_{\text{TEM-1}}$, *cmlA*, *tetA*, *qnrS*, *sul1*, *dhfrI*, *dhfrVII)* identified by PCR and six (11%) carried class 1 integrons. This study has shown that a diversity of NTS-clones are present in Ugandan poultry farm settings, while at the same time similar NTS-clones occur in different farms and areas. The presence of resistance genes to important antimicrobials used in human and veterinary medicine has been demonstrated, hence the need to strengthen strategies to combat antimicrobial resistance at all levels.

Keywords: antimicrobial resistance; genotypes; non-typhoidal *Salmonella*; poultry; genes; integrons; subtyping

1. Introduction

Salmonella enterica subsp. *enterica* include serotypes that are global foodborne pathogens significantly affecting public health and economy [1–3]. In humans, salmonellosis is classified into typhoid and non-typhoidal salmonellosis. Most cases of non-typhoidal *Salmonella* (NTS) disease are associated with consumption of contaminated foods of animal origin, particularly poultry, meat and in some instances vegetables [4–6]. Globally, NTS is estimated to cause 93.8 million cases of gastroenteritis annually, of which 80 million cases are foodborne and causing 155,000 deaths [7]. Although African countries have low estimated cases of NTS gastroenteritis compared to other parts of the world, they have a much higher level of invasive non-enteric NTS infections [7,8]. NTS bacteraemia

is an emerging opportunistic infection in individuals infected with HIV and is reported to be highly correlated with malaria, especially in children and elderly persons [9–13].

In poultry, transmission of NTS can occur by direct contacts with infected birds, consumption of contaminated feeds and water, and contact with environmental reservoirs [13]. Transmission can also occur through cross contamination anywhere along the production chain, and for specific serotypes, vertical transmission is also possible [14,15]. However, NTS infections in poultry is mainly asymptomatic [14], and may therefore not get the necessary attention with regard to prevention and control. The diversity of NTS circulating in poultry and livestock production environment in most developing countries is poorly understood, as very limited studies have been undertaken. Molecular typing is important for characterization of bacteria to establish genetic relatedness between isolates in order to elucidate the dynamics of the bacterial populations. Although whole genome sequencing is getting more established, pulsed-field gel electrophoresis (PFGE) technique is still considered an adequate molecular method suitable for subtyping of serotypes of *Salmonella*.

The increasing development of antimicrobial resistance (AMR) in NTS is complicating treatment of bacteraemia cases and results in poorer treatment outcomes. Even more worrying is the emergence of multidrug resistance (MDR) in NTS against commonly used antibiotics in human and animal treatment, which has become a serious public health challenge [15–18]. Resistance is increasing not only against first line antibiotics, but also against clinically important antimicrobial agents like fluoroquinolones and third generation cephalosporins [19]. Inappropriate use of antimicrobials in agriculture is known to be a key factor contributing to the development of AMR, and the influence of livestock environment in the development of MDR in NTS has been demonstrated [20]. Increased intensification of production in agriculture, use of antibiotics as feed additives, and prophylactic treatment are some of the practices that influence development of AMR [21,22]. MDR NTS can be transferred from the poultry reservoirs to humans through the food chain, but AMR can also be transferred from one bacterium to another through resistance genes associated with integrons and mobile genetic elements such as plasmids and transposons. Most studies on AMR in poultry are done in developed countries while in most developing countries, including Uganda, there are no surveillance and monitoring programs for important foodborne pathogens and AMR in primary production units. To date in Africa, only a few limited studies have documented AMR and corresponding genes in NTS isolated from humans, animal products, and poultry farms [23–29]. Therefore, data is scarce and the extent of NTS and AMR remains poorly known. As a result, development of appropriate mitigation measures and control efforts is compromised. The aim of this study was to characterize a collection of NTS isolates from poultry by using PFGE for molecular subtyping and to investigate the presence of integrons and acquired antimicrobial resistance genes from the phenotypically resistant isolates. The NTS were isolated from faecal samples collected from poultry farms in three districts (Wakiso, Lira, and Masaka) in Uganda between 2015 and 2016 [30].

2. Materials and Methods

2.1. The NTS isolate collection

The majority (75/85) of the NTS isolates used in this study were from a previous study by Odoch et al. [30]. The remaining 10 isolates originated from additional sampling. However, all 85 isolates were from fecal samples collected from poultry houses in three districts with high numbers of commercial poultry farms (Wakiso, Lira, and Masaka) in Uganda between 2015 and 2016, according to a sampling design and procedure described in Odoch et al [30]. A map of the study area is provided as Supplementary Materials Figure S1. NTS were isolated, identified, serotyped and tested for antimicrobial sensitivity according to standard methods as earlier described [30]: Culture and isolation of NTS were done according to ISO 6579:2002/Amd 1:2007, Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production [31]. Biochemical confirmatory tests were done by using the API-20E (BioMerieux, Marcy l'Etoile, France) identification system. All isolates were serotyped according to the Kauffman–White–Le–Minor technique at the Norwegian Veterinary Institute. Phenotypic

susceptibility testing of 13 antimicrobials (gentamicin, sulonamide, trimethoprim-sulfamethoxazole, ciprofloxacin, cefotaxime, meropenem, chloramphenicol, ceftazidime, ampicillin, amoxicillin/clavulanic acid, trimethoprim, tetracycline, and enrofloxacin) was performed by the disc diffusion test. The metadata, serotype and phenotypic resistance of the isolates are presented in the Supplementary Materials (Table S1).

2.2. Pulsed-Field Gel Electrophoresis (PFGE) and Bionumerics Analysis

The PulseNet standardized protocol for PFGE for molecular subtyping of *Salmonella* (https://www.cdc.gov/pulsenet/pathogens/pfge.html) was used on all the 85 isolates. Overnight cultures were used to prepare DNA templates according to the PulseNet protocol. DNA was digested with the restriction enzyme *XbaI* and *Salmonella* Braenderup H9812 was used as a molecular size standard in all PFGE investigations. Electrophoresis was performed with the CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA, USA) with the following set parameters: initial switch time 2.2 s, final switch time 63.8 s, voltage-6 V, time-19 h and temperature 14 °C. The gels were stained with ethidium bromide and the bands visualized under UV transillumination and captured by GelDoc EQ system with Quantity One®software (Version 4.2.1; Bio-Rad Laboratories, Hercules, CA, USA). PFGE banding patterns were compared using a combination of visual inspection and the BioNumerics software vers. 6.6.11 (Applied Maths, Ghent, Belgium). A dendrogram was generated using band-based dice similarity coefficient and the unweighted pair group method using a geometric average (UPGMA) with 1.2% position tolerance and 1.2% optimization. A cutoff of 97% similarity was used to define a PFGE pulsotype (PT).

2.3. Bacterial DNA Extraction

Total DNA for PCR were extracted using the boiled lysate method [32]. This was done by taking 200 µL of an overnight culture, mixing with 800 µL of sterile distilled water and boiling for 10 minutes. The resultant solution was centrifuged at 13,000 rpm for five minutes and the supernatant was used as a DNA template. This was kept at −20 °C for subsequent use.

2.4. Detection of Integrons and Antibiotic Resistance Genes

The isolates that were classified as resistant according to the results of the disc diffusion test (n = 54) were screened by PCR for the most relevant AMR genes corresponding to their phenotypic resistance pattern. In addition, all resistant isolates were screened by PCR for the presence of integron class 1 and 2 encoding genes. The isolates tested were *S.* Newport (n = 18), *S.* Bolton (n = 8), *S.* Hadar (n = 6), *S.* Mbandaka (n = 4), *S.* Heidelberg (n = 8), *S.* Typhimurium (n = 2), and *S.* Zanzibar (n = 8) serotypes. The existence of class 1 integron was investigated by PCR for the detection of genes encoding the variable part between the 5′ conserved segment and the 3′ conserved segment of the variable region [33]. Presence of class 2 integron was investigated by detection of *hep74* and *hep51* genes using primers and following PCR conditions previously reported [33]. Presence of 22 AMR genes (Table 1) known to confer resistance to six commonly used classes of antimicrobials (β-lactams, tetracyclines, phenicols, fluoroquinolones, trimethoprim, and sulfonamides) were investigated by PCR. The primer sets used for detection of integrons and AMR genes are shown in Table 1. Ampicillin resistant isolates (n = 4) were screened for four β-lactamase resistance encoding genes, and ciprofloxacin resistant isolates (n = 40) were screened for four fluoroquinolone plasmid mediated quinolone resistance (PMQR) determinant genes. Chloramphenicol resistant isolates (n=4) were screened for four phenicol resistance genes, tetracycline resistant isolates (n=12) were screened for three genes. Sulfonamide resistant isolates (n = 21) were screened for two genes and six trimethoprim resistant isolates were screened for five trimethoprim resistance genes. These genes were selected because they are the most frequently detected genes associated with the corresponding phenotypes of the NTS isolates [34]. All the integron PCR products were purified and sequenced (GATC Biotech, Cologne, Germany) and the sequence results were analysed using BLAST and compared to GenBank database (http://blast.ncbi.nlm.nih.gov/blast.cgi). Similarly, one PCR product from each of the AMR PCRs was sequenced to confirm the PCR results. Negative controls were included in all PCR analyses.

Table 1. PCR primers used for amplification of genes encoding integrons and antimicrobial resistance in non-typhoidal *Salmonella* isolates.

Target Category	Target Gene	Primer Sequence	Amplicon Size (bp)	Annealing Temp (°C)	Reference
Integron	Class 1 integron *5′-CS* *3′-CS*	GGCATCCAAGCAGCAAG AAGCAGACTTGACCTGA	Variable size	55	[33]
	Class 2 integron *hep74* *hep51*	CGGGATCCCGGACGGCATGCACGATTTGTA GATGCCATCGCAAGTACGAG	491	55	[33]
Resistance to ampicillin by detection of four β-lactamase genes	bla_{PSE-1}	CGCTTCCCGTTAACAAGTAC CTGGTTCATTTCAGATAGCG	419	57	[35]
	bla_{CMY-2}	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	462	64	[36]
	bla_{TEM-1}	AGGAAGAGTATGATTCAACA CTCGTCGTTTGGTATGGC	535	55	[37]
	bla_{OxA}	ACCAGATTCAACTTTCAA TCTTGGCTTTTATGCTTG	590	55	[38]
Resistance to ciprofloxacin by detection of four fluoroquinolone plasmid mediated quinolone resistance genes	*qnrA*	AGAGGATTTCTCACGCCAGG TGCCAGGCACAGATCTTGAC	580	54	[39]
	qnrB	GATCGTGAAAGCCAGAAAGG ATGAGCAACGATGCCTGGTA	476	53	[40]
	qnrC	GGGTTGTACATTTATTGAATCG CACCTACCCATTTATTTTCA	307	53	[40]
	qnrS	GCAAGTTCATTGAACAGGGT TCTAAACCGTCGAGTTCGGCG	428	54	[39]
Resistance to chloramphenicol by detection of four phenicol resistance genes	*floR*	AACCCGCCCTCTGGATCAAGTCAA CAAATCACGGGCCACGCTGTATC	548	60	[41]
	cat1	CTTGTCGCCTTGCGTATAAT ATCCCAATGGCATCGTAAAG	508	55	[42]
	cat2	AACGGCATGATGAACCTGAA ATCCCAATGGCATCGTAAAG	547	55	[42]
	cmlA	CGCCACGGTGTTGTTGTTAT GCGACCTGCGTAAATGTCAC	394	55	[42]

Table 1. *Cont.*

Target Category	Target Gene	Primer Sequence	Amplicon Size (bp)	Annealing Temp (°C)	Reference
Resistance to sulfonamide by detection of two dihydropteroate reductase genes	*sul1*	GCG CGG CGT GGG CTA CCT GATTTCCGCGACACCGAGACAA	350	65	[43]
	sul2	CGG CAT CGT CAA CAT AACC GTG TGC GGA TGA AGT CAG	720	52	[43]
Resistance to tetracycline by detection of three efflux pump genes	*tetA*	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	210	55	[35]
	tetB	TTGGTTAGGGGCAAGTTTTG GTAATGGGCCAATAACACCG	659	55	[35]
	tetG	CAG CTTTCG GATTCT TACGG GAT TGGTGA GGCTCG TTAGC	844	55	[35]
Resistance to trimethoprim by detection of five dihydrofolate reductase genes	*dhfrI*	AAGAATGGAGTTATCGGGAATG GGGTAAAAACTGGCCTAAAATTG	391	50	[37]
	dhfrV	CTGCAAAAGCGAAAAACGG AGCAATAGTTAATGTTTGAGCTAAAG	432	50	[37]
	dhfrVII	GGTAATGGCCCTGATATCCC TGTAGATTTGACCGCCACC	265	50	[37]
	dhfrIX	TCTAAACATGATTGTCGCTGTC TTGTTTTCAGTAATGGTCGGG	452	50	[37]
	dhfrXIII	CAGGTGAGCAGAAGATTTTT CCTCAAAGGTTTGATGTACC	294	50	[37]

The β-lactamase encoding genes (*blaPSE-1*, *blaCMY-2*, bla_{TEM-1}, *blaOxA*) encode production of β-lactamase enzyme that breaks the β-lactam antibiotic ring open and deactivates the molecule's antibacterial properties. The plasmid mediated quinolone resistance genes (*qnrA*, *qnrB*, *qnrC*, *qnrS*) encode pentapeptide repeat proteins that bind to and protects DNA gyrase and topoisomerases IV from the inhibition of quinolones. The phenicol resistance genes, (*cat1*, *cat2*) encode chloramphenicol acetyltransferase enzyme that inactivates chloramphenicol, chloramphenicol resistance gene, *cmlA* and florfenicol resistance gene *floR*, encode efflux pump proteins. Sulfonamide resistance genes *sul1* and *sul2* encode insensitive sulfonamide-resistant dihydropteroate synthase which cannot be inhibited by sulfonamide. Tetracycline resistance genes (*tetA*, *tetB*, *tetG)* encode membrane associated efflux pump proteins that export tetracycline from the cell and reduces drug concentration and thereby protecting ribosomes. Trimethoprim resistance genes *(dhfrI, dhfrV, dhfrVII, dhfrIX, dhfrXIII*) encode a drug-insensitive dihydrofolate reductase which cannot be inhibited by trimethoprim.

3. Results

3.1. Pulsed-Field Gel Electrophoresis Typing

A total of 75 Salmonella isolates were typable, and 15 PTs were identified (Figure 1) and the PFGE banding pattern of all isolates were included in a dendrogram as the Supplementary Materials (Figure S2). The 10 nontypable (NT) isolates belonged to different serotypes; Salmonella Bolton (n = 1), *S.* Newport (n = 3), *S.* Typhimurium (n = 1), *S.* Hadar (n = 4), and *S.* Heidelberg (n = 1). For the majority of the typable isolates, there was a complete association between serotype and PT. The 21 typable *S.* Newport isolates all belonged to PT (H), but were isolated from several farms in all districts (Figure 1). Ciprofloxacin resistant isolates were the majority and most diverse in terms of serotypes, pulsotypes and geographic distribution. Four *S.* Mbandaka isolates were characterized by the same PT (N) and phenotypic resistance pattern, but were isolated from three different farms in two districts. A similar distribution pattern was also observed for 10 *S.* Aberdeen isolates of PT (F); these were isolated from nine different farms from all districts. However, the isolates were fully sensitive in the disc diffusion test. The exceptions from the serotype-PT associations were *S.* Hadar and *S.* Heidelberg. A total of seven *S.* Hadar isolates were typable. Four of them with identical PT originated from the same district, but from two farms, and had same phenotypic resistance towards three antimicrobials. The other three *S.* Hadar isolates had three different PTs, however, two of these isolates were similar with only one band difference (Figure S2). The typable *S.* Heidelberg isolates consisted of two different PTs; one PT (A) with two isolates from the same district and one PT (B) with seven isolates from the other two districts. The isolates in PT (A) were fully susceptible in the disc diffusion test, while all in PT (B) expressed ciprofloxacin resistance and two also expressed sulfonamide resistance.

3.2. Detection of Integrons and Antibiotic Resistance Genes

Genes encoding class 1 integrons were only detected in six *S.* Hadar isolates, four belonging to PT (G) and two nontypable. The integrons were similar in size, with approximately 1700 bp. All the *S.* Hadar isolates that carried integrons originated from four farms in one district, Wakiso. Genes encoding class 2 integrons were not detected in any of the isolates. Sequencing of the six integron PCR products revealed the presence of *aadA1* and *dfrA15* genes that confer resistance to streptomycin/spectinomycin and trimethoprim, respectively.

AMR genes were detected in 31 (57.4%) of the 54 phenotypically resistant. Only seven genes *(*bla_{TEM-1}*, cmlA, qnrS, tetA, sul1, dhfrI, dhfrVII)* of the 22 AMR genes were detected among the selected phenotypically resistant isolates. These genes are known to confer resistance to six categories of antimicrobials (β-lactams, chloramphenicol, fluoroquinolones, tetracyclines, sulfonamides, and trimethoprim).

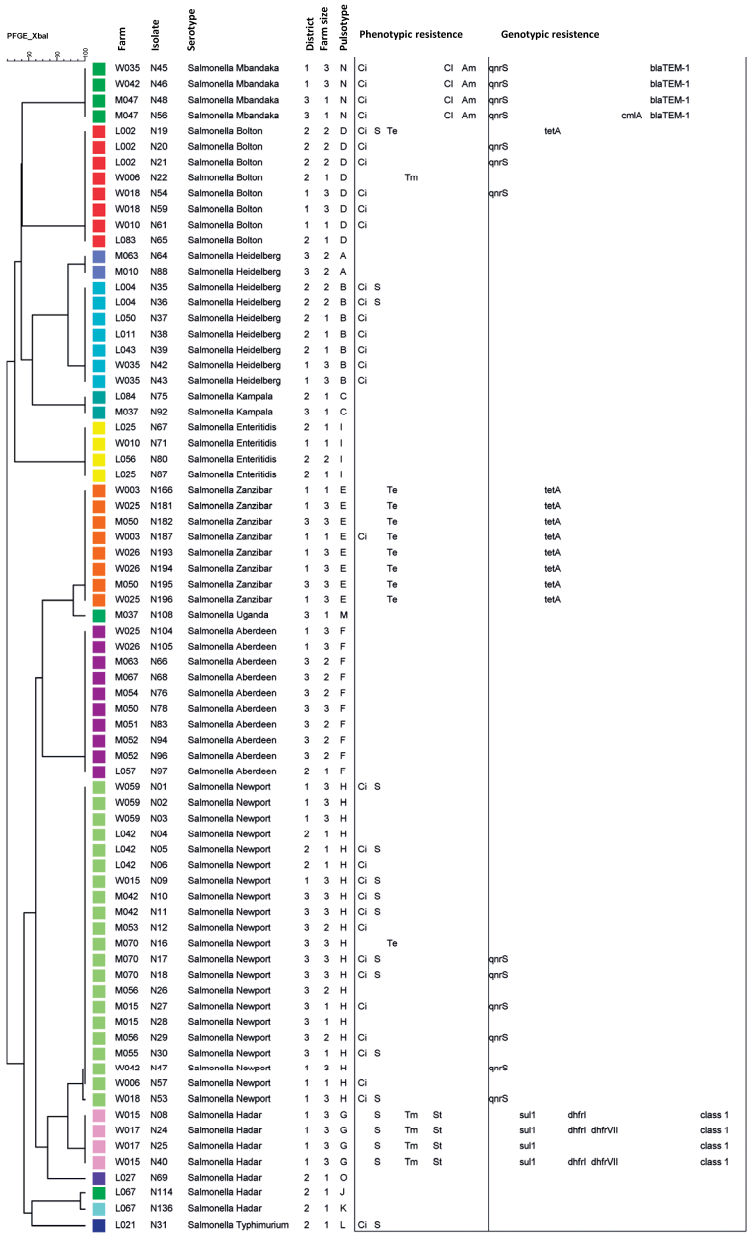

Figure 1. Dendrogram based on Pulsed-Field gel electrophoresis (PFGE) patterns of 75 non-typhoidal Salmonella from poultry from Uganda. A cutoff level of 97% similarity defines a PFGE profile. For each isolate the isolate number, PFGE profile, serotype, farm, size of farm, district, phenotypic resistance (Ci; ciprofloxacin, S; sulphonamide, Te; tetracycline, Tm; trimethoprim, St; sulphamethoxazole_trimethoprim, Cl; chloramphenicol, Am; ampicillin) and identified genotypic resistance genes (*qnrS*, *sul1*, *tetA*, *dhfrI*, *dhfrVII*, *cmlA*, *bla*$_{\text{TEM-1}}$, *integrons*, *dfrA15*, *aadA1*) have been included.

All four ampicillin and chloramphenicol resistant *S.* Mbandaka strains harbored the bla_{TEM} gene that confers resistance to β-lactams, but only one of them was harboring the chloramphenicol resistance gene *cmlA*. The PMQR gene *qnrS* was detected in 16 (18.8%) out of the total 85 isolates. Forty of these displayed ciprofloxacin resistance, of which 16 (40%) carried *qnrS*. All 13 tetracycline resistant isolates were positive for the *tetA* gene. The sulfonamide resistant gene *sul1,* was the only one identified in six of the 21 sulfonamide resistant isolates (*sul2* was not detected). Out of the six trimethoprim resistant *S.* Hadar strains, four were resistant to sulfonamide/trimethoprim and they all harbored the *dhfr*1 gene (Table S1). Three of the six harbored both *dhfr1* and *dhfrVII* (Figure 1).

4. Discussion

The diversity of NTS circulating in poultry in most developing countries is poorly understood, as few studies have been undertaken [44–46]. In this study, 15 PTs from 11 different serotypes of NTS isolates were identified, with most of the identified serotypes having only one PT implying they are clonally related. The PFGE dendrogram combined with the geographical origin of the isolates indicate that many related clones are circulating in geographically diverse areas. For example *S.* Newport, the most prevalent serotype of all, belonged to the same PT and was isolated from all the districts. This situation is not surprising considering the uncontrolled movement of poultry and poultry products in Uganda. In addition, most commercial farms share sources of chicks, feeds, feed ingredients, and live bird markets and these are all potential common sources of NTS contamination. A similar situation has been reported in Senegal [46]. Because NTS is known to persist in the environments for months [47,48], they can easily be spread over large geographical areas. Some of the NTS serotypes represented in this study have caused foodborne illnesses and outbreaks globally [49]. There were isolates with similar PTs that varied with regard to their content of resistance genes, the AMR genes tested for are acquired genes, and not through mutations in chromosomally encoded genes, therefore the genes might be spread among isolates due to their location on plasmids, transposons and integrons. Integration of these elements does not necessarily result in changes in PT.

Through this study, the occurrence of AMR genes among a diversity of NTS isolates from poultry farms in the study districts have been unveiled. The isolates were screened for the genes conferring resistance to the antibiotics to which the isolate revealed a resistance phenotype. The genes detected confer resistance to some of the most important antimicrobials used for treatment of bacterial infections in humans and animals [50]. However, among the 22 AMR genes that are commonly occurring within the *Enterobacteriaceae* family, only seven genes were identified. Discordance was seen where observed phenotypic AMR was not reflected by the detection of corresponding AMR genes. For example, neither *sul1* nor *sul2* genes were detected in the nine phenotypically sulfonamide resistant *S.* Newport isolates. This discordance could be due to presence of other and more unusual resistance mechanisms encoded by genes not included in this study.

Previous investigations on the occurrence of integrons in NTS isolates from animal sources have yielded varying results [51–53]. Class 1 integrons are known for their roles in the dissemination of AMR, especially in the carrying of multiple AMR genes. In this study, integrons were identified in six *S.* Hadar isolates and all of them were identified with *aadA1* and *dfrA15* genes that confer resistance to streptomycin/spectinomycin and trimethoprim, respectively. It is in agreement with studies and reports that most of these genes are found in gene cassettes located within class 1 and 2 integrons [41,51]. In addition, PCR identified four of these *S.* Hadar isolates with *dhfrI* genes with three of the four carrying both *dhfrI* genes and *dhfrVII* genes. More than 30 gene variants encoding dihydrofolate reductase have been identified [38] and *dfrA* are the most commonly genes identified from NTS.

Class I integrons are always associated with *sul1* genes. In this study, *sul1* gene was the only sulfonamide resistance gene identified in six of the 21 phenotypically sulfonamide resistant isolates. Previous studies have reported that in NTS, *sul1* is more common than *sul2* and *sul3* and these genes encode the dihydropteroate synthase [54]. As reported earlier, increase in resistance to

sulfonamides/trimethoprim in Uganda has serious public health implications as it is the main drug used to control opportunistic infections in HIV/AIDS patients [30].

The PMQR gene *qnrS* was the only PMQR gene detected from the NTS isolates that were phenotypically resistant to ciprofloxacin. This finding is in agreement with some similar studies undertaken previously [55–57]. It may, however, be noted that the detection of the *qnrS* genes was restricted to the serotypes *S*. Newport, *S*. Bolton and *S*. Mbandaka, while they were not detected in *S*. Zanzibar, *S*. Typhimurium, *S*. Heidelberg. PMQR genes are rapidly spreading globally, although their presence only mediate low levels of fluoroquinolone resistance, they can interact with genomic determinants to increase the minimum inhibitory concentrations of fluoroquinolones of the PMQR harboring bacteria [58]. Ciprofloxacin is an important fluoroquinolone used in Uganda and other countries for treatment of salmonellosis and other bacteraemic infections. It is often used as a last resort antimicrobial in the treatment of blood stream infections in children and is classified by World Health Organization (WHO) as critically important [50]. In the current study areas, a potential risk exits that ciprofloxacin resistance genes could get transferred to humans through contact with poultry, and consequently complicate the use of ciprofloxacin. The high occurrence of *qnrS* in NTS from poultry needs to be explored further to determine whether it could be associated with use of enrofloxacin in poultry. Enrofloxacin, also a fluoroquinolone, is sometimes used prophylactically and metaphylactically in combination with other drugs in some commercial poultry farms in Uganda [30]. As all fluoroquinolones have the same mechanism of inhibition of the topoisomerase genes, resistance to any one of them will confer resistance to all others. High presence of the plasmid-mediated quinolone resistance gene *qnrS* therefore shows the potential of horizontal transfer of resistance genes [59].

In this study, all the tetracycline resistant isolates carried *tetA* genes, they were all negative for *tetB* and *tetG* genes. This result is similar to what has been reported in previous studies undertaken in Thailand, Australia, Germany, Morocco, and Egypt [18,60–63]. However, the results is also in contrast to another study in Egypt [64]. Many genes responsible for tetracycline resistance have been identified and described [65]. The occurrence of *tetA* gene is known to be widespread in NTS and is associated with non-conjugative transposons. These genes are associated with efflux pump mechanisms implying that these are the predominant mechanisms for tetracycline resistance in NTS in these areas. High presence of *tetA* genes is not surprising as tetracycline is an extensively used drug in human and veterinary medicine, mainly because it is cheap and readily available [66].

All four *S*. Mbandaka isolates that were resistant to chloramphenicol were negative for phenicol resistance encoding genes *floR*, *cat1*, *cat2*, and only one was positive for *cmlA* genes. This finding is consistent with an earlier study [67]. The chloramphenicol exporter gene *cmlA* has been previously found in plasmid-located class 1 integrons in *S*. Typhimurium. Use of chloramphenicol for animal treatment is banned in many countries, including Uganda, due to health hazards associated with the persistence of residues in foods [68]. These same isolates of *S*. Mbandaka were identified with *qnrS* gene and $bla_{\text{TEM-1}}$ gene but were negative for all the other screened β-lactamase encoding genes($bla_{\text{PSE-1}}$, $bla_{\text{CMY-2}}$, $\text{bla}_{\text{OxA)}}$. The gene $bla_{\text{TEM-1,}}$ is reported to be the most widely distributed of the β-lactamase genes worldwide [52] and is mainly known to be spread by plasmids. Not much information is available on the occurrence of beta-lactamase encoding genes in isolates from poultry in Uganda, but similar results have been reported in studies elsewhere [69–71]. Carriage of the $bla_{\text{TEM-1}}$ gene is a threat to the potency of β-lactam antibiotics and in the case of Uganda, ampicillin is still widely used in human and veterinary medicine.

The interpretation of results from this study needs to be taken with a bit of caution, especially when looking at the bigger picture of the whole country. This study evaluated a limited number of resistance genes and only on phenotypically resistant isolates from a previous study [30], the sample size was quite small and samples were collected from only three districts that were purposively selected. However, as far as we are concerned, it is the first of its kind in Uganda and the data generated should make a significant contribution towards the national and international efforts to control antimicrobial resistance.

5. Conclusions

This study was a follow up of a previous study that determined prevalence, antimicrobial susceptibility and risk factors associated with NTS in Uganda [30]. The occurrence of AMR genes and integrons in *Salmonella enterica* isolates from Ugandan poultry has been unveiled, and through subtyping, the diversity of NTS isolates from three districts in Uganda has been explored.

The study has put into perspective the need to monitor use of antimicrobials and occurrence of AMR genes in farm ecosystems in developing countries, in order to institute measures to contain spread of AMR. Poultry keeping is predicted to continue growing in developing countries and in Uganda it will remain an important economic activity. However, as demonstrated, poultry farm environments remain a significant source of spread of AMR genes. Farmers have to be educated on the adoption of strict biosecurity measures, prudent use of antimicrobials and better management practices. More investigations need to be undertaken to further enhance understanding of the driving forces in farm ecosystems for the development of AMR in important foodborne pathogens like *Salmonella*. This study underscores the need for using the One Health approach to generate data on AMR in *Salmonella* organisms originating from humans, animals, and environmental samples.

Supplementary Materials: The following are available online at http://www.mdpi.com/1660-4601/15/2/324/s1, Figure S1: A map of the study areas, Figure S2: A PFGE dendrogram of all typable isolates including the PFGE banding pattern, Table S1: List of all Salmonella isolates with metadata.

Acknowledgments: Funding for this project was provided under the NORHED project No.UGA-13/0031, based at Makerere University and Norwegian University of Life Sciences (NMBU). Our sincere thanks and gratitude go to professor Eystein Skjerve for overall coordination and logistical support. We are grateful to Henning Sørum for providing the positive controls for the resistance genes. In addition, we acknowledge the technical support and contributions of Aud Kari Fauske, Gaute Skogtun, Kristina Borch-Pedersen, and Kristin O'Sullivan, all from NMBU.

Author Contributions: Terence Odoch, Camilla Sekse and Yngvild Wasteson conceptualized and designed the study; Terence Odoch and Clovice Kankya collected field data and samples, and were responsible for bacterial isolation procedure; Trine M. L'Abée-Lund, Yngvild Wasteson and Helge Christoffer Høgberg Hansen mobilized molecular laboratory reagents and supervised molecular laboratory procedures; Terence Odoch and Helge Christoffer Høgberg Hansen performed PCR and molecular subtyping techniques; Terence Odoch, Camilla Sekse and Yngvild Wasteson analyzed laboratory results; Terence Odoch, Yngvild Wasteson, Trine M. L'Abée-Lund and Terence Odoch wrote the paper with contributions from Clovice Kankya and Helge Christoffer Høgberg Hansen. All authors read and approved the final paper submitted.

Conflicts of Interest: The authors declare no conflict of interest.

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PAPER 3

Title

Additive Bayesian networks for antimicrobial resistance and potential risk factors in non-typhoidal *Salmonella* solates from layer hens in Uganda

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Abstract

Introduction

Multi-drug resistant bacteria are seen increasingly and there are gaps in our understanding of the complexity of antimicrobial resistance due to a lack of appropriate statistical tools. This hampers efficient treatment, precludes determining appropriate intervention points and renders prevention impossible.

Methods

We re-analysed data from a previous study using additive Bayesian networks. The data contained information on resistances against seven antimicrobials and seven potential risk factors from 85 non-typhoidal *Salmonella* isolates from laying hens in 46 farms in Uganda.

Results

The final graph contained 18 links between risk factors and antimicrobial resistances. Solely ampicillin resistance was linked to management practice and farm size. Systematic associations between sulfonamide trimethoprim and sulfametoxazole as well as between ciprofloxacine and sulfonamide and ciprofloxacin and trimethoprim resistances were detected. Ampicillin resistance was linked to sulfametoxazole and to chloramphenicol resistances. Variables which were highly connected to other variables were the size of the poultry farm, the vaccinating person and the presence of pets.

Discussion

Although the results needs to be interpreted with caution due to a small data set, additive Bayesian network analysis allowed a description of a number of associations between the risk factors and antimicrobial resistances investigated.

Keywords

47 *Salmonella*, antimicrobial resistance, multi-drug resistance, patterns of antimicrobial
48 resistance, additive Bayesian networks
49

Introduction

Antimicrobial resistance (AMR) is a serious global public health challenge putting the use of antimicrobials in jeopardy as microbes develop resistance to essential antimicrobials (Brown & Wright, 2016; WHO, 2014). Emergence and spread of AMR including multi-drug resistance (MDR) in bacteria are seen increasingly and gaps in our understanding of the complexity of AMR hampers efficient treatment, precludes determining appropriate intervention points and renders prevention impossible. There is growing evidence that use of antimicrobials in food producing animals contributes to AMR in Salmonella (Crump, Sjolund-Karlsson, Gordon, & Parry, 2015). Different mechanisms for antibiotic resistance in *Salmonella* isolates have been described (Frye and Jackson, 2013). The presence of multiple resistance determinants within bacterial isolates can be described as patterns of AMR. Due to biological and evolutionary mechanisms, different resistance genes might be linked to each other (e.g. if harboured on the same plasmid), thus their dissemination is being co-dependent. Therefore, systematic and distinct patterns rather than solely random patterns of AMR might be observed. In the context of risk factor analysis it is of interest to assess systematic statistical co-dependencies between multiple antimicrobial resistances.

The difficulty of assessing the role of relevant risk factors, and therefore defining efficient intervention points, can be (at least partly) explained by the lack of appropriate statistical tools for analysing such complex data. In classical risk factor studies, the multivariable regression techniques typically utilized have their origins in experimental research. Here, the investigator is able to fix all the factors of scientific interest at pre-defined levels – an option which is simply not available in observational studies. Additionally, the investigator will aim to obtain a balanced design, attempting to have similar numbers of individuals in different groups, to benefit from a higher statistical power compared to non-balanced designs. In observational studies, data are typically unbalanced, i.e. different numbers of individuals are being exposed and non-exposed to different risk factors. Unless specifically considered in the sampling plan

to assure that equal numbers of individuals are exposed and unexposed, frequently the issue of sparse data or data separation is encountered. When cross-tabulating binary variables, resulting 2x2 cross tables might have a zero in at least one of the four cells. In this situation, confidence intervals might go to infinity, and odds ratios are not estimable.

In an observational setting, risk factors are presumably interrelated, thus precluding the separation of single risk factors and differentiating between direct and indirect effects. Furthermore, in the context of AMR, the response variable consists of a number of different resistant phenotypes and/or genes, thus necessitating a multivariate approach in contrast to classical risk factor analysis with one single outcome, i.e. healthy or diseased. Most often, data on AMR with multiple patterns are analysed in a descriptive way. To quantify the association between antimicrobials, resistance and susceptibility indices have been proposed, which could also be adapted for multiple resistances, providing also confidence intervals (Ruddat et al. 2012, 2014). Attempts to include potential risk factors in the analysis are rare, with the notable exception of Agga and Scott (2015), who used generalized ordered logistic regression for the analysis of the effect of chlortetracycline and copper on antimicrobial resistance in *E. coli* of weaned pigs.

Additive Bayesian network (ABN) modelling, an approach originating from machine learning and not yet seen widely applied in veterinary epidemiology, appears to be a promising tool for the analysis of multivariate resistance data. The result is presented in the form of networks, consisting of nodes, representing the variables, and links, designating the conditional probabilities between the variables of interest. ABN modelling is specifically designed to deal with messy, highly correlated and complex data. It is suitable to disentangle direct from indirect statistical associations and can be understood as a generalisation of generalised regression models (GLMs). Thus, in contrast to classical regression approaches, the outcome and the predictors are not defined as such beforehand, but within the network different GLMs

applicable to the data at hand are evaluated. Consequently, the first step in an ABN analysis is to find the optimal or most complex network still supported by the data, based on a metric which is controlling for complexity, allowing for the maximum number of links or associations between all variables included. In a second step, measures are undertaken to adjust for potential overfitting and to trim off links that are not supported by the data, given a specific cut-off.

In the beginning of the coil of discovery within a field, such a holistic approach might help due to their ability to act as model proposition fully data driven. It is possible to view ABN modelling as a holistic approach, as it does not impose the classical but sometimes subjective paradigm to treat some variable as outcome and some variable as response. This has to be seen as a major difference compared to other approaches where the model is theory driven such as Structural Equation modeling.

In applied research with binomial (two states random variables) variables, data separation is a surprisingly common issue. It arises when one predictor predicts perfectly the outcome variable. Similarly, the term sparse data is used when only few observations of a possible combination is present in the dataset. Then classical approaches fail to accurately estimate the regression coefficient. The ABN approach requires to perform all possible regressions between the all the possible combination of the variables. Hence, sparsity of the dataset is a major concern and should be addressed properly. Another common feature of epidemiological dataset is the mixture between continuous, binomial and multinomial data (multiple state random variable).

The aim of this study was to determine if specific risk factors are associated with single AMRs and if specific AMRs are linked to each other. For this study we used a data set from previous studies (Odoch et al., 2018).

Material and methods

Sample collection and identification

Non-typhoidal *Salmonella* isolates used in this study were isolated from poultry fecal samples from three districts in Uganda. The study design and sampling is described in full and reported in Odoch et al., (2017). A standardized sampling scheme was adapted from previous studies. Culture and isolation followed ISO 6579:2002/Amd 1:2007 Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production (ISO, 2007)**.** These analyses were carried out at the food microbiology laboratory at the College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, Kampala Uganda. The isolates were serotyped at Norwegian Veterinary Institute, Oslo, using Kauffman–White–Le–Minor technique (Grimont & Weill, 2007).

Antimicrobial resistance testing

Phenotypic antimicrobial susceptibility testing was performed using Kirby-Bauer disk diffusion methods on Muller-Hinton agar (Odoch et al., 2017). The antibiotics were selected based on those commonly used in Uganda and those recommended by World Health Organization (WHO) for routine monitoring and surveillance.

Statistical analysis: Additive Bayesian Networks

The following seven risk factors were selected to be included in the ABN analysis: 1) Gender of the manager (binomial, baseline male or female), 2) "Pets", presence of pets (binomial, baseline no or yes), 3) "Farm size" of the poultry farm (multinomial, small with less than 500 birds, medium between 500 and 1000 birds and large with more than 1000 birds), 4) "Management", i.e. management practice (binomial, baseline free-range or semi-intensive versus intensive), 5) "Eggtrays", indicating if the eggtrays were re-used (binomial, baseline no or yes), 6) "Vaccinator" describing who vaccinates (multinomial, "private service", "self or

family member" or "employee"), 7) "Disposal" of dead birds (multinomial, "burrying", "burning", "throw away", "giving to animals (dogs and pigs)", and "drop in a pit"). Data on antimicrobial resistance against the following seven different antibiotics sulfonamide (SULFA), ciprofloxacin (CIPR), tetracycline (TET), trimethoprim (TRIM), sulfamethoxazole/trimethoprim (SXT), chloramphenicol (CHL) and ampicillin (AMP) were included as binary variables (baseline no resistance).

The entire analysis was conducted using R (R Core Team 2017). As ABN requires a complete dataset, under the assumption of missing at random, missing values were imputed with the R package missforest (Stekhoven 2013, 2017). ABN analysis was performed with the R package abn (Lewis 2016). Here, a scoring procedure (BIC) is implemented to identify the maximum a posteriori Bayesian network based on information theoretic metrics (Kratzer and Furrer, 2018) and controls internally for model complexity. Similar to classical regression analysis, e.g. linear or logistic, regression coefficients or odds ratios depending on the index variable can be obtained.

We used an exact search (Koivisto and Sood, 2004) to find first an optimal network, meaning the optimal level of complexity in terms of the simultaneous presence of different GLMs with potential covariates in the data at hand. In this approach, networks of different increasing complexity, i.e. allowing for more links or covariates to be included, were evaluated. For a plausibility check, the magnitude of the marginal likelihood for each model, i.e. individual GLMs, in the network was assessed visually. In order to adjust for overfitting, a non-parametric bootstrapping analysis with 10'000 bootstraps was performed. This means, a part of the data (95% thereof) was randomly selected, then the entire procedure to find the best network was applied. With the aim to obtain robust results, i.e. associations or links between variables being highly supported by the data, a 50 % threshold was applied.

Results

Descriptive analysis of risk factors and pattern of antimicrobial resistance

In Table 1, the proportions of the seven included risk factors are presented. Antimicrobial resistance testing of 86 isolates originating from 43 farms resulted in 11 different patterns of antimicrobial resistance (Table 2). Based on the resistance patterns which are at least present with a frequency of n = 10, at least 76 % originate from different farms, thus rendering a large clustering effect at farm level implausible. While 32 isolates (37.2%) were not resistant to any of the seven antibiotics tested, 27 isolates (31.4 %) showed resistance against one antibiotic, 16 isolates (18.6 %) against two antibiotics, 9 isolates (10.5 %) against three antibiotics and 2 against four antibiotics (2.3 %). In descending order the following proportions of isolates were found to be resistant against antibiotics: ciprofloxacin 46.5%, sulfonamide 24.4%, tetracycline 15.1%, trimethoprim and trimethoprim-sulfamethoxazole both 7.0%, chloramphenicol and ampicillin both 4.6 %.

Additive Bayesian networks

The results of the final adjusted network are presented graphically as well as numerically with odds ratios on the logit scale for binomial and multinomial variables. In the case of the latter ones, assuming three levels (e.g. vaccination performed by a private service, oneself or a family member, employee) the results first will be presented as private service versus oneself or a family member, then oneself or a family member versus an employee, but can be re-ordered to describe a private service versus an employee.

Six missing values (farm size n = 2, management n = 1, eggtrays = 3) were imputed. In Figures 1 and 2 the networks before (with 21 links) and after bootstrapping (with 18 links) are shown. Within this network of 14 variables comprising of seven antimicrobial resistances and seven risk factors, the variable which was linked to the highest number of other variables was

the farm size which was linked directly to ampicillin resistance, management, reusing eggtrays, disposal, presence of other pets and the person who vaccinates. Regarding the antimicrobial resistances, solely resistance to ampicillin was linked to farm size and management with large farms being more likely to display ampicillin resistance. Still this needs to be interpreted with caution as there were only four isolates with ampicillin resistance. The following antimicrobial resistance characteristics were linked to each other: : resistance towards trimethoprim was linked positively to resistance towards sulfonamide,sulfamethoxazole/trimethoprim and ciprofloxacin. Resistance towards sulfonamide was also linked positively to resistance to ciprofloxacin. There was also a positive association between resistance to chloramphenicol and ampicillin, with all isolates being either both susceptible or resistant (n=4). Negatively associated were resistance to ampicillin and to sulfamethoxazole-trimethoprim. Resistance to etracyclines was not linked to any other antimicrobial resistance.

Regarding the associations between the seven risk factors, median and large farms were less likely to have other pets compared to small farms. Large farms were less likely to reuse eggtrays than medium sized farms. Also associated with reusing eggtrays was the person who vaccinated: if an employee vaccinated compared to the manager him- or herself or a family member, it was less likely that eggtrays were re-used. If the manager him- or herself or a family member, compared to a private service, it was also less likely that eggtrays were re-used.

A female manager was more likely to vaccinate herself or charge another family member than to work with a private service compared to males. A female manager was also less likely to have an employee for the vaccinations.

In Table 3 the corresponding odds ratios on a log.odds scale of the graph before bootstrapping are displayed. Relatively large or small log.odds values are indicative of sparse data (at least

one zero in a contingency table) with leads to unstable estimation of the effect size. Although the magnitude of the effect size is not necessarily meaningful, the direction of the association is still relevant. In Table 4 the results of the 10'000 bootstraps are presented, indicating how many times specific links were retrieved.

Discussion

Based on the data from the previously published data (Odoch 2017), despite the presence of sparse data and data separation, it was possible to obtain networks including seven potential risk factors and seven antibiotic resistances. Due to sparse data the results need to be carefully interpreted. Solely resistance to ampicillin was found to be linked directly to the size of the farm and the management and indirectly to the person who vaccinates, to the reuse of eggtrays and disposal.

It is a well-known fact that many of the genes coding for AMR characteristics are located on mobile genetic elements, and that these genes are disseminated between related and unrelated bacteria through horizontal gene transmission mechanisms. However, we do not have any data on the location of the genes encoding the AMR characteristics in the bacterial isolates analysed in this study, and can therefore only speculate that one explanation for the AMR linkages observed in the ABN analysis is the physical linkage of genes on the same mobile genetic element. What we do know from the Odoch et al. 2018-study, is that six *S*. Hadar isolates harbored class1 integron genes (*int1*) that were also associated with the gene determinant *dfrA15* encoding trimethoprim resistance. As *int1* always are associated with the *sul1* determinant encoding for sulfonamide resistance, this *int1-sul1-dfrA15* linkage is a molecular explanation for the observed association. Use of antimicrobials is a main driver for development and dissemination of AMR. The very often standard simultaneous administration of trimethoprim and sulfonamides (trimethoprim-sulfamethoxazole) can probably be regarded as an important driver for evolution of this genetic linkage.

Although chloramphenicol is banned in poultry, still four isolates were found to be resistant, the source and mechanisms underlying this needs further investigations, although an earlier study identified chloramphenicol resistance encoding gene, *cmlA* in one of these isolates (Odoch et al., 2018). This requires further investigations.

To our knowledge the only two studies that relied an ABN for analysis on antimicrobial data are Ludwig et al. (2013) and Hidano et al. (2015). In both studies, not binary data (being resistant or not) but continuous data, assumed to be Gaussian, as zones of inhibition measured in mm were considered. In our study, due to recent adaptions in the abn code, it was possible to directly include the dichotomized antimicrobial resistance data, based on CLSI, without encountering the issue of sparse data. Still due to sparse data, inevitably present in a small data set, not all associations were estimable resulting in either missing values (NA) or very large estimates and standard errors. Another novelty lies in the opportunity to also include multinomial data.

Although, due to the small sample size and the relative low proportion of resistances against some antimicrobials, the results need to be considered carefully, we are confident, that the actual version of ABN allows for valuable insights in future analyses of larger data sets. The particular added value lies in the opportunity to disentangle the role of single risk factors on the multivariate outcome of antimicrobial resistance data.

Acknowledgement

We are greatly to the NORHED project No.UGA-13/0031 coordinated at

Makerere University and Norwegian University of Life Sciences (NMBU) for providing the financial support for this study. Sincere thanks to Dr Clovice Kankya for ensuring that the required resources were availed in time. Special thanks and gratitude to Professor Paul Togerson at University of Zurich (UoZ) for additional logistical that enabled data analysis to

b e done at UoZ We thank all the researhers and technical staff at the department of Food Safety and Infection Biology at NMBU for their contributions.

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Tables

Figures

386 Figure 1. Bayesian network graph before bootstrapping

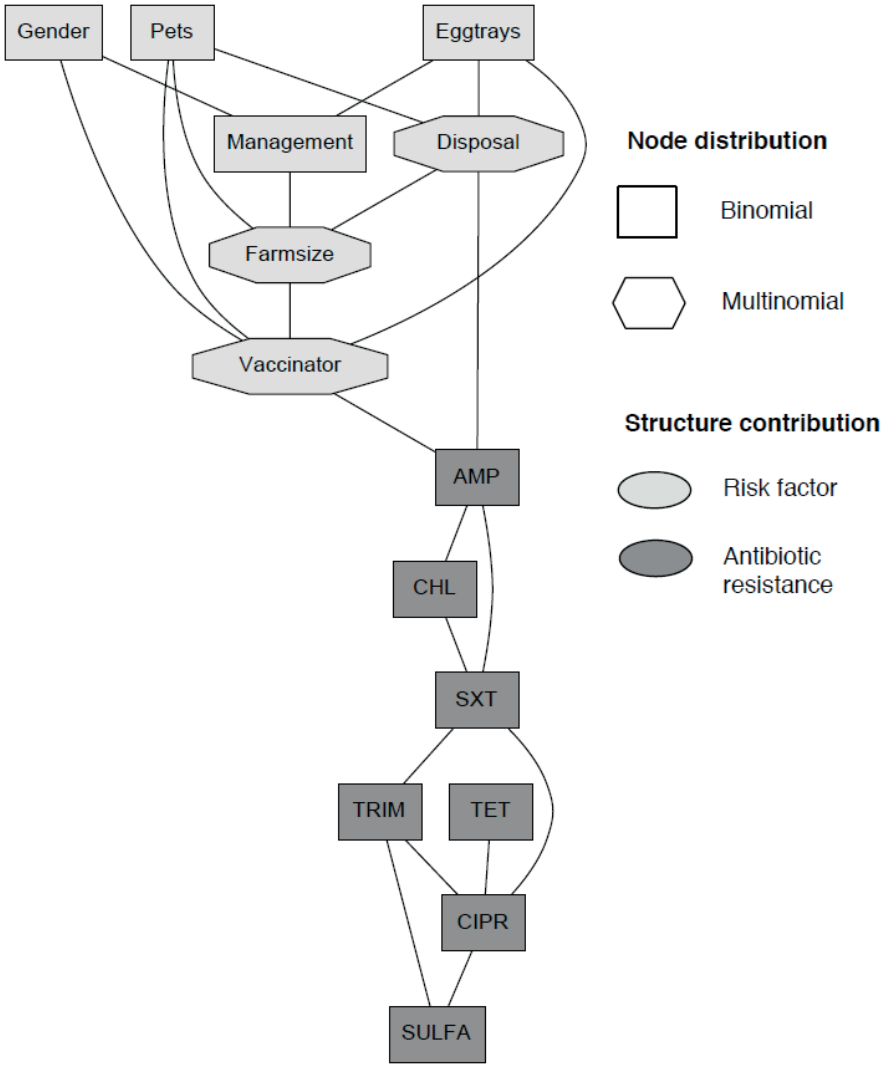

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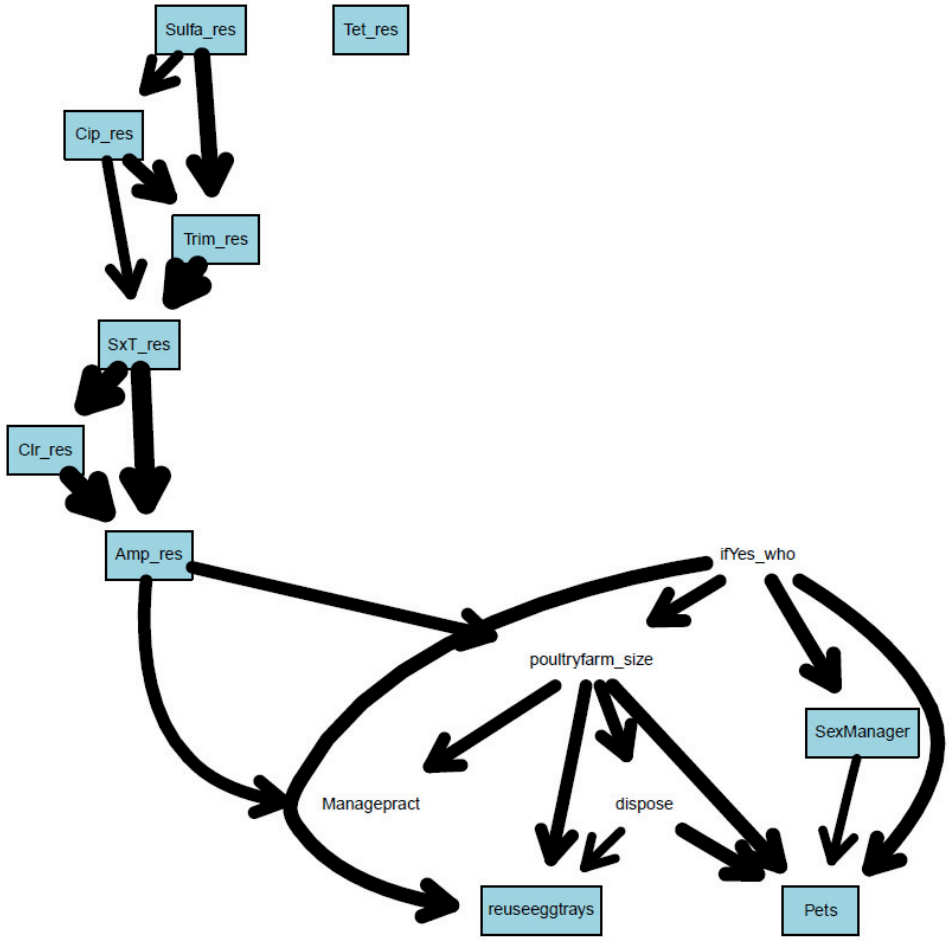
Figure 2. Bayesian network graph after bootstrapping



401 Table 1: Descriptive analysis of risk factors

Risk factor	Categories (n / %)					Missing data
Gender	Male (59 / 68.6)	Female (27 / 31.4)				-
Pets	Yes 29 (29 / 33.7)	No 57 (57 / 66.3)				-
Farm size	Small: 50-500 (31 / 36.0)	Medium: 501-100 (16 / 18.6)	Large: >1000 birds (37 / 43.0)			2
Management	Free range (2 / 2.3)	Semi intensive (24 / 27.9)	Intensive (39 / 45.3)			1
Eggtrays	Yes (52 / 60.5)	No (31 / 36.0)				3
Vaccinator	Private service (30 /34.9)	Self or family (38 /44.2)	Employee (18 /20.9)			-
Disposal	Burrying (46 / 53.5)	Burning (13 / 15.1)	Throwing away (20 / 23.2)	Giving to animals (dogs and pigs) (4 / 4.6)	Drop in a pit (3 / 3.5)	-

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405 Table 2 . Descriptive analysis of patterns of antibiotic resistance

Antibiotic resistance									
Sulfa	Cip	Tet	Trim	SxT	Clr	Amp	Frequencies resistance	Frequen-cies isolates	Number of farms
0	0	0	0	0	0	0	0	32	30
0	1	0	0	0	0	0	1	17	13
1	1	0	0	0	0	0	2	12	10
0	0	1	0	0	0	0	1	8	7
0	1	0	0	0	1	1	3	4	4
0	1	1	0	0	0	0	2	3	2
1	0	0	1	1	0	0	3	3	3
1	0	0	0	0	0	0	1	2	2
1	1	0	1	1	0	0	4	2	2
1	1	1	0	0	0	0	3	2	2
0	0	0	1	1	0	0	2	1	1

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Table 3

See separate file (Table 3_Odoch_PhDpaper3)

Table 4

See separate file (Table 4_Odoch_PhDpaper3)

PAPER 4

Emergence in poultry of non-typhoidal *Salmonella* with reduced susceptibility to ciprofloxacin but without classical mutations

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Abstract

Resistance to ciprofloxacin in *Salmonella* isolates is rapidly increasing worldwide and posing serious public health threats. Ciprofloxacin is the main drug of choice in treatment of multidrug resistant bacterial infections including *Salmonella* in most developing countries. The aim of this study was to determine the occurrence of reduced susceptibility to ciprofloxacin in non-typhoidal *Salmonella* (NTS) isolates and to detect for mutations in the quinolone resistance-determining region (QRDR). NTS isolates originated from fecal samples collected from poultry farms in Uganda. They had been isolated and identified through standard lab procedures. The minimum inhibitory concentrations (MIC) values for ciprofloxacin were determined by microboth dilution to assess for reduced susceptibility to ciprofloxacin. EUCAST cut of value of >0.06µg/mL was used. All isolates with reduced susceptibility to ciprofloxacin were screened for mutations in the quinolone resistance-determining region (QRDR) of the DNA gyrase (*gyrA, gyrB*) and topoisomerase IV (*parC, parE*) genes by PCR amplifications and sequencing. They were then analysed for their association with the district of origin and presence of *qnrS* genes. Reduced susceptibility to ciprofloxacin was detected in 33 (38.4%) of the 86 isolates of NTS and this was significantly associated with the district where they were isolated (p= 0.014) and presence of qnrS genes (p<0.01). Classical mutations at GyrA (S83F and D87N)/GyrA (S83F and D87G) and ParC (T57S and S80R) normally associated with quinolone resistance in *Salmonella spp*. were not detected. However, 7 isolates had point mutations at codon 67 of the QRDR. The study has shown significant presence of reduced susceptibility to ciprofloxacin in NTS isolates without the classical mutations. There is need for further investigations of other mechanisms underlying increasing resistance to fluoroquinolones in NTS and factors driving it.

Introduction

Salmonella enterica is an important public health pathogen causing enormous challenges to public health systems. It normally causes two forms of infections in humans; the typhoid fever caused by human adapted *Salmonella* Typhi, *S.* Sendai, *S.* Paratyphi A, B, C., and non-typhoidal *Salmonella* (NTS) caused by the majority of *Salmonella* serovars. NTS is estimated to cause 93.8 cases and 155,000 deaths annually *(36)*. An estimate by World Health Organization (WHO) put Disability Adjusted Life Years (DALYs) due to diarrheal and invasive infections due to NTS at 4.07 millions*(32)*. Although globally NTS cause less cases compared to typhoid fever, it is now estimated to cause more deaths than typhoid fever, especially in developing countries where it is the leading cause of bacteremia *(3, 17)*.

In addition to causing illnesses, there is an increasing worldwide concern about the development and spread of resistance in *Salmonella* organisms to fluoroquinolones, especially ciprofloxacin. This is posing global public health challenges *(4, 50)*. This trend is mainly attributed to the extensive use and misuse of fluoroquinolones in both human and veterinary medicine. Fluoroquinolones are normally the drugs of choice for many infections where first line treatment have failed. Ciprofloxacin is the main drug used to treat multi drug resistant Salmonellosis *(14)*. Unfortunately, the development of resistance by organisms to fluoroquinolones is diminishing their effectiveness.

Mechanisms for resistance to quinolones and fluoroquinolones in have been described *(13, 51)*. In bacteria, chromosomal point mutations in the quinolone resistance-determining region (QRDR) of the DNA gyrase (genes *gyrA* and *gyrB*) and DNA topoisomerase encoded by *parC* and *parE* genes is the main mechanism of resistance *(13, 16, 47)*. Particularly, in *Salmonella,* resistance to ciprofloxacin is mainly reported as a result of double mutations in gyrA and a single

mutation in parC *(7)*. However, there have also been reports of prevalence of ciprofloxacin resistant food borne pathogens without the typical classical mutations *(34, 52)*.

Emergence of drug resistance strains of *Salmonella* is a global concern posing serious challenges. Resistance in *Salmonella* is developing both in clinical and food isolates *(34, 57)*. Development of multidrug resistant (MDR) *Salmonella* is mainly attributed to misuse and overuse of antimicrobials in humans and animals. Multidrug resistant species of *Salmonella* are implicated in harboring and transferring antimicrobial resistance genes *(10, 11)*.

In Uganda, ciprofloxacin is not authorized for use in animals, but a related fluoroquinolone, enrofloxacin is used in some poultry products meant for prophylactic and metaphylactic treatment. In humans ciprofloxacin is used a lot to treat different bacterial infections. Not so many studies have been done on ciprofloxacin resistance in NTS isolated from farms in Uganda, the limited studies done is mainly on human isolates and focusing largely on prevalence and patterns of resistance *(38)*. Therefore, not much is known on the incidence and the state of resistance to fluoroquinolones in *Salmonella*, especially from the livestock sector. In an earlier study, where four commonly reported plasmid-mediated quinolone resistance (PMQR) genes were tested, only *qnrS* gene was detected *(41)*. This prompted the need for more investigations and follow up studies. The main aim of the current study was to analyze the occurrence of reduced susceptibility to ciprofloxacin in *NTS* isolates from poultry farms in Uganda and to detect for mutations in the quinolone resistance-determining region (QRDR) of the DNA gyrase (*gyrA, gyrB*) and topoisomerase IV (*parC, parE*) from isolates with reduced susceptibility to ciprofloxacin. As far as we are concerned no similar studies have ever been undertaken *Salmonella* isolates originating from poultry in Uganda.

Materials and methods

Bacterial isolates

During a period between 2015 and 2016, a research project collected fecal samples from poultry houses in the districts of Wakiso, Lira and Masaka in Uganda. The collection of fecal samples, identification of NTS and typing of isolates followed standardized protocols previously reported *(41, 42)*. Isolation was done at the food microbiology lab at the College of Veterinary Medicine Animal Resources and Bio security (CoVAB) of Makerere University Kampala, Uganda using ISO procedures for bacteriological isolation and identification*(30)*. The isolates were serotyped on the basis of somatic O and H flaggelar antigens by agglutination tests at Norwegian Veterinary Institute (NVI) according to Kauffman-White-Le Minor scheme for salmonella serotyping *(20)*. For each isolate, key variables of interests (serotypes, district of origin, presence of *qnrS* gene, pulsotype) were captured from the supplementary table of Odoch, T., C. Sekse *(41)*.

Susceptibility testing

Eighty six *Salmonella* isolates had their minimum inhibitory concentrations (MIC) determined for ciprofloxacin by broth microdilution using a commercially available dehydrated panel (SensiTitre® TREK EUVSEC, TREK diagnostics Ltd) and performed at Norwegian University of Life Sciences (NMBU), food safety pathogenic lab according to manufacturers' instructions. The intervals assayed ranged from 0.015 – 8µg/mL. The minimum inhibitory concentrations (MICs) were determined after 18hrs of incubation at 37°C and isolates with MIC >0.06µg/mL were considered to have reduced susceptibility to ciprofloxacin.. MIC values breakpoints were based on epidemiological cut off (ECOFF) and interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org).

PCR amplification and sequencing

Total DNA for PCR were extracted using the boiled lysate method as reported earlier *(2)*. This was done by taking 200 µl of an overnight culture, mixing with 800 µl of sterile distilled water and boiling for 10 minutes. The resultant solution was centrifuged at 13,000 rpm for five minutes and the supernatant was used as a DNA template.

The QRDR region of the genes *gyrA, gyrB, parC and parE* were amplified by PCR. The PCR primers (Sigma-Aldrich, UK) and conditions used were previously described (Table 1). The genes were confirmed by 1.5% gel electrophoresis. After amplifications, PCR products were purified by QIAGEN PCR purification kit according to the manufacturer and sequenced in Germany (GATC Biotech, Germany). The sequence data was imported into BioEdit program and inspected for mutations thru alignment. Mutations in the QRDR region were then examined by translation of the nucleotide sequence into proteins and then aligned with a reference sequence of the wild type *Salmonella* Typhimurium strain LT2 in the NCBI database (Accession Number AE006468).

Results

Thirty-three (38.4%) of the 86 tested *Salmonella* isolates had reduced sensitivity to ciprofloxacin by MIC cut off (>0.06µg/mL). The MIC results categorized as susceptible or reduced susceptibility against the serotypes are presented in Figure 1. Reduced susceptibility to ciprofloxacin was highest among Newport (n=9), Heidelberg (n=8) and Bolton (n=6) serotypes (Figure 1). *Salmonella* isolates with reduced susceptibility to ciprofloxacin were found to be associated with the district where the samples were isolated (p=0.014) with Wakiso district having the highest numbers (Figure 2).

Fourteen (42.4%) of the 33 *Salmonella* isolates with reduced susceptibility to ciprofloxacin harbored *qnrS* genes (Table 2). The MIC for isolates with reduced susceptibility ranged from

0.12 – 1.00µg/mL, an in indication of low-level resistance. Reduced susceptibility to ciprofloxacin was significantly associated with presence of *qnrS* genes ($p<0.01$). Fourteen (42.4%) of the 33 isolates were identified with *qnrS* gene distributed among three serotypes; Bolton (n=4), Mbandaka (n=5), and Newport (n=5). All the isolates of *S.* Mbandaka with reduced susceptibility to ciprofloxacin had qnrS gene, while it was 6/9 and 4/6 for Newport and Bolton respectively (Table 2).

All the isolates with decreased susceptibility (n=33) were tested for mutations in the quinolone resistance determining regions of *gyrA, gyrB, parC* and *parE* genes. The classical double amino acid substitutions in GyrA (S83F and D87N)/GyrA (S83F and D87G) and ParC (T57S and S80R) normally associated with quinolone resistance in *Salmonella spp*. were not detected. However, some 7 isolates had point mutations at codon 67 of the QRDR (table 2). The mutations were Ala67 Leu(n=2), Ala67Ser(n=2), Ala67Tyr (n=2), Ala67Pro.

Discussions

This study has demonstrated high prevalence of reduced susceptibility (38.6%) to ciprofloxacin in *NTS* isolates. However, the classical mutations at codon 83 and 87 of GyrA normally associated with quinolone resistance were absent. Some few isolates (7/33) had point mutations at codon 67 in the quinolone resistance determining region (QRDR) of the *gyrA* genes. The observed reduced susceptibility to ciprofloxacin were strongly associated with the presence of plasmid mediated quinolone resistance gene (PMQR), *qnrS* and the district of origin.

The strong association between the presence of *qnrS* gene with reduced susceptibility to ciprofloxacin have been well documented before. One similar study by Thong, K. L., S. T. Ngoi *(55)* detected only *qnrS1* gene but found silent multiple mutations at sites outside the *parE* QRDR. PMQR genes are known to play an important role in fluoroquinolone resistance in

Enterobacteriacae (52, 56). The main PMQR genes (*qnrA, qnrB, qnrC, qnrD and qnrS*) have been widely described to date. The PMQR gene is known to confer low-level resistance to ciprofloxacin, they are rapidly disseminated and therefore suspected to be responsible for the increasing resistance to fluoroquinolone *(43, 48, 53, 54)*. There have been suggestions that *qnr* proteins protect DNA gyrase and topoisomerase IV from quinolone inhibition and that isolates with *qnr* genes may be less likely to develop topoisomerase mutations than others *(9)*.

This study adds to a growing list of studies reporting fluoroquinolone resistant isolates without the typical mutations in the *gyrA* and *parC* genes *(8, 15, 23, 26, 52)*. A similar study in China *(6)* found 21% of *Salmonella* isolates resistant to ciprofloxacin and enrofloxacin without PMQR and QRDR genes and suggested that there could be a new mechanisms underlying resistance in *Salmonella* to fluoroquinolones. And in some cases presence of mutations have been reported outside the QRDR in some ciprofloxacin resistant mutants of *E*.coli in a study by Jaktaji, R. P. and E. Mohiti *(31)* and also by Friedman, S. M., T. Lu *(18)*. A similar result is reported in a previous study *(46)*. The observed point mutations at codon 67 did not seem to cluster with any variable in the study.

In *Salmonella*, the main mechanism associated with resistance to ciprofloxacin is the presence of double mutations and single mutations in *gyrA* and *parC* genes respectively *(11, 27, 45)*. Double mutations mainly reported are substitutions in *gyrA* (Ser83Phe and Asp87Gly)/gyrA (Ser83Phe and Asp87Asn) and single mutations in *parC* (Thr57Ser and Ser80Arg). Naturally, these double mutations are supposed to be spontaneous and rare events *(58)*. However, mutations in the *gyrB* and topoisomerase IV genes *parC* and *parE* are considered rare in *Salmonella (22, 25, 35, 44)*. But currently, the rapid global spread of fluoroquinolone resistance being observed cannot be explained by mutations in DNA gyrase and topoisomerase alone. Another great concern now is

the increasing prevalence of ciprofloxacin-resistant food-borne *Salmonella* strains harboring multiple PMQR elements but with no target gene mutations. The results of this study are indicative of the potential variations in the contributions of the different resistance mechanisms with the geographic regions, driven by differences in farm management and bio security practices. It has been suggested that the presence of several PMQR genes in *Salmonella* could act synergistically and cause resistance seen in isolates without mutations in the *gyrA* and *parC* genes*(34)*.

The suggestions that other mechanisms could be playing increasing roles in fluoroquinolone resistance is supported by this study and earlier studies *(49, 51)*. Currently, there are four known mechanisms of resistance to quinolone; gyrase and topoisomerase gene mutations, changes in expression of efflux pump, cell membrane alternations, and plasmid mediated quinolone resistance. All these can work discretely or in combination *(21)*. Quinolones were serendipitously discovered by products from chloroquine synthesis*(37)*, being purely synthetic, resistance to them was least expected to develop very fast. In human isolates, the rapid development of resistance to fluoroquinolones can be attributed to their wide scale use and misuse. A fluoroquinolone like ciprofloxacin is a life saving drug for severe and systematic infections *(28)*. It is usually the first line of drug for the treatment of most invasive salmonellosis and widely used in human and veterinary medicine*(14)*. Ciprofloxacin was at one time the most consumed antibiotic worldwide *(1)* and is still one of the most prescribed antimicrobial drugs listed as essential medicine and critically important*(12)*.

This study has shown that out of the PMQRs*, qnrS* gene is most likely contributing significantly to the reducing susceptibility to ciprofloxacin in Uganda, notwithstanding the roles of other resistance mechanisms that warrant further investigations. Past reports have associated resistance

to nalidixic acid to reduced susceptibility to ciprofloxacin, but increasingly a number of studies are reporting emergence in NTS and other *Enterobacteriacae* of isolates with reduced susceptibility to ciprofloxacin but sensitive to nalidixic acid *(5, 19, 23, 24, 29)*. The association of the occurrence of reduced susceptibility to ciprofloxacin to the districts of origin point to a number of factors at play. Wakiso, where the prevalence is highest is in the centre of the country, near the capital, and the hub of all commercial activities. This is where a lot of trade and movement of people, livestock and livestock products occur. Therefore, potential for horizontal transmission of PMQR genes, including direct or indirect contacts with the organisms is high.

This study has demonstrated the absence of classical mutations in the *gyrA* gene and *parC* gene in NTS isolates with reduced susceptibility to ciprofloxacin. Therefore signifying the roles other mechanisms could be playing in the rapid and world wide spread of AMR in *Salmonella.* This point to the importance of more understanding of fluroquinolone resistance mechanisms in *Salmonella.* Already. other mechanisms like efflux pump mediator mechanisms have been identified that are related to fluoroquinolone resistance*(51)*. In *Salmonella* at least nine of these pumps have been described *(39, 40)*. Therefore, since the classical mutations in the QRDR was was not detected in any of the isolates involved in this study, further investigations should focus on other resistance mechanisms like multi drug efflux pumps.

Our study only focused on the detection of classical mutations that is widely known to confer resistance in fluoroquinolone encoded by genes that cause mutations in gyrA and topoisomerases, therefore we could not explain the other mechanisms behind the observed resistance. More investigations in the interplay among resistance mechanisms is required including the roles of quorum sensing, and biofilm formation in development of resistance.

This study, as far as we are concerned is the first of its kind in Uganda. It has added data to growing pieces of evidence of the existence of high prevalence of low level resistance to ciprofloxacin in NTS without classical mutations. Moreover in NTS isolates from poultry farms in Uganda where ciprofloxacin is not normally used in animal production. Although the mechanisms underlying fluoroquinolone resistance has been widely studied, a lot is unknown especially from poor and developing countries. The multiple mechanisms that underlie fluoroquinolone resistance in *Enterobacteriaciae* makes characterizing fluoroquinolone resistance in resistant isolates a complicated issue, as the extent to which the different mechanisms contribute singly or in combination needs to be well established. In addition, drivers of the growing resistance are multifactorial and complex, a more regional and local outlook is required for developing countries that are lacking capacity and hence limited data are available for a sound scientific comparison. The urgent need to control fluoroquinolone resistance in *Salmonella* and other enteric organisms in poultry and livestock requires continuous surveillance, monitoring and rational use of drugs. These efforts should go hand in hand with epidemiological studies.

Acknowledgement

We are greatly to the NORHED project No.UGA-13/0031 that was based at

Makerere University and Norwegian University of Life Sciences (NMBU) for funding for this study. Special thanks and gratitude to Professor Eystein Skjerve and Associate Professor Clovice Kankya as coordinators for all the logistical support. We thank professor Henning, researchers, and all the technical staff from the labs at the department of food safety and infection biology at NMBU for their valuable time and resources put in to this project.

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Figure legend(s)

Figure 1. Susceptibility of NTS isolates from poultry to ciprofloxacin based on minimum inhibitory concentration (MIC) testing on all the isolates that comprised 11 serovars of NTS. MIC results were categorized as susceptible or reduced susceptibility and expressed as a percentage by districts

Figure 2. Susceptibility to ciprofloxacin based on minimum inhibitory concentration (MIC) testing in percentage by districts for the NTS isolates. MIC results were categorized as susceptible or reduced susceptibility and expressed as a percentage by districts

Table 1. List of primers used in this study

Gene	Primer name	Primer sequence (5′ to 3′)	Amplicon (bp)	AT in ℃	References
gyrA	GyrA-FP	AAATCTGCCCGTGTCGTTGGT	344	58	*(26)*
	GyrA-RP	GCCATACCTACTGCGATACC			
gyrB	GyrB-FP	GAATACCTGCTGGAAAACCCAT	446	57	*(26)*
	GyrB-RP	CGGATGTGCGAGCCGTCGACGTCCG			
parC	ParC-FP	AAGCCGGTACAGCGCCGCATC	395	57	*(26)*
	ParC-RP	GTGGTGCCGTTCAGCAGG			
ParE	ParE-FP	TCTCTTCCGATGAAGTGCTG	240	55	*(33)*
	ParE-RP	ATACGGTATAGCGGCGGTAG			

Table 2. Minimum inhibitory concentration (MIC) values of NTS isolates with reduced susceptibility to ciprofloxacin, gyrA nutations and qnrS status

Isolate_id	District of Origin	Serovar	*gyrA mutations*	*qnrS*	MIC
PSUG01	Lira	Bolton	-	+	0.25
PSUG02	Lira	Bolton	-	-	0.12
PSUG03	Lira	Bolton	-	+	0.12
PSUG04	Wakiso	Bolton	-	+	0.25
PSUG05	Wakiso	Bolton	*Ala67Tyr*	+	0.25
PSUG06	Lira	Bolton	-	-	0.12
PSUG07	Wakiso	Hadar	-	-	0.12
PSUG08	Waksio	Hadar	-	-	0.12
PSUG09	Lira	Heidelberg	*Ala67Tyr*	-	0.12
PSUG10	Lira	Heidelberg	-	-	0.12
PSUG11	Lira	Heidelberg	-	-	0.12
PSUG12	Lira	Heidelberg	-	-	0.12
PSUG13	Lira	Heidelberg	-	-	0.12
PSUG14	Wakiso	Heidelberg	-	-	0.12
PSUG15	Wakiso	Heidelberg	-	-	0.12
PSUG16	Wakiso	Heidlberg	-	-	1.0
PSUG17	Newport	Mbandaka	-	+	0.12
PSUG18	Wakiso	Mbandaka	-	+	1.0
PSUG19	Wakiso	Mbandaka	-	+	1.0
PSUG20	Masaka	Mbandaka	-	+	0.50
PSUG21	Masaka	Mbandaka	-	+	0.50
PSUG22	Wakiso	Newport	*Ala67Leu*	-	0.12
PSUG23	Lira	Newport	*Ala67Ser*	-	0.25
PSUG24	Wakiso	Newport	-	-	0.12
PSUG25	Wakiso	Newport	-	+	0.25
PSUG26	Wakiso	Newport	-	+	0.12
PSUG27	Wakiso	Newport	-	+	0.25
PSUG28	Masaka	Newport	*Ala67Pro*	+	0.12
PSUG29	Wakiso	Newport	-	+	0.12
PSUG30	Wakiso	Newport	-	-	0.12
PSUG31	Lira	Typhimurium	-	-	0.25
PSUG32	Lira	Typhimurium	*Ala67Leu*	-	0.25
PSUG33	Wakiso	Zanzibar	*Ala67Ser*	-	0.12

Figures 1. Susceptibility of *Salmonella* isolates from poultry to ciprofloxacin based on MIC testing

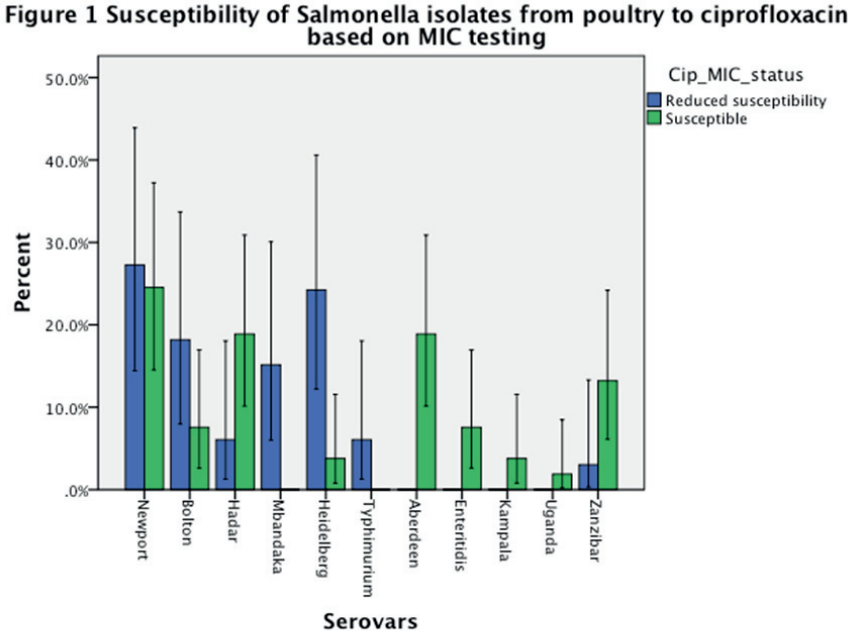
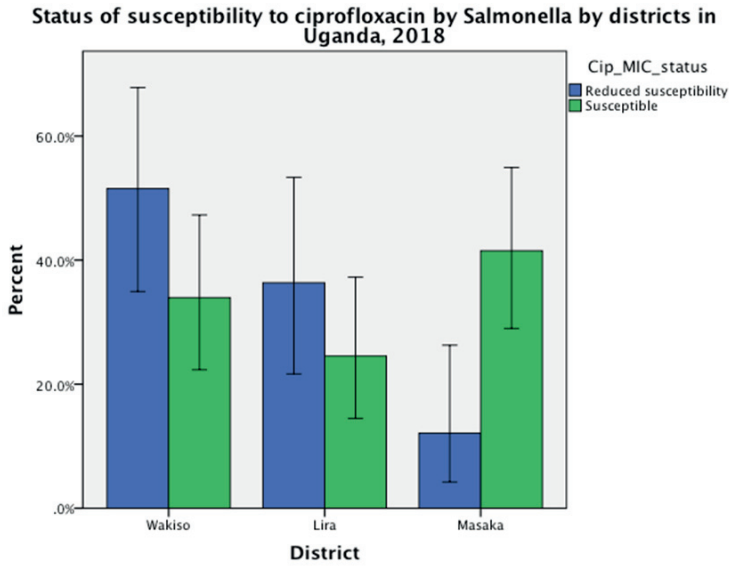


Figure 2. Susceptibility to ciprofloxacin based on minimum inhibitory concentration (MIC) testing in percentage by districts for the NTS isolates



16 APPENDIX II: QUESTIONNAIRES

QUESTIONNAIRE

Interview Date: _____

Identification number

A. PERSONAL INFORMATION OF THE FARMER/FARM MANAGER/ATTENDANT

A.1. Name of the farmer

A.2. Location of the farm GPS Position. . ……

A.2.1. Village (LC 1)…… A.2.2. Parish ……..

A.2.3. Sub-County…… A.2.4. District……

A.3. Sex of farmer □ M □ F

A.4. Education level of the farmer

(1) None (2) Primary (3) Secondary (4) Tertiary (Specify main area)……..

A.5. Education Level of the farm manager/attendant

(1) None (2) Primary (3) Secondary (4) Tertiary (Specify main area)

A.6 Gender of the farm manager/attendant:

(1) Male (2) Female

A.7 Age (in years) of the farm manager/attendant

(1) <20 (2) 20 – 35 (3) 36 – 50 (4) >50

A.8 Any other occupation of the farmer: ……..

B. POULTRY MANAGEMENT PRACTICES ***(You may tick more than one choice where necessary)***

B.1. What species of Livestock do you keep?.....

B.2. Do you have pets (1) Yes (2) No If yes go to B3 if no skip B3

B.3. What pets do you have (1) Dogs (2) Cats (3) Both dogs and Cats (4) Others (specify)……..

B.4. What is the size of your poultry farm?

(1) Small: 50 – 500 birds (2) Medium: 501 - 1000 birds (3) Large >1000

B.5. What poultry species are kept in this farm/home?.....

(1) Ducks (2) Turkeys (3) Pigeon (3) Guinea Fowls (4) Others (Specify)……..

B.6. What are the main breeds of chicken kept on the farm

(1) Local-native (2) Exotic (Layers) (3) Exotic (Broilers) (4) Mixed (Local and exotic)

(5) Exotic Layers and Exotic Broilers) (6) Others……..

B.7. What is the age of the current flock (in weeks)? (Multiple answers allowed)

(1) Less than 8 weeks (2) 9 – 20 weeks (3) 21 – 45 weeks (4) More than 45 weeks

B.8. How many poultry houses do you have?.....

B.9. What is the capacity of each of the poultry house? (multiple answers allowed).....

B.10. Do you keep birds of different ages at the farm (1) Yes (2) No

If yes go to B11, if no skip B12

B.11. Do you keep birds of different ages in the same house? (1) Yes (2) No

B.12. Where do you obtain your poultry?

(1) Gift (2) Buy from market (3) Hatchery (4) Dealers of day old chicks (Agents) (5) Others (Specify)…….

B.13. What type of poultry management do you apply

(1) Free range (2) semi- intensive (3) intensive (4) Others (Specify)…….

B.14. If management is intensive or semi intensive, who is the main person who takes care of the poultry?

(1) Husband (2) Wife (3) Children (4) Relative (5) Employee (6) Others (specify)……

B.15. Do you practice ***all in all out*** system of management? (1) Yes (2) No

B.16. What is the type of housing for your poultry?

(1) Kitchen (2) Chicken house (3) Main House (3) Garage (5) Others (specify)……

B.17. Do your poultry share housing with other birds, animals or humans? (1) Yes (2) No

If yes go to B18, if no skip B18

B.18. If yes, which species

……

B.19. Do your poultry intermingle or mix freely with birds/ animals in the neighborhood? (1) Yes (2) No

B.20. Do you restrict persons entering the poultry house? (1) Yes (2) No

B.21. If yes, what type of restrictions? ……

B.22. Do you control rodents and flies in your poultry house?

(1) Yes (2) No If yes, go to B.22 and B.23, if no go to B24

B.23. What do you do to control rodents?.....

B.24. What do you do to control flies?.....

B.25. Have ever seen a rodent on this farm in the last 6 months? (1) Yes (2) No

B.26. What is your main source of feeds?

(1) Ready to use commercial feeds (2) Home mixed rations (3) Kitchen wastes

(4) Mill bye products (brans etc.) (5) Whole grains (maize, millet sorghum etc)

(6) Others (specify).... . . . . .

B.27 Where do you buy the feeds or feed ingredients from?.....

B.28 How often do you replace litter?

B.29. What is your main water source (sources)?

(1)Family tap water (2) Public tap water (3) Bore hole (4) Open well (5) Surface water (spring, pond, river)

(6) Others (specify)……

B.30. How long have you been in poultry farming? (1) less than 1 year (2) 1 – 5yrs (3) More than 5 yrs.

B.31. Do you keep records on this farm? (1) Yes (2) No

B. 32. What types of records do you keep?

(1) Treatment records (2) Vaccination records (3) Disease records (4) Production records (5) Sales records

(6) Others (Specify)……

C. POULTRY DISEASES PREVENTION MEASURES

C.1. Do you have a written biosecurity plan (1) Yes (2) No

C.2. Do you apply measures to prevent diseases in your poultry? (1) Yes (2) No

If yes, list the measures (eg. Quarantine, cleaning, disinfection, vaccination)

.....

C.3. Do you use disinfectants in your poultry house? (1) Yes (2) No (If yes go to C3)

C.4. What type of disinfectants? (May show you the containers)

……

……

C.5. How often do you disinfect your poultry house?

……..

- How do you carry disinfection?

.....

C.6. Do you clean and disinfect between flock? (1) Yes (2) No

C.7 Do you provide footbath at the entry of each poultry house (1) Yes (2) No

C.8. Do you have a disinfectant for vehicles entering your farm (1) Yes (2) No

C.9.Do you have dedicated personnel clothing and equipment for poultry production? (1) Yes (2) No

C.10. Do you put on protective clothing (Gumboots, overalls etc.) while in the poultry house? (1) Yes (2) No

C.11. Do you have a separate hand washing facilities for farm use (1) Yes (2) No

C.12. What is your down time (time between clearing the house and bringing new stock)?

C.13. Do you use egg trays (1) Yes (2) No

C.14. Do you reuse egg trays? (1) Yes (2) No

C.15. How do you clean egg trays?.....

……..

C.16. Do you vaccinate your poultry? (1) Yes (2) No

If yes, go to C 11 if no go to C11

C.17. Who does the vaccination?

(1) Government/Local Government worker (2) Non- Governmental organization worker (3) Private provider

(4) Neighbor (4) Family member (5) Others (specify)……

C.19. What is the source / supplier of the vaccines

(1) Government (2) Non- Governmental organization (3) Private sector

C.20. What diseases do you vaccinate your poultry against?

(1) New Castle Disease (2) Gumboro (3) Fowl Typhoid (4) Fowl pox (5) Mareks disease (6) Infectious Laryngitis
(7) Others (Specify)

C.21. Do you have a vaccination schedule that you follow? (verify by seeing it) (1) Yes (2) No

C.22. Do you have a separate poultry house for new birds? (1) Yes (2) No

C.23. Do you have a separate poultry house for sick birds? (1) Yes (2) No

C.24. Do you sometimes see the presence of wildlife/wild bird present in areas where your birds are housed (1) Yes (2) No

C.25. Do other animals share water sources with your poultry? (1) Yes (2) No

D. POULTRY DISEASES AND MANAGEMENT

D.1. Do you get poultry diseases on this farm? (1) Yes (2) No If yes go to D2,

D.2. What are the diseases do you get on your farm ?
……
……
.....

D.3. What disease signs do you normally see? (eg Cough, diarrhea, loss of weight, blood in feces, discharges from nose, eyes)
……
……
……

D.4. When disease occurs, which is the most affected age group?

(1) Chicks (Less than 4 weeks) (2) Growers/Pullets

(3) Adults (4) All age groups

D.5. What do you do when birds get sick?

(1) Eat (2) sell off (3) Isolate them from healthy birds (4) Seek diagnosis/ treatment

(5) Others (specify)

D.6. If you treat your poultry, who mainly does the treatment?

(1) Self with traditional medicine
(2) Self with modern medicine
(3) Government Veterinary/ Animal health worker
(4) Private Veterinary/ Animal health worker (5) NGO Veterinary/ Animal health worker
(6) Community Animal Health worker (
(7) Traditional medicine man (woman)

D.7. What is the main outcome of this treatment?

(1) Recovered (2) Died (3) Still sick (4) Others (Specify)

D.8. What is the extent of losses/deaths over one year?

(1) None (2) moderate (less than 50%) (3) Severe (above 50%)

D.9. What do you do when birds die?

(1) Dispose off (2) Eat (3) Others (Specify)……..

D.10. What are the sources of your drugs supplies?

(1) Government (2) Non- Governmental organization 3) Private sector Vet/Agrovet Drug shop

D.11. How do you dispose dead birds?

……

D.12. Do you access animal health extension services?

(1) Yes (2) No

D.13. If yes, who provides?

(1) Government (2) Non- Governmental organization (3) Private

D.14. Who mainly pays for this service?

(1) Government (2) Non- Governmental organization (3) Self

THANKS

ISBN: 978-82-575-1767-0
ISSN: 1894-6402

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