// Abstract

BALL, TAKIYAH ASHA. Implementing and Sustaining Antimicrobial Resistance Surveillance Programs in Developing Countries. (Under the Direction of Dr. Paula Cray and Dr. Maria Correa)

The antimicrobial surveillance programs which have been sustained over a period of time are important as they provide data which illustrate changes in antimicrobial resistance (AMR) and allow for trend analysis to be conducted; they are also useful as they may help identify newly emerging or re-emerging resistant pathogens. A surveillance system can also aid in informing possible threats and illness burdens with populations by detecting various shifts in susceptibility in organisms. One example of these types of surveillance systems is the National Antimicrobial Resistance Monitoring Program (NARMS) here in the US. The ecosystem of AMR involves many components including food animals and associated meatstuffs, the environment, and the human population; this makes AMR a critical and relevant One Health and One World Issue. When assessing surveillance data that may be available on resistance in the food chain and food-producing animals, major gaps in data are noted. In this study, we evaluate the process it takes for a laboratory in Uganda to implement an AMR surveillance program successfully in countries that have the economic and educational capacity to sustain. The specific objectives of this project are to: evaluate laboratory capacity and provide recommendations for the development and implementation of an AMR surveillance system in food animals at the University of Makerere in Kampala, Uganda; conduct a pilot study examining the prevalence and phenotype of *Salmonella* and *E. coli* isolated from cattle and chicken farms in the Wakiso district of Uganda two times a year; and genotypically characterize *Salmonella* and *E. coli* isolated from cattle and chicken farms in the Wakiso district of Uganda. Our overall goal is to assist in the implementation of an antimicrobial resistance surveillance programs in developing countries,

such as Uganda, will encourage successful, sustainable and harmonized programs that will result in international integrated data systems to monitor AMR.

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Implementing and Sustaining Antimicrobial Resistance Surveillance Programs in Developing Countries

By

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Dedication

This thesis is dedicated to my son, Nathaniel Jayce. All my work has led to being able to bring you into this world and be able to give you the world. You are my pride and joy, and I cannot wait to see you grow up.

Biography

Takiyah Ball MS, MPH grew up in Kennesaw, GA, and later graduated from The University of Georgia with a double BS in Microbiology, and Cellular Biology, an MS in Animal Science focused on Reproduction and Physiology. She has an MPH with a focus in Prevention Science from the Rollins School of Public Health at Emory University. Her previous work includes being a microbiological technician in the NARMS program at the USDA-ARS in Athens, GA. Her Ph.D. project is to assist in implementing a surveillance system in Uganda for antimicrobial resistance of *Salmonella* and *E.coli* in collaboration with the World Health Organization, Makerere University Veterinary School, and the Ugandan Public Health Department. Upon completion of her Ph.D., Ms. Ball would like to pursue a career in international outbreak investigation and implementation of surveillance systems in developing countries about antibiotic resistance.

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I would like to thank my mentor for over 17 years, Dr. Paula Fedorka-Cray. She has been the one who has shaped my career and my life. It will be sad to finally go off on my own, but I am sure our paths will cross many times in the future. I would also like to thank my committee, Dr. Maria Correa, Dr. Awa Aidara-Kane, Dr. Megan Jacob, and Dr. Andrew Stringer for helping to guide me through this process. No matter how much education I have, there is always more to learn, and they showed me that. I would also like to acknowledge all those that played a part in this process, without them it would not have been possible.

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Chapter 1: Introduction

Introduction

The antimicrobial surveillance programs which have been sustained over a period of time are important as they provide data which illustrate changes in antimicrobial resistance (AMR) and permit trend analysis to be conducted; they are also useful as they may help identify newly emerging or re-emerging resistant pathogens (Masterton, 2008). Examples of emerging pathogens include *Shiga.toxin Escherichia coli* (Griffin & Karmali, 2017) and *Salmonella* 4,[5],12:i:- (Arnott et al., 2018). A surveillance system can also aid in informing possible threats and illness burdens with populations by detecting various shifts in susceptibility in organisms (Bax et al., 2001; A. P. Johnson, 2015). When using surveillance data, it may also be possible to develop mitigation strategies to control AMR. Not only does surveillance provide invaluable information on resistance, but it also provides information on pathogen incidence and prevalence (Masterton, 2008). Use and in particular misuse of antimicrobials are just some contributing factors for resistant to develop (Bronzwaer et al., 2002). Monitoring the usage of antimicrobial drugs can be one means to help control the increasing problem of AMR (Bronzwaer et al., 2002). Although surveillance programs seem like the ideal way to manage AMR, many factors must be considered before implementing a surveillance program. Factors include the populations to be studied, program funding, political and economic public policies, sampling methods and associated costs, the organisms to be studied, the methodology of susceptibility testing, and dissemination of results (Bax et al., 2001).

When assessing surveillance data available on resistance in the food chain and food-producing animals, major gaps in data are seen. Data sharing is one aspect that can impact analytics of surveillance data which in turn can impact the health and well being of animals and humans, especially when it relates to treatment. An integrated (and harmonized) surveillance

program to compare data from both humans and animals, which includes data sharing, will be an important tool in combatting the AMR problem (WHO, 2014).

The ecosystem of AMR involves many components including food animals and associated meatstuffs, the environment, and the human population; this makes AMR a critical and relevant One Health and One World Issue (Robinson et al., 2016). In this study, we evaluate a process for a laboratory in Uganda to develop a framework to implement an AMR surveillance program successfully. The objectives of this project are to:

- Evaluate laboratory capacity and provide recommendations for the development and implementation of an AMR surveillance system in food animals at the University of Makerere in Kampala, Uganda
- Conduct a pilot study examining the prevalence and phenotype of *Salmonella* and *E. coli* isolated from cattle and chicken farms in the Wakiso district of Uganda over a one year period
- Genotypically characterize *Salmonella* and *E. coli* isolated from cattle and chicken farms in the Wakiso district of Uganda

The overall goal is to assist in the implementation of antimicrobial resistance surveillance programs in developing countries, such as Uganda, which will encourage successful, sustainable and harmonized programs that will result in international integrated data systems to monitor AMR.

Chapter 2: Literature Review

Literature Review

Antimicrobials and Development of Resistance

Antimicrobials

The terms ‘antibiotic’ and ‘antimicrobial’ are often used interchangeably in the literature. However, between the two there are important differences. Antibiotics are naturally produced from molds or bacteria. Antimicrobials, however, can be chemically synthesized (Quinn, Markey, Carter, Donnelly, & Leonard, 2002; Salyers & Whitt, 1994). In addition to killing (bactericidal) or inhibiting (bacteriostatic) fungi and bacteria, (Quinn et al., 2002; Salyers & Whitt, 1994) they can also kill some viruses particularly when chemical sanitizers are used. For this thesis, the term ‘antimicrobial’ is used.

Alexander Fleming reported the first antibiotic, penicillin, a compound naturally produced from a mold. Florey and Chain later purified penicillin for clinical use (Quinn et al., 2002). Interestingly, most antibiotics used in human medicine occur naturally. Chemically synthesized antimicrobials include sulfonamide drugs, which were discovered in the 1930’s, quinolones in the 1960’s, and oxazolidinone in the 2000’s (Walsh, 2003).

Since their discovery, antimicrobials were/are categorized as either broad or narrow spectrum antimicrobials. They can also be categorized further into classification schemes such as penicillins, macrolides, cephalosporins, and fluoroquinolones (B. Berger-Bachi, 2002; Salyers & Whitt, 1994). Broad-spectrum antimicrobials work on Gram-negative and Gram-positive bacteria; Gram-positive bacteria are typically more sensitive to their action. While broad-spectrum antimicrobials can kill pathogens, they can also disrupt resident microflora resulting in a microbial population change in gut composition (Acar, 1997). Narrow-spectrum

antimicrobials are typically directed toward a target bacterium and minimize disturbance against the microflora of the body (Yao et al., 2016).

Antimicrobials are sorted into classes based on their mechanism of action against bacteria. Antimicrobial classes include β-Lactams, glycopeptides, aminoglycosides, tetracyclines, macrolides and lincosamides, quinolones, and trimethoprim and sulfonamides (B. Berger-Bachi, 2002; Salyers & Whitt, 1994).

Beta-Lactams are bactericidal antimicrobials that inhibit cell wall synthesis. They consist of penicillins, cephalosporins, carbapenems, and monobactams. All contain a β-Lactam ring and inhibit the last step in peptidoglycan synthesis of microorganisms. They account for approximately one.half of all antimicrobials used, considered broad spectrum, and are effective against Gram-negative and Gram-positive bacteria (B. Berger-Bachi, 2002; Salyers & Whitt, 1994). Cephalosporins are traditionally divided into four generations which have pharmacokinetic differences. The first generation is known to be more effective against Gram-positive organisms, while the second generation is more effective against Gram-negative organisms. Third generation cephalosporins are effective against Gram-negative, but not effective against β-Lactamase enzymes in Gram-negative organisms. Fourth generation act as the same as the third generation, but are also effective against Gram-positive organisms and are more stable against β-Lactamase enzymes (Scholar, 2007).

Glycopeptides are antimicrobials that inhibit peptidoglycan synthesis and include vancomycin and teicoplanin. Their primary targets are Gram-positive bacteria including MRSA (B. Berger-Bachi, 2002; Salyers & Whitt, 1994). Vancomycin is very effective against life.threatening Gram-positive bacteria, where others are less toxic and not effective, for example, *Clostridium difficle* (Kuriyama, Karasawa, & Williams, 2014)

Aminoglycosides are produced by strains of Streptomyces, Micromonospora, and Bacillus species and account for about three percent of all antimicrobials used (Quinn et al., 2002; Salyers & Whitt, 1994). Common aminoglycosides include kanamycin, gentamicin, streptomycin, and neomycin (Salyers & Whitt, 1994). These antimicrobials inhibit protein synthesis by preventing the 30S subunit of the bacterial ribosome from binding to the 50S subunit (Quinn et al., 2002; Salyers & Whitt, 1994) They are active against Gram-negative bacteria and are typically bacteriocidal. Aminoglycosides are usually held in reserve until treatment failure of other antimicrobials occurs (Salyers & Whitt, 1994).

Tetracyclines are broad-spectrum antimicrobials that inhibit the 30S subunit of bacterial ribosomes by distorting the tRNA's A site where it cannot align with the mRNA codon. They are typically derived from individual tetracyclines include chlortetracycline and oxytetracycline, commonly used in animal production to promote growth. Tetracyclines are bacteriostatic and most often used in human medicine to treat acne and Lyme disease (Salyers & Whitt, 1994).

Macrolides include erythromycin, oleandomycin, spiramycin, and tylosin and account for 11% of antimicrobials used. Erythromycin is often used for treatment in people with allergies to penicillin. They prevent the elongation of the 50S subunit and the translocation of the ribosome and are derived from a strain of actinomycetes. Macrolides are bacteriostatic and bacteriocidal against Gram-positive bacteria (B. Berger-Bachi, 2002; Salyers & Whitt, 1994).

Quinolones are bactericidal antimicrobials that include ciprofloxacin, nalidixic acid, enrofloxacin and all newer fluoroquinolones. Quinolones inhibit nucleic acid synthesis by inhibiting the β subunit of the DNA gyrase from supercoiling in DNA replication (B. Berger-Bachi, 2002; Quinn et al., 2002; Salyers & Whitt, 1994). They also inhibit topoisomerase IV, which helps in dividing the chromosome for replication (Axelson, 2002). They do not appear to

completely degrade when excreted and may affect the development of and/or maintenance of resistance in farm environments, particularly in poultry (Mandell & Tillotson, 2002)

Trimethoprim and sulfonamides act as competitors to bacteria and prevent the production of tetrahydrofolic acid (THF) (B. Berger-Bachi, 2002; Quinn et al., 2002; Salyers & Whitt, 1994). Sulfonamides inhibit dihydrofolic acid synthesis by binding to dihydropteroate synthetase, while trimethoprim inhibits THF by binding to dihydrofolate reductase (Gleckman, Blagg, & Joubert, 1981; Kalkut, 1998).

Sometimes antimicrobials are not effective enough to treat alone, especially against multi.drug-resistant bacteria. The lack of efficacy is mainly due to misuse of antimicrobials leading to resistance. Therefore, antimicrobials are potentiated to increase the efficacy (Corbett et al., 2017; Hare, 1960). Examples of these antimicrobials are augmentin, which combines amoxicillin (β-Lactam antibiotic) with clavulanic acid (β-Lactamase inhibitor). Together they inhibit cell wall biosynthesis (Worthington & Melander, 2013).

As previously mentioned, bacteria have developed resistance against antimicrobials throughout the years. Examples of resistance mechanisms include the absence of the structure for antimicrobials to inhibit (i.e. cell walls), microorganisms that are impermeable to antimicrobials, microorganisms that inactivate the antimicrobial, modification of the antimicrobial's target, and utilizing an efflux pump to expel the antimicrobial (B. Berger-Bachi, 2002; Salyers & Whitt, 1994). Bacteria have a way of resisting the effects of the antimicrobials found within all antimicrobial classes. For example, bacteria can carry a β-Lactamase enzyme which cleaves the β-Lactam ring causing inactivation of the antimicrobial (Berger-Bachi, 2002; Shaikh, Fatima, Shakil, Rizvi, & Kamal, 2015). Penicillin.binding proteins can also be altered which do not allow the antimicrobial to bind while permitting peptidoglycan synthesis to

continue (Georgopapadakou, 1993). Microorganisms harbor genes that do not allow binding of glycopeptides which allows transpeptidase to continue (Pootoolal, Neu, & Wright, 2002). Cytoplasm proteins, enzymatic inactivation, and efflux proteins are used against tetracyclines (Speer, Shoemaker, & Salyers, 1992) and bacteria can methylate the 23S subunit on the rRNA inhibiting binding of macrolides and lincosamides (Nakajima, 1999). Bacteria also have point mutations to alter the affinity of gyrase, which confers resistance to quinolones (Hooper & Jacoby, 2015). Point mutations are also commonly found which affects the mode of action of trimethoprim and the sulfonamides (Skold, 2000).

Infections associated with antimicrobial resistance (AMR) have been increasing yearly, and resistant infections are predicted as the cause of over 10 million deaths per year by 2050 with an associated cost over $100 trillion (Mckenna, 2014). In the last 30 years, there have not been any new antimicrobials developed to treat infectious disease until recently. Teixobactin is a recently marketed antimicrobial used to inhibit the cell wall synthesis of Gram-positive bacteria (Ling et al., 2015).

Methodology for Determining Antimicrobial Resistance

Antimicrobial resistance testing methods have evolved particularly as technology has improved, and automation occurred. One early testing method still in use in many developing countries is disk diffusion, also known as Kirby Bauer testing. Disk diffusion includes dissolving antimicrobials in a liquid or agar medium then soaking paper disks with the solution before placement on top of a bacterial lawn (Jorgensen & Ferraro, 2009; Schoenknecht, 1973). A clear zone, called the "Zone of Inhibition," will occur following incubation and growth of the agar plate; this represents the diffusion and effect of the drug. The zone is related to the degree inhibition has occurred; the lower the susceptibility of the bacterial population under test, the

larger the clear area will be (Axelson, 2002; Clinical and Laboratory Standards Institute, 2009). In contrast, resistance to the antimicrobial is recorded when bacterial growth occurs at the edge of the antimicrobial disk or the clear zone is below a certain millimeter cut-off.

Another testing method includes determination of the minimal inhibitory concentration (MIC), which gives the highest dilution to inhibit growth (Axelson, 2002; Clinical and Laboratory Standards Institute, 2009). The early testing methodology included 2-fold serial dilutions of the antimicrobial in test tubes followed by inoculation with a standard amount of bacteria (typically calibrated by nephelometer). The tubes were incubated overnight, and the concentration of the antimicrobial in the last tube inhibiting growth is considered the MIC. New methods include the use of custom-made 96-well panels with different concentrations of antimicrobials. Microorganisms are standardized using a McFarland standard in broth followed by standardized inoculation into the panel. Incubation occurs for 18-24 hours an automated, calibrated reader will determine the MIC level of resistance (Thermofisher Scientific). Conversely, the panel can also be manually read and results recorded. The most recent means of determining resistance is to determine the presence of antimicrobial resistance genes using next-generation sequencing (Koser, Ellington, & Peacock, 2014). Having the bacterial genome sequenced allows AMR determinants to be identified within its genome. Concordance between the resistance phenotype using susceptibility testing methodology compared to sequencing has been reported (McDermott et al., 2016).

Plasmids

Resistance genes are often located within the chromosome or on a plasmid which can be transferred between bacteria. Plasmids contain genes which confer resistance to antimicrobials and can stimulate conjugation, passing the resistant plasmid to another susceptible

microorganism (Stone, 1975). For example, with aminoglycosides, genes within the R plasmids can phosphorylate or acetylate the drugs resulting in a lack of efficacy. In penicillins, genes on R plasmids enable the split of the β-Lactam ring. Each gene in an R plasmid is specific for rendering a particular antimicrobial ineffective (Salyers & Whitt, 1994).

No one factor in human treatment or animal production has been identified which can alter or prevent the development of resistance once an antimicrobial has been used. Using antimicrobials typically results in the development of a resistant population of bacteria. Human and animals are reliant on the immune system to clear infectious bacteria. However, if a population of resistant bacteria is retained, they can re-emerge in the presence of low doses of antimicrobials or when antimicrobials are used for treatment. In humans, a weakened immune state may cause resistant bacteria to proliferate compounding treatment options. Some animal husbandry practices may affect the likelihood that resistance will develop or persist. Animal husbandry can contribute to resistance due to the overcrowding (stress) and poor hygiene of the animals (Watts & Lindeman, 2006). Preventing antimicrobial resistance can include combining therapy, when two antimicrobials that are unrelated are used in treatment (Salyers & Whitt, 1994), discontinuing antimicrobials as growth promoters and educating the farmers about the advantages of efficacious vaccines or other immunotherapies (Emborg, Ersboll, Heuer, & Wegener, 2001).

Gram-Negative Bacteria

Salmonella

Salmonella, named by the veterinarian and bacteriologist Daniel Salmon is a Gram-negative, rod-shaped, non-lactose forming facultative anaerobe. It is a member of the non-spore

forming bacillus in the family of *Enterobacteriaceae* (Cima G, 2013; Guthrie, 1991). There is a 90% molecular homology between *Salmonella* and *E. coli* (Salyers & Whitt, 1994). Most of the time, it is hard to distinguish between *Salmonella* and *E.coli* microscopically; on some media, they also exhibit similar morphologies (Jay, Davos, Dundas, Frankish, & Lightfoot, 2003). Phenotypically, they appear as raised colonies on agar and *Salmonella* are about 2.4 mm in diameter with round and smooth edges (Gast, Porter, & Hold, 1997).

Salmonella growth requirements include a pH ranging from 4-9 in temperatures ranging from eight to 45°C on various media (Gast et al., 1997; Jay et al., 2003). The recommended temperature for growth is 37°C (Gast et al., 1997; Guthrie, 1991; Jay et al., 2003). *Salmonella* is typically cultured from feces. However, since it is ubiquitous, it has also been recovered from environmental sources including sewage, feed, and water (Guthrie, 1991). Recovery from septicemia via blood occurs less often (Guthrie, 1991).

Enrichment, used to increase the number of *Salmonella* cells, is widely advocated and various media are available for use. Selective enrichment is preferred to block the growth of unwanted bacterial species (Gast et al., 1997). Typical enrichment broths that are used for culture of salmonellae include Gram-negative Hajna (GN), tetrathionate (TT) (with or without supplement), and Rappaport-Vassiliadis (RV) Broths (Fedorka-Cray, Bush, Thomas, Gray, & McKean, 1996; Jay et al., 2003). GN, RV, and TT are recommended at incubation temperatures of 37°C for 18 to 48 hours (Fedorka-Cray et al., 1996). Agar medium, used to visualize the growth of salmonellae*,* include Brilliant green (BG), Xylose-Lysine-Tergitol 4 (XLT.4), Lysine Iron (LIA), and Triple Sugar Iron Agar (TSI) (Fedorka-Cray et al., 1996). Agar media typically relies on a chemical reaction to components within the media, such as a black appearance on XLT-4 agar, a result of hydrogen sulfide production (Hardy Diagnostics, 2017).

Salmonella can be destroyed by heating to temperatures above 70°C or irradiation (Gast et al., 1997; Guthrie, 1991). However, heat tolerance has been reported and may affect killing (Shachar & Yaron, 2006). Disinfectants such as hydrogen peroxide (Hebrard, Viala, Meresse, Barras, & Aussel, 2009), chlorine (H. Wang & Ryser, 2014), and trisodium phosphate are very effective against *Salmonella* (Sarjit & Dykes, 2015).

Salmonella is characterized by three antigens; the O (liposaccharide layer), H (flagellar antigen), and Vi (virulence antigen) and over 2500 serovars, typically named after the geographic location from where it was recovered, have been reported (Gast et al., 1997; Giannella, 1996; Jay et al., 2003).

Serotypes can be further broken down into two species, *Salmonella enterica* which is further divided into six subspecies and *Salmonella bongori* (Popoff & Le Minor, 1997). Within these subspecies are many serotypes, some that are host specific. Host specific examples include *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Paratyphi in humans, *Salmonella enterica* serovar Pullorum and *Salmonella enterica* serovar Gallinarum in poultry, *Salmonella enterica* serovar Dublin in cattle, and *Salmonella enterica* serovar Cholereasuis in pigs. The most common serotypes worldwide originating in both humans and animals include *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium (WHO, 2016).

Virulence Factors enhance the ability of microorganisms to cause disease. These factors include hydrolytic enzymes, bacterial cell proteins and carbohydrates that protect them from killing by the host, surface proteins that enable bacteria attachment, and toxins (L. Chen et al., 2005). *Salmonella* has several virulence factors that allow it to invade the host cell. One type of factor includes the type three secretion system (TTSS) which allows *Salmonella* to secrete its effector

proteins through a needle-like structure into the host cell. Other virulence factors include adhesion factors which allow better attachment to the membrane of the intestine. Pathogenicity islands are factors that have specific phases in the development of infection. Two of these phases include SP1 which allows invasion of the epithelial cell and SP2 which mediates macrophage survival (Groisman & Ochman, 1996). Another virulence factor of *Salmonella* is the lipopolysaccharide (LPS) that contains lipid A endotoxin that triggers inflammatory mediators to induce degranulation (Rosenberger, Scott, Gold, Hancock, & Finlay, 2000).

Disease resulting from infection with *Salmonella* is usually called salmonellosis (WHO, 2016). Animals can be a perfect host in which bacteria can survive and facilitate the transfer of bacteria from animals to humans, human to human, and animal to animal (Clarke & Gyles, 1986). This transmission between animals and humans make *Salmonella* a zoonotic pathogen. This transfer can occur from eating contaminated foodstuffs, direct contact, water or aerosolization. Most human infections result from consuming contaminated foods (Fedorka-Cray et al., 1996; Guthrie, 1991). Infections begin in the mucous membranes including the mouth, urinary, respiratory, and gastrointestinal sites. Within the gastrointestinal tract, the stomach harbors the fewest colonies of bacteria due to its low pH, whereas the colon harbors a larger number of bacteria. Disease as a result of *Salmonella* infection in humans are typically gastroenteritis; however, infection with certain serovars results in typhoid or enteric fever (Madigan, Martinko, & Parker, 1999). Symptoms of gastroenteritis include nausea, fever, abdominal pain, diarrhea, depression, anorexia, and pneumonia after 12 to 14 hours of ingestion of foods containing an infectious dose of *Salmonella*. Symptoms are typically self-limiting and last for two to seven days. The elderly, young and immunocompromised persons are at the highest risk for severe infection (WHO, 2016). A severe case of infection leads to dehydration, loss of electrolytes, and acid-base

imbalance which may result in death (Clarke & Gyles, 1986). After the infection has resolved, the person can become an asymptomatic carrier of *Salmonella* (Guthrie, 1991).

Escherichia coli

Theodore Escherich, a German bacteriologist, discovered the *Escherichia coli* (*E. coli*) bacterium in 1885, from the colon of a human (Shulman, Friedmann, & Sims, 2007). *E. coli* is a species within the coliform group along with *Klebsiella, Enterobacter,* and *Citrobacter.* These coliforms are Gram-negative, rod-shaped, facultatively anaerobic bacteria (Singleton, 1999). *Escherichia coli* often resides in the intestines of warm-blooded organisms and are shed through fecal matter (Russell & Jarvis, 2001; Singleton, 1999).

Growth conditions for *E. coli* include incubation temperatures ranging from 36°C to 49°C. Enrichment broths, such as lysogeny broth (LB), and bacterial media that contain ingredients such as ammonium phosphate, dibasic acid, glucose, magnesium sulfate, monobasic acid, potassium phosphate, sodium chloride, and water are used to culture *E. coli* (Ingledew & Poole, 1984). A common media broth for the growth of *E. coli* is LB, Tryptone, Terrific broths (Lessard, 2013). *Escherichia coli* can be killed by the same means as *Salmonella* including high temperatures and use of strong disinfectants.

Escherichia coli is categorized by serotype based on their antigenic formula, which includes the O antigen (lipopolysaccharide layer), the K antigen (capsule) and the O antigen (flagellin) (Brenner, Krieg, & Staley, 2005; L. Wang, Rothemund, Curd, & Reeves, 2003). These are few virulence determinants harbored by some pathogenic *E. coli*.

Virulence properties and serological characteristics aids in classifying *E. coli*. These features include ways that the bacterium attaches to the host cell, produce toxins, affect the attachment of

host cell, and invades the host cell. Based on these characteristics, the bacteria is virotyped. Examples of *E. coli* virotypes include the following Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), and Enterohemorrhagic *E. coli* (EHEC) cause diarrhea in animals and humans. Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), and Adherent. Invasive *E. coli* (AIEC) cause diarrhea only in humans (Salyers & Whitt, 1994). Other virulent strains of *E. coli* include Shiga-toxin producing *E.coli* (STEC), otherwise known as *E. coli O157: H7*, urinary tract *E.coli* (UTEC), and neonatal meningitis *E. coli* (NMEC) (Todar, 2008). The most common virulence factors known to assist in invading host cells include toxins, pili, and fimbriae (Salyers & Whitt, 1994).

Diseases of *E. coli* include gastroenteritis, urinary tract infections, and neonatal meningitis. Virulent strains can cause septicemia, diarrhea, hemolytic-uremic syndrome, and pneumonia (Todar, 2008). Disease typically require treatment with therapeutics or supportive care.

Surveillance Programs

With the background information presented in the previous sections for AMR and pathogenic Gram-negative bacteria such as *Salmonella* and *E. coli,* programs to monitor antimicrobial use and susceptibility of bacteria to commonly used antimicrobials in humans and animals have been implemented worldwide to the emerging and increasing resistance issue. These programs are most often denoted as surveillance systems.

The medical definition of surveillance is a “close and continuous observation or testing”(Webster Dictionary). The Center for Disease and Prevention (CDC) definition of surveillance is “Epidemiologic surveillance is the ongoing and systematic collection, analysis, and interpretation of health data in the process of describing and monitoring a health

event"(CDC, 1988). It involves collecting and analyzing data to report any threats related to disease and the health of the public (A. P. Johnson, 2015). There are many programs which monitor disease risk on a large scale and other programs that focus on specific disease threats around the world (Bax et al., 2001). In the last 30 years, a limited number of country surveillance programs have been implemented to monitor threats against antimicrobial resistance. Surveillance programs that currently exist which focus on antimicrobial resistance include DANMAP (Denmark), NARMS (United States), CIPARS (Canada), GERM.VET (Germany), JVARM (Japan), NORM/NORM.VET (Norway), SWEDRES (Sweden), NETHMAP/MARAN (Netherlands), ONERBA (France), and FINRES_VET (Finland) (A. P. Johnson, 2015; WHO, 2014). To properly make informed decisions regarding treatment of these infections, knowledge of resistance among these pathogens, which is ascertained over time through data collected in these surveillance systems, is critical for the formulation of treatment plans (A. P. Johnson, 2015).

Implementing a surveillance system for antimicrobial resistance requires a number of considerations. One major question is – "What is the purpose of the surveillance?'. This drives the type of information collected by clinicians, researchers, drug formularies, and policy regulators. These data must be available for use within all boundaries including local, national, and global levels (Bax et al., 2001).

Roadblocks will occur throughout that the process of implementing a surveillance program. It is important to design the study correctly from inception. For example, will the study include disease-based research or just the organism that causes the disease? Choosing the right population to sample is also important. Once these are known, next steps are to choose the sampling methods, the organisms to be studied, the susceptibility testing method(s), how the

results will be handled, what statistical programs will be used, and how the study will be funded (Bax et al., 2001). Communicating the results is the final step which includes disseminating findings to policymakers and the public.

Another question to answer after the surveillance has been implemented for a period of time is whether the program has achieved its goal(s). For example, is there a better control for resistance or antimicrobial to use in the testing platform?

Other concerns in implementing surveillance programs that have to be taken into account or sampling biases in AMR surveillance. The definition of biases is "systematic errors that may occur in collecting or interpreting data." In AMR surveillance six biases have to be taken into consideration when implementing, and they include the following: denominator data, case definition, case ascertainment, sampling biases, multiple counting, and lab practices and disease (Rempel & Laupland, 2009).

Program Evaluation

The best means for evaluation is to have program evaluations performed by an outside organization. The definition of program evaluation is "the systematic application of scientific methods to assess the design, implementation, improvement or outcomes of a program" (Rossi & H.E., 1993). There are many purposes for conducting an evaluation. A list of a few is as follows: (Short, Hennessy, & Campbell, 1996).

- "Demonstrate program effectiveness to funders
- Improve the implementation and effectiveness of programs
- Better manage limited resources
- Document program accomplishments
- Justify current program funding
- Support the need for increased levels of funding

- Satisfy ethical responsibility to clients to demonstrate positive and negative effects of program participation
- Document program development and activities to help ensure successful replication."

After the program evaluation is completed, there will be recommendations for those leading the surveillance program related to concerns, need for change, or recommendation for improvement. These recommendations benefit the organization and allow them to improve or achieve success and maintain a sustainable program (CDC, 1988).

Public Health Impact of Surveillance Systems for Antimicrobial Resistance

The World Health Organization (WHO) has reported that the rates of bacterial resistance are increasing and causing community.acquired and health-care associated resistant infections. They noted that there is also a lack of coordination, data sharing, and standardized methods leaving significant gaps in surveillance information, globally (WHO, 2014). The World Health Assembly initiated the Global Action Plan (GAP) in 2014, to ensure effective treatment and prevent infectious diseases, by using medications correctly. Since the inception of the GAP, countries were encouraged to implement their national action plan according to GAP guidelines, which includes combating AMR (WHO, 2015a). From here, the Global Antimicrobial Resistance Surveillance System (GLASS), was implemented by WHO, to foster AMR data collection and to report globally (WHO, 2017a). As mentioned in previous sections, AMR surveillance is critical for public health and for generating mitigation and control approaches to combat AMR. Through actions of implementing appropriate drug-use policies, improved diagnostics practices, reduced rates of infection transmission, and preventing and controlling AMR in agriculture public health can be improved (CDC, 2012; Masterton, 2008).

The WHO has been working diligently with the Food and Agriculture Organization (FAO) and The World Organization for Animal Health (OIE) in a tripartite coalition to attack the

AMR problem through a multidisciplinary and intersectoral collaboration at the global level. In 2008, the WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (WHO.AGISAR) was formed to support WHO in lessening the impact of resistance attributed to antimicrobial use in food-producing animals. AGISAR is also responsible for continuously updating the Critically Important Antimicrobials list. These collaborations help gather data and information to support policies at the national and global levels to develop good practices for animal husbandry (WHO, 2014). AGISAR provides grant funding to selected developing countries to support focused research projects and country pilot projects related to AMR. Uganda is one of several countries that received funding for a country research project, which will be discussed in the next section.

Uganda

Demographics

Uganda is situated on the east side of Africa, right on the equator, bordering Kenya, Rwanda, South Sudan, Democratic Republic of Congo, and Tanzania. Uganda is comprised of 111 districts and the capital city, Kampala. As of today, the population is 41.5 million, of which 49.9% is male, and 50.0% is female. Uganda has a multi-party political system and has been led by President Yoweri K. Museveni, since 1986. Table 2.1 highlights the demographics of Uganda in 2016.

Table 2.1: Demographics of Uganda

Population **Male** **Female**	41.49 Million 49.9% 50.0%	**Area (sq*km)**	241,550.0
Life Expectancy at Birth (Years)	59.9	**Population Growth Rate**	3.3%
GDP Growth rate	4.6%	**Languages**	English, Swahili, Luganda
Literacy Rates **Males** **Females**	78.4% 85.3% 71.5% (2015)	**Per Capita Income**	660 USD

Source: (http://databank.worldbank.org/data/reports.aspx?source=2&country=UGA).

Population Health Status

There are many health.related infections and diseases affecting the health of the Ugandan population. From the Global Burden of Disease (GDB) Compare, a tree map of the world's health levels and trends from 1990-2015, the top 10 death related causes in Uganda are listed in Table 2.2 (Institute for Health Metrics and Evaluation, 2016).

Table 2.2: Top 10 Causes of Death in Uganda (2015)

Top 10 Causes of Disease in Uganda (2015)	Percentage (%)
HIV	10.43
Lower Respiratory Infection	7.3
Malaria	6.63
Diarrhea	5.17
Neonatal Encephalopathy	4.76
Congenital Birth Defects	4.54
Neonatal Preterm	4.07
Meningitis	3.57
Neonatal Sepsis	3.14
Hemoglobinopathies and Hemolytic Anemias	2.89

Source: http://vizhub.healthdata.org/gbd.compare/

Agriculture Demographics

Uganda is approximately 19,997.38 hectares (ha). Figure 2.1 (FAO, 2015) shows the distribution of land use throughout the country. As shown, arable land (land used for growing crops) accounts for the largest land area covering approximately 32.4% of available land.

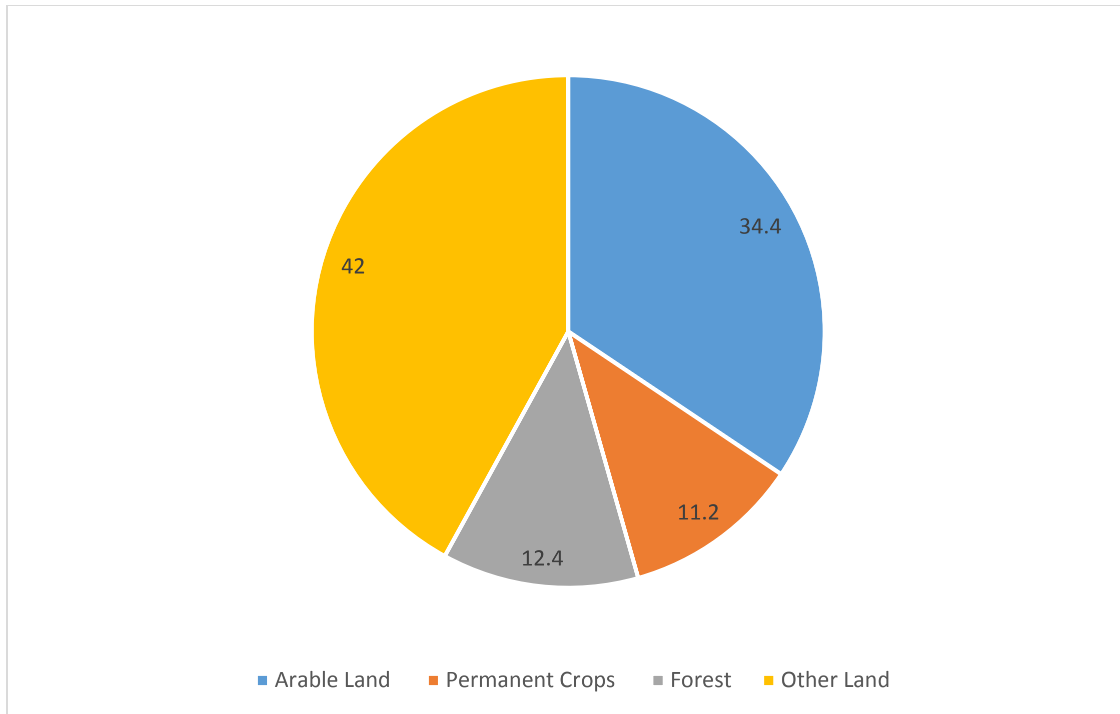

Figure 2.1: Land Use in Uganda (2012)
Source:http://uganda.opendataforafrica.org/gallery/agriculture

The primary food animal commodities in Uganda include cattle, sheep, goats, pigs, and poultry. Figure 2.2 below shows the livestock numbers from 2012-2014. As a result of exporting, sheep, goat, and pig numbers have decreased. Cattle and poultry demand in the country has increased and seems to be continuing (Uganda Bureau of Statistics, 2015).

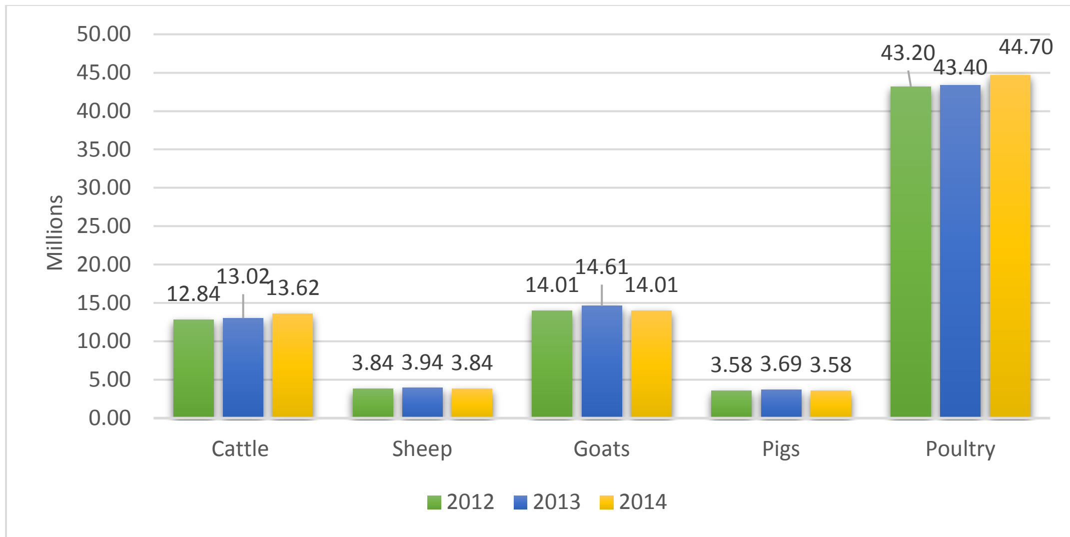

Figure 2.2: Livestock Numbers in Uganda (2012.2014)

Figure 2.3 displays the cattle production distributed throughout the country of Uganda (Balikowa, 2011). Poultry production has not had a census since 1991, so there is a huge gap in data to determine how many poultry producing farms are in Uganda (FAO, 2008).

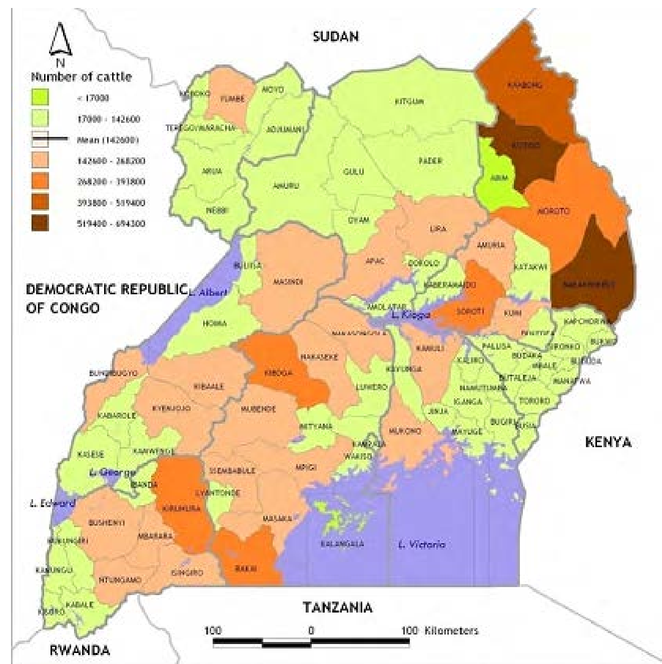

Figure 2.3: Cattle Distribution in Uganda

Uganda Antimicrobial Use and Resistance

Antimicrobial Use: There are no available records in Uganda, regarding the quantities of imported and exported antimicrobials. Existing reports indicate that many antimicrobials used in Uganda were manufactured primarily in India. The antimicrobials used most often include ciprofloxacin and amoxicillin (Foster, Sosa, Najjuka, & Mwenfa, 2011). Although the antimicrobials are being imported, many believe that these drugs are of substandard quality (UNAS, CDDEP, GARP-Uganda, Mpairwe, & Wamala, 2015). The most commonly used antimicrobials in veterinary practice are tetracycline and penicillin (UNAS et al., 2015). As far as the quality of antimicrobials, approximately 94% are acceptable but counterfeit drugs are encountered, and companies from India have been banned from importing as a result. Due to limited resources, The National Drug Administration (NDA) cannot control all the counterfeit drugs coming in from Asia, Middle East, and Europe (UNAS et al., 2015).

The use of antimicrobials in agriculture, poultry, veterinary practice, milk and meat products all have the same issues, which include inadequately trained staff, farm managers, veterinary assistants, animal husbandry officers and veterinary practitioners. Use of antimicrobials is often accomplished with little regard to proper evaluation and regulations for treating animals. The Ministry of Agriculture (MOA) has the authority over antimicrobial use in Uganda, but due to lack of resources and human power, there is limited control and enforcement on how antimicrobials are being regulated. Because of the lack of regulation, farmers, veterinarians, and managers take it upon themselves to diagnose and treat disease (UNAS et al., 2015). Antimicrobials are accessible countrywide, often over-the-counter. Farm to table supervision that has not been implemented as recommended by the Food and Agriculture Organization (FAO). There is also a lack of veterinary diagnostic services, and few laboratories

are available to perform analysis, including antimicrobial susceptibility testing, on microorganisms (UNAS et al., 2015).

Many modern testing platforms for conducting susceptibility testing are used by economically advantaged countries. These include expensive, high throughput equipment such as VITEK® (Biomeruix Inc) or Sensititre (Thermofisher Scientific). Inexpensive methods including broth or agar dilution tests and disc diffusion are often used in resource-limited countries like Uganda. Disk diffusion is routinely used and easy when performing quality assurance. A set of standards for conducting disk diffusion as well guidelines provide interpretive criteria for determining breakpoints of antimicrobials. North America uses the Clinical Laboratory Standards Institute (CLSI) recommendation and guidelines for susceptibility methodology; however, CLSI guidelines are not readily available to everyone because of costs (Gelband, 2016). Other guidelines are available for free such as the European Committee on Antimicrobial Susceptibility Testing (EUCAST) documents. However, both programs are not identical, and CLSI is more widely used around the world (Gelband, 2016). To subside the costs for countries with limited resources, WHONET was created as a free, user-friendly and technically supported software program that includes all CLSI, EUCAST, and other interpretive criteria needed to analyze susceptibility data (Gelband, 2016).

Uganda prescribers follow guidelines created by the Ministry of Health (MOH), Uganda Clinical Guidelines. The MOH states “It is designed to provide updated, practical and useful information for both upper and lower level health facilities on the diagnosis and management of common conditions present in Uganda” (Ministry of Health, 2014). It was later reported that these guidelines were never used because many questioned the source of information (UNAS et al., 2015).

According to UNAS et al., three important factors are required for accelerating the mitigation of antimicrobial resistance. These factors include economic power, knowledge, and information. Uganda lacks economic power and consumer's knowledge regarding many aspects of antimicrobials including mode of action, misuse of over the counter drugs, and incorrect self-medication (UNAS et al., 2015). Many healthcare workers do not have the knowledge or information to provide proper health care to patients and animals. It was reported that almost all (99%) of the healthcare workers did not know the guidelines related to prescription of effective antimicrobials (Kamulegeya, William, & Rwenyonyi, 2011).

The MOH recognizes the need for surveillance for antimicrobial, antimalarial, and antiretroviral resistance. They believe it will require minimizing importation of substandard drugs, regulating use by prescribers, and self.medication controls (UNAS et al., 2015).

Antimicrobial Resistance: It is believed that the first antimicrobials introduced to Uganda were sulphonimides. Table 2.3 was reported by UNAS et al. showing when antimicrobials were first introduced into Uganda (UNAS et al., 2015).

Table 2.3: Introduction of antimicrobials to Uganda

Antibiotic agent	Estimated year of use in Uganda	Major Impact of the Antibiotics
Sulphonamides	Late 1940s	Prevalence of tropical ulcers and gonococcal urethral stricture went down; yaws eliminated
Penicillin G Streptomycin	Late 1940s or early 1950s	
Chloramphenicol, Tetracycline, Neomycin, Nitrofurantoin, Metronidazole	Mid 1950s	Reduction of mortality due to enteric fevers, septicemias, and UTIs
Ampicillin, Co-trimoxazole, Erythromycin, Gentamicin	1960s	
Tobramycin, Rifampin	1980s	Management of gonorrhea and tuberculosis, which had grown resistant to earlier drugs
Cephalosporins, Fluoroquinolones	1990s	Management of bacterial resistance to earlier drugs
Vancomycin, Oleandomycin, Imipenem, Linezolid, Teicoplanin Daptomycin and other newer antibiotics	2000s	

For *Salmonella,* several antimicrobial resistance studies utilizing human, cattle, and other food samples were conducted from 1995 through 2014 (UNAS et al., 2015). It was reported that resistance during that period was observed most often to co-trimoxazole, ampicillin, tetracycline, and chloramphenicol. Resistance was observed in 50.85% of the isolates collected. About 20% of the isolates were resistant to amikacin and ceftriaxone (UNAS et al., 2015). For *E. coli*, studies show that there was a 40% resistance to the majority of the antimicrobials tested from 2007-2011(UNAS et al., 2015). Extended-beta lactamase (ESBL) testing has not been conducted in Uganda. The prevalence from published studies ranged from 10.75% among Gram-negative isolates. There is need surveillance on ESBL producing bacteria Uganda as it is an emerging public health concern (UNAS et al., 2015).

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Chapter 3: Phenotypic Characterization of *Salmonella* and *E. coli* from Cattle and Chicken Farms in the Wakiso District, Uganda

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Abstract

Background: Antimicrobial resistance (AMR) is a global concern with over 10 million deaths and $10 trillion in costs estimated to occur annually by 2050. The development of AMR in food-borne pathogens is of particular concern as treatment options may be compromised. Surveillance programs are needed to monitor resistance trends over time and guide treatment options.

Objectives: Estimate the prevalence of *Salmonella spp.* and commensal *E. coli* on cattle and chicken farms in the Wakiso District of Uganda and determine AMR phenotype of recovered isolates. Determine if seasonal differences in prevalence are significant.

Methods: Forty chicken and dairy farms were visited each season (rainy and dry); ten fecal environmental samples were collected per farm. Samples were cultured using standard laboratory methods for *Salmonella* and *E. coli*. AMR profiles were determined using the Sensitire^{TM} system per manufacturer's directions. Using a logistic regression model seasonal differences in prevalence were calculated.

Results: *Salmonella* and *E. coli* were recovered from 379 and 400 chicken and cattle samples, respectively. From chicken, *Salmonella* and *E. coli* prevalence was 13.5% (N=51) and 88.1% (N= 334), respectively. From cattle, *Salmonella* and *E. coli* prevalence was 1.3% (N=5) and 96.3% (N=385), respectively. *Salmonella* Enteritidis (31.7%) and Kentucky (21.6%) were most often recovered on chicken farms. Collectively, *Salmonella* and *E. coli* were most often resistant to tetracycline and sulfisoxazole. Eighty.nine percent and 23% of chicken and cattle isolates, respectively, were multi-drug resistant. All *Salmonella* Kentucky isolates were resistant to ciprofloxacin. ESBLs were detected in eight *E. coli* isolates. Resistance to the cephems, quinolones, and macrolides was observed among both organisms. A significant seasonal difference between chicken sampling periods was observed ($p= 0.0017$).

Conclusions: The emergence of MDR among both *Salmonella* and *E. coli* and the presentation of ESBLs among *E. coli* requires further investigation and monitoring over time. Further characterization of these isolates at the genotypic level is warranted.

Introduction

Salmonella is a food-borne pathogen and is one out of four leading causes of diarrheal diseases worldwide. Salmonellosis has been linked to more than 33 million deaths annually. Conversely, while most food-borne infections caused by *Escherichia coli* are less severe, some strains are associated with severe disease (WHO, 2016).

In Uganda, there is no reporting of death related to food-borne diseases caused by *Salmonella* spp. and commensal *E. coli* (UNAS et al., 2015). Unfortunately, most reports only indicate numbers of diarrhea-related deaths of unknown etiology. Therefore, it is difficult to estimate mortality linked to food-borne illnesses. Additionally, there is a lack of information on antimicrobial resistant (AMR) food-borne pathogens and no coordinated AMR surveillance system exists.

As a result of these information gaps, the World Health Organization (WHO) released a call for research proposals in developing countries to collect data on the prevalence and AMR of food-borne pathogens, including *Salmonella* and *E. coli,* in food animals, humans, and the environment (WHO, 2015a). Antimicrobial resistance is a growing issue that can render current treatment regimens ineffective resulting in higher health care costs for patients with resistant infections. AMR is also associated with increased morbidity and mortality (WHO, 2017a). If no changes or efforts are made to control AMR, by 2050, it is estimated that over 10 million deaths per year will be related to AMR infections (Mckenna, 2014).

Antimicrobial resistance is common among many different bacterial genera that humans, animals, and the environment harbor. Since it is difficult to separate the cause and effects of resistance among humans, animals, and the environment, studies need to employ One Health

concepts. Increased use of antimicrobials, including misuse among physicians and veterinarians, the ready availability of antimicrobials in developing countries, and exposure of the environment to antimicrobial and antimicrobial-like compounds often contribute to the resistance problem (UNAS et al., 2015).

In developing countries like Uganda, owning livestock is one means of income and employment. The income derived from livestock ownership contributes to economic survival among the majority of Ugandans. However, as food animals are often treated like family and production may occur near the family living quarters, environmental pollution and transfer of disease and/or zoonotic bacteria often occur (Herrero et al., 2013).

Since surveillance practices are absent in Uganda, the prevalence of *Salmonella* and *E. coli* in food animal production, humans, and the environment is largely unknown. The lack of an AMR surveillance system in Uganda can compromise treatment options trends in resistance go unmonitored. One study reported the presence of *Salmonella* in water systems in Uganda ranged from 53% in wastewater to 60% in stormwater (Afema et al., 2016). This study also reported AMR among *Salmonella* isolates recovered from ruminants, poultry, and swine (Afema et al., 2016). Resistance was most often observed among poultry with resistance most often observed to ampicillin, amoxicillin/clavulanic acid, chloramphenicol, kanamycin, quinolones, streptomycin, sulfonamides, and tetracyclines. Using this knowledge, we can design a pilot study to determine the prevalence, including AMR prevalence, of *Salmonella* and commensal *E. coli* among chickens and cattle, the two most common animal protein sources in the area.

The objective was to implement a pilot AMR surveillance system in one of 111 districts in Uganda over a one-year period. The Wakiso district was chosen because of its proximity to Kampala. Also, chicken and cattle production is prevalent in this district, and these food animals

serve as a major food source for the people of Kampala. The prevalence and AMR profiles of *Salmonella* spp. and commensal *E. coli* will be determined, from environmental fecal samples collected on chicken and cattle farms. This pilot study will serve as a baseline for implementation of similar surveillance systems in surrounding districts.

Methods

Experimental Design and Sample Collection

This study was cross-sectional design over a one-year period. Sampling occurred over two seasons, the rainy season that began in March ending in September, and the dry season that began in June ending in December. Enrollment in the study occurred through contact with producers throughout the Wakiso district. A total of 20 each, chicken and cattle producers agreed to participate in the study. On-farm sampling was conducted once during the rainy and dry seasons totaling 40 collection periods per animal source. Ten samples per farm were collected at each visit totaling 400 samples per animal species.

For chicken, ten drag swabs were used per farm. Drag swabs (3" x 3" sterile gauze pads) in sterile skim milk was the preferred collection tool (Hardy Diagnostics, Inc., Santa Maria, CA). A sampling schematic was pre-drawn to ensure maximum sampling of the house floor environment including inside diagonals, feeding and water containers, coops, and wall to wall samples. Swabs were individually placed in a sterile whirl-pak bag; the bag was kept in a cooler on ice prior to transport to the laboratory.

For cattle, tongue depressors were used to collect fresh fecal droppings from the pasture environment. Care was taken to ensure each sample represented one cow; contact with the ground did not occur during sampling. Each sample was placed in a sterile whirl-pak bag and kept in a cooler on ice prior to transport to the laboratory.

Bacterial Culture and Isolation

Three hundred seventy-nine drag swab samples were collected from chicken farms while 400 environmental fecal samples were collected on cattle farms. Two chicken producers dropped out of the study after the first sampling. Following transport to the laboratory, culture methods for recovery of both *Salmonella* spp. and commensal *E. coli* were implied.

For *Salmonella* isolation from cattle feces, approximately 1 gm of sample was transferred each to 9 ml GN Hajna and Tetrathionate broth. For drag swabs, 90 ml of buffered peptone water was added to each bag, incubated overnight at 37ºC, and 1 ml each of the liquid was transferred to 9 ml GN Hajna and Tetrathionate broth. Culture continued as described (Fedorka-Cray et al., 1996). Presumptive positive *Salmonella* colonies were confirmed using slide agglutination followed by PCR for the InvA gene. For *E. coli*, one sterile cotton tip applicator (100 µl) of liquid from the GN broth was struck to a Chromagar plate for isolation; plates were incubated overnight at 37ºC. The following day, well-isolated presumptive *E.coli* colonies were struck to another Chromagar plate to ensure purity. Plates were then incubated at 37ºC overnight. *Escherichia coli* isolates were confirmed using the Kovac's Indole testing (Sigma, Inc. St. Louis, MO).

One isolate was struck to a 4.0 ml trypticase soy agar tube prior to shipping to the US for additional testing. Whole genome sequencing (WGS) was used to determine the serovar of *Salmonella*. DNA was extracted using a Qiagen blood and tissue kit (Qiagen, Inc. Valencia, Ca). Sequencing was performed according to manufactures' instructions, (Illumina Inc., San Diego CA). *Salmonella* serotypes were derived using the sequence data from the WGS procedure. Sequences were imported into SeqSero (Center for Genomic Epidemiology; http://www.genomicepidemiology.org/) for serotype interpretation.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was determined for all confirmed *Salmonella* and *E. coli* isolates. Broth microdilution (Sensititre™,Thermo Fisher Scientific, Waltham, MA) was used to measure the Minimum Inhibitory Concentration (MIC) of 14 antimicrobials including: ampicillin, amoxicillin/clavulanic acid, azithromycin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim/sulfamethoxazole. Quality control strains included ATCC 29213 *Staphylococcus aureus*, ATCC 25922 *E. coli,* ATCC 27853 *Pseudomonas aeruginosa*, and ATCC 29212 *Enterococcus faecalis.* The Clinical and Laboratory Standards Institute procedures were followed for interpretation of the MIC (Patel, 2017).

Statistical Analysis

The prevalence of *Salmonella* and *E. coli* were analyzed using WHONET and Microsoft Excel. A logistic regression model was used in SAS® software (SAS® Cary, NC) where season (rainy and dry) served as the factor. Farm was included as a random effect.

Results

Prevalence of *Salmonella* and *E.coli* from Cattle and Chicken

During the second collection of the chicken samples in the rainy season, two farms withdrew from the study. Additionally, nine instead of ten samples were collected on one farm resulting in a total of 379 samples.

From the 379 chicken samples, 334 (88.1%) were positive for *E. coli* while 385 of 400 samples (96.3%) were positive from cattle farms. For *Salmonella*, 51 of 379 samples (13.5%)

were positive from chicken farms, and *E. coli* was also recovered from 49 of the 51 samples (96.1%). From cattle farms, five of 400 samples (1.3%) were positive for *Salmonella,* and *E. coli* was also recovered from four of the five positive samples (80%).

Tables 3.1 and 3.2 show the *Salmonella* serotypes distribution from chicken and cattle farms. In Table 1, *Salmonella* serovar Enteritidis (31.7%) and Kentucky (21.6%) were most often identified among the isolates from chicken. Although there were very few isolates collected from cattle, *Salmonella* serovar Guildford was identified most often (4/5; 80%).

Prevalence of *Salmonella* and *E. coli* by season

No statistically significant difference in seasonal prevalence for *Salmonella* and *E. coli* among cattle sampling periods were shown. However, on the chicken farms, the prevalence of *Salmonella* and *E. coli* was statistically significantly different between the two seasons ($p=0.0017$). Prevalence was higher during the rainy season.

Antimicrobial Resistance

Tables 3.3 and 3.4 show the minimum inhibitory concentration (MICs) distribution and percent resistance for *E. coli* in chicken and cattle and *Salmonella* in chicken. All *Salmonella* recovered from cattle were pan-susceptible, exhibiting no resistance to any antimicrobial tested. Table 3.3 shows percent resistance of *E. coli* from chicken in the following antimicrobials: tetracycline (93%), sulfisoxazole (85%), trimethoprim-sulfamethoxazole (70%), streptomycin (56%), ampicillin (48%), nalidixic acid (28%), ciprofloxacin (21%), chloramphenicol (20%) and azithromycin (16%). In cattle, the resistance among *E. coli* was observed for tetracycline (17%), sulfisoxazole (17%), streptomycin (13%), trimethoprim-sulfamethoxazole (9%), ampicillin (7%), and chloramphenicol (3%). Table 3.4 shows the percent resistance of *Salmonella* in chicken to

the following antimicrobials: tetracycline (51%), nalidixic acid (39%), ciprofloxacin (24%), sulfisoxazole (24%), streptomycin (24%), azithromycin (12%), ampicillin (8%), and trimethoprim-sulfamethoxazole (4%).

Multiple drug resistance (MDR) by antimicrobial class is shown in Tables 3.5 and 3.6 for *E. coli* and the *Salmonella*, respectively. Seventy-nine percent of the *E. coli* isolates from cattle were pan-susceptible. Most of the remaining 21% of isolates exhibited MDR. Multi-drug resistance to three classes of antimicrobials was observed most often (7.0%), followed by resistance to four (4.2%) and two (3.9%) classes of antimicrobials.

Conversely, *E. coli* isolates from chicken were most often resistant to multiple drug classes. Twenty-four percent of the isolates were resistant to four classes of antimicrobials followed by resistance to three (18.9%), two (17.4%), and five (13.8%) classes of antimicrobials.

Salmonella results for cattle in Table 3.6 show that all five samples were pan-susceptible. Thirty-seven percent of *Salmonella* isolates from chicken were pan-susceptible. The MDR profiles from most resistant to least resistant are: 21.6% were resistant to two classes of antimicrobials, 13.7% were resistant to four classes, 3.9% were resistant to five classes, and 2% were resistant to three classes of antimicrobials. Overall, about 50% of the *E. coli* isolates and 38% of *Salmonella* from chicken were resistant to two or more classes of antimicrobials.

Tables 3.7 through 3.9 show the top resistant patterns of *E. coli* from cattle and chicken and *Salmonella* from chicken, respectively. For cattle isolates, the most frequent resistance pattern for *E. coli* was streptomycin, sulfisoxazole, and trimethoprim-sulfamethoxazole (3.6%), followed by the combination of ampicillin, streptomycin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole (2.9%), and resistance to tetracycline alone (2.1%). For chicken isolates, the most frequent resistance patterns for *E. coli* were ampicillin, streptomycin,

sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole (12.6%), followed by resistance to sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole (12.0%). Resistance to tetracycline alone (5.4%), and ampicillin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole (5.4%) were the third most common patterns observed.

Salmonella from chicken isolates were most often resistant to the combination of nalidixic acid and tetracycline (17.6%) which was represented by *Salmonella* serovars Virchow and Newport. Resistance to tetracycline (13.7%) alone was represented by *Salmonella* serovar Zanzibar and Marienthal while *Salmonella* serovar Kentucky was most often resistant to ciprofloxacin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline (13.7%).

Discussion

Initially, the laboratory in Uganda routinely used Tetrathionate (Tet) and Rappaport-Vassiliadis (RV) broths, and Xylose Lysine Deoxycholate (XLD) agar for *Salmonella* isolation with low recovery (data not shown). As previously described (Fedorka-Cray et al., 1996), a more sensitive method for the recovery of *Salmonella* was used as recovery using Tet, RV, and XLD resulted in identified only 18 isolates as *Salmonella*, 32% of the isolates recovered. There is a lack of information that is available for *Salmonella* and *E. coli* prevalence and AMR in Uganda on cattle and chicken farms. One study reported 6.6% prevalence in fecal samples from poultry live bird markets in Kampala, between 2012 and 2013 (Afema et al., 2016). Compared to our study and methodology, this is 50% less than the 13.5% we reported. In Kenya, one study presented an even lower prevalence of 3.6% in cloacal swabs from chicken in Nairobi, Kenya (Nyabundi et al., 2016). Conversely, in the US, NARMS 2014 report an 18% recovery of *Salmonella* from chicken ceca samples (FDA, 2014). Methods used by the NARMS program are similar to culture methods used in this study. We speculate that the increased prevalence may be

due to the enhanced culture methods used to recover isolates and highlight an important need when establishing surveillance systems: harmonization of methods (WHO, 2017b). An important consideration when discussing the pros and cons of particular bacterial culture methods is recognition that unlike some studies in which media comparisons were conducted on the same sample (Davies et al., 2000), there is always the possibility that the prevalence of *Salmonella* has merely increased over time. Additional comparative culture studies and surveillance efforts would increase confidence in one or the other supposition.

With regard to recovery of *E. coli*, there seemed to be comparable results using the ChromAgar (the medium used for this study) and ChromOccult media (medium the lab has routinely used) for culture and isolation. Approximately 88.1% of our samples were positive for *E. coli*. This compares well to the recovery of *E. coli* from cecal samples in the US; in 2014, approximately 95% of samples were positive for *E. coli* as reported by NARMS (FDA, 2014).

Although the primary focus of this study was to look at the prevalence and AMR of *Salmonella* and *E. coli*, we also wanted to see if there was a seasonal effect. In the US, an increase in human infections associated with *Salmonella* occurs during the summer months, which correlates with an increased prevalence of recovery in animal samples (FDA, 2014). Seasonal differences are well described to also occur in early winter (October-November) (Lal, Hales, French, & Baker, 2012), likely due to holiday season.

Since there is little temperature variation between Winter, Spring, Summer, and Fall in Uganda, we elected to look for differences between the rainy and dry seasons. Uganda typically has a rainy season that occurs between March to May and October to December (Kigozi et al., 2016). For recovery from cattle farms, no significant difference was observed for *E. coli* ($p=0.4298$) and *Salmonella* ($p=0.9973$). Conversely, for recovery from chicken farms, a

significant difference (p=0.0017) for recovery of *Salmonella* between the rainy and dry seasons as a higher prevalence of *Salmonella* was observed. During the rainy season, there is an increase in humidity as well as moisture which has been reported to influence the recovery of several bacterial species in poultry (Akil, Ahmad, & Reddy, 2014).

The serotype distribution in this study indicated that *Salmonella* serovars Enteritidis and Kentucky were most often recovered from chicken samples. This is comparable to the most commonly seen serotypes in chickens reported in the US (United States Department of Agricullture, 2014). Kentucky has previously been reported in Uganda in humans, poultry, and environment (Afema et al., 2016).

We also recovered three isolates of *Salmonella* serovar Marienthal a serovar that is rarely reported globally and *Salmonella* with an antigenic formula of 42:r:z39 which has been reported a study by Afema et al. (Afema et al., 2016),

A higher percentage of AMR was observed for *E. coli* recovered from chicken for all antimicrobials tested compared to cattle *E. coli*. For both chicken and cattle *E. coli*, the highest percent resistance was most often observed to tetracycline, 83%, and 17%, respectively. After, utilizing survey data (IRB 17745) on the classes of antimicrobials used on their farms, oxytetracycline was used most often to treat ill animals. Tetracycline is reported as the most commonly used antimicrobial for the treatment of infections, as additives in feed and for prophylaxis (UNAS et al., 2015). Without further knowledge and study, a causal relationship between tetracycline use and resistance to tetracycline in *E.coli* can only be speculated.

We also observed decreased susceptibility to antimicrobials such as ciprofloxacin (21%) and azithromycin (16%). This is considered of unusual phenotypes as reported by NARMS

(FDA, 2015). In Africa, fluoroquinolones and azithromycin are commonly used antimicrobials to treat infections such as *Neisseria* gonorrhea, human plague, and Syphilis (Apangu et al., 2017; Janier M & Develoux M, 2006). Fluoroquinolones are also now commonly used in veterinary practice in Uganda but are not being appropriately used for treatment (UNAS et al., 2015).

It has been established that the lack of proper withdrawal strategies for sulfonamides residues in chicken drinking water has negatively impacted resistance in eggs as one report observed 98% resistance of sulfonamides (Sasanya, Okeng, Ejobi, & Muganwa, 2005). We were aware of any sulfonamide use in feed or water in this study, but resistance to sulfisoxazole and trimethoprim-sulfamethoxazole were the second only to tetracycline resistance.

We also observed many *E. coli* isolates from both cattle and chickens with multi-drug resistance (MDR), and more MDR from chickens than cattle. Approximately 88% of the isolates from chickens were resistant to more than two classes of antimicrobials, including four isolates which were resistant to ten antimicrobials. Using ceftriaxone and/or ceftiofur resistance criteria to test for possible extended-spectrum beta-lactamases (ESBL), we noted eight *E. coli* isolates meeting the criteria; of those three were observed to have an uncommon phenotype of ceftriaxone resistance and ciprofloxacin and/or nalidixic acid resistance. Further characterization of these isolates is warranted.

As previously mentioned, all five cattle *Salmonella* isolates were pan-susceptible. Note that pan-susceptible does not mean the absence of resistance genes which requires additional characterization of these isolates. However, among chicken isolates, as observed for *E. coli*, *Salmonella* also presented with MDR phenotypes to the antimicrobials tested. Approximately 38% of the isolates were resistant to two or more classes of antimicrobials, including two isolates resistant to seven antimicrobials. We noted above that the two most common serotypes were

Salmonella serovar Enteritidis (31.7%) followed by *Salmonella* serovar Kentucky (21.6%). In the US, *Salmonella* serovar Kentucky surpassed Enteritidis in broiler slaughter samples which have not been observed for over two decades (Ladely, Meinersmann, Ball, & Fedorka-Cray, 2016). The *Salmonella* serovar Kentucky isolates in this study presented MDR to over five (ciprofloxacin, nalidixic acid, streptomycin, sulfisoxazole, and tetracycline) or seven (chloramphenicol/ampicillin, ciprofloxacin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole) antimicrobials. All *Salmonella* serovar Kentucky isolates resistant to ciprofloxacin. Since early 2000's, ciprofloxacin resistance for *Salmonella* serovar Kentucky has been on the rise, especially from travelers to northern and eastern Africa (Rickert-Hartman & Folster, 2014). In one study, 9% of the *Salmonella* serovar Kentucky isolated from travelers were ciprofloxacin resistant. It is of interest to note that poultry as thought to be a reservoir for these resistant strains (Rickert-Hartman & Folster, 2014; Weill et al., 2006). Cases of ciprofloxacin resistant Kentucky have been seen in the US from travelers from India resulting in seven infected with one death (Rickert-Hartman & Folster, 2014).

Conclusion

In conclusion, we have studied the prevalence and AMR of *E. coli* and *Salmonella* isolates within the Wakiso district of Uganda. It would be beneficial to expand this study, using the same methods, with a larger sample size to cover the entire district. The limitations associated with expansion include transportation to collect the samples, funding, and the lack of trained personnel to culture large numbers of samples. Human data which is collected at the same time is needed to make any correlations between observed prevalence and resistance in animals that could be transferred to the human population or vice versa.

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Table 3.1: *Salmonella* serotype distribution among chicken isolates (N=51)

Serotype from Chicken	**Number (%)**
Salmonella serovar Enteritidis	16 (31.7)
Salmonella serovar Kentucky	11 (21.6)
Salmonella serovar Virchow	8 (15.7)
Salmonella serovar Zanzibar	5 (9.8)
Salmonella serovar Marienthal	3 (5.9)
Salmonella 42:r:z39	3 (5.9)
Salmonella serovar Newport	2 (3.9)
Salmonella serovar Typhimurium	2 (3.9)
Salmonella serovar Barranquilla	1 (2.0)

Table 3.2: *Salmonella* serotype distribution among cattle isolates (N=5)

Serotype from Cattle	**Number (%)**
Salmonella serovar Guildford	4 (80.0)
Salmonella serovar Enteriditis	1 (20.0)

Table 3.3: Distribution of MICs and Resistance by Animal Source among *E. coli*, 2016

Antimicrobial	Source (# of Isolates)	%I[1]	%R[2]	95% CI[3]	Distribution (%) of MICs (µg/ml)[4] .016	.032	.064	.125	.25	.5	1	2	4	8	16	32	64	128	256	>256
Aminoglycosides																				
Gentamicin	Chicken (334)	0.3	**0.6**	0.1-2.4					4.2	73.4	20.4	1.2		0.3		0.6				
	Cattle (385)	0.0	**0.0**	0.0-1.2					4.2	78.2	16.9	0.8								
Streptomycin*	Chicken (334)	0.0	**56.3**	50.8-61.7								0.3	8.4	24.9	10.2	5.4	2.4	48.5		
	Cattle (385)	0.0	**12.8**	9.6-16.5									34	48.3	4.9	2.6	3.4	6.8		
β-Lactam/β-Lactamase Inhibitor Combinations																				
Amoxicillin-Clavulanic Acid	Chicken (334)	6.3	**0.6**	0.1-2.4							1.5	17.7	38.3	35.6	6.3	0.3	0.3			
	Cattle (385)	1.3	**0.0**	0.0-1.2							2.6	9.1	72.7	14.3	1.3					
Cephems																				
Cefoxitin	Chicken (334)	1.5	**0.6**	0.1-2.4							0.9	27.5	53	16.5	1.5		0.6			
	Cattle (385)	0.5	**0.0**	0.0-1.2							0.3	24.4	61.3	13.5	0.5					
Ceftiofur	Chicken (334)	0.3	**1.2**	0.4-3.3				2.1	38	55.1	3.3		0.3		1.2					
	Cattle (385)	0.0	**0.8**	0.2-2.5				0.8	26.2	70.1	1.6	0.5			0.8					
Ceftriaxone	Chicken (334)	0.0	**1.2**	0.4-3.33					98.5	0.3						0.6		0.6		
	Cattle (385)	0.0	**1.1**	0.3-2.8					99				0.3			0.3		0.5		
Folate Pathway Inhibitors																				
Sulfisoxazole	Chicken (334)	0.0	**84.7**	80.3-88.3											1.2	2.1	5.4	4.5	2.1	84.7
	Cattle (385)	0.0	**16.6**	13.1-20.8											12.7	9.9	45.2	8.3	7.3	16.6
Trimethoprim-Sulfamethoxazole	Chicken (334)	0.0	**70.4**	65.1-75.2				15.3	6	6.6	1.8			70.4						
	Cattle (385)	0.0	**8.8**	6.3-12.2				85.7	3.4	2.1				8.8						
Macrolides																				
Azithromycin	Chicken (334)	2.0	**15.6**	41.9-52.8								4.8	47.9	29.3	2.4	15.6				
	Cattle (385)	0.0	**0.5**	15.0-23.0							0.3	10.1	70.9	17.7	0.5	0.5				
Penicillins																				
Ampicillin	Chicken (334)	0.0	**48.2**	42.7-53.7							3.9	30.5	16.5	0.9		0.3	47.9			
	Cattle (385)	0.0	**7.3**	5.0-10.5							3.6	36.6	49.9	2.3	0.3		7.3			
Phenicols																				
Chloramphenicol	Chicken (334)	4.2	**19.5**	15.5-24.2								0.3	21.3	54.8	4.2	4.5	15			
	Cattle (385)	5.5	**2.6**	1.3-4.9								0.3	24.2	67.5	5.5	0.5	2.1			
Quinolones																				
Ciprofloxacin*	Chicken (334)	34.4	**21.3**	17.1-26.2	41.6	2.7		0.9	20.1	13.5	3.6	1.8	1.2	14.7						
	Cattle (385)	3.4	**0.0**	0.0-1.2	84.4	12.2	1.3		1.3	0.9										
Nalidixic Acid	Chicken (334)	0.0	**27.8**	23.1-33.0							2.4	35.3	12.6	14.4	7.5	2.4	25.4			
	Cattle (385)	0.0	**0.3**	0-1.7							3.9	75.6	19	1.3			0.3			
Tetracyclines																				
Tetracycline	Chicken (334)	0.0	**92.5**	89.0-95.0									7.5			9.6	82.9			
	Cattle (385)	0.3	**17.4**	13.8-21.6									82.3	0.3		0.8	16.6			

[1] Percent intermediate suceptibility

[2] Percent resistant

[3] Using the Wilson interval with continuity method, the 95% confidence intervals for percent resistant (%R) were calculated

[4] The range of dilutions confirmed for each antimicrobial is represented in the unshaded areas. The breakpoints for susceptibility are represented by the single vertical bars indicate, while breakpoints for resistance is represented by the double vertical bars. The proportions of isolates with MICs greater than the highest tested concentrations are the numbers repersented in the shaded area. The proportions of isolates with MICs equal to or less than the lowest tested concentration are represented by the numbers listed for the lowest tested concentrations. CLSI breakpoints were used, although there are no CLSI breakpoints for streptomycin.

Table 3.4: Distribution of MICs and Resistance of Chicken* among *Salmonella*, 2016 (N=51)

Antimicrobial	%I[1]	%R[2]	95% CI[3]	Distribution (%) of MICs (µg/ml)[4]															
				.016	.032	.064	.125	.25	.5	1	2	4	8	16	32	64	128	256	>256
Aminoglycosides																			
Gentamicin	0.0	**0.0**	0.0-8.7					56.9	41.2	2									
Streptomycin	0.0	**23.5**	13.2-37.8								21.6	7.8	21.6	25.5	**9.8**		**13.7**		
β-Lactam/β-Lactamase Inhibitor Combinations																			
Amoxicillin-Clavulanic Acid	0.0	**0.0**	0.0-8.7							88.2	3.9	3.9	3.9						
Cephems																			
Cefoxitin	0.0	**0.0**	0.0-8.7							2	66.7	27.5	3.9						
Ceftiofur	0.0	**0.0**	0.0-8.7				2		25.5	66.7	5.9								
Ceftriaxone	0.0	**0.0**	0.0-8.7					100											
Folate Pathway Inhibitors																			
Sulfisoxazole	0.0	**23.5**	13.2-37.8												3.9	11.8	47.1	13.7	**23.5**
Trimethoprim-Sulfamethoxazole	0.0	**3.9**	0.7-14.6				76.5	17.6	2				**3.9**						
Macrolides																			
Azithromycin	88.2	**11.8**	4.9-24.6									88.2	**11.8**						
Penicillins																			
Ampicillin	0.0	**7.8**	2.5-19.7							82.4	9.8					**7.8**			
Phenicols																			
Chloramphenicol	0.0	**2.0**	0.1-11.9								2	17.6	78.4			**2**			
Quinolones																			
Ciprofloxacin*	17.6	**23.6**	13.2-37.8	27.5	31.4			11.8	5.9	**2**			**21.6**						
Nalidixic Acid	0.0	**39.3**	26.2-53.9							2	7.8	49		2	**2**	**37.3**			
Tetracyclines																			
Tetracycline	0.0	**51.0**	36.8-65.1									49				**51**			

*Cattle (N=5) was Pan.susceptible for *Salmonella*

[1] Percent intermediate suceptibility

[2] Percent resistant

[3] Using the Wilson interval with continuity method, the 95% confidence intervals for percent resistant (%R) were calculated

[4] The range of dilutions confirmed for each antimicrobial is represented in the unshaded areas. The breakpoints for susceptibility are represented by the single vertical bars indicate, while breakpoints for resistance is represented by the double vertical bars. The proportions of isolates with MICs greater than the highest tested concentrations are the numbers repersented in the shaded area. The proportions of isolates with MICs equal to or less than the lowest tested concentration are represented by the numbers listed for the lowest tested concentrations. CLSI breakpoints were used, although there are no CLSI breakpoints for streptomycin

Table 3.5: MDR *E. coli* from cattle and chicken, 2016

Source	**Cattle (N=385)**	**Chicken (N=334)**
Resistance Pattern	**N (%)**	**N (%)**
No Resistance Detected	304 (79.0)	15 (4.5)
Resistance =1 CLSI Class[1]	17 (4.4)	26 (7.8)
Resistance = 2 CLSI Classes[1]	15 (3.9)	58 (17.4)
Resistance = 3 CLSI Classes[1]	27 (7.0)	63 (18.9)
Resistance = 4 CLSI Classes[1]	16 (4.2)	80 (24.0)
Resistance = 5 CLSI Classes[1]	4 (1.0)	46 (13.8)
Resistance = 6 CLSI Classes[1]	1 (0.3)	33 (9.9)
Resistance = 7 CLSI Classes[1]	1 (0.3)	12 (3.6)
Resistance = 8 CLSI Classes[1]	0 (0.0)	1 (0.3)

[1]CLSI: Clinical and Laboratory Standards Institute M100 Document

Table 3.6: MDR *Salmonella* from cattle and chicken, 2016

Source	**Cattle (N=5)**	**Chicken (N=51)**
Resistance Pattern		
No Resistance Detected	5 (100.0)	19 (37.3)
Resistance = 1 CLSI Class[1]	0	11 (21.6)
Resistance = 2 CLSI Classes[1]	0	11 (21.6)
Resistance = 3 CLSI Classes[1]	0	1 (2.0)
Resistance = 4 CLSI Classes[1]	0	7 (13.7)
Resistance = 5 CLSI Classes[1]	0	2 (3.9)

[1]CLSI: Clinical and Laboratory Standards Institute M100 Document

Table 3.7: Top 8 Resistance patterns of *E. coli* from cattle (N=385)

Profile	Number (%)
STR SOX TCY	19 (4.9)
AMP STR SOX TCY SXT	11 (2.9)
TCY	8 (2.1)
SOX TCY SXT	7 (1.8)
SOX	4 (1.0)
STR TCY	4 (1.0)
STR SOX	3 (0.8)
AMP SOX TCY SXT	3 (0.8)

AMC=Amoxicillin-Clavulanic Acid, AMP=Ampicillin, AZM=Azithromycin, FOX=Cefoxitin, TIO=Ceftiofur, CRO=Ceftriaxone, CHL=Chloramphenicol, CIP=Ciprofloxacin, GEN=Gentamicin, NAL=Nalidixic Acid, STR=Streptomycin, SOX=Sulfisoxazole, TCY=Tetracycline, SXT=Trimethoprim-Sulfamethoxazole

Table 3.8: Top 10 Resistance patterns of *E. coli* from chicken (N=334)

Profile	Number (%)
AMP STR SOX TCY SXT	42 (12.6)
SOX TCY SXT	40 (12.0)
TCY	18 (5.4)
AMP SOX TCY SXT	18 (5.4)
STR SOX TCY	16 (4.8)
AMP AZM STR SOX TCY SXT	13 (3.9)
STR SOX TCY SXT	12 (3.6)
AMP CHL CIP NAL STR SOX TCY SXT	10 (3.0)
AMP AZM CHL CIP NAL STR SOX TCY SXT	9 (2.7)
AMP CHL STR SOX TCY SXT	8 (2.4)

AMC=Amoxicillin-Clavulanic Acid, AMP=Ampicillin, AZM=Azithromycin, FOX=Cefoxitin, TIO=Ceftiofur, CRO=Ceftriaxone, CHL=Chloramphenicol, CIP=Ciprofloxacin, GEN=Gentamicin, NAL=Nalidixic Acid, STR=Streptomycin, SOX=Sulfisoxazole, TCY=Tetracycline, SXT=Trimethoprim-Sulfamethoxazole

Table 3.9: Top Resistance patterns of *Salmonella* from chicken (N=51)

Profile	Number (%)
NAL TCY	9 (17.6)
TCY	7 (13.7)
CIP NAL STR SOX TCY	7 (13.7)
STR	3 (5.9)
AMP SOX	2 (3.9)
CIP NAL	1 (2.0)
AMP CIP NAL SOX	1 (2.0)
CHL CIP NAL STR SOX TCY SXT	1 (2.0)
AMP CIP NAL STR SOX TCY SXT	1 (2.0)

AMC=Amoxicillin-Clavulanic Acid, AMP=Ampicillin, AZM=Azithromycin, FOX=Cefoxitin, TIO=Ceftiofur, CRO=Ceftriaxone, CHL=Chloramphenicol, CIP=Ciprofloxacin, GEN=Gentamicin, NAL=Nalidixic Acid, STR=Streptomycin, SOX=Sulfisoxazole, TCY=Tetracycline, SXT=Trimethoprim-Sulfamethoxazole

Chapter 4: Genotypic Characterization of *Salmonella* from Cattle and Chicken Farms in the Wakiso District in Uganda

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Genotypic Characterization of *Salmonella* from Cattle and Chicken Farms in the Wakiso District in Uganda

Takiyah A. Ball, Siddhartha Thakur, Joy Horovitz, Awa Aidara-Kane, Francis Ejobi, and Paula J. Fedorka-Cray*

Abstract

Background: *Salmonella* is a food-borne pathogen that causes diarrheal diseases worldwide, particularly in the young, elderly and immunocompromised. The development of antimicrobial resistance (AMR) in foodborne pathogens is of particular concern as treatment options may be compromised. Additionally, antimicrobial resistance genes may be transferred from *Salmonella* to other zoonotic or commensal bacteria compounding the resistance issue.

Objectives: Estimate the prevalence of *Salmonella spp.* and determine the AMR phenotypes and genotypes of isolates recovered from cattle and chicken farms in the Wakiso District of Uganda.

Methods: Fift-six isolates from cattle (n=5) and chicken (n=51) farms were cultured for *Salmonella* using standard laboratory methods. AMR profiles were identified for all chicken and cattle *Salmonella* using the Sensitire^{TM} system per manufacturer's directions. Of the 56 isolates, ten from chickens were MDR to three or more classes of antimicrobials. All *Salmonella* isolates were screened for 28 replicon type plasmids and Class I Integrons by PCR; PFGE was also conducted on all isolates to determine relatedness.

Results: *Salmonella* was recovered from 51/379 (13.5%) and 5/400 (1.3%) of chicken and cattle samples, respectively. Five replicon plasmids were identified among all chicken and cattle *Salmonella* including *Inc*FIIS 18/56 (32.1%), *Inc*I1α 12/56 (21.4%), *Inc*P 8/56 (14.3%), *Inc*X1 8/56 (14.3%), and *Inc*X2 1/56 (1.8%). The Class I integron, *Int1* was observed in one chicken isolate presenting with multidrug resistance (MDR) to five antimicrobial classes. PFGE cluster analysis of all isolates showed 17 distinctive cluster types and displayed distinct clusters by replicon-types *Inc*P, *Inc*X, *Inc*FIIS, and *Inc*I1α. Heterogeneity was noted among the isolates.

Conclusion: MDR may be associated with particular plasmids presented in this study. Interestingly, the replicon-type plasmids exhibited associations with certain *Salmonella* serotypes, and MDR was observed in isolates when replicon-types were identified.

Introduction

Antimicrobial resistance (AMR) has developed as a major concern globally and particularly in developing countries. Uganda is one of numerous developing countries that are beginning implementation of a countrywide surveillance system to monitor AMR using a One Health approach in which animals, the environmental, and humans are sampled temporally. In recent years, one study from Uganda also demonstrated that AMR *Salmonella* could also be recovered in public water systems (Afema et al., 2016). In our previous study, of the 56 *Salmonella* positive isolates recovered from chicken and cattle, 41.1% of the chicken isolates exhibited multidrug resistance (MDR) (Manuscript Submitted).

Not only is AMR of concern, but the emergence of MDR is also of particular concern and within many *Salmonella* serovars (FDA, 2014, 2015). With no new classes of antimicrobials on the horizon, antimicrobials are no longer efficacious for treating infectious diseases (Ling et al., 2015). Only one antibiotic has been manufactured in the last 30 years, Teixobactin, which is used to inhibit cell wall synthesis in gram-positive bacteria (Ling et al., 2015). In *Salmonella*, genes that are associated with AMR are usually located on plasmids that are transferable. The plasmid platform in which genes are reasserted and assembled, allows a bacterium to survive in areas that are typically hazardous (Bennett, 2008). Bacterial plasmids are particularly adept at transferring between cells. Most plasmids that carry resistance are conjugative plasmids, promoting the transfer of DNA from cell to cell (Bennett, 2008).

Integrons are typically found within conjugative plasmids and utilize these plasmids for the transfer of genetic material between cells (Rowe-Magnus & Mazel, 2001). Class I Integrons distribute AMR genes through an assortment of gene cassettes. These gene cassettes typically

have resistance determinants of aminoglycosides, folate pathway inhibitors, and β-Lactams (Fluit & Schmitz, 2004; Mazel, 2006).

Extended-spectrum beta-lactamases (ESBLs) occur globally in *Enterobacteriaceae*. Examples of common ESBLs include $bla_{CTX\text{-}M}$, bla_{OXA}, bla_{TEM}, bla_{CMY}, and bla_{SHV} (Bradford, 2001; Gniadkowski, 2001). $bla_{CTX.M}$ has been commonly observed in *Salmonella* and *E. coli* isolates (Paterson, 2001). Reports indicate that ESBLs evolved from the *Kluverya* spp. chromosome as a result of a mutation and gene transposition (Humeniuk et al., 2002). ESBLs are also common on integrons and transposons, which aids in the transfer of genetic material from one bacterium to another (Bradford, 2001; Gniadkowski, 2001).

Previously, we reported on the phenotypic characterization of *Salmonella* and *E. coli* isolates from cattle and chicken farms within the Wakiso District of Uganda (Manuscript submitted). Based on the high prevalence of MDR in isolates collected, we sought to characterize these isolates further. All 56 isolates were subjected to pulsed-field gel electrophoresis (PFGE), plasmid replicon typing was conducted, and isolates were screened to the Class 1 Integron, *Int1*. The *Salmonella* isolates were also screened for ESBL genes based on their resistant profiles.

Methods

Bacterial Isolates

Previously we reported on the phenotypic characterization of *Salmonella* isolates from cattle and chicken farms in the Wakiso District of Uganda (manuscript submitted). All 56 isolates previously were frozen at -80°C were struck for isolation to Tryptic Soy Agar (TSA) with 5% sheep blood (BAP) and incubated overnight at 37°C to ensure purity. Lysates were prepared by suspending a loopful of well-isolated colonies into 200 µl of molecular grade water

and vortexed at maximum speed for several seconds. The suspension was boiled at 100°C for 10 minutes, centrifuged at 13 X 1000 rpm for 60 seconds, and the supernatant was collected for use as the DNA template.

Replicon Plasmid Screening using Polymerase Chain reaction (PCR)

The 56 *Salmonella* DNA templates were screened using the PCR based replicon typing (PRBT) system (Diatheva, Cartoceto, Italy) which included 28 replicons. The PBRT kit is composed of an eight multiplex PCR for amplification of the replicons, which represents the incompatibility group which is recognized in resistant plasmids among *Enterobacteriaceae.* The replicons include: HI1, HI2, I1α, M, N, I2, BO, FIB, FIA, W, L, P, X3, I1γ, T, A/C, FIIS, U, X1, R, FIIK, Y, X2, FIC, K, HIB.M, FIB.M, and FII. Manufacturer's procedures were followed as described: (https://www.diatheva.com/images/DATASHEET/MBK_MBR/MBK0038%20kit%20%20IVD%20CE.pdf).

Integron 1 PCR Screening

The 56 *Salmonella* DNA templates were screened for Class 1 integrons by PCR. The target gene was *intI1 with* (5' CAGTGGACATAAGCCTGTTC 3') as the forward primer and (5' CCCGAGGCATAGACTGTA 3') as the reverse primer. Thermal cycler parameters were as follows: an initial cycle at 94°C for ten minutes, 29 cycles of 94°C for one minute, 54°C for one minute, and 72°C for two minutes, elongation cycle at 72°C for ten minutes, and 4°C to hold (Dillon, Thomas, Mohmand, Zelynski, & Iredell, 2005).

PCR screening of Extended-Spectrum β-Lactamase (ESBL)

To examine molecular mechanisms of isolates presenting β-Lactam resistant isolates that presented resistance to ceftiofur (MIC≥8 µg/ml and/or ceftriaxone (MIC≥4 µg/ml) antimicrobials were screened for ESBL producing genes. The Hotstar Taq Master Mix kit was used for the PCR (Qiagen Inc, Valencia, CA). If any isolates met the criteria, DNA templates were screened with primers and conditions for the presence of five genes: bla_{CTX}, bla_{TEM}, bla_{CMY}, $bla_{OXA.1}$, and bla_{SHV} (Bonnet et al., 2003; Brinas, Zarazaga, Saenz, Ruiz-Larrea, & Torres, 2002; S. Chen et al., 2004).

Pulse Field Gel Electrophoresis

The 56 *Salmonella* isolates were subjected to PFGE using the "Standard Operating Procedure" as described for the Pulsenet System (https://www.cdc.gov/pulsenet/pdf/ecoli.shigella.salmonella.pfge.protocol.508c.pdf). All gels were analyzed using the BioNumerics software version 7.5 (Applied Maths, A Biomérieux Company).

Results

Antimicrobial Resistance

From our previous study, 51 (379/400;13.5%) chicken and 5 (5/400;1.3%) cattle *Salmonella* isolates were available for this study (Manuscript Submitted). AMR testing indicated that 10 of the 56 isolates, all from chickens, presented with MDR to three or more antimicrobial classes. All five cattle isolates were pan-susceptible.

Replicon Plasmids

Five of the 28 plasmids that were screened were observed in multiple isolates: *Inc*FIIS was positive for 32.1% of the isolates followed by *Inc*I1α (21.4%), *Inc*P (14.3%), *Inc*X1 (14.3%), *Inc*X2 (1.8%). Table 4.1 demonstrates the numbers and percentages of these replicon-types. Table 4.2 shows the resistance patterns for each replicon-types and the number of isolates that presented the patterns and plasmid types. Two isolates with resistance to both ampicillin and sulfisoxazole were each positive for *Inc*I1α and *Inc*FIIS. Both isolates originated from the same farm. Eight isolates which were resistant to nalidixic acid and tetracycline were positive for replicon-types *Inc*P and *Inc*X1 (seven from one farm) while one isolate with the same resistant pattern was positive for replicon-type *Inc*X2. All 28 replicon plasmid types were negative in the isolates presenting with resistance to ciprofloxacin and nalidixic acid and isolates with resistant patterns to ciprofloxacin, nalidixic acid, streptomycin, sulfisoxazole, and tetracycline.

IntI1

Only one chicken isolate was positive for the *IntI1* gene. This isolate exhibited an MDR pattern of chloramphenicol, ciprofloxacin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole. The *Inc*I1α gene was also present within this isolate from the PCR screening.

ESBL

No *Salmonella* isolates tested presented decreased susceptibility to the ceftiofur and/or ceftriaxone during screening. Therefore, no further ESBL characterization took place.

PFGE

PFGE showed 17 distinctive clusters and certain serotypes, and replicon types were observed within each cluster (Figure 4.1). *Inc*P and *Inc*X1-positive strains exhibited resistance to nalidixic acid and tetracycline, with intermediate (approaching resistance) for ciprofloxacin. This resistance pattern was observed among *Salmonella* serovar Virchow which appeared in two clusters. *Inc*I1α-positive strains exhibited tetracycline resistance in the majority of isolates, and these isolates were comprised of serovars Marienthal, Zanzibar, and Kentucky; these serovars were distributed among seven PFGE clusters. All of the *Salmonella* serovar Enteritidis isolates were susceptible to all antimicrobials. However, *Inc*FIIS was observed in all isolates which were part of three PFGE clusters. One isolate, *Salmonella* serovar Newport, was *Inc*X2 positive and exhibited resistance to ciprofloxacin, nalidixic acid, and tetracycline.

Discussion

In this study, the objective was to explore *Salmonella* isolates obtained from cattle and chicken farms in the Wakiso District of Uganda at the molecular level. Characterization included PFGE genotypes, plasmid replicon typing, integron screening, and analysis of ESBL genes. A correlation between AMR phenotypes and replicon.type was observed for some isolates (*Inc*1α displayed MDR followed by *Inc*P, *Inc*X1, and *Inc*X2). The predominate replicon type observed was *Inc*FIIS (n=18; 32.1%) followed by *Inc*1α (n=12; 21.4%). Studies have shown that bacterial isolates containing $bla_{CTX.M.1,}$ harbor the *Inc*FIIS along with other incompatibility plasmids (Zurfluh, Jakobi, Stephan, Hachler, & Nuesch-Inderbinen, 2014). *Inc*1 plasmids are known to be distributed throughout many serotypes of *Salmonella* and predominate in both *E.coli* and *Salmonella* (Dierikx, van Essen-Zandbergen, Veldman, Smith, & Mevius,

2010; Garcia-Fernandez et al., 2008; Lindsey, Fedorka-Cray, Frye, & Meinersmann, 2009). In this study, *Inc*1α was observed among *Salmonella* serotypes such as Zanzibar, Marienthal, Kentucky, and Typhimurium. Both serovars Zanzibar and Marienthal isolates were from the same farms. This could suggest the plasmid is being spread between the two serovars. All isolates from Kentucky came from the same farm, as well as isolates with serovar Typhimurium.

*Inc*P and *Inc*X1 were the next most common plasmids seen in this study. Both were present in the *Salmonella* serovar Virchow isolates. It has been reported that *Inc*P can spread through groups of bacteria via conjugative transfer and code for broad range antimicrobial resistance. *Inc*P is highly likely to be found in manure, wastewater, and soil (Popowska & Krawczyk-Balska, 2013). *Inc*X1 is commonly found as a narrow host-range plasmid in *Enterobacteriaceae,* also spreading to other bacteria via conjugative transfer (Norman, Hansen, She, & Sorensen, 2008).

In this study, no *Salmonella* presented resistance to ceftriaxone or ceftiofur or any other antimicrobials tested, therefore, were not screened for β-Lactamase genes. This does not mean that the ESBL genes are not present. Further characterization using whole genome sequencing (WGS) could identify these ESBL genes. In previous studies (McDermott et al., 2016), discrepancies were seen between phenotypic resistance and genotypic analysis using WGS. It was reported that a MIC might not reach the breakpoint, but resistance genes were present (McDermott et al., 2016).

The PFGE analysis displayed 17 clusters that were distributed amongst the different serotypes represented in the study. Most of the clusters were associated with particular *Salmonella* serotypes. *Salmonella* serovars Virchow, Guildford, Enteriditis, Kentucky, and antigenic formula 42:r:z39 displayed a high homogeneity profile. This shows that each of these

serovars has a close genetic relationship. Interestingly, *Salmonella* serovars Zanzibar and Marienthal shared a homogeneous profile. There is little reporting on *Salmonella* serovar Marienthal. One report isolated Marienthal from feeds and feed ingredients during the early 80's (Durand, Giesecke, Barnard, Vanderwal, & Steyn, 1990). Because of the limited information on this serovar, it is not known how it is closely related to Zanzibar, except they both share the same O antigen and phase one H antigen based on the

(Source: https://www.pasteur.fr/sites/default/files/veng_0.pdf). Further characterization will have to be explored with this serovars to see relatedness.

Class 1 integron integrase *int1* was only detected in one chicken isolate. Although most integrons are found on plasmids and transposons, they can also be found in chromosomes. WGS sequencing was not performed for this study, but it is suspected that more Class 1 integrons could be detected with WGS. Replicon type plasmid *Inc*1α was also detected as mentioned earlier in this isolate. *Int1* may be found as a part of this plasmid, but further characterization will have to be done.

In the cattle isolates collected, phenotypically, all were susceptible to the 14 antimicrobials. There was very few *Salmonella* isolated (n=5), four of which were cultured from the same farms and shared the same serovar Guildford. There is little reporting on resistance of serovar Guildford which could suggest why we did not see any resistance. Although these isolates, along with the other isolates for chicken, phenotypically display susceptibility, it does not suggest that resistance is not there, as esistance genes could be seen genetically. Future analysis will compare both the phenotypic and genotypic (WGS) profiles.

Conclusion

These *Salmonella* strains may be harboring particular plasmids conferring resistance to particular antimicrobials. Future work with these isolates will include looking at the sequences through WGS and analysis of antimicrobial use.

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Table 4.1: Prevalence of plasmid replicon types (%) from chicken and cattle *Salmonella* Isolates (n=56)

FIIS	18 (32.1)
Ilα	12 (21.4)
P	8 (14.3)
X1	8 (14.3)
X2	1 (1.8)

Table 4.2: Antimicrobial resistance profiles and associated Inc replicon types of *Salmonella* from chicken and cattle (N=56)

Resistance Profile[a]	**Replicon Type**	**Number of Isolates**
Pan-Susceptible	FIIS	16
TCY	I1α	7
AMP SOX	I1α.FIIS[b]	2
CIP NAL	N/A[c]	N/A
NAL TCY	P.X1, X2	8, 1
AMP CIP NAL SOX	I1α	1
CIP NAL STR SOX TCY	N/A	N/A
AMP CIP NAL STR SOX TCY SXT	I1α	1
CHL CIP NAL STR SOX TCY SXT	I1α	1

[a]AMC=Amoxicillin-Clavulanic Acid, AMP=Ampicillin, AZM=Azithromycin, FOX=Cefoxitin, TIO=Ceftiofur, CRO=Ceftriaxone, CHL=Chloramphenicol, CIP=Ciprofloxacin, GEN=Gentamicin, NAL=Nalidixic Acid, STR=Streptomycin, SOX=Sulfisoxazole, TCY=Tetracycline, SXT=Trimethoprim-Sulfamethoxazole
[b]Replicon types with dashes are combination replicon types with the same pattern in the same number of isolates.
[c]N/A are resistant patterns that showed replicon types as negative in the screening.

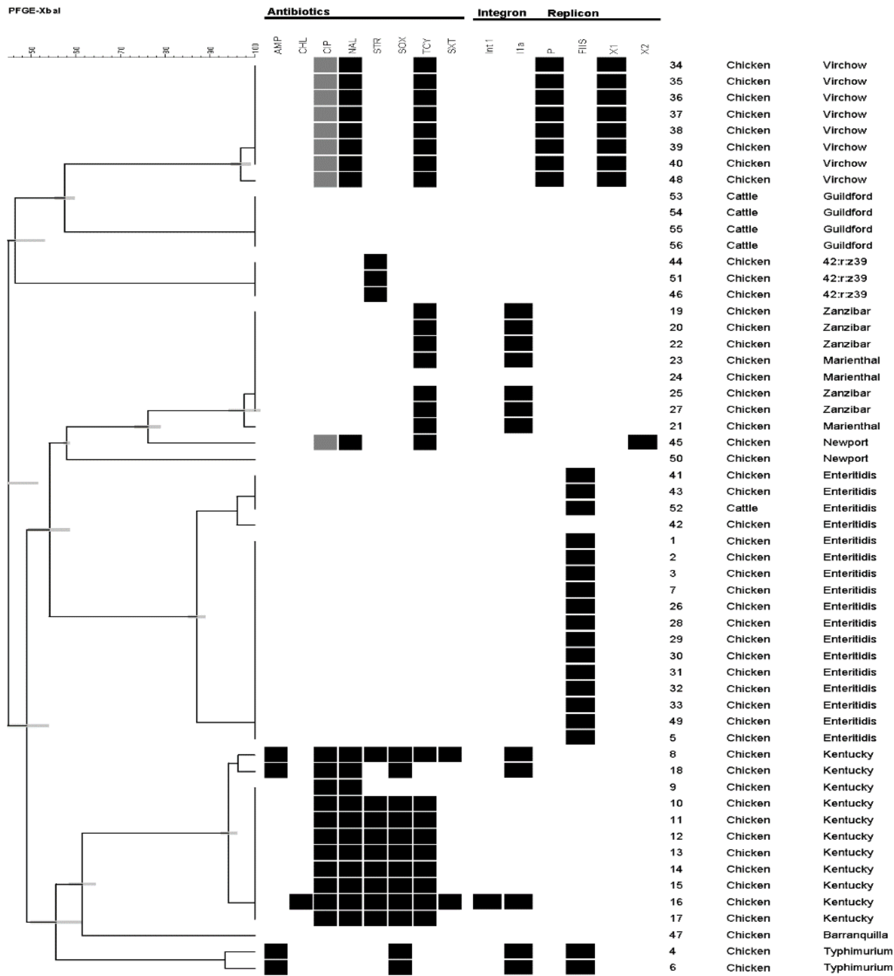

Figure 4.1: PFGE Dendogram of chicken and cattle (N=56),AMR profile, Class 1 Integron, Replicon Typing, source, and Strain serovar. For PFGE analysis, BioNumerics software was used. AMR profiles, Class 1 Integron Int1, and Replicon typing is to the right of the dendogram. For the AMR the black resistance to that antimicrobial and the gray indicates intermediate resistance to that antimicrobial. For integron and replicon, typing black indicates a positive trait. The animal source and *Salmonella* serovar are listed in the last two columns. AMC=Amoxicillin-Clavulanic Acid, AMP=Ampicillin, AZM=Azithromycin, FOX=Cefoxitin, TIO=Ceftiofur, CRO=Ceftriaxone, CHL=Chloramphenicol, CIP=Ciprofloxacin, GEN=Gentamicin, NAL=Nalidixic Acid, STR=Streptomycin, SOX=Sulfisoxazole, TCY=Tetracycline, SXT=Trimethoprim-Sulfamethoxazole

Chapter 5: Molecular Characterization of Extended-Spectrum β-Lactamase Escherichia coli from Cattle and Chicken Farms in the Wakiso District of Uganda

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Molecular Characterization of Extended-Spectrum β-Lactamase *Escherichia coli* from Cattle and Chicken Farms in the Wakiso District of Uganda

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Abstract

Background: Extended-Spectrum β-Lactamase (ESBL) are enzymes that confer resistance to the β-lactam antibiotics. Many Gram-negative organisms carrying ESBL genes cause serious infections in humans and animals that are difficult to treat. Illness associated with ESBL producing bacteria can cause increased morbidity lasting for months to years. Some strains of *Escherichia coli* (*E. coli*) carry ESBLs.

Objectives: Characterize ESBL-producing *E. coli* isolated from cattle and chicken farms in the Wakiso District of Uganda at the molecular level.

Methods: Antimicrobial susceptibility testing was conducted using broth microdilution (Thermo Fisher Scientific, Waltham, MA). Four *E. coli* isolates each from cattle (4/385; 1%), and chicken (4/334; 1.2%) farms presenting resistance to ceftriaxone and/or ceftiofur were screened by polymerase chain reaction (PCR) for five ESBL genes: $bla_{CTX\text{-}M}$, bla_{SHV}, bla_{CMY}, bla_{OXA}, and bla_{TEM}. Whole genome sequencing (WGS) was conducted for identification of antimicrobial resistance determinates. Replicon type plasmids and Class I integrons were screened via PCR. The isolates were also subjected to pulse field gel electrophoresis (PFGE) to determine relatedness.

Results: All eight isolates displayed multiple drug resistance (MDR) to a panel of 14 antimicrobials. Collectively, six replicon-type plasmids were identified among cattle and chicken; one of four cattle isolates were positive for *Inc*HI1, *Inc*FIB, *Inc*P, *Inc*X3, and *Inc*FII while two of four cattle isolates were positive for *Inc*HI2. Two of four chicken isolates were positive for *Inc*FIB and *Inc*FII. The Class I integron *Int*1 was detected among cattle (1/4), and chicken (4/4) isolates. PFGE cluster analysis showed seven distinctive cluster types. ESBL genes were identified as follows: cattle ($bla_{CTX\text{-}M;}$ n=3/4, $bla_{TEM.12}$; n=2/4, and bla_{SHV}; n=1/4) and chicken ($bla_{CTX\text{-}M}$; n=3/4, $bla_{TEM.12}$; n=3/4, and $bla_{oxa.1}$; n=2/4). Concordance between WGS results and PCR was observed except one chicken isolate in which WGS detected $bla_{TEM,}$ but not during PCR screening.

Conclusions: We conclude ESBL-producing *E.coli* may harbor plasmids that carry MDR genes for most of the 14 antimicrobials tested including the cephalosporins. WGS analysis was more sensitive when identifying AMR genes as compared to phenotypic characterization.

Introduction

Escherichia coli (*E. coli*) are Gram-negative organisms that can exist in a host as commensal or virulent populations of bacteria. Commensal *E. coli* typically resides in the gastrointestinal tract and can be a component of healthy gut flora. Virulence is largely dependent on the presence of different molecular attributes (toxin genes and resistance determinants in particular) that are responsible for increased morbidity and mortality (Lee et al., 2010; Li, Qu, Hu, & Shi, 2012). When *E. coli* are associated with human infections, infection is often considered less severe when compared to *Salmonella* (WHO, 2016).

While commensal *E. coli* colonize the human gastrointestinal tract without causing disease, the transfer of virulent genes and can result in infection. One report indicates that commensal strains of *E. coli* typically acquired virulence factors through the transfer of genetic elements from pathogenic microorganisms (Donnenberg, 2002). These virulent factors are typically encoded on bacteriophages, plasmids, or pathogenic islands (J. R. Johnson, Delavari, Kuskowski, & Stell, 2001).

Extended-spectrum β-lactamases (ESBLs) are enzymes that inactivate the β-lactam ring of antibiotics such as penicillin and cephalosporin, causing resistance to these antibiotics. ESBLs are classified into four Ambler molecular classification schemes, A through D (Paterson & Bonomo, 2005). They are also classified into four classes of the Bush-Jacoby-Medeiros scheme based on their functionality similarities (Paterson & Bonomo, 2005). *E. coli* and *Kluyvera* species are suspected to be the originating organisms for the ESBL resistant mechanisms described with the original genes acquired via transposition and mutation (Humeniuk et al., 2002). Reports indicate that ESBLs are derived from TEM and SHV genes by mutating through mutation of amino acids in β-lactam ring near the activation site (Paterson &

Bonomo, 2005). Resistance is known to transfer via plasmids, which often carry resistance to many other antibiotics. This compromises treatment options, leaving most treatment regimens highly ineffective (Paterson & Bonomo, 2005). Today, there are more than 200 genes characterized as ESBLs from over 30 different countries (Paterson & Bonomo, 2005).

In African countries, such as Nigeria, South Africa, Israel, Saudi Arabia, and Kenya, the first reported outbreaks of ESBL-producing organism originated in *Klebsiella* species. Only outbreaks were reported as no surveillance system were in place to track ESBLs in Africa (Paterson & Bonomo, 2005). This is slowly changing as the WHO has worked with developing countries in Africa to implement surveillance systems through the Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR). Some African countries involved include Cameroon, Tanzania, Kenya, Uganda, and Ethiopia (WHO, 2013).

In this study, we focused on the commonly reported ESBLs, including bla_{SHV} (referring to sulfhydryl variable), bla_{TEM} (known as Temoneira), bla_{OXA} (named for oxacillin.hydrolyzing abilities), $bla_{\mathrm{CTX.M}}$ (named for hydrolytic activity against cefotaxime) and other ESBL types, such as bla_{CMY} (Evans & Amyes, 2014; Jamali, Shahid, Sobia, Singh, & Khan, 2017). Previously we reported on the recovery of *E. coli* isolates collected from chicken and cattle farms in the Wakiso District of Uganda (manuscript submitted). Eight isolates presented as potentially harboring ESBL gene were subjected to plasmid analysis and screened for Class I integrons, Pulsed-field gel electrophoresis (PFGE) was also conducted. All isolates will be subjected to Whole Genome Sequencing (WGS) to determine concordance between AMR phenotypic and genotypic analysis.

Methods

Bacterial Isolates

Previously we reported on the phenotypic characterization of *E. coli* isolates from cattle and chicken farms in the Wakiso District of Uganda (manuscript submitted). All isolates (cattle, n=385 and chicken, n=334) were screened for resistance to ceftriaxone (MIC ≥4 µg/ml) and/or ceftiofur (MIC ≥8 µg/ml). Of these, 4/385 (1.0%) cattle and 4/334 (1.2%) chicken *E.coli* isolates were resistant to these antibiotics. These eight isolates were then characterized as described below. For all PCR screenings, boiled lysates were used as DNA templates. Isolates previously were frozen at -80ºC were struck for isolation to Tryptic Soy Agar (TSA) with 5% sheep blood and incubated overnight at 37ºC to ensure purity. Lysates were prepared by suspending a loopful of well isolated colonies into 200 µl of molecular grade water and vortexed at maximum speed for several seconds. The suspension was boiled at 100°C for 10 minutes, centrifuged at 13 X 1000 rpm for 60 seconds, and the supernatant was collected for use as the DNA template.

PFGE Analysis

The Pulsenet protocol developed at the CDC for PFGE analysis was conducted on all eight isolates (https://www.cdc.gov/pulsenet/pdf/ecoli.shigella.salmonella.pfge.protocol.508c.pdf). BioNumerics 7.5 software program was used to analyze the results (Applied Maths, A Biomérieux Company). A dendogram was constructed, and 85% was used as the cut-off for determining relatedness between isolates.

Class 1 Integron *IntI1* screening

All eight *E. coli* isolates were screened for the class 1 integron integrase gene *intI1*. The primers used for the target integrase gene *intI1* were forward primer (5’

CAGTGGACATAAGCCTGTTC 3') and reverse primer (5' CCCGAGGCATAGACTGTA 3'). The PCR conditions were as follows: 94°C (10 minutes) for the initial cycle; 29 cycles of 94°C (60 seconds), 54°C (60 seconds), 72°C (2 minutes); 72°C (10 minutes) for elongation cycle; and 4°C to hold (Dillon et al., 2005)

Replicon Plasmid Screening

All isolates were screened for 28 replicon plasmids using a PCR based replicon typing (PBRT) system. The PBRT Kit (Diatheva, Cartoceto, Italy) is a multiplex PCR that used for molecular typing of plasmids based on incompatibility groups of plasmids associated with antimicrobial resistance in *Enterobacteriaceae*. The PCR is comprised of eight multiplex groups and include the following replicons: HI1, HI2, I1α, M, N, I2, BO, FIB, FIA, W, L, P, X3, I1γ, T, A/C, FIIS, X1, U, R, FIIK, Y, X2, K, FIC, , HIB.M, FIB.M, and FII. The test was conducted as per manufacturer's procedures. This procedure is available at (https://www.diatheva.com/images/DATASHEET/MBK_MBR/MBK0038%20kit%20%20IVD%20CE.pdf).

ESBL Screening

All eight isolates were tested for the presence of the five most common ESBL genes: bla_{CTX}, bla_{TEM}, bla_{CMY}, $bla_{OXA.1}$, and bla_{SHV} (Bonnet et al., 2003; Brinas et al., 2002; S. Chen et al., 2004) using the Hotstar Taq Master Mix kit (Qiagen Inc, Valencia, CA) according to manufacturer's instructions. To examine molecular mechanisms of isolates presenting β-lactam resistance, isolates that presented resistance to ceftiofur (MIC≥8 µg/ml) and/or ceftriaxone (MIC≥4 µg/ml) antimicrobials were screened for ESBL producing genes. Boiled lysates were used as DNA templates as described above for bacterial isolates.

Whole Genome Sequencing

Whole genome sequencing was conducted to determine antimicrobial determinates using the blood and tissue kit from Qiagen (Qiagen, Inc. Valencia, CA) to extract the DNA from the eight isolates. Isolates were sequenced using manufacture's procedures for the Illumina Miseq (Illumina, Incorporated, San Diego, CA). All gene sequences were uploaded and analyzed in the CLC Genomics Workbench (Qiagen).

Results

Bacterial Isolates

Resistance to ceftriaxone (MIC ≥4 µg/ml) and/or ceftiofur (MIC ≥8 µg/ml) was used as a presumptive positive screening method to detect the presence of ESBLs. A total of eight isolates (cattle n=4 and chicken n=4) were resistant to ceftriaxone. Interestingly, only three cattle isolates were resistant to ceftiofur while all four chicken isolates were also resistant to ceftiofur. Multi-drug resistance was observed for all isolates and resistance to as many as seven different classes of antimicrobials were observed among these isolates (Figure 5.1 and Table 5.1). In general, chicken isolates exhibited a larger number of MDR attributes than cattle isolates.

PFGE Analysis

Analysis of the PFGE profiles indicated the presence of seven clades. Homology was only observed for two of the eight isolates. These two chicken isolates expressed identical homology across all tests. A summary of these findings are shown in Figure 5.1.

Class 1 Integron *Int1* screening

The class 1 integron integrase gene *intI1* was observed in all chicken isolates, and only one cattle isolate as seen in Figure 5.1.

Replicon plasmid screening

As many as three replicon types were found among the isolates primarily distributed among two cattle isolates. Two chicken and one cattle isolate did not harbor replicon-type plasmids (Figure 5.1 and Table 5.1) while two other cattle isolates were positive for *Inc*FIB and *Inc*FII. No homology was observed in replicon-type for the three additional cattle isolates.

ESBL Screening

One to three ESBL genes were among seven of the eight isolates. One chicken isolate was negative for ESBLs and replicon.type plasmids while harboring the integrase gene. $bla_{\text{CTX.M}}$ was the most common ESBL gene observed among the isolates as seen in Figure 5.1 and Table 5.1.

WGS screening

WGS presented results which identified phenotype/genotype discordance among one isolate for antimicrobial determinants. Tetracycline resistance was seen genetically with WGS but was not present phenotypically with microdilution as seen in Table 5.1.

Discussion

The development of antimicrobial resistance is most often mediated by the use of antimicrobials. Farm records on antimicrobial use in Uganda were incomplete and lacked detail making associations between use and resistance difficult. Most notably, there is no on-going surveillance system in Uganda, which captures veterinary or human data regarding resistance over time. This lack of historical information makes it nearly impossible to determine when or how resistance and associated attributes first emerged. This study was the first attempt to

establish a surveillance system in the Wasiko District of Uganda. On-going surveillance will be invaluable over time as mitigation strategies are developed. What is most important is the observance of transmissible plasmids harboring multiple resistance to drugs which may no longer be effective against some infectious diseases of cattle and chicken.

In this study, the objective was to characterize the presumptive ESBL *E. coli* observed in our previous study. We were able to confirm through PCR and WGS the presence of ESBL-producing *E. coli* among our isolates collected from the chicken and cattle farms in the Wakiso District of Uganda.

The cephalosporin antimicrobials used for screening in this study included ceftiofur which is for veterinary use in swine and ruminants (respiratory infections), horse (foot rot and metritis), and poultry (mortality infections) (Hornish & Kotarski, 2002); and ceftriaxone which is for human use to treat many bacterial infections (Source: https://www.drugs.com/search.php?searchterm=ceftriaxone&a=1). In this study, one isolate displayed susceptibility to ceftiofur and resistance to ceftriaxone. There is limited to research of this discordance, and it is unknown as to why. Isolates were tested more than once with the same results. Further investigation on the molecular level to know the reason for unlikely results of this isolate.

The PFGE analysis in this study shows that there is little relatedness to the ESBL *E.coli* isolates. The heterogeneity was evident in the ESBL *E.coli*; different clonal types were recovered from the various farms. The isolates clustered together were isolates that originated from the same farm. As in other studies, PFGE is used as the gold standard for confirming similarity between isolates, but *E. coli* prevents this due to high diversity in the genes (Anvarinejad et al., 2012).

Of the eight ESBL *E. coli*, five were positive for the class I integron integrase gene *IntI1*. The presence of integron gene cassettes harboring antimicrobial resistance genes is important for ESBL-producing organisms (Mehdipour Moghaddam, Mirbagheri, Salehi, & Habibzade, 2015). It has also been reported that in clinical samples, there was no relation between ESBL genes and the presence of Class I integrons except for *bla*$_{CTX.M.24}$, which is shown to be located on the platform of integrons consisting of a gene for the integron integrase *(intI)*; an integron.carried promoter (Pc); the integron.associated recombination site *(attI)*; and gene cassettes (Gillings, 2014; Machado et al., 2005). We did observe this among our samples as *Int1* was present in isolates harboring several ESBL genes including *bla*$_{TEM.1B}$, *bla*$_{CTX.M.15}$, *bla*$_{CTX.M.24}$, and *bla*$_{OXA.1.}$ We will have to explore the relationship more between *Int1* and these ESBL genes in future work by analyzing the sequences more. We also see that three isolates where *Int1* was not observed and in another isolate *Int1* was present, but no replicon plasmids were present. In this study, we did not further investigate the presence of *Int1* and replicons using WGS to see if there was concordance with the PCR, but will in future studies.

Variations between the sources (cattle and chicken) were observed with replicon-type plasmids. In our study, six of the 28 plasmids screened were present including *Inc*FII, *Inc*P, *Inc*HI1, *Inc*HI2, *Inc*X3, and *Inc*FIB. It has been shown that *bla*$_{CTX.M.15}$ has carried *Inc*F plasmids which include *rep*FII alone or *rep*FIA and *rep*FIB in combination (Sykora, 1992). As seen in this study the *Inc*FII plasmids have concordance with the *bla*$_{CTX.M.15}$ genes. *Inc*F plasmids are known to carry multiple replicons, where one is usually preserved by selective pressure carried out by the plasmid duplication (Sykora, 1992). Some reports indicate that *bla*$_{CTX.M.1}$ ESBLs from chicken and human *Enterobacteriaceae* samples harbor many incompatibility plasmids and

report seeing *Inc*HI1 for the first time (Zurfluh et al., 2014). Dissemination of *Inc*HI2 has also been seen in the spread on $bla_{CTX.M.9}$ (Carattoli, 2009).

We saw in the WGS that all eight isolates had at least one of the following ESBL genes present: $bla_{TEM.1B}$, $bla_{CTX.M.15}$, $bla_{CTX.M.24}$, $bla_{SHV.12,}$ and $bla_{OXA.1.}$ Discordance was seen in one isolate where no ESBLs or replicon plasmids were observed in the standard PCR. However, the ESBL genes were observed when screened through WGS. The isolate was screened again using PCR and ended with the same results. We also wanted to explore if there were any genes present genotypically through WGS as compared to the traditional PCR methods. In previous studies, discrepancies were seen in phenotypic resistance compared to genotypic resistance using WGS for beta-lactamase antibiotics cefoxitin, ceftiofur, and ceftriaxone. It was seen phenotypically that the MIC did not reach the breakpoints but had the resistant genes (McDermott et al., 2016). In this study, there was concordance for the β-lactamase antibiotics cefoxitin, ceftiofur, and ceftriaxone. Most of the genotyped isolates were in concordance with the PCR screenings. One chicken isolate was discordant with respect to tetracycline resistance. Phenotypic susceptibility was observed using the broth microdilution while WGS showed resistance to tetracycline. This isolate was retested with the same result.

It has been reported that although WGS is becoming the "gold standard" method of testing for antimicrobial determinants, discrepancies between phenotypic and genotypic results are observed. This may be due to the antibiotic breakpoints. Isolates approaching an intermediate or resistant range may present as phenotypically absent while the gene is observed by WGS. This is most often observed for the quinolone antimicrobials, streptomycins, and some first and second generation cephalosporins (McDermott et al., 2016).

Conclusion

We conclude ESBL-producing *E.coli* may harbor plasmids among that carry MDR genes for most of the 14 antimicrobials tested including the cephalosporins, a drug of choice for treatment of foodborne infections. Using WGS, we observed that a more extensive analysis of resistance and virulence attributes were possible among the ESBL isolates reported. Genotypic characterization was more sensitive than phenotypic characterization. This is important as resistance genes may be present but may not express themselves phenotypically. Additionally, the presence of MDR and ESBL- producing *E. coli* warrants continual monitoring through the establishment of a sustainable surveillance system.

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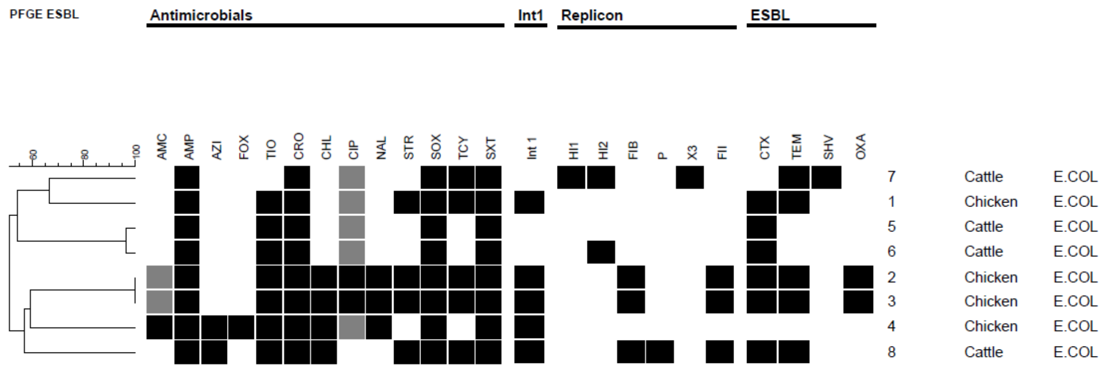

Figure 5.1: Summary of phenotypic and genotypic characteristics of ESBL *E. coli* isolates from Cattle and Chicken Farms in the Wakiso District of Uganda. PFGE Dendogram, resistance profile, Class 1 Integron screening, Replicon Typing, ESBL PCR gene screening, and isolate source. BioNumerics software was used to construct the PFGE dendogram with 85% similarity used as the cutoff. Black cubes are positive for the presence of the attribute; gray cubes indicate intermediate resistance. AMC=Amoxicillin-Clavulanic Acid, AMP=Ampicillin, AZM=Azithromycin, FOX=Cefoxitin, TIO=Ceftiofur, CRO=Ceftriaxone, CHL=Chloramphenicol, CIP=Ciprofloxacin, GEN=Gentamicin, NAL=Nalidixic Acid, STR=Streptomycin, SOX=Sulfisoxazole, TCY=Tetracycline, SXT=Trimethoprim-Sulfamethoxazole

Table 5.1: Phenotype and genotype WGS comparison of ESBL *E. coli* from cattle and chicken farms in Wakiso District of Uganda

Sample	Antimicrobial Resistance Phenotype (Sensititre)	Resistance by Antimicrobial Class by Genotype (WGS)	Antimicrobial Genotype (WGS)	ESBL (PCR)	Class I Integron (PCR)	Replicon Type Plasmids (PCR)
***E. coli*.1 (Chicken)**	AMP, TIO, CRO, CIP*, STR, SOX, TCY, SXT	aminoglycoside, beta-lactamase, quinolone, sulphonamide, tetracycline, trimethoprim	strA, strB, blaTEM.1B, blaCTX.M.15, QnrS1, sul2, tet(A), dfrA14	CTX.M, TEM	Int1	
***E. coli*.2 (Chicken)**	AMC*, AMP, TIO, CRO, CHL, CIP, NAL, STR, SOX, TET, SXT	aminoglycoside, beta-lactamase, quinolone, phenicol, rifampicin, sulphonamide. tetracycline trimethoprim	aadA5, aac(6').Ib, strA, strB, blaTEM.1B, blaCTX.M.24, blaOXA.1, aac(6')Ib.cr, , aacA4, catA1, catB3, ARR.6, sul2, sul1, tet(B), dfrA17	CTX.M, TEM, OXA.1	Int1	FIB, FII
***E. coli*.3 (Chicken)**	AMC*, AMP, TIO, CRO, CHL, CIP, NAL, STR, SOX, TET, SXT	aminoglycoside, beta-lactamase, quinolone phenicol, rifampicin sulphonamide, tetracycline trimethoprim	aadA5, strA, strB, aac(6').Ib, blaOXA.1, blaTEM.1B, blaCTX.M.24, aac(6')Ib.cr, , aacA4, catB3, catA1, ARR.6, sul1, sul2, tet(B), dfrA17	CTX.M, TEM, OXA.1	Int1	FIB, FII
***E. coli*.4 (Chicken)**	AMC, AMP, AZI, FOX, TIO, CRO, CHL, CIP*, NAL, SOX, SXT	aminoglycoside, beta-lactamase, phenicol, quinolone, sulphonamide, tetracycline, trimethoprim	strA, strB, blaTEM.1B, catA2, QnrS1, sul2, tet(A), dfrA14		Int1	
***E. coli*.5 (Cattle)**	AMP, TIO, CRO, CIP*, SOX, SXT	aminoglycoside, beta-lactamase, quinolone sulphonamide	strA, strB, blaCTX.M.15, QnrS1, sul2	CTX.M		
***E. coli*.6 (Cattle)**	AMP, TIO, CRO, CIP*, SOX, SXT	aminoglycoside, beta-lactamase, quinolone sulphonamide, trimethoprim	strA, strB, blaCTX.M.15, QnrS1, sul2, dfrA14	CTX.M		HI2
***E. coli*.7 (Cattle)**	AMP, CRO, CIP*, SOX, TCY, SXT	aminoglycoside, beta-lactamase, fosfomycin quinolone, sulphonamide Tetracycline, trimethoprim	strA, strB, blaSHV.12, blaTEM.1B, fosA, QnrS1, sul2, tet(A), dfrA14	TEM, SHV		HI1, HI2, X3
***E. coli*.8 (Cattle)**	AMP, AZI, TIO, CRO, CHL, STR, SOX, TCY, SXT	aminoglycoside, beta-lactamase, macrolide phenicol, sulphonamide tetracycline, trimethoprim	strA, strB, blaCTX.M.27, blaTEM.1B, mph(A), catA2, catA1, sul1 sul2, tet(A), dfrA7, dfrA16	CTX.M, TEM	Int1	FIB, P, FII

*Intermediate result, AMC=Amoxicillin-Clavulanic Acid, AMP=Ampicillin, AZM=Azithromycin, FOX=Cefoxitin, TIO=Ceftiofur, CRO=Ceftriaxone, CHL=Chloramphenicol, CIP=Ciprofloxacin, GEN=Gentamicin, NAL=Nalidixic Acid, STR=Streptomycin, SOX=Sulfisoxazole, TCY=Tetracycline, SXT=Trimethoprim-Sulfamethoxazole

Chapter 6: Antimicrobial Resistance Project: Evaluation of Makerere University College of Veterinary Medicine Laboratory Surveillance Capability

Executive Summary

Antimicrobial resistance (AMR) is detrimentally impacting public health on a global basis as reports increasingly reflect rises in mortality, morbidity, and health costs associated with AMR associated infections. By the year 2050, 10 million deaths are projected to be due to AMR, costing over $100 trillion globally. While inappropriate use in both human and veterinary medicine has contributed to the rise in AMR, it has also contributed to an increase in awareness of the problem.

The World Health Organization (WHO) established the Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) to advise and support WHO in the fight against AMR in food-animals in 2008. In the US, an executive order was issued to combat AMR in 2014. Notable gaps in AMR data are observed from the lack of information available from underdeveloped countries. This prompted the WHO to develop a call for grants to fund pilot projects for countries to develop AMR surveillance systems.

This report presents an evaluation to assess the quality and sustainability of an AMR surveillance program with the College of Veterinary Medicine, Animal Resources, and Bioterrorism (CoVAB) at the Makerere University, in Kampala, Uganda, who was awarded a country pilot project. The Centers for Disease Control and Prevention (CDC) framework for Program Evaluation Standards was used as a guide for this evaluation. A logic model was used to emphasize the outcomes and sustainability of the program. Some information on AMR and surveillance in Uganda was provided in the literature review. Through stakeholder collaboration, this evaluation addressed issues to determine if CoVAB met the project goals and determined if any changes should be made to sustain the program. An observational study design was used to gather data about the project design, implementation, and analysis. Following this evaluation,

these findings will be disseminated via a PowerPoint presentation and oral presentation to WHO. Recommendations for improvements to the project will be provided to WHO to publish.

Background

Statement of the problem

Antimicrobial resistance (AMR) is estimated to cause over 10 million deaths per year, costing over $100 trillion, globally (Mckenna, 2014). Antimicrobials consist of antibacterials, antifungals, and antivirals. There are many reasons attributed to the emergence of resistance which includes spontaneous mutations in microorganisms, evolutionary events, and use and misuse of antimicrobials (Masterton, 2008). Surveillance programs have been implemented most often in developed countries to combat resistance. These surveillance programs help to identify newly emerging and re-emerging pathogens (Masterton, 2008); a component of these programs may include monitoring for resistance. Data from surveillance programs can aid in the development of mitigation and control strategies (Bronzwaer et al., 2002).

Despite the implementation of surveillance programs in developing countries, there is a paucity of AMR surveillance data from underdeveloped countries, particularly in food-producing animals.

In 2008, the Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) was established by the World Health Organization (WHO) (WHO, 2017b). This group is comprised of experts around the world, including physicians, veterinarians, epidemiologists, data analysts, consumer representatives, and microbiologists. The primary goal of AGISAR is to support WHO in their efforts to protect public health by minimizing the development of AMR in food-producing animals. The AGISAR strategic framework to accomplish this is aligned with the five objectives of the Global Action Plan on Antimicrobial Resistance: " to improve awareness and understanding of antimicrobial resistance through effective communication, education and training; to strengthen the knowledge and evidence

base through surveillance and research; to reduce the incidence of infection through effective sanitation, hygiene and infection prevention measures; to optimize the use of antimicrobial medicines in human and animal health; to develop the economic case for sustainable investment that takes account of the needs of all countries and to increase investment in new medicines, diagnostic tools, vaccines and other interventions (WHO, 2015b)." Starting in 2010, WHO released a call for proposals to underdeveloped countries to pilot small and countrywide AMR surveillance projects to gather data on AMR within their area in an attempt to fill gaps in AMR data. This data will be included in the Global Antimicrobial Surveillance System (GLASS) (WHO, 2017b). The pilot proposals asked for two types of projects and included the following descriptions:

1. **"Small grants for focused projects"**

   These projects should include characterization of foodborne pathogens in at least two of the following sectors; human, food and animal. Characterization should include antimicrobial susceptibility testing. The duration of the project is one year and the total amount requested from WHO should not exceed 15,000 USD.

2. **"Country pilot projects"**

   These projects implemented at country level should include:

   - Sampling and characterization of foodborne pathogens (*Salmonella* and *Campylobacter* as a minimum) in human, food and animal sectors.
   - Antimicrobial susceptibility testing should be performed in pathogens and indicators (*E.coli, Enterococcus*).
   - Monitoring of antimicrobial usage in animals and humans (WHO, 2015b).

Developing countries around the world submitted applications for these projects, and over the past seven years, the WHO has collected data from these countries. With this information, the prevalence and incidence data were collected, emerging pathogens were detected, and antimicrobial usage was monitored.

Makerere University College of Veterinary Medicine Fights Antimicrobial Resistance

The Makerere University College of Veterinary Medicine, Animal Resources and Biosecurity (CoVAB) was founded in 1922 and is located in Kampala, which is the capital city of Uganda. The mission of the college is "to drive transformative knowledge, skills, innovations, and services for the continuous improvement of society." Their vision is to have healthier, wealthier and safer societies through animal value. (Makerere College of Veterinary Medicine, 2017)."

In 2014, Makerere University signed an Agreement of Performance of Works with WHO, to launch a countrywide pilot surveillance project for AMR. A workshop was held in March 2015. The objectives were as follows:

1. Launch the research projects of AMR in Uganda
2. Share information about the project to key stakeholders and solicit input
3. Share information on antimicrobial usage and the impact of resistance in animal and human health
4. Have a model for strategies to control and prevent AMR in humans and food animals.

In collaboration with North Carolina State University (NCSU) College of Veterinary Medicine (CVM), this project officially began in March 2016. The original objectives of this project included the following:

1. “To establish the trends in quantities of antimicrobial agents imported to Uganda in the period 2009.13
2. To identify the major distribution pathways and geographic destinations of antimicrobial agents imported to Uganda
3. To isolate *Salmonella and E. coli* organisms in samples from: a) humans, b) cattle and broiler chicken, and c) retail chicken meat and beef in Uganda
4. To test the antimicrobial susceptibility of bacterial isolates stated in above (Patel, 2017).”

For one year, four cross-sectional studies were conducted to collect fecal and environmental samples from cattle and chicken farms in the Wakiso District of Kampala. Samples were collected and cultured for the presence of *Salmonella* and *E. coli* at the CoVAB lab. Isolates were to be stored and later tested for AMR, and this information was to be submitted to WHO for inclusion in GLASS. The laboratory that was to perform this work is a teaching lab for students in the college where student learn different microbiological procedures related to outbreak samples. The lab is also used for student projects related to the epidemiology of microbial outbreaks.

Program Description

Antimicrobial Resistance Surveillance Programs

The Centers for Disease Control and Prevention (CDC) defines surveillance as follows: “Epidemiologic surveillance is the ongoing and systematic collection, analysis, and interpretation of health data in the process of describing and monitoring a health event (CDC, 1988)”. It involves collecting and analyzing data to report any threats related to the disease that affects the health of the public (A. P. Johnson, 2015). In the last 30 years, surveillance programs have been

implemented to monitor threats related to antimicrobial resistance, and they have been limited in number. Current antimicrobial resistance surveillance programs include DANMAP (Denmark), NARMS (United States), CIPARS (Canada), GERM.VET (Germany), JVARM (Japan), NORM/NORM.VET (Norway), SWEDRES (Sweden), NETHMAP/MARAN (Netherlands), ONERBA (France), and FINRES_VET (Finland) (A. P. Johnson, 2015; WHO, 2014).

The need for AMR surveillance is particularly important when infectious diseases require treatment with antimicrobials; resistant pathogens evade the effects of antimicrobials rendering treatment ineffective. Therefore, it is critical to assess the susceptibility of microorganisms prior to initiating treatment. To properly make informed decisions on treatment of these infections, knowledge of antimicrobial resistance from these pathogens can be ascertained over time through surveillance systems (A. P. Johnson, 2015).

Many countries lack surveillance systems, including Uganda, this project will provide the initial groundwork to implement an AMR surveillance program within the country of Uganda.

Logic Model

Figure 1 represents the proposed logic model representing an envisioned AMR surveillance program for this pilot project. The model provides the activities, outcomes, and impact of what the surveillance project will have the following implementation of a countrywide AMR surveillance program that will eventually include all sectors-food animals, humans, and the environment.

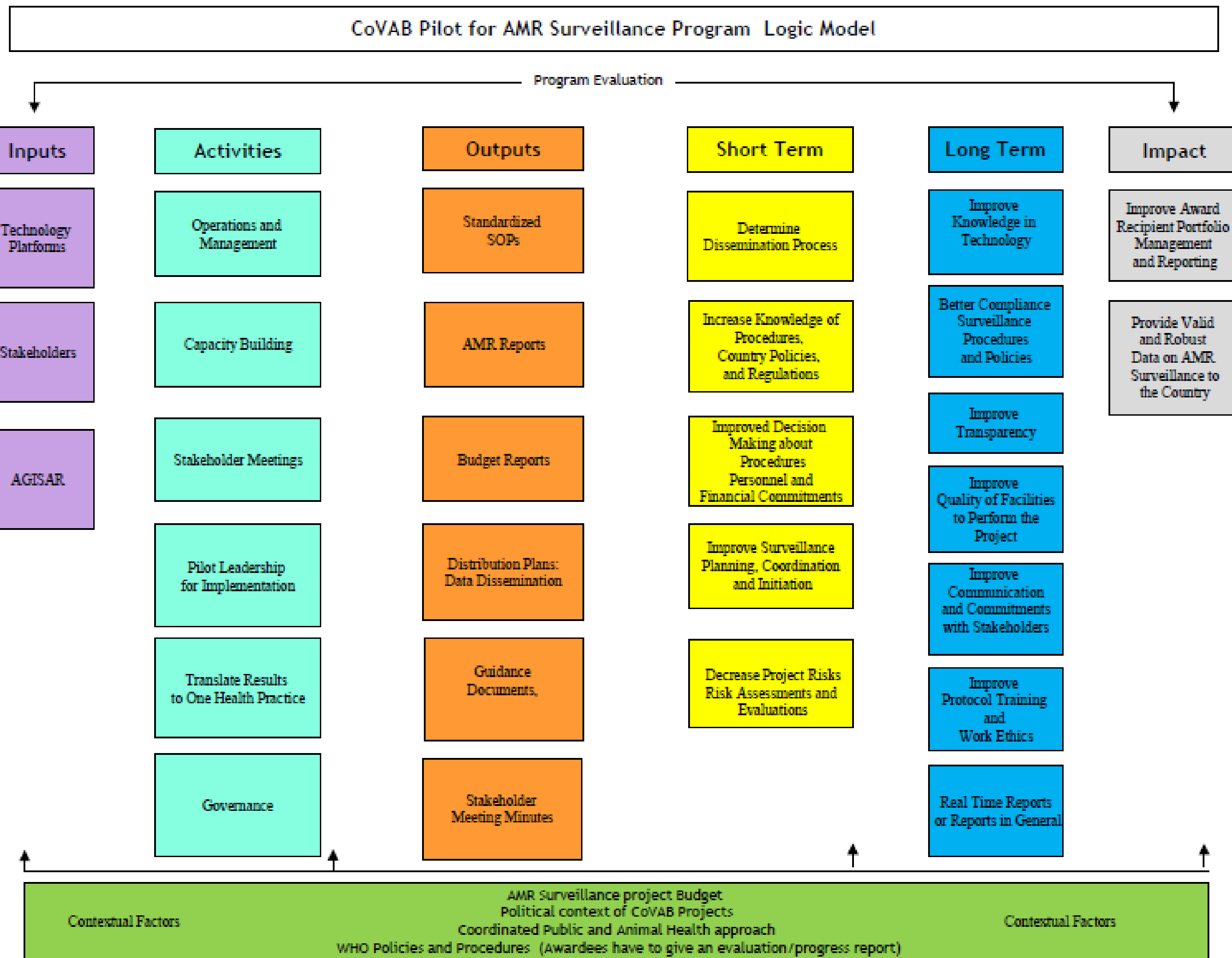

Figure 6.1: Logic Model

Stakeholders

An AMR surveillance project focusing on food-producing animals will be implemented by CoVAB staff (i.e., principal investigator, lab manager, laboratory staff, and Deans of the college) and the WHO Department of Food Safety and Zoonosis (i.e., AGISAR secretariat). These are the primary stakeholders of the AMR surveillance project as they developed and submitted the grant for funding. Another major stakeholder is the collaborator from North Carolina State University (NCSU) College of Veterinary Medicine (CVM), Raleigh, NC, USA, who will help with training and implementation of the surveillance system along with dissemination of the results through reports and publications. Other stakeholders include the Ugandan Ministry of Health (MOH), Ugandan Ministry of Agriculture (MOA), Animal Industry and Fisheries (MAAIF), National Drug Administration (NDA), International Livestock Research Institute (ILRI), Ugandan Veterinary Association (UVA), the World Animal Health Organization (OIE), Uganda National Council for Science and Technology (UNCST), Ugandan consumers and the Wakiso district farm owners. Because this project will be implemented countrywide, building the system requires a broadly collaborative effort to determine what information to collect, how to use it, and how to support many policymakers, farm owners, and researchers that need access to the data to make better informed public health decisions. Stakeholders engage in teleconference calls to provide forward-thinking, guidance, and consultation on the project plans and implementation goals. Also, the stakeholders recommend plans to enhance the AMR surveillance based on knowledge gained and a broad and overall understanding of the policies, practices, and procedures of the WHO and Ugandan food production systems.

The expected outcome of this AMR surveillance program is to improve the capability of the principal investigator to manage and report the acquired information as well as to provide a

valid and robust dataset on AMR surveillance to all stakeholders to improve animal and public health within Uganda. Short and long-term outcomes will be defined, and a timeline will be established.

The short-term outcomes are to:

By December 2016

- Determine the best means to disseminate information
- Increase knowledge of procedures, country policies, and regulations surrounding AMR
- Improve decision making about procedures, personnel, and financial commitments
- Improve surveillance planning and initiation
- Decrease project risks

Once short-term outcomes are accomplished the following long-term outcomes are projected:

- Improve knowledge related to data capture and information technology (e.g., WHOnet, GLASS)
- Better compliance with established surveillance procedures and policies
- Improved transparency
- Improved quality of facilities to perform the project
- Improved communication and commitments with stakeholders
- Improved protocol training and work ethic

Literature review

This literature review was conducted with two primary goals: 1) identify published research on AMR within the country of Uganda and 2) identify AMR previously conducted at the CoVAB lab. Using Google Scholar and Pubmed, the following keywords were used to search for published articles and studies: antimicrobial resistance in Uganda, food.borne pathogens in Uganda, antimicrobial use in Uganda, antimicrobial resistance in food.borne pathogens in Uganda, antimicrobial surveillance systems in Uganda, antimicrobial surveillance of food.borne pathogens in Uganda and evaluation of AMR surveillance systems.

The literature supports the use of the logic models to assist in the evaluation of the CoVAB lab, to be successful in implementing a pilot system for AMR Surveillance Program. The literature supports the need to focus on short-term goals in the early implementation period and then move on to the long-term goals to make this a sustainable system (W.K. Kellogg Foundation, 2004). There is a deficiency of information in the literature on research conducted on food-pathogens at a larger scale in Uganda, which will limit accomplishing the activities and outputs needed for implementation of this pilot project. The few research publications that were found were small projects that do not have enough information to support the validity of what is occurring concerning the development of AMR in food-pathogens within the country (UNAS et al., 2015). Other reviews that were found explain the lack of projects published in Uganda and lack of regulation to manage the emergence of AMR and its use (UNAS et al., 2015). All reviews and articles expressed the need for a more robust system for the country to monitor AMR to control reported increased resistance and deaths related to AMR infections. The most common issue identified the misuse of antimicrobials in animals and humans but acknowledged that there are no enforced regulations to stop the misuse. Another issue is the lack of personnel

and funding in the MOH, MOA, and NDA to enforce the regulations that are already established (UNAS et al., 2015). Many researchers are conducting experiments related to AMR but have yet to publish the data to inform others of their results. Research initially started with the intentions to contribute to a surveillance program but was never sustained due to the lack of funding and trained personnel. Because of the lack of surveillance systems and research related to AMR systems, there were few evaluations conducted regarding projects on AMR.

After reviewing all available literature, we concluded that due to the lack of information on AMR surveillance systems in Uganda, our logic model would aid us when evaluating this pilot project and when assessing the difficulty of implementation and long-term sustainability.

Evaluation

Evaluation Purpose

The purpose of evaluating this pilot project on implementation of an AMR surveillance system is to determine how to ensure the quality of the program remains high as the program grows. The stakeholders are specifically interested in the functionality and quality of program activities and outcomes. The evaluation results will determine how this program should be enhanced to improve performance.

- the evaluation will serve intended users the information they need."

Evaluation Stakeholders

The primary evaluation stakeholders and intended users include the Department of Population Health and Pathobiology Department Head of NCSU CVM, The Principal Investigator of the proposed AMR Surveillance lab at Makerere CoVAB lab, and the AGISAR secretariat. This evaluation will provide information on the implementation, progress, and recommendation of this project, to assist in any improvement(s) to ensure future sustainability.

Evaluation Questions

The following will guide the evaluation of the program:

1. What type of framework is required to choose a suitable applicant successfully?
2. What are the expectations of the applicant to perform and sustain a surveillance laboratory?
3. Will the applicant's lab be able to efficiently collect samples throughout the grant to obtain enough isolates and perform AMR testing?
4. Is there adequate trained staff available to collect and process samples?
5. Is the lab equipped to perform quality testing for antibiotic resistance including the use of appropriate controls, purchase lab reagents as necessary, and participate in proficiency testing?
6. Will the data be of sufficient quality and quantity for use in the integrated international database?
7. How will the funding agency monitor progress throughout the term of the project to ensure sustainability of the program?
8. What is the process of evaluating both the applicant and the funding agency after the project is completed?

Methods

Study Design: Observation and Qualitative Inquiry

To gather information for this evaluation, informal interviews took place with the CoVAB laboratory team that conducted the AMR project. They were asked fundamental questions about conventional methods, daily operations, financial situations, infrastructure, and work environment. They were also requested to identify any challenges they come across before

and after implementation of this project. The evaluation also included observational information the staff may not have addressed. The following is a collection of qualitative data that was collected:

- The condition of the facility in which the project will be conducted including building infrastructure, laboratory conditions and layout
- How equipped is the laboratory to conduct the project including collection, culturing, and testing equipment and supplies
- Transportation to and from collection sites including distance, road conditions, and type of transportation
- The knowledge and training of the personnel about the project
- The project design and how it fulfills the grant's requirements
- The methodology proposed for the project and how it was used or modified
- The timeliness of the project according to the duration of the grant
- The communication between all personnel involved in the project from collection, culturing, testing, and dissemination
- The methods used for data analysis and dissemination
- The ability of the laboratory to continue the project after the completion of the grant.

Evaluation Participants

This evaluation will include participation from stakeholders from CVM and the WHO using communications that have taken place through emails, teleconferences, and workshops to help to inform the evaluation.

Data collection and Analysis

As mentioned earlier, to collect the information for an informed evaluation an informal interview will be held with personnel at the CoVAB lab. This information will be recorded and logged. Observations of the conditions outside of interviews will be recorded and logged. Other observational information is the involvement of the CoVAB lab with the stakeholders during all communication such as emails, teleconferences, and workshops.

Recommendations and Dissemination of Results

After all of the information is collected and analyzed, recommendations will be formulated and provided to all participants involved in the project which include the CVM, the WHO secretariat, and the Makerere CoVAB. There will be a Microsoft PowerPoint slide show presentation created, and this written report of the evaluation will be included.

Evaluation Results

Qualitative and Observational Analysis

For one year, CVM assisted on-site with four visits working in collaboration with the CoVAB lab on this AMR surveillance project. During this time, questions were addressed, and observations were made and collected. The following results were collected to address the inquiries listed:

The condition of the facility in which the project will be conducted including building infrastructure, laboratory conditions, and layout: The Makerere University College of Veterinary Medicine was built in the 1980's and is the only Veterinary School in Uganda. The facilities have not been updated in years to keep up with other top universities and cannot accommodate new and innovative equipment for conducting state-of-the-art research. The labs

which were renovated were done with outside funds from nonprofit organizations such as the Walter Reed Foundation. Many of the labs are in dire need of renovations to properly conduct any research let alone high impact research. The infrastructure of the college has many issues with power. There are no backup generators for most of the building; therefore precluded most research from continuing until power is restored. Restoration of power could take hours up to days. Delays of this nature and duration often preclude completion of the work and affect the quality of the data if the work continues off schedule. The laboratory that was initially used for this project was located on the top level (4th floor) of the building that does not have elevator access. This makes it challenging to transfer equipment and supplies to the lab for routine use. Most of the equipment and supplies that will have to move up to the top level needed for the project will require payment for help to assist in safely transporting materials up long flights of stairs. The lab is sizable and adequate to carry out the work needed for the project, but some limitations compromise quality for use as a microbiology lab. When performing microbial work, the lab is required to be clean so as not to contaminate any samples or personnel in the lab. The lab will need to have limited access, a temperature-controlled environment with continuous power and equipment hooked up to a backup generator. This lab was not temperature controlled and had open windows. This allowed insects and dust to come into the lab on a daily basis. Because of this, the lab had to be cleaned on a daily basis. The lighting was very dull as some of the lights were out and never replaced. Many unused items in the lab should have been discarded to allow room for other more useful and needed items.

How equipped is the laboratory to conduct the project including collection, culturing, and testing equipment and supplies: There was equipment, but not enough for the amount of work required for this project. Equipment included a small benchtop autoclave capable of holding a

two-liter flask, two incubators (neither of which had been calibrated), two refrigerators (one working), a -20°C freezer (full with samples), and a -40°C freezer (empty but contaminated with mold). Glassware was limited and reused even though they were disposable. All glassware, pipettes, and tips were washed by hand and autoclaved afterward. No double deionized water was available for use in the lab. The media that was available for the project was limited, and some bottles had expired. There were no sterile supplies available for collecting the samples. These items were purchased for proper collection and culture the samples. After the supplies and equipment were obtained, an attempt to culture the samples in the lab was initiated, but due to the open windows, all samples and media were contaminated. Therefore, a request to use an enclosed and cleaner lab was made, and all work was subsequently moved to this lab even though it was very small and not large enough for this type of work. Additionally, use of the cleaner lab was dependent upon the schedules and needs of other personnel who used it.

Transportation to and from collection sites including distance, road conditions, and type of transportation: Because of lack of funding, a personal vehicle was used for transportation to the farms to collect samples. The vehicle was a Toyota Rav4 which was the largest vehicle available; it held four passengers. This vehicle lacked air conditioning for personal comfort and storage space for the supplies and collected samples. The SUV was not the best vehicle for the distance required for travel to the farms to collect the samples; the road conditions were also appalling and large, deep ruts were the norm. Once on the country dirt roads, the SUV could not traverse the dirt road due to deplorable conditions. There were two occasions where the vehicle got stuck on these roads and assistance was needed to get pulled it out of the ditch. To properly address this situation, a 4x4 truck would have been a better choice to use to overcome road conditions.

The knowledge and training of the personnel about the project: The purpose of CVM's involvement in the project was to provide expert training on the collection and culture of the samples and testing of isolates which is required to implement and sustain an AMR surveillance system. Upon arrival at the lab, it was expected that a permanent staff member would be there to train to collect samples, perform culture and isolation and conduct susceptibility testing on the isolates. Because of a lack of communication and funding, this was not the case. There was no staff to train for sample collection; therefore, CVM, the driver, and district veterinarians who were not affiliated with the university did all sample collections. The driver was a family member of the PI, and the district veterinarian was under contract to help with collections. Once samples were transferred back to the lab, the lab manager was to be trained to culture the sample and conduct susceptibility testing on the isolates. During the majority of the project, the lab manager and other staff were not working or available for training for reasons such as university strikes (which occurred the majority of the time during the project), lack of transportation to work, and family emergencies. To replace them, untrained undergraduate students were paid to help CVM personnel in the lab. These students were not permanent staff and would not be able to continue the project once CVM left. Without reliable, well-trained staff sustainability was questionable.

The project design and how it fulfills the grant's requirements: As mentioned previously, the objectives of this project were:

1. "To establish the trends in quantities of antimicrobial agents imported to Uganda in the period 2009.13
2. To identify the major distribution pathways and geographic destinations of anti.microbial agents imported to Uganda

3. To isolate *Salmonella and E. coli* organisms in samples from: a) humans, b) cattle and broiler chicken, and c) retail chicken meat and beef in Uganda
4. To test the antimicrobial susceptibility of bacterial isolates stated in above (Ejobi, 2017)."

At the end of this project, only two objectives (three and four) were partially completed. CVM was able to help complete collection of samples and culture of *Salmonella* and *E. coli* from cattle and chickens. Due to lack of funding, planning, and communication human and retail meat samples were not included in the final study. The lab did not procure any AMR testing supplies or quality controls for susceptibility testing. Therefore, all isolates were shipped to the CVM lab in North Carolina, US for AMR testing and further characterization of the isolates. *Campylobacter* and *Enterococcus* were omitted from sample cultures as a result of the lack of personnel, supplies, and equipment.

After many attempts from Ugandan collaborators to obtain antimicrobial import, distribution and use information, the CoVAB PI finally was able to obtain some information for objective one, but it was not complete information. This was partially due to the paucity of regulated imports of antimicrobials into the country (UNAS et al., 2015). Objective two was not addressed by the PI.

The methodology proposed for the project and how it was used or modified: The CoVAB had done previous work culturing food-borne pathogens using diagnostic laboratory culture procedures. CVM wanted to determine if the culture methods for *Salmonella* and *E. coli* were as sensitive and specific as those used at the CVM. A small pilot comparison study was conducted and determined that the CoVAB methods lacked sensitivity and specificity. A 40% increase in recovery was observed when the CVM culture protocol was used.

It should also be noted that the CVM culture protocol was modified because all reagents were not available locally. Prior to the inception of the actual project a decision was made that CVM would have the supplies and media ordered and shipped to the lab in Uganda. This is of major concern for future continuation and sustainability of the project unless there are other local companies who have the needed supplies at a reasonable cost.

Concerning AMR testing, the CoVAB lab usually used disk diffusion to test for antimicrobial susceptibility. The lab did not have the antimicrobials on disk available for testing nor did they have the required quality controls. Therefore, all isolates were shipped back to the CVM for phenotypic and genotypic characterization.

The timeliness of the project according to the duration of the grant: As mentioned earlier, only two of the objectives were partially completed. The grant was extended to allow more time to complete all objectives. There was some hesitation from the PI to get things done in a timely matter due to lack of funding, university closings as a result of strikes, lack of help, and not having the information required to finish the project. While all objectives were not fulfilled, some useful data did result from the AMR testing.

The communication between all personnel involved in the project from the collection, culturing, testing, and dissemination: Communication was a huge concern in every aspect of this project. The PI had to explain the project objectives to the granting agency prior to implementation of the project. While the proposal was initially written well, it did lack in some detail. As this project was implemented and progressed, it became quite obvious that the PI was ill-equipped to complete the project as described. Not only did the project take more resources than the PI anticipated, there PI had not prepared or seemed to have thought about, contingency plans.

Interestingly, the project was communicated very well in a workshop led by the PI to stakeholders and collaborators. Everyone was on board, and the project seemed very promising. Agencies, government officials, researchers, and media were informed of the proposed project and approved of the idea to implement an AMR surveillance system for the country of Uganda.

However, since the project began, there was miscommunication about the personnel who were to be involved in this project. The expectation was to train permanent staff to do the work, but none was available when the project began. There was also miscommunication on the condition of the lab and availability of supplies and equipment to perform the work. The PI and the lab manager did not seem to have a similar understanding of what their roles were for this project which led to the CVM to handle all the work. There was also lack of communication with the PI and staff about financial payments that resulted in some of the staff not working because they were not paid. The PI lacked transparency on daily work schedules and operations, which also hindered timely completion of the work

Communication to other stakeholders such as the district veterinary, driver, and farm owners were not as strong as it could have been in many circumstances. There was little communication to confirm visits to the farms for sample collection. Many farm owners did not know they were being sampled, and others did not have an understanding of why they were being sampled. The PI did not speak the dialect of the majority of the farm owners and relied on the district veterinarian to communicate both during and after visits. This worked until there were scientific terms that the district veterinarian did not understand in order to translate to the farm owners and managers. There were many disconnects in information communicated from PI to district veterinarian to farm owners and managers. The PI was also hesitant about taking directions from CVM staff to perform the project. There was miscommunication on the meaning

of some English phrases that were difficult to get across to both parties. The PI also was not appreciative of a CVM staff member of lower rank who asked questions about plans and daily operations. Position rank and/or gender may have been an issue in the relationship.

Communication between CVM and the WHO after the project ended continued through the next year. The PI continued to ascribe blame to the CVM as well as to the WHO who did not send funding quickly.

Overall, communication was a huge concern throughout this project. Transparency was lacking, and many cultural differences created stressful and challenging conversations that did not serve the project well.

The methods used for data analysis and dissemination: The PI did not communicate which methods were going to be used for data analysis. What was transmitted from the PI was an informal report which did not provide any detail relating to antimicrobial use on the farm. The WHO provided training to the PI on WHOnet which is the preferred software to capture antimicrobial susceptibility information. The PI was hesitant about learning this system for data analysis but eventually took the training; no further analysis was conducted by the PI even with the training. As a result, the isolates were shipped back to CVM for AMR testing, and all data analysis was done at the CVM. Data analysis was also completed at the CVM using WHOnet and Microsoft Excel. CVM conveyed dissemination of the results of the project. Dissemination methods include powerpoint presentations, poster presentations, and publications. To date, the work was presented as a poster at the Med.Vet.Net Association 5th International Scientific Conference, June 2017, in Surrey, England, the NCSU CVM Research symposium, September 2017, the 2nd Annual Uganda National AMR Conference, November 2017, the International Society of Disease Surveillance Conference, January 2018, the Consortium of Universities in

Global Health Conference, March 2018, and the North Carolina State Graduate Research Symposium, March 2018. Three manuscripts were written using project data and will be submitted.

The ability of the laboratory to continue the project for the duration of the grant: After evaluating the lab environment and the personnel work ethics, there is very little chance of this project continuing and sustaining after the period of the grant. The personnel was not there for training the majority of the time. The lab is not up to standards for microbiological work. The communication and education about the project and the methodology needed improvement. Recommendations to improve the project to ensure sustainability will be provided in the recommendations section. If work does continue with no improvements, the quality and validity of the data are questionable.

Evaluation Questions

After going through the qualitative and observational analysis, we can now answer the evaluation questions we wanted to answer before project implementation.

What type of framework is needed to choose a suitable applicant successfully?

The WHO AGISAR secretariat already has an application process in place for proposals on the small grants and countrywide grants. To receive better-qualified applications, additional information on the work that the PI has done in the past will be useful when assessing the PI's capability to implement and sustain projects like this. Providing peer-reviewed publications, first author publications, and a detailed protocol and budget for the project should be required. If any of the applicants are qualified using this additional criterion, a site visit should be made to visually inspect the lab, ensure supplies can be obtained, and that well.trained staff is in place. On-site visits, observations should be made and recorded of the infrastructure, the lab conditions,

the equipment and supplies available (locally and elsewhere) and access to help. If everything is acceptable, then the applicant is qualified for funding.

What are the expectations of the applicant to perform and sustain a surveillance laboratory?

The expectations for the applicant are to implement and sustain a surveillance system ensuring they are using the newest and most innovative means to conduct the study. They must continue training and education to develop new ideas for sustainability of the system including knowledge of methodology, data analysis, and reporting. Communication and networking with others that are successful in this field are required. Building collaborations on other projects is a way of capacity building that strengthens the program and allows for new ideas to improve performance and sustainability.

Will the applicant's lab be able to efficiently collect samples throughout the grant to obtain enough isolates and perform AMR testing?

The PI of this project does not have the trained personnel or the appropriate vehicle to continue collection of the samples. Because of this, CVM had to intervene by using their own trained personnel with collecting the samples to obtain the isolates needed for this project. The lab that the PI attempted to use for culturing and AMR was not appropriate for microbiological work and therefore had to be moved to a more appropriate lab. Because the staff was not aware of the proper procedures to do AMR testing using quality controls, the AMR testing was moved to CVM to provide more efficient testing to give better quality results.

Is there enough trained staff available to collect and process samples?

As mentioned earlier, there was no trained staff available to collect and process samples. The staff that was available were not permanent staff to ensure program continuity nor did they

have the expertise or education to maintain a quality laboratory system. The staff member that was permanent was not available for training the majority of the time and did not participate in the collection and processing of samples. CVM assumed this responsibility.

Is the lab equipped to perform quality testing for antibiotic resistance, purchase lab reagents as necessary, and participate in proficiency testing?

The lab was not able to perform any AMR testing. They were not supplied with the appropriate supplies and equipment to do AMR testing, nor was the staff trained in proficiency testing. The staff did not have the proper training to conduct disk diffusion and or quality control testing. The lab did not have the appropriate quality controls to run with the AMR testing. Reagents could be purchased locally, but there was not enough funding to do this after purchasing collection and culture supplies. To obtain quality controls and antimicrobials for testing was another issue because they were not available locally. These would have had to have been ordered outside of Uganda and required import and export permits. Some, but not all antimicrobials could be purchased for disk diffusion procedures within the country.

Will the data be of sufficient quality and quantity for use in the integrated international database?

Since the AMR testing was done at the CVM facilities, the data will be of sufficient quality and quantity for use in the international database. Once all data has been analyzed and written in reports and manuscripts, raw data will be sent to the WHO AGISAR secretariat for importing into the GLASS database. CVM, with the help of the WHO, has provided the CoVAB PI an individual report for each farm with all the AMR data related to their farms. These reports also included recommendations and guidance from the WHO on improving the hygiene of their farms to decrease AMR. We hope that these reports were distributed to all farms by the PI.

How will the funding agency monitor progress throughout the term of the project to ensure sustainability of the program?

Throughout the project, the WHO and CVM have had ad hoc meetings with the PI in CoVAB to get updates on the status of the projects and encourage and give advice on the methodology. There was hesitation from the PI at times to continue the project due to lack of funding and issues with strikes at the university. With encouragement from WHO and CVM, the project continued with no interruptions. The funding agency also requested reports from the PI regarding updates on what had been done and what was left to do.

What is the process of evaluating both the applicant and the funding agency after the project is completed?

The CVM has elected to do this evaluation of the process of this project as an outside organization. The hope is that this evaluation would provide insight and recommendations to make improvements for future grant applicants.

Strengths and Limitations

Strengths

Overall the strengths of this project were 1) the need for a surveillance system on AMR in the country of Uganda and 2) this was the start of implementing the surveillance system. The idea for the proposal was great and was very promising. The collaborators and stakeholders were on board and ready to make this initiative happen. All stakeholders that were needed for this project were aware of what was necessary for this project to happen.

With the help of the CVM staff, this project did ensure high-quality data were available for dissemination to inform future projects and provide a map for others to follow and continue. Only then would this be considered a sustainable surveillance system.

One thing that was observed was when there were obstacles; the CoVAB lab found a way to make things work to the best of their ability to continue the daily process during the project. There were a lot of encounters that came about that could have hindered the project, but they were all worked out to continue the project.

Limitations

There were many limitations of this project that resulted in modification of many procedures. Sampling was one example. Because most of the sampling farms were in rural areas, the road conditions made it difficult to get to all locations, especially during the rainy season. Most of the roads were one lane dirt roads with very large potholes that made travel difficult to impassable. The vehicle got stuck in large ruts at times and extended the time to get to each farm. The weather was an issue during the rainy season as runoffs from dirt roads made it difficult for travel and increased the potential for the vehicle to get stranded in the potholes. The vehicle was not fit enough for this type of travel.

As mentioned earlier, the overall communication was an issue. Some farmers were not aware of the project we were conducting and hesitated about allowing us to collect samples. There were also many dialects spoken that were not understood by the PI, and the district veterinarian had to be on the premises to communicate to the farm owners and managers and serve as the translator. There was also supposed to be a prepared questionnaire supplied by the PI with IRB approval; the questionnaire was unavailable although IRB approval was received, CVM surveyed the farm owners.

Because of high temperatures within the country throughout the year, extra caution was employed when transporting the samples back to the laboratory. Everything had to be on ice and sealed properly to ensure the viability of the samples when cultured.

Once in the laboratory, there were many limitations because the lab was not appropriate for microbiological work. The original lab issue included antiquated equipment, dust and insects coming in from the open windows, lack of trained staff on collection, methodology and data analysis, lack of lab supplies, poor communication, poor work ethics, and limited access and understanding to current literature on methodology.

Recommendations

Below are the following recommendations issued to improve upon to make this project sustainable if allowed to continue in the future:

Funding Agency (WHO)

- Request a full profile of the applicant that includes previous work and peer.reviewed publications
- Request work experience from staff members that will be assisting in the project
- Request a detailed protocol for the project proposed
- Request a detailed budget for the project proposed
- Conduct a mandatory site visit
    - Verify facilities are accepted to perform the project
    - Verify the equipment is fully functional and calibrated for use for this project
    - Have a meeting with the staff that will be involved in the project
- Better effort to distribute funds to the awardees in a timely matter
- Continue evaluation checks to determine the laboratory are on track with the project

The CoVAB Laboratory

- Will need to hire more permanent staff to work on the project

- All staff will need proficiency training in methodology and data analysis. The methods used for this project are state-of-the-art methods that are used by CVM, and it is recommended to continue with the same methods to ensure a valid and robust data set to GLASS.
- Communication needs improvement between the staff and farm owners/managers from the PI to understand better what the project is about; expectations for all parties need to be conveyed.
- Better transparency between the PI and stakeholders
- PI needs a better accounting process for financial expenditures throughout the project
- Instead of ad.hoc reporting and meetings with stakeholders, meeting needs to be scheduled. For example, quarterly meetings with the funding agency for updates and quarterly meetings with farm volunteers to keep them informed about the project.
- An entire new laboratory facility needs to be used for this project. The lab and building are not suitable for this type of project on a continuous basis in the condition it is in. Either the lab has to be renovated, or the work should be moved to a different facility that is fully functional.
- The equipment is very antiquated, and most pieces are not in a working condition to ensure validated data. New equipment is needed, and the proper equipment is required. If the grant funding is not enough to purchase new equipment, the PI needs to find funds that will allow the project to carry on

- PI is not familiar with the data analysis format the WHO recommends using the global integrated database. More training is required.
- Because the CVM performed all the analysis and reporting for this project, the PI needs to become familiar with the format used and improve upon reporting skills, which relates back to improve on communication.
- CVM will disseminate the data for this project, so it is recommended that PI follows the same procedures in future.
- PI needs to better schedule projects and make sure there is financial stability to perform what is being proposed. The experience and communication skills were lacking, and this hindered the successful execution of this project.
- It is recommended to procure better transportation to handle the travel required for sampling for this project, especially if it is to increase on a larger scale. A 4x4 utility truck is needed for better travel and handling of the road and weather.
- Everyone at the CoVAB lab needs to have a better understanding of what is being asked for in this project and what is expected of them. There was no information given to the staff about what they were doing and why; this affects implementing new procedures. This leads back to the lack of communication and transparency.
- The funding for this grant was insufficient for this project; in particular, this laboratory lacked supplies and equipment that were accessible. In other labs, the funding would have been sufficient. For this lab, other funding sources will be needed to continue and make this project successful and sustainable in the future.

Conclusions

Overall, the objectives of this project were very promising and can still be implemented if the above recommendations are accepted and improved upon in the future. Currently, the PI of the CoVAB for this project has received funding from the Food and Agriculture Organization (FAO) to continue work on implementing an AMR surveillance program in Uganda. There is uncertainty if the same procedures will be used or if any of these recommendations will be taken. It is understood that implementing a surveillance system countrywide can take years and this is just the beginning stage in Uganda. We hope there is an improvement in the future and a sustainable program in years to come.

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Chapter 7: Discussion

Discussion

In 2014, Makerere University was offered an opportunity to start a program aimed at improving the health system in Uganda. This opportunity was a chance to implement an AMR surveillance program for food pathogens. As AMR was already of global concern, and likely to contribute to increased morbidity and mortality each year, implementing an AMR surveillance program would provide health and related professionals in the country timely access to emerging resistance information. This information would be invaluable when developing mitigation and control measures. The WHO was aware of the growing resistance problem globally and initiated a grant program for developing countries to improve their own AMR surveillance. Countries that have been involved in the WHO program include India, Ethiopia, Cameroon, Bangladesh, Tanzania, Ghana, and in Latin America. It could take many years to get a program up and running as obstacles such as funding, political buy-in and consumer buy-in, trained staff and well-equipped facilities are often problematic at program inception.

At an initial workshop at Makerere University, the project was introduced to many stakeholders including the cattle and chicken producers and had support from both the Ministry of Health and the Ministry of Agriculture. During the workshop, CVM and WHO collaborators visited laboratory and farm facilities and were assured that laboratory capacity and equipment necessary was available, and that permissions to visit farms will be granted. However, several issues hindered the progress of the project: poor coordination from the local PI, inadequate facilities, lack of communication between involved parties, and promises by the PI beyond the reality of the facilities or available funding. An evaluation was performed to determine the strengths and limitations of the project management at Makerere, laboratory facilities, personnel

support necessary to continue and complete the pilot study, and accuracy of the data collected and how to report these data to a global integrated database.

The project was re-planned to increase performance and changes made consisted on: moving to a different laboratory, training train the staff on field data collection methods and culture of *Salmonella* and *E. coli* from samples obtained from cattle and chicken farms, overseeing farm visit coordination, and sample processing. Nothing was easy due to infrastructure problems (e.g., windows opened during dry season, poor sample refrigeration, and insects), constant power outages, and impassable roads during the rainy season. Improvements were made and the project proceeded. The participating farmers spoke many different dialects and communication became a problem. Farmers have little knowledge of what antibiotics were and confused them with vitamins. These farmers were promised information that was not delivered and a few were lost to follow up. Shipping samples and equipment was also a hassle.

At this point, objectives three and four (collection and culture) were satisfied by our team at CVM. Objectives one and two have not been completed by our Makerere partners.

How does this story end? What have I learned from this work? Data was collected and samples processed at our laboratory at NC State; peer-review articles are being prepared for publication; and data will be submitted to the WHO GLASS global integrated database. Our findings are similar to what it is seen in other countries. For instance, the major *Salmonella* serovars in our study were Kentucky and Enteriditis. All *Salmonella* serovar Kentucky isolates exhibited ciprofloxacin resistance. Ciprofloxacin-resistant *Salmonella* serovar Kentucky had been a part of an outbreak in early 2000's from poultry in East Africa. It will be of interest to see if any of the isolates from this study are related to the ones in the outbreak. We also observed the majority of the isolates exhibiting resistance in tetracycline (81% *E. coli* and 51% *Salmonella*

and chicken), which compares to what is reported from other countries. The majority of the farmers in our study use oxytetracycline to treat their animals. Farmers and farm managers do not call the veterinarian due to cost and diagnose the animals themselves and medicate.

Other findings include azithromycin resistance, which is atypical in the US. It would of interest to investigate further, what caused this resistance, especially in the animal population since this is an antibiotic used in humans.

WGS was performed on a few of the isolates in this study for the ESBL *E. coli* to compare to the PCR results. We determined that for the majority of the isolates, the methods were comparable. Since the cost of WGS has decreased tremendously, the WHO has decided to supply labs in developing countries to do WGS in the near future. This will allow for faster and quality results. The idea behind using WGS is to analyze resistance genes and associated plasmids and integrons.

Although my involvement in this project was limited, I got a sense of the difficulties in conducting international projects aimed at improving global surveillance of AMR. It is not only the desire to conduct a project that is needed or thinking that funding will suffice to make the project successful. There is coordination at different levels and stages of the project, learning from set-backs, constant evaluation and re-defining goals, and above all, adapting to the reality of the country and problem to be resolved.