EVALUATION OF A CLIMATE-SENSITIVE DISEASE CONTROL STRATEGY AND INVESTIGATION OF MULTIDRUG RESISTANCE IN INFECTIOUS BACTERIAL DISEASES: A US-AFRICA EXPERIENCE

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Title

Evaluation of a climate-sensitive disease control strategy and investigation of multidrug resistance in infectious bacterial diseases: A US-Africa experience

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ABSTRACT

This paper presents two research projects that explore avenues of controlling infectious diseases both in Africa and the United States. In Uganda, a retrospective study of Otuboi Sub County patient data to evaluate the impact of Stamp Out Sleeping sickness (SOS) intervention was performed. Polymerase Chain Reaction to detect the conjugatively transferred virulence factors from MDR *E. coli* to *Salmonella* was performed in North Dakota.

Human African Trypanosomiasis prevalence was significantly reduced at intervention year (2006) compared to the pre-intervention years; 2004 (P = 0.00024) and 2005 (P = 0.000001). Of the 22 screened virulence factor genes, eight genes were PCR detected in MDR *E. coli* 2077 isolate. Six of the detected genes were found to be received by *Salmonella* transconjugates.

The protective effect of SOS intervention was sustained for only two years (2007 and 2008) post intervention. MDR *E.coli* 2077 isolate conjugatively transferred its virulence factors to *Salmonella* strains.

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I would like to thank all my mentors from both institutions; College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University and Department of Veterinary and Microbiological Sciences; College of Agriculture, Food Systems, and Natural Resources; North Dakota State University. Not to forget of course the American people for through USAID I was sponsored to attain this Master's degree.

DEDICATION

I dedicate this piece of work to my sons Batrum, Baron, and Baggio, for their patience when I have been away most of the time trying to achieve my academic dreams.

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CHAPTER ONE. GENERAL INTRODUCTION

Human African Trypanosomiasis (HAT), also known as sleeping sickness, has been identified by the Wildlife Conservation Society (New York) as one of the twelve infectious climate-sensitive diseases that is likely to spread to other parts of the globe due to climate change (31). Previously, HAT has been endemic only in African countries (5). The adverse effects of climate change have not only affected global food security but also global health security (18) as they affect the epidemiology of the climate-sensitive diseases. The adverse effects of climate change also affect the biota and physical aspect of the globe (37). Even though HAT has been grouped among the climate-sensitive diseases, the effect of climate variability on HAT epidemiology has not been explored (27; 31; 32). Apart from being a climate-sensitive disease, HAT has also been classified as a neglected tropical disease. Detailed studies are needed to successfully implement its control.

It is well established that climate information has been incorporated in the development of early warning systems for climate-sensitive diseases (32). The challenge is to have convincing evidence of the impact of climate variability on disease epidemiology (32). The Stamp Out Sleeping Sickness (SOS) campaign is one control strategy for HAT that has been used in Uganda (40). The SOS campaign presents a cost effective initiative for controlling a climate-sensitive disease like sleeping sickness (20). Even though the effects of SOS were evident in reducing the outbreaks in the northeastern Uganda, there is a need to evaluate this campaign for documentation and establishment of its impact on HAT incidence. It is this baseline information that will possibly guide future applications in the control of the climate-related diseases. The objective of this research is to evaluate the SOS campaign by studying its effects on the prevalence of sleeping sickness.

Multidrug resistance (MDR) by bacteria is a problem in the therapeutic use of antibiotics (7). Over the past ten years, the spread of MDR in *E. coli* in North Dakota cattle has made control of calf scours very difficult and costs the producers great economic losses. In an effort to understand the transfer mechanisms underlying this MDR using *E. coli* isolate 2077, we proposed that *E. coli* isolate 2077 could transfer its genes for antibiotic resistance and virulence to *Salmonella*. In this study we investigated the spread of MDR and virulence factors in bacterial infectious diseases. We used *E. coli* 2077 isolate as the donor of the MDR and virulence factors and *Salmonella* 2253-6A isolates as the recipients. Both the *E. coli* 2077 and *Salmonella* 2253-6A isolates were obtained from the Veterinary Diagnostic Lab (VDL - ND) at North Dakota State University. This *E. coli* has been associated with multidrug resistance in hundreds of cases of calf scours in North Dakota (16). In this study, we hypothesized that the MDR *E. coli* 2077 can transfer the multidrug resistance and virulence factors to other bacteria such as *Salmonella* by sharing a plasmid on which these MDR and associated virulence factors are located.

Understanding the fundamental processes underlying antibiotic resistance and virulence within bacteria and the resulting impact on humans, animals, and the environment forms an important basis for influencing these processes and outcomes (6; 7). These investigations could be useful in addressing the problem of transfer of virulence and multidrug resistance in *E. coli* and *Salmonella* in North Dakota.

1.1. Problem statement for project 1: control of climate-sensitive diseases

Presently, intergovernmental organizations are working to reduce the effects of climate change. Mitigation, adaptation and resilience to the effects of climate change require documentation to allow for successful implementation of control strategies (35). But there is doubt as to whether these same solutions will be relevant in stopping the spread of the infectious

climate-sensitive diseases, especially those that may cross international boundaries. HAT has been identified as one the climate-sensitive diseases that is likely to spread to other parts of the world in addition to Africa, where it is endemic (31). Evaluation of possible strategies to control diseases that are climate-sensitive is part of the struggle; otherwise it may be of no surprise to see outbreaks of tropical diseases in formerly cold environments. Without evaluation and monitoring of these intervention campaigns, we may lag behind in the ability to mitigate disease outbreaks. The SOS campaign against sleeping sickness has been successfully implemented, but is not a yet widely known disease control program (20; 40). SOS used the "one health, one world" concept in the control of HAT in Uganda. Although the evaluation of SOS is expensive, there has been a need for it to be done in order to guide future interventions.

1.2. Problem statement for project 2: spread of antimicrobial resistance

In the case of the developed countries like the United States, the systems are working to track drug resistance in bacteria to direct responsible use of antimicrobial agents in the food animals (7). There is much that needs to be known in order to efficiently manage the global public health problem of the increasing antibiotic resistance (15). *Escherichia coli* has become a factory for drug resistance and virulence genes and it shares these genetic elements with other pathogens (1; 16; 23). Recently there have been isolates of *Salmonella* identified with relatively similar patterns of drug resistance as those of *E. Coli* (1; 15). Our research seeks to determine the mechanisms by which other pathogens obtain MDR and virulence factors. Using this basic science, we may be able to address the problem of drug resistance associated with calf scours, and perhaps other diseases, more efficiently.

1.3. General purpose of the research

This master's paper consists of two projects that were intended to give the candidate of the IDM program an experience of the international platform in the arena of research to control infectious diseases and also to benefit the communities through the dissemination of the research findings.

1.4. Main objective

To investigate the approaches to infectious disease control both locally and internationally.

1.4.1. Specific objectives

1) To determine the impact of Stamping Out Sleeping sickness (SOS) Campaign in Uganda.

2) To investigate the transfer of virulence factors associated with multidrug resistance in *E. coli* in the state of North Dakota.

1.5. Literature review

According to the United Nations Forum for Climate Change Convention (UNFCCC), climate change has been defined as a result of certain anthropogenic activities that increase emissions of greenhouse gasses (GHG) (34; 36). Given the current scientific, technical, and socio-economical knowledge, there is evidence that global warming is a reality (37). The World Bank (in the world development report 2010) estimated the costs associated with climate-sensitive diseases and their impact on health to be as high as 9 % of the gross domestic product in some countries (46). Several infectious diseases have been seen to spread to new places in different continents where the climatic conditions previously did not favor their vectors. For example, West Nile Virus was first isolated in Uganda but now has spread to different countries in the tropics and even to the temperate regions of Europe and North America (8). West Nile

Virus is currently prevalent in the United States. The spread of West Nile Virus may be attributed to the change in climatic conditions that could have favored the vectors.

The traditional way of controlling climate-sensitive diseases has been by direct intervention by treatment of the infected animals and humans. However, research has shown that interventions that take the approach of dealing with the source of the disease are very effective (44). In Uganda, 75% of trypanosome pathogens in the reservoirs (cattle) in the infected areas also infect humans (44). Uncontrolled movement of cattle in Uganda has been linked to sleeping sickness outbreaks, given that these cattle were found to be the reservoirs of the infectious trypanosomes (11).

This project presented an interesting scenario of the natural history of HAT in Otuboi Sub County where HAT outbreaks were newly reported during 2004. According to Nathan *et al.*, 2004, the environment was vacated for over a decade (20; 24), which allowed the vector (tsetse fly) population to increase. The study area was under political insurgences that led to displacement of the people. The area was covered with bush that provided a very good habitat for the tsetse fly. The first case of HAT in Otuboi Sub County was registered in 2004 when the people began to return to their homes in Otuboi Sub County (4; 20).

1.5.1. Influence of climate change on infectious diseases

The most important issue about the influence of climate change on infectious diseases is to understand the influence of climate variability and change on the disease incidence, outbreak and spread (5; 27; 32; 46). Despite the difference in the regional contribution to climate change, poor countries are the most vulnerable to its adverse effects (34; 35; 45; 46). This is because they have fewer resources to adapt socially, technologically and financially (18; 35). HAT is

considered one of the climate-sensitive diseases that impose a heavy burden on resource hungry areas in Africa (5; 44). Changes in climate can have a major impact on human health by affecting the environmental conditions in which disease vectors live and reproduce (27; 32). The commonality in both the climate systems, temperate and tropical, is that the mechanisms of disease spread are influenced by the climatic conditions (5).

1.5.2. Control of HAT by the SOS Campaign

In Uganda, control of HAT has been attempted several times by international and national programs, but regardless of these interventions, HAT outbreaks continue to occur (42). An organized approach to control HAT using the "one health one world" initiative was used by SOS campaign (40) in 2006.

According to World Veterinary Association (Hamburg, Germany), the "one health one world" concept is a worldwide strategy for expanding interdisciplinary collaborations and communications in all aspects of health care for humans, animals, and the environment. The control of HAT in the country has attracted involvement of professionals from different fields including, but not limited to, veterinarians and medical professionals (40). A detailed description of SOS activities have been published elsewhere (17).

Briefly, the project involved use of a Restricted Application Protocol whereby cattle were sprayed with insecticides at specific points to act as live baits for tsetse flies (40). Additionally, bushes were cleared to interrupt the breeding ground for the tsetse flies, and massive prophylactic treatment of cattle (reservoirs) with trypanocidal drugs was conducted to clear the cattle of trypanosomes so that when the tsetse flies fed on them, they would not acquire the infection (17).

In addition to the above mentioned SOS activities, sensitization and mobilization of the community was part of the strategy in increasing public awareness in order for patient to continue seeking treatment. In the SOS campaign, mass treatment of cattle with trypanocidal drugs was carried out to clear the cattle of trypanosomes so that when the tsetse flies feed on them, they do not acquire the infection (40). Doctoral students from Edinburgh University, whose projects have helped map the origins and the spread of the disease in the region, have also been part of the control initiative (4).

1.5.3. Investigation of multi-drug resistance and virulence factors in *E. coli*

Antimicrobial resistance is defined as the ability of the bacterium to resist the effects of an antibiotic agent. A bacterium can acquire resistance when a genetic mutation occurs within the organism or it acquires existing resistance genes from another organism. Conjugative plasmids can carry these genetic elements. For example, pOLA52 that confers resistance to chloramphenicol, ampicillin and a growth promoter olaquindox has been characterized in bacteria isolated in swine manure (2).

According to Chen *et al.*, 2011, bacteria have varying pathogenicity, and their likelihood of causing disease is dependent on their virulence factors. Virulence factors refer to the properties (gene products) that enable the bacteria to establish infection and enhance their ability to cause disease. Some examples of virulence factors include: toxins, hydrolytic enzymes, and cell surface proteins. The pathogenic island that carries these virulence genes may be located on the bacterial chromosome, on a plasmid, or other genetic elements (3). The ability of a bacterium to share these virulence factors with other bacterial pathogens depends upon the location of the virulence genes within the genome of the pathogen and the involvement of the transferable elements such as transposons and conjugative plasmids (1; 2; 15; 23).

1.5.4. Use of chloramphenicol in the treatment of animal diseases versus human diseases

In accordance to the World Health Organization (WHO), several countries including the European Union imposed a ban on the use of chloramphenicol in food animals. The use of chloramphenicol is restricted to treatment of nonfood animals to protect the animals and humans (47) from the carcinogenic effects associated with its use. In the past ten to fifteen years, chloramphenicol was commonly used in aquaculture and the treatment of several terrestrial animals in veterinary practice.

The decision to ban of chloramphenicol from food animal use was based on the side effects associated with its use rather than the resistance that may be developed by the microorganisms that are exposed to it (17; 47). However, the number of isolates of *Salmonella* resistant to chloramphenicol isolated from cattle manure in the United States is increasing (47). Prudent use of chloramphenicol serves as an example of the recommendations that have been suggested in the control of antimicrobial resistance in bacteria (17; 47).

1.5.5. Laboratory investigation of multidrug resistance and virulence factors in bacteria

Conjugation refers to the transfer of plasmid DNA from a donor cell to the recipient cell by physical contact and use of the type IV secretory system found in Gram-negative bacteria. Conjugation experiments can be performed in the laboratory to determine what genetic material these bacteria share with the recipient bacteria *in vitro* (29).A number of studies performed in research laboratories to characterize antibiotic resistance profiles of different *E. coli* isolates from various sources (30). Clonal MDR *E. coli* capable of crossing the host-species barriers have also been characterized both in the veterinary hospitals and from field isolates (13). MDR *E. coli* from different animals and humans have been found to have relatively similar antibiotic resistance profiles (13).

1.5.6. Mechanisms of drug resistance by bacteria

There are a number of ways bacteria can resist the action of antibiotics which include: *de novo* resistance due to mutations that occur in the genes of the bacterium (1), acquisition of transferable resistance gene cassettes that enable inactivation of the antibiotic (1), efflux pumps that allow export of the antibiotic after it gains entrance into the bacterium (16), cell membrane permeability alterations, and target alteration as in the case of gyrase targeted by the quinolones. A number of research projects have characterized genes associated with different mechanisms of drug resistance in bacteria (1; 16). The ways of acquiring resistance and virulence factors genes can be by transduction, transformation or conjugation (1; 15).

1.5.7. Control of drug resistance

The CDC acknowledges that addressing antimicrobial resistance requires a multifaceted approach. Prevention of disease transmission, development of new agents, and reduction of inappropriate use are some of the suggestions put forth (7). Their activities surround prevention of emergence and prevention of spread of resistant organisms. The developed world is following the trend of intercontinental collaborations in the control of the antimicrobial resistance (7).

CHAPTER TWO. RETROSPECTIVE STUDY OF CLINICAL CASES OF HUMAN AFRICAN TRYPANOSOMIASIS IN OTUBOI SUB COUNTY BETWEEN 2004 AND 2010: EVALUATION OF SOS IN UGANDA

2.1. Introduction

Human African Trypanosomiasis (HAT), also known as sleeping sickness, is a vector-borne disease caused by protozoa belonging to the Genus *Trypanosoma*. These parasites are transmitted to humans by the bite of an infected tsetse fly (Genus *Glossina*). The tsetse fly acquires its infection by feeding on animals or other humans harboring the parasites. In Sub-Saharan Africa, HAT takes two forms, acute or chronic, depending on the parasite involved. Uganda has the dubious distinction of being the only country where both forms exist (4).

In Uganda, the chronic form of HAT occurs in the north-western part, near the border with Southern Sudan while the acute form was confined to the south-eastern part of the country. Since the 1980s, acute HAT has been spreading north, posing a real risk that the two forms of the disease will overlap, and thereby complicating the diagnosis and treatment for HAT (40). According to the SOS press kit, 2006, Stamp Out Sleeping Sickness (SOS) campaign is a Public-Private Partnership that was launched in Uganda on 11th October 2006 to keep the two forms of HAT from merging.

2.1.1. Chronic form versus the acute form of sleeping sickness

The two forms of sleeping sickness (chronic and acute) are caused by two genetically different but morphologically indistinguishable trypanosome strains (4). *Trypanosoma brucei gambiense* causes the chronic form (gambiensis) of sleeping sickness while the *Trypanosoma brucei rhodensiense* causes the acute form (rhodesiensis) (41). The two strains can be

differentiated in the laboratory by polymerase chain reaction (42). Both trypanosome strains are transmitted by tsetse flies. The epidemiology of the gambiensis involves the humans as the carriers of the causative trypanosomes while cattle have been found to be the reservoirs for rhodesiensis (41). Control of gambiensis requires active surveillance because humans can harbor the trypanosomes for more than twenty years without showing signs of the disease (42).

2.1.2. Clinical presentation of HAT and management of the disease

HAT generally causes a disruption of the sleeping cycles in humans hence the name sleeping sickness. The clinical picture of both acute and the chronic forms of HAT are indistinguishable in humans (4; 11; 40). The difference between the two forms of the disease is the time of onset of the clinical signs.

Humans appear apparently healthy when infected with *T. b. gambiense* for more than ten years and the disease takes a nervous form that culminates into coma but the incubation period for the Rhodesian trypanosomiasis ranges between 8 to 28 days (4; 42). Both forms of the disease are 100% fatal unless treated (11; 42; 44).

Disease management is performed in three steps, which involve: screening for potential infection, diagnosis and staging of the disease (4; 40; 41; 42). The screening involves the use of serological tests and or checking for clinical signs – generally swollen cervical glands, diagnosis to show whether the parasite is present and staging to determine the state of progression of the disease which entails examination of the cerebral spinal fluid obtained by lumbar puncture (4; 42).

The staging step is used to determine the course of treatment. Diagnosis must be made as early as possible before the neurological stage in order to avoid the complicated, difficult and

risky treatment procedures (42). The long, asymptomatic first stage of *T. b. gambiense* sleeping sickness is one of the factors that require the use of exhaustive active screening of the population at risk in order to identify patients at an early stage and reduce transmission (42).

Exhaustive screening of exposed population requires a major investment in human and material resources (42). In Africa such resources are often scarce, particularly in remote areas where the disease is mostly found. As a result, many infected individual may die before they can ever be diagnosed and treated (40; 41; 42).

2.1.3. Objectives of the study

The main objective of this project was to evaluate the impact of SOS on the incidences of HAT in Uganda. The specific objective was to study the effect of SOS activities on the incidences of HAT in Otuboi Sub County,

2.2. Materials and methods

In Uganda, we carried out a practicum to acquire research skills. We choose to revisit the data we had collected from Lwala hospital, which is the sentinel center for the surveillance of HAT. This data was available in the appendix of the dissertation I had submitted in for my undergraduate degree at Makerere University.

We had previously attempted to use the same data to evaluate the SOS. In the previous analysis, the two phase of SOS intervention in 2006 and 2010 were considered together. We did not account for the two intervention phases of SOS separately. In the current analysis, the impact of SOS has been analyzed based on only the first phase of intervention that occurred in 2006. This is because the study period considered in this study ends in 2010 when the second SOS intervention had just been concluded.

2.2.1. Conceptual frame work

Based on the previous research knowledge and experiences, we analyzed the HAT situation in Otuboi Sub County and developed a conceptual framework to provide guidance in the evaluation of the SOS intervention. Otuboi Sub County is found in Kaberamaido District that is one of the newly affected Districts in Uganda, and therefore presents an interesting scenario of the natural history of HAT in for the cases of the pre-intervention years (2004 and 2005).

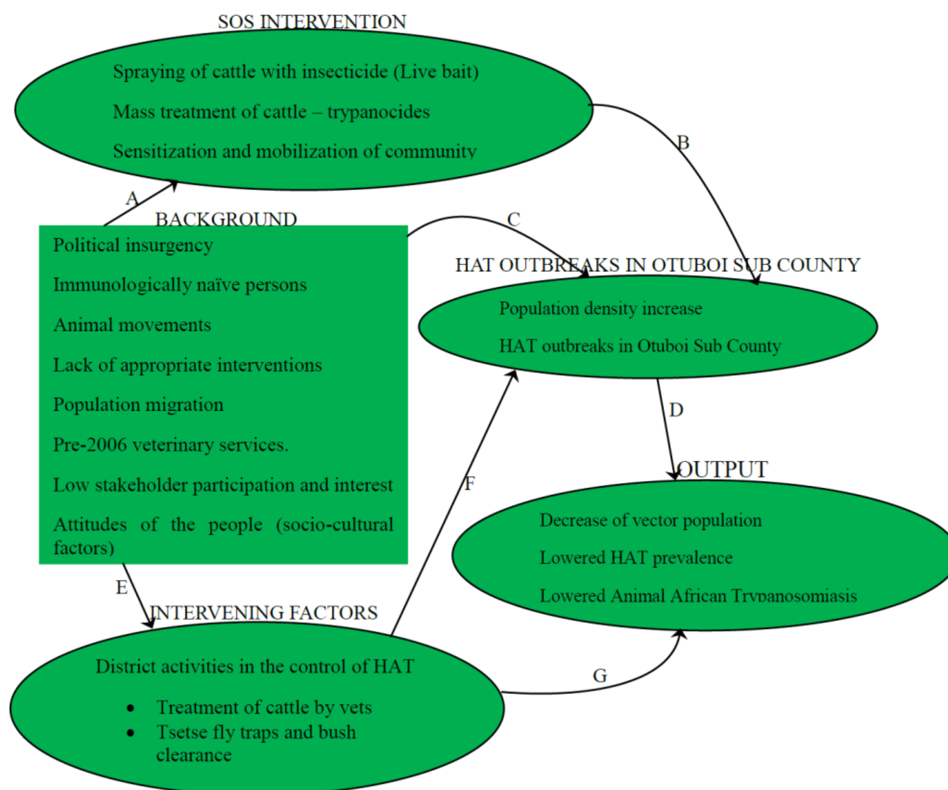

Figure 1. Conceptual framework for HAT situation analysis in Otuboi Sub County. The alphabetic letters represent the processes: A – major intervention response to need, B – major mitigation process due to SOS intervention, C- cause for HAT, D – primary effect process, E – minor intervention process, F – minor mitigation process and G – secondary effect process.

The outcomes of the intervention provide the measurable indicators of the SOS impact on HAT situation in Otuboi Sub County between 2004 and 2010 (Figure 1). These outcomes shown

as output in the conceptual framework include the reduction in HAT prevalence, Animal African Trypanosomiasis prevalence, and reduction in HAT incidence rate and tsetse fly population density in Otuboi Sub County (Figure 1).

2.2.2. Study area

The study was performed in Otuboi Sub County area that is nearest to sentinel center (Lwala hospital) where almost all cases of HAT are reported in the region. This area is found in Kaberamaido District (Figure 2) in northeastern Uganda; (coordinates 1°, 50’ N and 33° 10’ E) and has a total human population of 24,013 (33). The major activities are cattle keeping for both beef production and draft power (land tilling and fetching of water.) The area had been under rebel attacks that led to many people to be displaced to internally displaced people camps (IDP) until 2004 (20; 24).

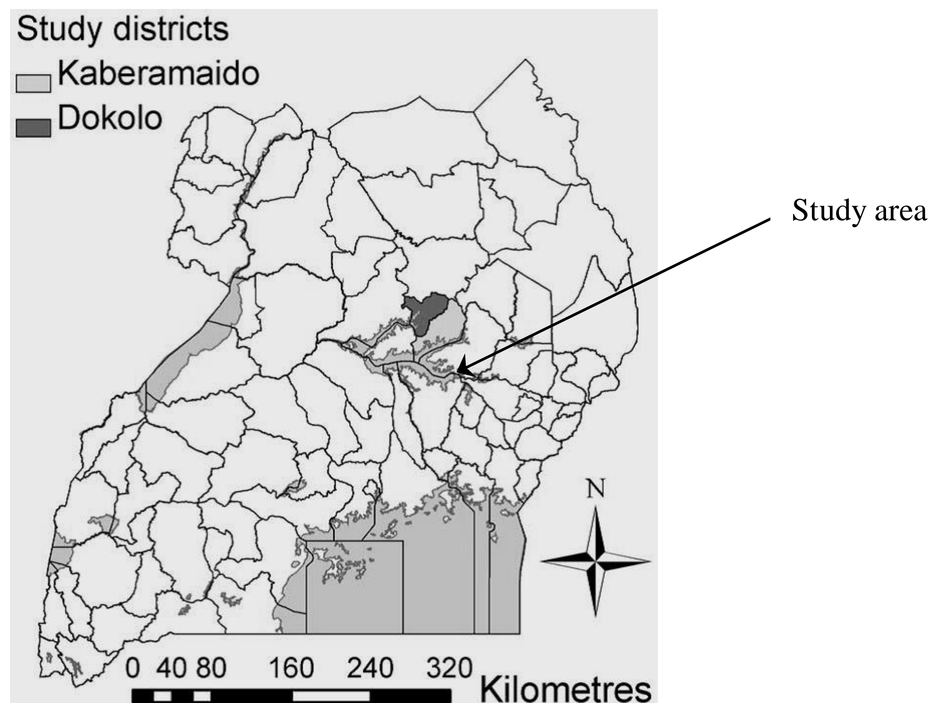

Figure 2. Map of Uganda showing the study area.

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2.2.3. Climate of the study area

The area experiences a bimodal rain fall that has one phase of March to May which peaks in April/May and the second phase of July – October that has its peak in September. A brief dry spell occurs in June and the longer dry spell is experience mid/late November to late February/early March. The average rainfall in the area is 1225mm per year. The highest temperatures up to 31˚C occur in the long dry season while the lower temperatures are experienced in the rainy seasons (4; 27).

2.2.4. Data source

Individual patient records of the confirmed cases of HAT from 2004 to 2010 were from Lwala hospital were used. These data were already available at the Library of the College of Veterinary Medicine, Animal Resources and Biosecurity (CoVAB), Makerere University in an unpublished undergraduate thesis by the author (A retrospective study of clinical cases of Human African Trypanosomiasis in Otuboi Sub County between 2004 and 2010: a partial evaluation of SOS campaign, 2010).

At the time of data collection, these records were found handwritten in a special logbook for sleeping sickness cases. The variables included the patient's name, age, sex, village of residence (as well as parish, Sub County and district), date of admission and discharge. Only patient's records of Otuboi Sub County were retrieved and names of patients were not recorded but replaced with serial numbers to protect patient confidentiality as was required by the institutional review board (IRB) of North Dakota State University (NDSU) in order to adhere to the international ethical guidelines for biomedical research that involves human subjects. The activity records of SOS intervention for both phase I (year 2006) and phase II (year 2010) were collected from Professor Charles Waiswa at the College of Veterinary Medicine, Animal

Resources and Biosecurity (CoVAB), Makerere University. These records as part of the SOS data included the number of cattle sprayed and treated during the first SOS phase (2006) and the second SOS phase (2010) in Otuboi Sub County.

2.2.5. Data analysis

Data was entered in Excel and exported into SAS version 9.2 for further analysis. Both descriptive and inferential analyses were performed. Descriptive statistics entailed both graphical and proportions. Using the procedure frequency in SAS, relative risk (RR) and the associated confidence intervals were estimated. The attributable fraction was also computed. All analyses were performed at a 5% level of significance. Attributable fraction and relative risk were used to quantify the risk of disease. Using2006 as the reference year, the Uganda pre- and post-intervention incidences of HAT were analyzed. For purposes of data analysis, 2004 and 2005 were considered pre-intervention period, 2006 as the intervention year and 2007 to 2010 as post intervention period. The attributable fraction represents the fraction of HAT cases saved when comparing the reference year (2006) to the population exposed during the year in question. A high percentage of attributable fraction shows a high protective effect of the intervention for the intervention year as compared to the year in question. In this research we indirectly measured the impact of SOS by analyzing the effect of intervention in cattle and vector population on the incidence rates of HAT during the study period.

2.3. Results for the project carried out in Uganda

A total of 149 cases of HAT were reported between 2004 and 2010 from Otuboi Sub County. The distribution of the cases by age is shown in Table 1. The mean age most affected was 30.9 years with a standard deviation of 5.82 (Table 1). The elderly were least affected by HAT in Otuboi Sub County. The gender most affected by HAT during the study period was

found to be males (58%) compared to the females (42%). These results give the description of the population at risk of exposure to HAT as associated to age and indirectly to the gender roles of the people in Otuboi Sub County.

Table 1. Distribution of HAT cases by age during the study period (2004 – 2010)

AGE GROUP	FREQUENCY	PERCENTAGE
0 - 14	39	26
15 - 29	44	30
30 - 44	25	17
45 - 59	24	16
60 - 74	14	09
75 - 89	03	02
Total	149	100

The HAT trend (temporal) for the pre-intervention years (Figure 3) indicate that the first recorded HAT cases occurred during July 2004 and reported cases increased for the rest of the months up to the end of the year. Generally pre-intervention year HAT incidence present decreased HAT incidence mid-year and increased cases early- and late-year (Figure 3).

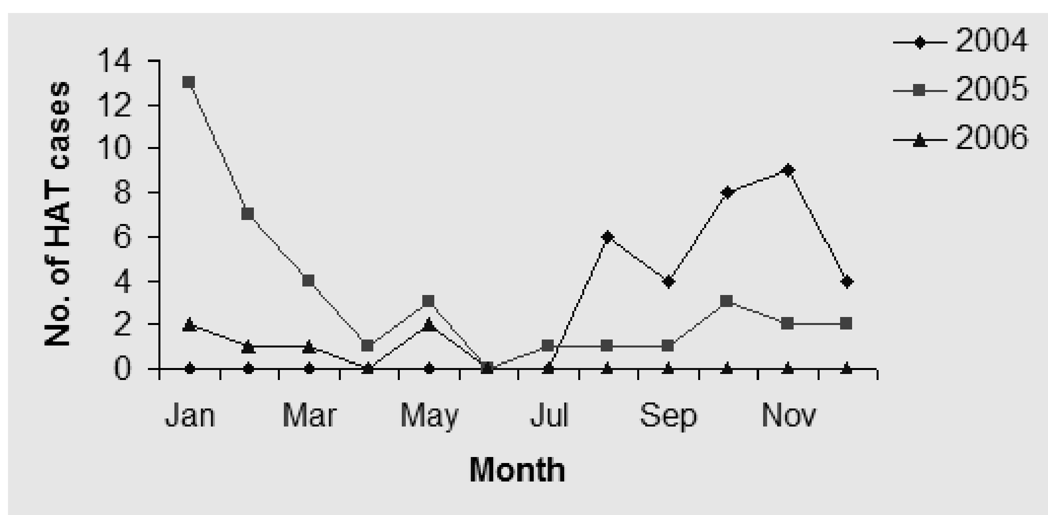

Figure 3. HAT monthly cases for the pre-intervention years: 2004, 2005 and 2006. The monthly number of HAT cases reported from Otuboi Sub County for the years before SOS intervention were plotted in this figure. SOS intervention occurred during 2006.

However the trend of HAT incidence changed to give a lowered reported HAT incidence. This was true for the year 2006, 2007, 2008 and 2009 except for the month of December of 2009 where there was an increase in HAT cases reported (Figure 4).The HAT cases recorded for 2010 only covered the initial months up to May when the study was carried out. The cases recorded during these months showed an increase in the HAT cases reported (Figure 4).

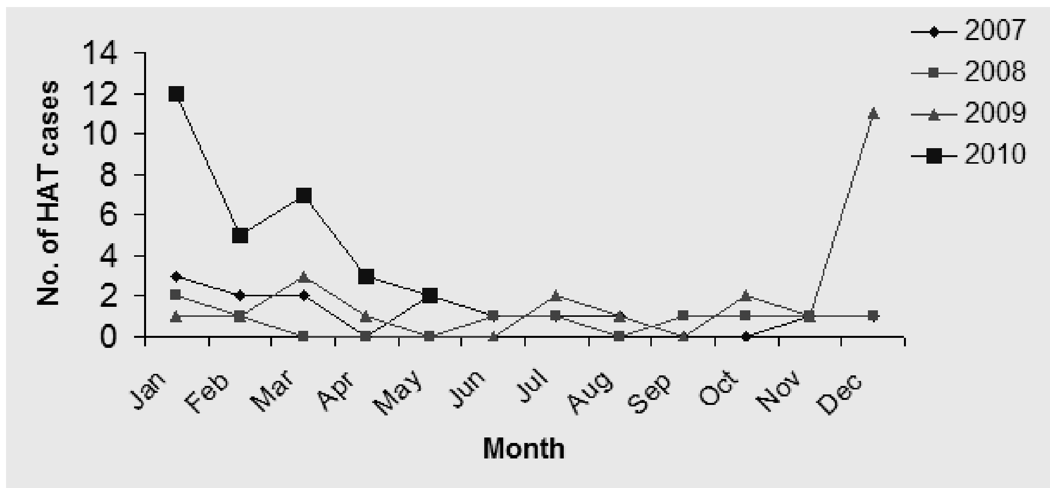

Figure 4. HAT monthly cases for the post-intervention years: 2007, 2008, 2009 and 2010. The monthly number of reported HAT cases from Otuboi for the years after SOS intervention were plotted in this figure.

By expressing the incidence as simply the number of new cases that occurred in a month, the average monthly HAT incidence for the pre-intervention years (Figure 5) and the post-intervention years were presented (Figure 6) to show the effect of SOS activities on the seasonal occurrence of the HAT. On average there was an increase in HAT incidence during the long dry season of the pre-intervention years. This trend was maintained for the post-intervention years except HAT cases were clustered only in a few months of the dry period; December and January (Figure 6).

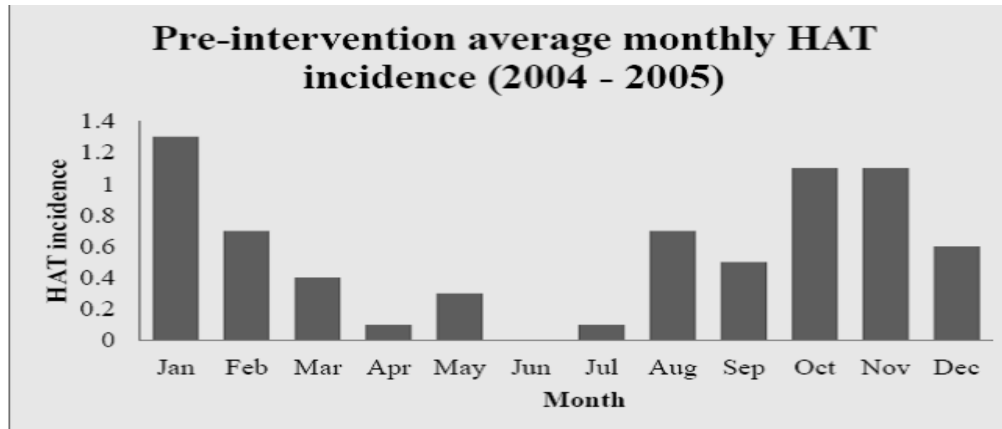

Figure 5. Average monthly HAT (seasonal) HAT incidence for pre-intervention years: 2004 and 2005. To obtain the average monthly HAT incidence, the sum of monthly HAT incidence for both the two years pre-intervention (2004 and 2005) was divided by the number of years (2) considered.

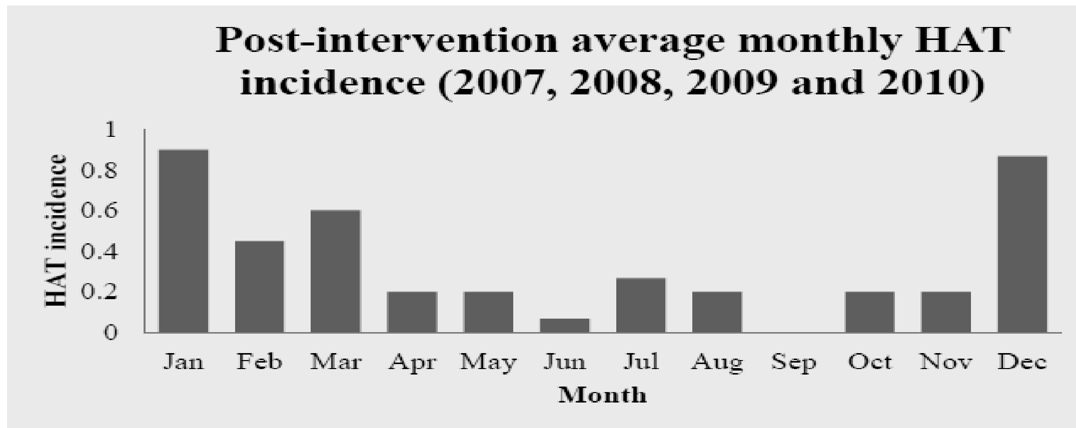

Figure 6. Average monthly HAT incidence for post-intervention years: 2007,2008,2009 and 2010. To obtain the average monthly HAT incidence, the sum of monthly HAT incidence for all the years post-intervention (2007, 2008, 2009 and 2010) was divided by the number of years (4) considered.

From the pairwise comparison analysis, using 2006 as the reference year, 2005 (P = 0.000024) and 2004 (P = 0.000001) had increased HAT cases of HAT reported. Comparing with 2004 HAT incidence, there was a high protective effect (AT = 80.7%) by the SOS intervention. This effect was still significant when compared to 2005 HAT incidence. However, when it came to the immediate years post-intervention (2007 and 2008), not much protection by the intervention (AF = 49.7% and AF = 11.9) was registered (Table 2).

Table 2. Results of the pairwise comparison of HAT incidence for intervention year (2006)

Year	Attributable Fraction (%)	95% CI Attributable Fraction	Relative Risk (RR)	95% CI (RR)	P value
2004	80.7%	53.61,91.93	0.19	0.08, 0.46	0.000024
2005	84.2%	62.65,93.33	0.16	0.07, 0.371	0.000001
2007	49.7%	-30.97, 80.66	0.5	0.19, 1.31	0.181329
2008	11.9%	-153.85, 69.44	0.88	0.31, 2.54	1
2009	69.4%	24.76, 87.53	0.31	0.12, 0.75	0.007961
2010	87.9%	70.74, 94.96	0.12	0.05, 0.29	<0.0001

Population size; year 2004 – 2006; N = 24,013, 2007 – 2010; N = 28,200. CI – Confidence interval. The HAT incidences were calculated for each year and person years were considered for each subsequent year.

In reference to 2006 HAT incidence, the results (Table 2) show no statistical difference for these years, 2007 (P = 0.181) and 2008 (P = 1). In reference to the HAT cases in 2006, the results for years 2009 (P = 0.00796) and 2010 (P = 0.0001) show a statistically significant difference. The outbreaks of HAT reported during 2009 and 2010 explains for the observed differences and increase in the attributable fractions.

2.4. Discussion

The idea of controlling a disease from the source rather than intervening after its spread is the underlying principle for the SOS intervention. Due to the available knowledge of the HAT epidemiology, the tsetse flies are considered “innocent” vectors if the source of the infection is removed. The mass treatment of cattle with trypanocides was intended to clear the cattle of the trypanosomes such that the tsetse flies do not acquire the infection during feeding. Immediately

following intervention, therefore we expect a low population density of vectors (tsetse flies), lowered infection in the cattle population and decreased HAT incidence. These are out come variables that we investigate to measure the impact of SOS intervention. In this study, we indirectly measured the impact of SOS by using one output; HAT incidence or number of cases in humans during the study period (2004 – 2010).

As shown in the conceptual framework (Figure 1), there were other minor interventions that preceded the SOS Campaign. These intervening factors were arguably considered minor interventions in this study unfortunately we did not account for their compounding effects. The SOS campaign presents the major mitigation process we are investigating. The systematic control approach used by SOS in controlling HAT had a foreseen positive impact that warranted assessment to guide the future interventions (20; 40). The SOS campaign used a “one world one health” approach in which different professions were included in the community engagement programs.

The results of this study agree with those of the previous analysis in the fact there was a post-intervention significant decrease in HAT cases. Previously, we did not account for the two (2006 and the 2010) SOS intervention phases separately. This analysis, rather clearly accounts for the effects of the first SOS intervention phase that occurred during 2006.The current analysis also considered data of 2006 as the baseline to which the data of the pre-intervention and post-intervention were compared.

This study provides a platform to analyze the natural history of HAT due to the fact that Otuboi Sub County was a newly infected area. The residents of this area had been displaced for more than a decade and they were returning home in 2004 from IDP (Internally Displaced People) camps (4; 20; 24). The arrival of people at that time of the year (mid-2004) could explain

the absence of cases in early-2004 (Figure 2) as well as the occurrence of outbreaks and registration of HAT cases in Otuboi Sub County in mid-2004 (Figure 2). Major factors contributing to the epidemiology of HAT in this region include: the re-establishment of homes and restocking animals (20), mostly cattle, without proper veterinary services and the arrival of immunologically naïve humans into a highly infested area.

During the study period, incidences of HAT were found to be higher in the most active age group (average age groups affected = 30.5 years). The males were most affected and this may be due to the fact that the gender roles in the study area dictate that males come in contact with the tsetse flies as they graze their cattle in bushy areas. These results are in agreement with the research that was carried out during the study period (25). The research reported men to be the most involved in livestock production in this area and also livestock ownership to be in the hands of people aged 18 to 60 years.

By comparing the trends of HAT in Otuboi Sub County (Figure 5) with trends of HAT post intervention (Figure 6), the bimodal pattern of the disease incidence was distorted. According to the UBOS statistical abstract 2010, the long term whether pattern of the study area has been maintained relatively unchanged. Based on this fact, we may attribute the observed seasonal difference in the HAT incidence to SOS intervention. We see a decrease in HAT incidence (Figure 5) during the wet seasons of the year (Apr - Sept) and an increase of HAT incidence during the dry seasons (Jan – March and Oct – Dec). The descriptive analysis for the post-intervention years (Figure 6) presents a general decrease in HAT incidence concentrated in the months of January and December. Since HAT epidemiology depends partly on the vector's ability to survive in warmer conditions (27; 32), the change in the trend of HAT incidences between the pre- and post-intervention years could have been due to the SOS intervention. The

effect of SOS on the yearly seasonal distribution of HAT incidences needs additional research to establish this connection definitively.

The intervention resulted in a statistically significant decrease of HAT incidences reported during 2006. The low relative risk (RR = 0.19) confirms that the percentage of the human population in Otuboi Sub County at risk of becoming infected was higher during 2004 compared to 2006. The protective effect of the SOS intervention in 2006 was higher when compared to 2004. This is shown by the high attributable fraction (AT = 80.7%), the attributable fraction represents the proportion of cases saved from contracting HAT by the intervention. A high percentage of the attributable fraction shows a high protective effect in the intervention year when compared to the year of interest. The results for 2005 have the same trend with that shown in the 2004. However, when it came to the immediate years post-intervention (2007 and 2008), there was not much protection (AF = 49.7% and AF = 11.9). This means that it would be a waste of resources if the interventions activities such as mass treatment of cattle with trypanocidal drugs were performed at this time in the study period. However, the spraying of cattle with insecticides at this time would be protective.

The relative risk was increasing as we approached 2006 as evidenced in Figure 2. This implies that as the incidences of HAT for a particular year were as low as the HAT incidence in the base year (2006), the relative risk increases towards one (RR = 1). For instance the year 2008 has a RR = 0.88, showing that the cases were as low as those that were registered during the intervention year (2006). During 2007 and 2008of the study period, we expected the vector population to be low due the environmental spraying of the insecticides that was done during the 2006 intervention. If there were tsetse flies in the area, it is possible that a few where infected with trypanosomes. Sensitization of the public could have created public awareness and mass

treatment of the reservoirs (cattle) may have cleared the cattle from trypanosomes that would be acquired by the tsetse flies as they feed.PCR detection of trypanosomes in the blood samples collected before and after SOS intervention could provide evidence to this.

The outbreaks of HAT registered during 2009 and 2010 explains for the observed differences and increase in the attributable fractions. The observed increase in the HAT incidence during 2009 and 2010 may be attributed to the delayed intervention. The SOS phase II intervention was performed after four years following the first phase. This delay could have allowed introduction of untreated cattle in the area among other factors that led to reinfection of both the tsetse flies and the cattle. All these highlighted factors could have contributed to the resurgence of HAT in the study area.

2.5. Conclusion

The 2006 SOS intervention helped to maintain low incidence of HAT during the intervention year and two years (2007 – 2008) post-intervention. The incidence of HAT was significantly reduced at intervention (2006) and post intervention for two years (2007 and 2008). Following 2008, HAT incidence increased in 2009 and 2010. Based on this analysis, intervention for the second phase SOS intervention scheduled for 2009 would likely have been protective.

This analysis is in agreement with the previous analysis. The intervention by targeting the source of the HAT outbreak impacted positively in reducing HAT incidences. The SOS campaign is a disease control strategy that provides relevant approaches in the control of climate-sensitive disease in areas with relatively similar climatic conditions. Our evaluation SOS as a climate-sensitive disease control strategy comes at the right time when climate change is a global problem expected to impact negatively to the poor communities of the world where these diseases are endemic.

CHAPTER THREE. PCR DETECTION OF TRANSFERRED MULTIDRUG RESISTANCE AND PLASMID BORNE VIRULENCE FACTORS IN BACTERIAL ISOLATES FROM SCOURING CALVES IN NORTH DAKOTA

3.1. Introduction

Bacterial conjugation is the transfer of genetic material in the form of plasmid DNA or transposons from a donor cell to the recipient. This occurs by physical contact. The type IV secretory systems of bacteria encoded by specific transfer genes enable the cells to conjugate. *Escherichia coli* is a normal inhabitant of the alimentally canal of all warm-blooded vertebrates. *E. coli* can cause disease if it gains access to otherwise sterile areas of the body. *E. coli* is capable of developing drug resistance to antibiotics and can share these genetic elements with other infectious bacteria like *Salmonella* (1; 15; 19). This is exacerbated by the acquisition of virulence genes that could allow the organism to bypass the host immune system or secrete toxins resulting in disease. *E. coli* can confer multi-drug resistance to other bacteria by sharing conjugative plasmids to which the genes associated with drug resistance and virulence factors are carried (1). The mapping of drug resistance is being developed in Dr. Gibbs' laboratory at North Dakota State University. In this research, we have worked on detecting virulence and antimicrobial resistance genes transferred from *E. coli* to *Salmonella* by conjugation experiments. Virulence factors were detected by PCR and resistance to antibiotics was determined by antibiograms. The objective of this project is to determine which genes and antibiotic resistances are transferred during conjugation to the *Salmonella* strains.

3.2. Methodology

3.2.1. Experimental design

A three step PCR experiment was performed screening for the 22 virulence factors in all bacterial isolates that were cryopreserved following the conjugation experiments.

PCR detection of the virulence factors conjugated

Step 1: PCR detection for 22 genes in *E. coli* 2077 (donor)

↓

Step 2: PCR detection for detected genes in step1; WT2253-6A-NA

↓

Step 3: PCR detection for detected genes in step 1; TC2253-6A: 2077

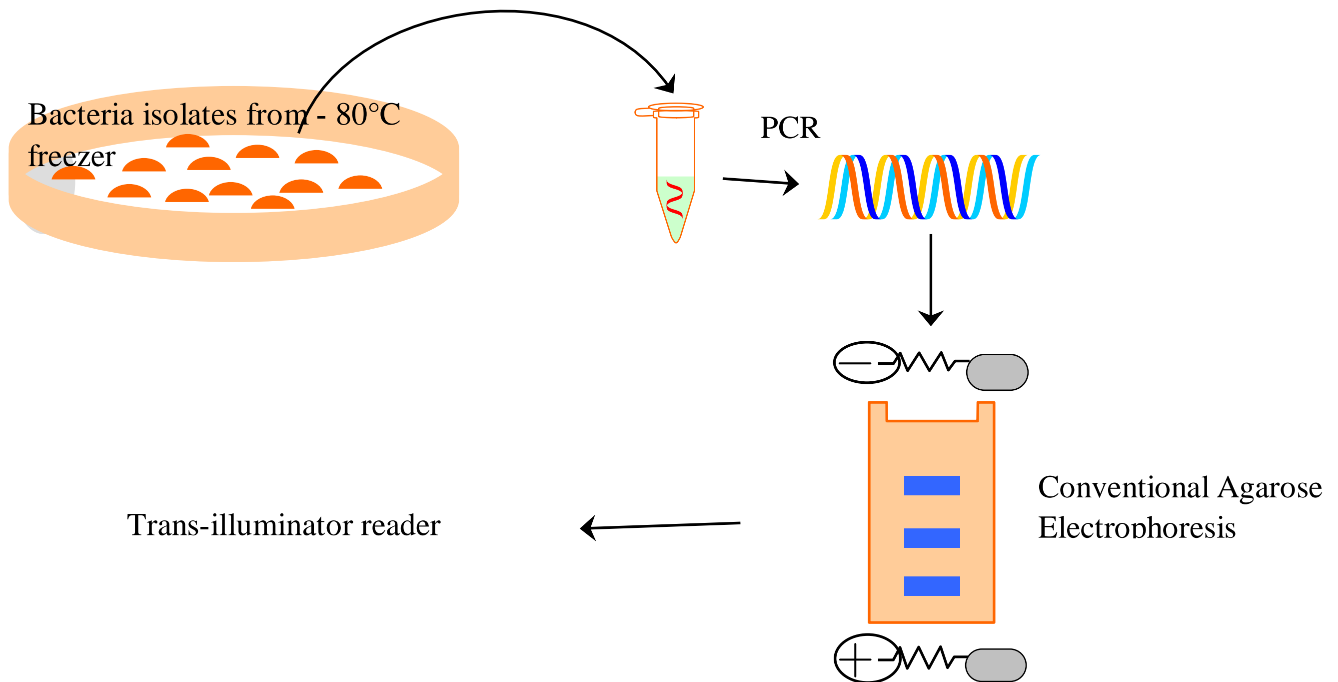

Figure 7. Experimental design for MDR and virulence factors investigation

The experimental design of this study is as follows: virulence factors in the *E. coli* 2077 isolates were screened in step one. The virulence factors detected in *E. coli* 2077 isolates were

screened in step two for the wild type *Salmonella* (2253-6A), and finally step three included screening of the for the detected genes in the transconjugates (TC2253-6A: 2077).

3.2.2. Bacterial isolates used

Bacterial isolates that were used in this study are summarized in Table 3**.** The isolates were obtained from the North Dakota Veterinary Diagnostic Laboratory at North Dakota State University (ND-VDL). The isolates were cryopreserved until use. The multidrug resistant *E. coli* 2077 donor was isolated from a scouring calf submitted to ND-VDL.

Table 3. Description of bacterial isolates used in this study

Bacterial isolate	**Purpose**	**Description**	**Reference**
E. coli 2077	Donor of antibiotic resistance/virulence factor plasmid	Confers drug resistance and virulence factors via conjugative plasmid	Veterinary Diagnostic Lab. At North Dakota State University (ND-VDL)
Salmonella WT2253-6A	Recipient of drug resistance plasmid	Isolate of wild type *Salmonella*	ND-VDL
Trans-conjugate TC2253-6A:2077	Tested for transferred virulence factors	Trans-conjugates	ND-VDL

3.2.3. Protocol

At step one of the experimental design (Figure 7) we carried out PCR (Polymerase Chain Reaction) detection of 22 virulence factors in *E. coli* 2077 isolates. The primers used are provided in Table 4. We used conventional agarose gel electrophoresis to visualize the amplicons using a 1.5% agarose gel. In step 2, the wild type *Salmonella* isolates (WT2253-6A) were screened for the genes that were detected in *E. coli* 2077.We obtained ten isolates of transconjugatesTC2253-6A:2077, which were created previously in Dr. Gibbs' laboratory at

NDSU. The transconjugates were used in step three (Figure 7). The transconjugates were screened for the presence of virulence factors that were detected in the donor (*E. coli* 2077).

Table 4. PCR primers used in this study

Gene	Gene size (base pair)	Primer (Sequences; 5' to 3')	Reference
astA	111	East11a: CCATCAACACAGTATATCCGA East11b: GGTCGCGAGTGACGGCTTTGT	Kaufman M, *et al.*, (2006)
hlyE	889	EHLY1: GAGCGAGCTAAGCAGCTTG EHLY2: CCTGCTCCAGAATAAACCACA	Janben *et al.*, (2001)
hlyA	351	HLYA-F: GTCCATTGCCGATAAGTTT HLYA-R: AAGTAATTTTTGCCGTGTTTT	Janben *et al.*, (2001)
iucD	692	AERA-F: ACAAAAAGTTCTATCGCTTCC AERA-R: CCTGATCCAGATGATGCTC	Janben *et al.*, (2001)
papC	482	PAPC-F: TGATATCACGCAGTCAGTAGC PAPC-R: CCGGCCATATTCACATAA	Janben *et al.*, (2001)
tsh	804	TSH-F: GTGATAAACAAGTCGGCAACA TSH-R: GCATTGAGACATCCATTCC	Janben *et al.*, (2001)
fimC	476	FIMC-F: GGGTAGAAAATGCCGATGGGTG FIMC-R: CGTCATTTTGGGGGTAAGTGC	Janben *et al.*, (2001)
eaeA	384	EAEA-F: GACCCGGCACAAGCATAAGC EAEA-R: CCACCTGCAGCAACAAGAGG	Paton and Paton, (1998)
vat	981	VAT-F: TCCTGGGACATAATGGTCAG VAT-R: GTGTCAGAACGGAATTGT	Ewers *et al.*, (2005)
irp2	413	IRP2-F: AAGGATTCGCTGTTACCGGAC IRP2-R: AACTCCTGATACAGGTGGC	Ewers *et al.*, (2005)
Irp1	1691	IRP1.A: GCGATGTTTAACCCCGATT IRP1.B: TGCCTGGAAACCCTGAGACT	Bach *et al.*, (2000)
Sfa/foc	410	Sfa/foc-F: CTCCGGAGAACTGGGTGCATCTTAC Sfa/fac-R: CGGAGGAGTAATTACAAACCTGGCA	Watt *et al.*, (2003)
afa	750	Afa-F: GCTGGGCAGCAAACTGATAACTCTC Afa-R: CATCAAGCTGTTTGTTCGTCCGCCG	Watt *et al.*, (2003)
ibeA	171	ibeA-F: TTACCGCCGTTGATGTTATCA ibeA-R: CATTAGCTCTCGGTTCACGCT	Watt *et al.*, (2003)
iucC	269	iucC-F: AAACCTGGCTTACGCAACTGT iucC-R: ACCCGTCTGCAAATCATGGAT	Watt *et al.*, (2003)
fimH	508	fimH-F: TGCAGAACGGATAAGCCGTGG fimH-R: GCAGTCACCTGCCCTCCGGTA	Watt *et al.*, (2003)
cnf	693	CNF-F: TTATATAGTCGTCAAGATGGA CNF-R: CACTAAGCTTTACAATATTGA	Usein, C.R, (2001)
est-1a/b	123	ST-1B: CTTCTTGACTCTTCAAAAGAGAAAATTAC ST-1C: GATTACAAGAAAGTTCACAGCAGT	Janben *et al.*, (2001)
elt-1a/b	365	LT-I/1: TCTCTATGTGCATACGGAGC LT-I/2: CCATACTGATTCCCGCAAT	Janben *et al.*, (2001)
stx1	614	SLTI-F: ACACTGGATGATCTCAGTGG SLTI-R: CTGAATCCCCCTCCATTATG	Gannon *et al.*, (1992)
stx2	779	SLTII-F: CCATGACAACGGACAGCAGTT SLTII-R: CCTGTCAACTGAGCACTTTG	Gannon *et al.*, (1992)
int1	280	INT1-F: CCTCCCGCACGATGATC INT1-R: TCCACGCACTGTCAGGC	Goldstein *et al.*, (2001)

3.2.3.1. Polymerase Chain Reaction (PCR) protocol

Two micro liters of the bacterial sample lysate was added to 23µL of the master mix in the PCR tube for each gene screened for. The polymerase chain reaction for each gene was run with specific programs on the thermo cycler. The resulting gene PCR amplicons were then loaded on the agar gel for separation.

Table 5. Antibiotic sensitivity profiles for the bacterial isolates used in this study

Antibiotic	2077	2253	111	112	113	114	115	116	117	118	119	120
Ampicillin	R	S	R	R	R	R	R	R	R	R	R	R
Clavamox	R	S	R	R	S	R	S	R	R	R	R	R
Amikacin	S	S	S	S	S	S	S	S	S	S	S	S
Chloramphenicol	R	S	R	R	S	I	S	R	R	R	R	R
Cephalothin	R	S	R	R	S	S	S	R	R	R	R	R
Ciprofloxacin	R	S	S	S	S	R	S	S	S	S	S	S
Gentamicin	I	S	S	S	S	R	S	S	S	S	S	S
Sulfisoxazole	R	S	R	R	R	R	R	R	R	R	R	R
Kanamycin	R	S	R	R	R	R	R	R	R	R	R	R
Nalidixic acid	S	S	I	I	I	I	I	I	I	I	I	I
Streptomycin	R	S	R	R	R	R	R	R	R	R	R	R
Ampicillin	R	S	R	R	R	R	R	R	R	R	R	R
Amoxicillin	R	S	R	R	S	R	S	R	R	R	R	R

R – Resistant to the antibiotic, S – sensitive to the antibiotic, I – intermediate, MDR *E. coli* – 2077, wild type Salmonella isolate – 2253 and the isolates 111 – 120 were the trans-conjugates. Measuring the diameter of the inhibition zones on the growth agar plates and comparing them with the standard diameters of inhibition zones to decide whether the isolate was sensitive, resistant or intermediate obtained the results in table 5.

All the bacterial isolates that were used in this study were previously screened (Kirby-Bauer method) for their antibiotic resistance and based on the Antibiogram, results of antibiotic

resistance were generated (Table 5). This method has been published elsewhere (9). Briefly Kirby-Bauer method also known as the disc diffusion method is used for antibiotic susceptibility test. The method relies on the inhibition of bacterial growth measured under standard conditions.

3.3. Results

Figure 8 shows a representative gel image of the gene profile for the amplicons from the bacterial isolate. The PCR products were separated on a 1.5% agarose gel. Of the 22 virulence factor-associated genes screened for in this study, eight genes (*fimH, fimC*, *papC, iucC*, *int-1, Esta11, st-1*, and *iucD)* were detected in *E. coli* 2077 isolates. None of the eight genes were detected in the wild type *Salmonella* strain. Of the eight genes detected in the *E. coli* 2077, six genes (*fimH*, *fimC, st-1, int-1, iucC* and *iucD)* were detected in the transconjugates (TC2253-6A:2077). These results are summarized in Table 6.

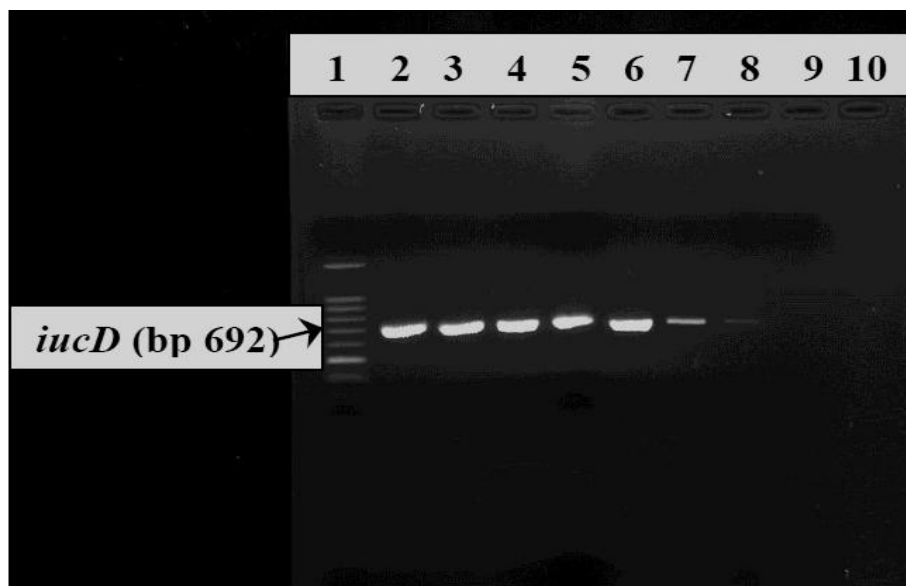

Figure 8. Gel image of *iucD* in the amplicons of *E. coli* isolate 2077. Lane 1 – 100 base pair DNA ladder (molecular weight marker), lanes 2 and 3 contained V1 (positive control), lanes 4, 5, 6,7 and 8 contained PCR amplified products of *E. coli* 2077, lanes 9and 10 contained *E. coli* DH5α (negative control).

Table 6. Summary of the PCR results for the 22 screened genes in this study

Gene	2077	2253	111	112	113	114	115	116	117	118	119	120
astA	+											
hlyE	-											
hlyA	-											
eaea	-											
papC	+	-	-	-	-	-	-	-	-	-	-	-
tsh	-											
fimC	+	-	-	+	+	+	+	+	+	+	+	+
fimH	+	-	-	+	+	+	+	+	+	+	+	+
vat	-											
irp2	-											
irp1	-											
Sfa/foc	-											
afa	-											
ibeA	-											
iucC	+	-	-	+	+	+	+	-	-	+	+	-
iucD	+	-	-	+	+	+	+	-	-	+	+	-
cnf	-											
est1a/b	+	-	+	+	+	+	+	+	+	+	+	+
elt1a/b	-											
stx1	-											
stx2	-											
int1	+	-	+	+	+	+	+	+	+	+	+	+

The detected genes (+), genes not detected (-). Since many genes were not detected in *E. coli* 2077, so we limited the genes tested in the wild type *Salmonella* and transconjugates to those present in *E. coli* 2077. Key: 2077 – *E. coli* 2077 isolate, 2253 – wild type *Salmonella*, 111 to120 – transconjugates.

3.4. Discussion

Of the 22 genes screened in 2077 *E. coli* (donor), only eight genes (*iucC, iucD, fimH, fimC, int1, st1, papC, astA)* were detected. The genes that were detected in the donor isolates

were the only genes tested in transconjugates. The results of the overall experiment suggest a higher percentage of transfer of the genes that were detected in 2077, except for *papC*.

The interesting observation is that the transfer of the genes fall under three different groups. The genes with similar patterns of transfer were arranged together as shown in Table 6. The possible explanation of this pattern may be derived from the fact that these genes are borne on the same plasmid (23) but probably flanked by different insertion sequences. In this way to transfer of each group is independent of the other. For example, it has been established that the *iuc* genes belong to a group of four genes (*iucA* to *iucD*) that are located on the same operon found on a ColV-K30 plasmid (23). Research has reported that these genes are conserved in the species of *E. coli* (23). This study may provide relevant information to explain the observed different in the frequency of transfer of *iucC* and *iucD* compared to other transferred genes in this project.

The presence of a gene on a transferable element aids in its ability to be potentially shared with other bacteria (1). The chromosomally located genes could only be transferred if they were incorporated in F' plasmids (1). The genes that are found on the pathogenic island can also be transferred by transposons and plasmids. If the pathogenic island is located on these mobile genetic elements, then it becomes easy for these virulence factor genes to be transferred.

In this experiment, we hypothesized that the donor cell is conferring multidrug resistance and virulence factors via conjugation. In this study, development of highly virulent MDR in *E. coli* associated with calf scours is linked to the transfer of the plasmid. Pulsed Field Gel Electrophoresis (PFGE) results previously performed on these isolates proved the presence of this plasmid (results not shown). The PFGE results were suggestive of non-clonal *E. coli* strains.

According to the results obtained in chapter three of this paper, there were differences observed in the rates of transfer of these tested genes. The *iucC* and *iucD* may be flanked by the similar insertion sequences and are transferred together independent of the rest of the genes. Supporting evidence was observed for the transmission of *fimC* and *fimH* that were received by the rest of the transconjugates except transconjugate 111.

The *papC* gene that is required in the formation of adherent pili in bacteria (26) was not received by any of the transconjugates. This gene is also part of an operon that consists of other *pap* genes. The plausible explanation for its failure to transfer could be derived from the fact that the operon is found on a different plasmid or the same plasmid but at a portion that was not transferred.

Since most of the resistant genes are localized on transferable elements (1), it is possible that these genes are carried together with the genes that encode for antibiotic resistance (2; 3). This is due to the fact that when gained multidrug resistance profiles of the different transconjugates are compared with the transferred genes (Table 6), the transconjugate 111 obtained almost similar resistance as other trans-conjugates (Table 5) regardless of the difference in the transferred virulence factors detected by PCR (Table 6).The question of whether there is a linkage between the transferred virulence factor genes and plasmid-mediated drug resistance presents the opportunity for the future research.

3.5. Conclusion

E.coli strain 2077 is capable of spreading several virulence factors and antibiotic resistances via its plasmid to other bacterial pathogens by conjugation. The plasmid on which these genes are carried has not yet been characterized. The connection between the transferred

multidrug resistance and the transferred virulent factors has not been established. The pattern of transfer of the virulence factors was suggestive of the presence of these genes on transferable genetic elements. These transferable elements are transferred independently from each other. The relationship between the detected virulence factor genes and multidrug resistance require more research to characterize the conjugative plasmid shared by *E. coli* 2077.

CHAPTER FOUR. GENERAL DISCUSSION

Humans act as carriers for the chronic form of HAT and the humans that carry these trypanosomes (*T.b gambiense*) appear apparently normal for a long period of time (42). By the time these infected people show signs of chronic HAT, the disease has already reached the late stage which presents as a nervous problem (42). At this stage the prognosis is poor. Therefore control of this form of HAT requires active surveillance whereby health teams go out in the field to screen humans for trypanosome infection (4). PCR is one of the modern techniques that can be used to differentiate the two strains of trypanosomes (morphologically indistinguishable) that cause the two forms of HAT (4; 42). The two research projects presented in this paper give us the opportunity to apply the acquired skills and knowledge in the control of HAT. We used PCR in the screening of genes associated with virulence in *E. coli*, the same technique is used in the characterization of trypanosomes. Trans-boundary diseases do not respect geographical borders that are known to humans (40). Relevant interventions that have managed to control diseases of international concern should be documented for future application. The experience we have obtained in exploring these projects in this research present the baseline knowledge that may be required in the control of international infectious diseases and especially those that are climate-sensitive.

Antimicrobial resistance is a public health problem that requires international collaborations. The establishment of trans-Atlantic taskforce for antimicrobial resistance by the U.S. and EU collaboration to combat antimicrobial resistance is a relevant example of the international and inter-continental participation in solving these global problems (7).

During this research, we have observed that the spread of antibiotic resistance as well as climate change are global issues. The spread of drug resistance in most cases is non-clonal which

makes further research in this field relevant if we are to address the problem of the emerging MDR in bacteria. Animals harbor many of the bacteria that cause disease to humans and the drug resistance developed by these bacteria in animals can spread to humans. This is also true for the bacterial drug resistance that develops in humans. The problem of drug resistance associate with nosocomial infections is probably caused by the misuse of antibiotics both in human and animal medicine. Prudent use of antibiotics is a strategy that may reduce the rate at which antimicrobial resistance in bacteria is increasing.

The observed reduction and maintenance of low HAT incidence for two years post intervention in this research puts SOS on the list of those few approaches that provide relevant control approaches to vector borne diseases in areas with similar climatic conditions. The results of our research suggest a timely intervention to be done every after two consecutive years if the disease is to be controlled effectively. The results of this project suggest that there was a delayed SOS intervention between phases one and two. This led to the HAT out outbreaks that were registered in 2009 and 2010. Other researches about SOS intervention have been published but none has report the impact of the intervention in this Teso farming region. (20; 40; 42)

Despite these interventions, current studies show existence of silently infected children and adult humans in these areas (42).The chronic form of HAT may be the cause of these discovered infections. Active disease surveillance of human population in the infected areas is the effective of stimulating interventions. This is because individuals remain apparently healthy for more than ten years (42) even though they are harboring the infective trypanosomes.

CHAPTER FIVE. GENERAL CONCLUSION AND RECOMMENDATIONS

Strategic control targeting lowering vector density towards the end of year would be most appropriate and cost effective in the control of HAT incidences in Otuboi Sub County. The tsetse fly population seems to increase at the beginning of the long dry season (Sep – Nov). Therefore an intervention targeting this period of the year would be more effective in killing the adult flies rather than spraying of cattle when many of the flies are at their dormant stage (pupa stage) of their life cycles. Intervention by SOS at a two-year interval would be cost effective and appropriate in the control of HAT in Uganda. The idea of screening cattle as well as the humans in these infected areas to establish the level of infection has been suggested by other researchers (4; 42). They suggest active surveillance that is done before the intervention is started and after it is done to guide in the effective evaluation of the SOS intervention.

It is hypothesized that *E. coli* is capable of spreading drug resistance with its virulence factors with ease to *Salmonella*. In this experiment we were able to demonstrate that *E. coli* isolate 2077 passes virulence factors to *Salmonella* by conjugation with a high frequency. However we were not able to establish the relationship between the transferred virulence associated genes to the multidrug resistance that was transferred. Therefore prudent use of antibiotics especially in food animals should be followed since animals act as reservoirs for many of the pathogenic and drug resistant bacteria that cause disease in humans. In addition, monitoring of nosocomial drug resistance in hospitals both for humans and animals should be made part of the management requirement both for developed countries and developing countries.

The observed patterns of gene transfer were suggestive of presence of a plasmid that carry several operons (1) for these the different virulence genes. The genes on the same operon

are probably transferred together and independently from other genes on another portion of the plasmid. The *iucC* and *iucD* genes are located on the same plasmid (23) and are transferred together all the time while *fimC*, *fimH*, *int1* and *st1* may be found on the same plasmid but flanked with separate insertion sequences. Despite the difference in the pattern of transfer of the virulence factor genes, we were not able to find a positive correlation between the transferred antibiotic resistance and the transfer pattern of virulence genes from *E. coli* 2077 to *Salmonella*. The question of whether there is a linkage between the transferred virulence factors and plasmid-mediated drug resistance present the opportunity for the future research.

REFERENCES

1. Alessandra Carattoli, (2003). Plasmid-Mediated Antimicrobial Resistance in *Salmonella enterica*. Caister Academic Press. (Accessed August 2012) http://www.horizonpress.com/cimb/v/v5/10.pdf
2. Anders Hay Sørensen, Lars Hestbjerg Hansen, Elsebetta Johannesen, Søren J. Sørensen, (2003). Conjugative plasmid conferring resistance to olaquindox. Antimicrobial Agents and Chemotherapy 47(2): 798–799
3. Bach S, de Almeida A, Carniel E, (1999). The Yersinia high-pathogenicity island is present in deferent members of the family Enterobacteriaceae. Journal of international microbiology. 2:161 – 167.
4. Batchelor NA, Atkinson PM, Gething PW, Picozzi K, Fe`vre EM *et al*., (2009). Spatial predictions of Rhodesian Human African Trypanosomiasis prevalence in Kaberamaido and Dokolo, Two Newly Affected Districts of Uganda. PLoS Neglected Tropical Diseases 3 (12): e563.
5. Birgitta Evengard and Rainer Sauerborn, (2009). Climate change influences infectious diseases both in the Arctic and the tropics: joining the dots. Global Health Action.
6. Center for Disease Control and Prevention, (2011). A public health action plan to combat antimicrobial resistance. Interagency task force on antimicrobial resistance. (Accessed August, 2012) http://www.cdc.gov/drugresistance/pdf/public-health-action-plan-combat-antimicrobial-resistance.pdf
7. Center for Disease Control and Prevention, (2011). Recommendations for future collaboration between the U.S. and the EU. Transatlantic taskforce for antimicrobial

resistance. (Accessed August 2012). http://www.cdc.gov/drugresistance/pdf/tatfar-report.pdf

8. Center for Disease Control and Prevention, (2011). Virus history and distribution. West Nile Virus. Division of vector-borne diseases. (Accessed August 2012). http://www.cdc.gov/ncidod/dvbid/westnile/background.htm
9. Center for Disease Control and Prevention. Antibiogram surveillance method using cumulative susceptibility data. (Accessed August 2012).http://www.cdc.gov/abcs/reports-findings/downloads/antibiogram-method.pdf
10. Ewers C, Janßen T, Kießling S, Philipp H and Wieler LH, (2005). Rapid Detection of Virulence-Associated Genes in Avian Pathogenic Escherichia coli by Multiplex Polymerase Chain Reaction. American Association of Avian Pathologists; Avian Diseases. (2) 49: 269-273.
11. Fe'vre EM, Coleman PG, Odiit M, Magona JW, Welburn SC, Woolhouse MEJ, (2001). The origin of a new Trypanosoma brucei rhodesiense sleeping sickness outbreak in eastern Uganda. Lancet, 358: 625 – 628
12. Gannon VPJ, King RK, Kim JY, and Thomas EJ, (1992). Rapid and sensitive method for detection of Shiga-like toxin-producing *Escherichia coli* in ground beef using the polymerase chain reaction. Applied and environmental microbiology. P. 3809 – 3815.
13. Gibson JS, Cobbold RN and Trott DJ, (2010). Characterization of multidrug-resistant Escherichia coli isolated from extra-intestinal clinical infections in animals. Journal of medical microbiology. 59:592 – 598.
14. Goldstein C, Lee MD, Sanchez S, Hudson C, Phillips B, Register B, Grady M, Liebert C, Summers AO, White DG, 4 and Maurer JJ, (2001). Incidence of Class 1 and 2 Integrases

in Clinical and Commensal Bacteria from Livestock, Companion Animals, and Exotics. Antimicrobial agents and chemotherapy. P.723 – 726.

15. Health Canada, (2002). Uses of antimicrobials in food animals in Canada: impact on resistance and human health. A report of the advisory committee on animal uses of antimicrobials and impact on resistance and human health. (Accessed August 2012). http://publications.gc.ca/collections/collection_2008/hc-sc/H164-68-2002E.pdf
16. Heather M. Vinson, Ablesh Gautam, Susan Olet, Penelope S. Gibbs, and Robert Barigye, (2010). Molecular analysis of porin gene transcription in heterogenotypic multidrug-resistant Escherichia coli isolates from scouring calves. Journal of antimicrobial and chemotherapy 65: 1926 – 1935.
17. International agency for research on cancer, 1990. Summaries and evaluations. Chloramphenicol. (50): 169
18. International Food Policy Research Institute, (2011). 2011 global food report.
19. Janßen T, Schwarz C, Preikschat P, Voss M, Philipp H, Wieler LH, (2001). Virulence-associated genes in avian pathogenic *Escherichia coli* (APEC) isolated from internal organs of poultry having died from colibacillosis. International Journal of Medical microbiology. (291): 371 – 378.
20. John Morton, 2010. The innovation trajectory of sleeping sickness control in Uganda: research knowledge in its context. A discussion paper. Research into use. (Accessed August 2012). http://www.researchintouse.com/resources/riu10discuss08ssickcntrl-ug.pdf
21. Kaufmann M,Zweifel C,Blanco C,Blanco JE,Blanco J,Beutin L,and Stephan R, (2006). *Escherichia coli* O157 and non-O157 Shiga toxin–producing *Escherichia coli* in fecal

samples of finished pigs at slaughter in Switzerland. Journal of food protection. (2) 69:260 – 266.

22. Lihong Chen, Zhaohui Xiong, Lilian Sun, Jian Yang and Qi Jin, (2011). Virulence Factor Data Base 2012 update: toward the genetic diversity and molecular evolution of bacterial virulence factors. (Accessed August 2012). http://nar.oxfordjournals.org/content/40/D1/D641.full.pdf+html
23. Marta Herrero, Victor De Lorenzo, and J. B. Neilands, (1988). Aerobactin operon and topology of its product studied with phoa and lacz gene fusions. Journal of bacteriology. 170:56-64.
24. Nathan N, Tatay M, Piola P, Lake S, Brown V, (2004). High mortality of displaced populations of northern Uganda. The lancet volume 363: 1402 (accessed on 07/24/2012) www.thelancet.com
25. Nenghanjwa Betty Esenu, (2005). Gender relations in livestock production and ownership: Implications for household food security in the Teso Farming System (TFS). A master's thesis. (Accessed August 2012). http://www.dfid.gov.uk/r4d/PDF/outputs/R8108e.pdf
26. Norgren M., Bága M., Tennent J.M., (2006). Normark Nucleotide sequence, regulation and functional analysis of the papC gene required for cell surface localization of Pap pili of uropathogenic Escherichia coli. (Accessed August 2012). http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2958.1987.tb00509.x/abstract
27. Omwata Charles Opio, (2011). Diagnosis of the relationship between weather variables and occurrences of measles in Soroti and Serere districts.

28. Paton JC and Paton AW, (1998). Pathogenesis and Diagnosis of Shiga Toxin-Producing *Escherichia coli* Infections. Journal of Clinical Microbiology Reviews. P 450 – 479.
29. Rodrigo G, Villar MD, Mark DM, Shawna S, Peggy SH, Marcia JG, Jay HL, Lynnell LR, Denny H, Marianne P, Paul SM, (1999). Investigation of multidrug-resistant salmonella serotype typhimurium DT140 infections linked to raw-milk cheese in Washington State. Journal of American Medical Association 281(19): 1811 – 1816.
30. Sanchez S, Stevenson MAM, Hudson CR, Maier M, Buffington T, Dam Q, Maurer JJ, (2002). Characterization of Multidrug-Resistant *Escherichia coli* Isolates Associated with Nosocomial Infections in Dogs. Journal of Clinical microbiology. (40): 3586 – 3594.
31. Sean Moore, Sourya Shrestha, Kyle W. Tomlinson, Holly Vuong, (2011). Predicting the effect of climate change on African trypanosomiasis: integrating epidemiology with parasite and vector biology. (Accessed August, 2012) http://rsif.royalsocietypublishing.org/content/early/2011/11/01/rsif.2011.0654.full.pdf
32. Stephen Connor, (2006). Climate information for health: managing climate sensitive disease. World Meteorological Organization task force on social and economic applications of public weather services. World Meteorological Organization (accessed August, 2012). http://www.wmo.int/pages/prog/amp/pwsp/documents/StephenConnor_health.pdf
33. Uganda Bureau of Statistics. Uganda population and housing census, years**:** 1980, 1991, and 2002. Kampala: Government of Uganda.
34. United Nations (2002), United nations framework convention on climate change, unfccc/informal/84 (1992), art.2.article 2 (accessed on 07/23/2012) http://unfccc.int/resource/docs/convkp/conveng.pdf

35. United Nations Framework Convention on Climate Change, (2007). Climate change: impacts, vulnerabilities and adaptation in developing countries. (Accessed August 2012).http://unfccc.int/resource/docs/publications/impacts.pdf
36. United Nations, (1998). Kyoto protocol to the United Nations framework convention on climate change.
37. United Nations, (2011). Compilation of economy-wide emission reduction targets to be implemented by Parties included in Annex I to the Convention.
38. Usein CR, Damian M, Tatu-Chitoui D, Capusa C, Fagaras R, Tudorache D, Nica M, Bouguenec CL, (2001). Prevalence of virulence genes in Escherichia coli strains isolated from Romanian adult urinary tract infection cases. Journal of cellular and molecular medicine. (3) 5:303 – 310.
39. Vincent Delespaux and Harry P. de Koning, (2007). Drugs and drug resistance in African trypanosomiasis. 10: 30 – 50.
40. Waiswa C and Kabasa JD, (2010). Experiences with an in-training community service model in the control of zoonotic sleeping sickness in Uganda. Journal of veterinary medical education 37(3): 276 – 281
41. Waiswa C, Olaho-Mukani W, Katunguka Rwakishaya E, (2003). Domestic animals as reservoirs for sleeping sickness in three endemic foci in southeastern Uganda.
42. Wastling SL, Picozzi K, Wamboga C, Wissmann B, Amongi-Acup C, Wardrop NA, Stobard JR, Kakembo A and Welburn SC (2011). Latent Trypanosoma brucei gambiense foci in Uganda: a silent epidemic in children and adults? Parasitology 138(12): 1480 – 7
43. Watt S, Lanotte P, Mereghetti L, Moulin-Schouleur M, Picard B, and Quentin R, (2003). *Escherichia coli*Strains from Pregnant Women and Neonates:Intra-species Genetic

Distribution and Prevalence ofVirulence Factors. Journal of clinical microbiology. p. 1929 – 1935.

44. Welburn SC, Coleman PG, Maudlin I, Fe’vre EM, Odiit M, Eisler MC, (2006). Crisis, what crisis? Control of Rhodesian sleeping sickness. Trends in parasitology. Science direct. 22(3): 123 – 128
45. World Bank, (2004). Montreal protocol: successful partnerships to ozone protection - the case of China. Washington D.C. - The World bank. (Accessed on 07/24/2012) http://documents.worldbank.org
46. World Bank, 2010. Development and climate change. World development report 2010. (Accessed August 2012). http://siteresources.worldbank.org/INTWDR2010/Resources/5287678-1226014527953/WDR10-Full-Text.pdf
47. World Health Organization, (2004). Who food additives series 53. Chloramphenicol. FAO/WHO expert committee on food additives.