MAKERERE  **UNIVERSITY**

CHARACTERISATION OF SHIGA TOXIGENIC *ESCHERICHIA COLI* FROM MILK AND ASSOCIATED RISK FACTORS AMONG SMALL HOLDER DAIRY FARMS IN MITYANA DISTRICT

BY

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DECLARATION

I, MUWEREZA Nelson declare that the work described in this dissertation is my original and has never been presented to any university or any other Institution for any award of a degree. Other scientists' work has been appropriately cited.

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DEDICATION

I dedicate this work to my Parents, Mr. and Mrs. George William Ntalo Kalungu, my wife Mukuunya Cathy and my children (Garvin, Pauline, Carlton and Calvin) for the spiritual and moral support they rendered me during my studies.

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LIST OF ACRONYMS AND SYMBOLS

bp	Base pairs
DAEC	Diffusely adherent *Escherichia coli*
DDA	Dairy Development Authority
DNA	Deoxyribonucleic acid
EAAPP	Eastern Africa Agricultural Productivity Project
EAAPP-PCU	Eastern Africa Agricultural Productivity Project -Project Coordination Unit
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohemaorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
et al	And others
ETEC	Enterotoxigenic *Escherichia coli*
HC	Heamolytic Colitis
HUS	Heamolytic Ureamic Syndrome
LT	Labile Toxins
mM	Millimolar
NM	Non Motile
PCR	Polymerase Chain Reaction
Stx	Shiga Toxin
STEC	Shiga Toxin Producing *Escherichia coli*
TTP	Thrombotic Thrombocytopenic Purpura
µl	Micro litres

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ABSTRACT

Strains of Shiga toxigenic *Escherichia coli* are some of the milk-borne zoonotic pathogens that globally cause sickness to millions of people each year. While Shiga-toxigenic *Escherichia coli* has generally been isolated in the environment, cattle and human faecal samples in Uganda, no study has specifically isolated these pathogens from bovine milk in the country. This study aimed at isolation and characterization of Shiga toxigenic strains, quantification and determination of antibiotic sensitivity of *E.coli* isolates from milk from Mityana district. The amount of *E. coli* in the 200 collected samples was quantified based on the number of colony forming units (CFUs) per 1mL of the sample. Antibiotic sensitivity was assessed using the Bauer-Kirby disc diffusion method. Up to six Shiga toxigenic field strains were isolated and characterized by a multiplex PCR. The average CFUs/mL of milk was 1.68 x 10^5. Of the Eighty (80) *E.coli* isolates included six STEC and 74 non-STEC strains. Among the six STEC isolates, four had all the three genes (*stx_1, Stx_2 and EHEC-hly*), while the other 2 isolates had either Shiga toxin $_1$ (*Stx_1*) and Shiga toxin $_2$ (*Stx_2*) or *Stx_1* and Enteroheamolysin (*EHEC-hly*). All the STEC (6) and non-STEC (74) isolates were 100% susceptible to Ciprofloxacin and resistant to and Cloxacillin. Shiga toxigenic *E. coli* occurs in milk from Mityana district, presenting a risk of human infection if milk is consumed raw.

CHAPTER ONE
INTRODUCTION

1.1 Background

Bovine milk is highly nutritious and contains proteins, lipids, vitamins, carbohydrates and minerals essential for the nutritional requirement of a growing calf. Owing to these nutritional properties, milk is also a good growth matrix for a variety of spoilage and potentially pathogenic microorganisms (Millogo et al., 2005). Milk is contaminated at different points in the milk value chain. This can be at farm level and during transportation to local or main urban markets. At the farm level, contamination can result from improper cleaning of milking utensil, unhygienic milking personnel, improper washing of the cow's udder and using unclean water for washing udder or milking utensils. Sometimes the wagging of a feacally contaminated tail by the milking animal during milking can also contaminate milk (Gleeson et al., 2009).

Milk is usually transported to collection centres in different types of containers like Jerrycans, metallic cans or Jugs and some of these containers are not ease to clean thus predisposing it to contamination. Milk from collection centres is transported to major market either in insulated trucks that allow minimal temperature changes from 4^0C to 10^0C or metallic cans in which temperatures are not regulated and this results in proliferation of microbes to about 10 fold (Grimaud et al., 2006). Different microbes can contaminate milk but of interest in this study is Shiga toxigenic *E. coli*. Shiga toxigenic *E. coli* strains are common inhabitants of the gut of humans and other warm blooded animals and a majority are usually harmless to the hosts (Nataro and Kaper, 1998). Though *E. coli* strains are generally harmless, normal non pathogenic *E.coli* can cause infection in the debilitated or immunosuppressed hosts, or when gastrointestinal barriers are violated (Nataro and Kaper, 1998). Some *E. coli* strains have acquired unique virulence factors resulting in pathogenicity to both humans and animals. The clinical syndromes resulting from these *E.coli* pathogens manifests in three main types; the enteric and diarrhoeal diseases, sepsis and meningitis as well as urinary tract infections. *E. coli* that cause enteric diseases also called diarrheagenic *E. coli* have been divided into at least six pathotypes, based on their virulence factors and mechanisms by which they cause disease (Kaper, Nataro, & Mobley, 2004). These pathotypes include Enterotoxigenic *E. coli* (ETEC); Enteropathogenic *E. coli* (EPEC); Enteroaggregative *E. coli* (EAEC), Enteroheamorrhagic *E. coli* (EHEC), Enteroinvassive *E. coli* (EIEC) and Diffusely adherent

E. coli (DAEC) (Nataro and Kaper, 1998). EHEC is a subgroup of Shiga Toxigenic *E. coli* (STEC) or Verotoxigenic *E. coli* (VTEC) characterised by presence of Shiga toxin. The STEC has two most important virulent factors which include the two phage- encoded cytotoxins called Shiga toxin $_1$ and Shiga toxin $_2$ (Law, 2000).

1.2 Problem statement

While milk is commonly produced as a sterile product, it usually suffers contamination with pathogens including *E.coli* during milking, storage and transportation. Milk is supposed to be consumed after boiling or pasteurization, but in rural communities, it is taken as fresh or raw which poses a risk to the consumers if it is contaminated with pathogenic bacteria. Despite the fact that most of the *E. coli* is normal flora, some *E. coli* pathotypes possess virulence genes that lead to production of toxins which result in severe life threatening sickness in the form of bloody diarrhoea, Haemorrhagic Colitis (HC) or a much fatal Haemolytic Uremic Syndrome (HUS) to infants or immune compromised individuals. It is based on the above background that this study was carried out to establish the presence of Shiga toxigenic *E.coli* in milk from Mityana district and its susceptibility to the commonly used antibiotics.

1.3 Justification

Milk is composed of all the nutrients which are required for normal growth and this makes it a good media for growth of micro organisms. Different micro organisms including Shiga toxigenic *E. coli* contaminate milk at different levels of handling and transportation. Without the knowledge on the level and factors that influence contamination, it would be difficult to minimize contamination of milk with *E. coli*. Determining the level of contamination of milk with *E. coli* would provide a baseline information against which effects of control strategies could be measured. Equally, establishing the factors, which influence milk contamination, would be useful in the development of strategies for reducing contamination. Isolation of *E.coli* and characterization of the different pathotypes in milk from Mityana district would provide information on the occurrence of the different pathotypes contaminating milk in the study area. Determination of the antibiotic susceptibility of the isolated *E. coli* pathotypes would help in guiding the health practitioners on the class of antibiotics to be used in treatment of milk borne infection resulting from STEC.

1.4 Overall Objectives

To examine the microbiological quality of milk using *E.coli* as a sentinel for small holder dairy farmers in Mityana district.

1.5 Specific Objectives

1. To determine the prevalence *E. coli* in milk from Mityana district
2. To determine the presence of Shiga toxigenic *E. coli* pathotype in milk.
3. To determine the susceptibility of the isolated Shiga toxigenic *E. coli* to commonly used antibiotics in Uganda.
4. To establish the factors that favour contamination of milk with *E. coli.*

1.6 Research questions

1. What is the level of *E. coli* contamination of milk from Mityana?
2. Does Shiga toxigenic *E. coli* pathotype contaminate milk from Mityana district?
3. What commonly used antibiotics are the isolated Shiga toxigenic *E. coli* isolates susceptible to?
4. What factors influence contamination of milk from Mityana district with *E.coli*?

CHAPTER TWO
LITERATURE REVIEW

2.1 Milk and milk composition

In healthy cows, milk inside the mammary gland is sterile due to the presence of immunoactive substances like lysozyme, lactoperoxidase, lactoferrins and immunoglobulins, and these have anti-microbial properties (Harding, 1999). Milk contains approximately 87.4% water and 12.6% Solids (Goff, 2010).While milk solids consists of 3.9% fat, 3.2% protein, 4.6% lactose and 0.9% others components like minerals, vitamins and nitrogen (FAO, 1986). In general, the composition of milk also depends on or is related to stage of lactation, milking interval, plane of nutrition of the milking cow, quantity of milk produced, seasons of the year and health status of the cow (Kahuta, 2013).

2.2 Milk production in Uganda

Uganda is divided into five milk regions/sheds; Eastern, Southwestern, Western, Northern, and Central. There are differences in the milk sheds in terms of the economic importance of the dairy industry to the region, herd population and production levels, farm size, grazing systems, practices, and cattle breeds used for milk production (Choudhary and Englen., 2011). Milk production in Uganda is concentrated in the cattle corridor (regions with high concentration of dairy animals) and these areas extend from just below 1° latitude in the North to Kabale in the South and from Mbale in the East to Kabarole in the West (Okwenye, 1994).

Uganda's annual milk production was estimated at 1.6 billion litres in 2011 representing an increment of 3% from 2009. Of the 1.6 billion litres produced annually 20% is retained at the farms and only 1.05 billion litres is commercially traded and of which 90% is marketed unprocessed as raw milk (DDA, 2011). Every year, milk consumption has improved in Uganda up to 50 litres per person, providing the 1.6 billion litre milk industry with new market heights (DDA, 2011). The milk produced only meets approximately 20% of the population's nutritional requirements and as such, methods need to be sought to increase milk production in the region (DDA, 2011). This is because over 85% of the milk produced is from indigenous cattle that yield an average of 3 litres of milk per day and are always on open grazing (Masinde, 2013). This level of milk production is inadequate for the existing human population who would require at least 103 million metric tons/year (Mubiru, 2007).

The raw milk market is organized into two steps; viz; traders get the milk from farms and may sell it to fellow traders, processors or directly to consumers. Traders then sell it to big processors or cool it and sell to consumers directly (Mbabazi, 2005). This is associated with high losses in milk. For instance, 27% of the milk produced is wasted or lost; with 10% lost to spoilage during transportation, 11% during handling and marketing, while 6% is lost at farm level which translates into significant loss to the industry (FAO, 1996) indicates that in Uganda.

2.3 Sources of bacterial contaminants of milk

While milk within the mammary glands is usually sterile, pathogens within the surrounding environment can get into the cow's milk through the feed or waters before the animal is milked and during milking. Also bacteria on the inside or outside of the udder can get into the milk (Grimaud *et al.*, 2006). The milking personnel can also contaminate milk if they don't wash and disinfect their hands before milking. Some of the microbes contaminating milk include *Staphyloccocus*, *Streptococcus*, *Escherichia*, *Campylobacter*, *Listeria, Brucella, Mycobacterium* and many others (Grimaud *et al.*, 2006). Although, *Escherichia coli* is part of the non pathogens forming the normal micro flora of the intestinal tract of man and animals, some strains have acquired virulence factors that enable them cause intestinal and extra intestinal diseases in man (Kaper *et al.*, 2004). *E.coli* strains that cause enteric infections have been categorized based on their virulence factors and mechanism by which they cause disease (Nataro & Kaper, 1998).These strains have been categorized into five main pathotypes and these are; Enteropathogenic *E.coli* (EPEC), Enterotoxigenic *E.coli* (ETEC), Enterohaemorrhagic *E.coli* (EHEC), Enteroinvasive *E.coli* (EIEC) and Enteroaggregative *E.coli* (EAEC) (Thorpe, 2004, Gyles, 1998). Generally, sources of milk contaminants can be categorized into four as follows; from the Udder infections, dirt on the udder and teats, contaminated milker's hands and udder wiping towels or from the milking equipment (Grimaud et al., 2006).

Shiga Toxigenic *E.coli* is transmitted from cattle which are the main reservoirs to man through the oral route. This is either by consumption of meat, milk, water or foods contaminated with cattle manure or bathing contaminated water and also direct contact with cattle (Gyles, 2007).

Shiga Toxigenic *E.coli* is acquired through the consumption of contaminated food or water (Armstrong *et al.*, 1996). Ruminants are the primary sources of Shiga Toxigenic *E. coli* shed in faeces contaminating carcasses during slaughter (Kaper *et al.*, 2004). The main sources of

infection are raw milk, water and fresh vegetable contaminated with bovine manure. Undercooked minced beef is also an additional source of infection. Human to cattle transmission has so far not yet been reported but Human to human transmission occurs (Armstrong *et al*., 1996).

In a study by Majalija (2009), Shiga toxin producing *Escherichia coli* was isolated and characterized from cattle, water sources and diarrhoeagenic children in the pastoral community of Nyabushozi county, Kiruhura district (Majalija, 2009).

2.4 Pathotypes of E.coli associated with milk contamination

Pathogenic *E.coli* was commonly used to refer to all diarrhoea associated *E.coli*. Due to advance in diagnostic methods, these groups are now classified based on their unique virulence factors acquired during evolution (Kaper *et al.*, 2004). The pathogenic groups include ETEC, EHEC, EPEC, EIEC, EAEC and DAEC (Feng *et al.*, 1998).

2.4.1 Enteropathogenic E.coli (EPEC)

On ingestion of milk contaminated with Enteropathogenic *E. coli,* the organism adheres to the epithelial tissue of the intestines resulting in watery or bloody diarrhoea and this is associated with infant diarrhoea in the developing countries (Nataro and Kaper, 1998). It is a major cause of childhood mortality in areas with poor hygiene. The virulence factors responsible for the virulence mechanism of this pathotype are by the *eae* gene that codes for intimin and *bfp* gene coding for the bundle forming pili(Nataro and Kaper, 1998b). Pathogenesis of EPEC involves intimin protein that causes attaching and effacing lesions and also a plasmid encoded protein called EPEC adherence factor (EAF) which facilitates localized adherence of bacterial to intestinal cells (Tobe, 1999). Watery diarrhoea is associated with the attachment to and physical alteration of the integrity of the intestines. Bloody diarrhoea is associated with attachment and an acute tissue destructive process (Field, 2003).

2.4.2 Enteroinvassive E.coli (EIEC)

Enteroinvassive strains closely resemble Shigella biochemically, genetically and pathogenetically and cause an invasive dysenteric form of diarrhoea in humans (Nataro and Kaper, 1998). Pathogenicity of EIEC is due to its ability to invade and destroy the colonic tissue. EIEC are believed to have evolved from a similar lineage but during evolution, they diverged into different pathovars (Hicks *et al.*, 1996). The pathogenesis of EIEC involves; epithelial cell penetration, lysis of the endocytic vacuole, intracellular multiplication,

directional movement through the cytoplasm and extension into the adjacent endothelial cells. An ulceration is manifested grossly when a severe infection elicits a strong inflammatory reaction (Kaper *et al.*, 2004).

2.4.3 Enteroaggregative E.coli (EAEC)

Enteroaggregative *E.coli* is the major cause of persistent diarrhoea in children and adults worldwide (Mbonye, 2004). This *E. coli* pathotype contains *aggR* genes which encode for adherence factors responsible for its virulence mechanism. Consumption of milk contaminated with this pathotype will result in its adherence to the intestinal mucosa facilitated by aggregative adherence fimbriae I and II (AAF I /AAF II) proteins encoded for by *aggR* genes (Kagambèga *et al.*, 2012). This is followed by enhanced mucus production resulting in deposition of thick mucus containing biofilm encrusted with EAEC. This culminates into elaboration of an EAEC cytotoxin which results in damage of the intestinal cells (Nataro and Kaper, 1998). EAEC has become a major cause of diarrhoea in patients infected with Human Immunodeficiency Virus (HIV) (Durrer *et al.*, 2000).

2.4.4 Diffusely adherent E.coli (DAEC)

Diffusely adherent *E.coli* causes diarrheal disease in all humans of all age groups. This pathogen has surface fimbriae proteins that form diffuse adherent patterns on cultured epithelial cells (Bilge *et al.*, 1989). The surface fimbriae proteins are encoded by genes located on a conjugative plasmid or in the chromosome.(Nataro, 1992;Yee *et al.*, 1996)

2.4.5 Enterotoxigenic E.coli (ETEC)

Enterotoxigenic *E.coli* is the causative agent for traveller's diarrhoea characterized by watery diarrhoea with little or no fever. ETEC contains *E.coli* strains that produce at least one of the members of the two plasmid encoded enterotoxins: heat labile toxin (LT) and heat stable toxins (ST) (Nataro and Kaper, 1998). The heat labile toxin produced by ETEC is very similar in size (86 KDa), sequence, antigenicity and function to the cholera toxin (CT). ETEC is the major cause of weaning diarrhoea, a watery type of diarrhoea among infants in developing countries (Dutta *et al.*, 2011).

2.4.6 Enterohaemorrhagic E.coli (EHEC)

Enteroheamorrhagic *E.coli* is a subgroup that causes Haemorrhagic Colitis (HC) or bloody diarrhoea and this can potentially progress to fatal Heamolytic uremic Syndrome (HUS). EHEC is identified by the production of Verotoxins or Shiga toxins (Stx_1 and Stx_2). The genes encoding for the Shiga toxins are phage mediated and a particular strain may contain one or both genes (Nataro and Kaper, 1998a). EHEC infections are mostly food and waterborne and

infections have resulted from consumption of raw milk, minced beef, water or unpasteurized apple juice (CDC. 1996). Shiga toxins are the major virulent factors and a defining characteristic for EHEC (March *et al.*, 2001).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study design

A cross sectional study was conducted to investigate the factors favouring contamination of milk by *E.coli* and the presence of Shiga toxigenic *E.coli* in milk from Mityana district (Fig: 1). The level of contamination of this milk with *E.coli* and susceptibility of *E.coli* isolates to common antibiotics was also determined. The lists of farms with their respective owners and the estimated numbers of milking animals on a given farm/ zero-grazing unit in the Kakindu Subcounty and Mityana Town council were given by the respective veterinary officer. Farms in Kakindu Subcounty that had more than 3 milking animals were selected while in Mityana Town council, farms with cattle at any stage of lactation was included in the study.

Map of Mityana district with the different sub-counties

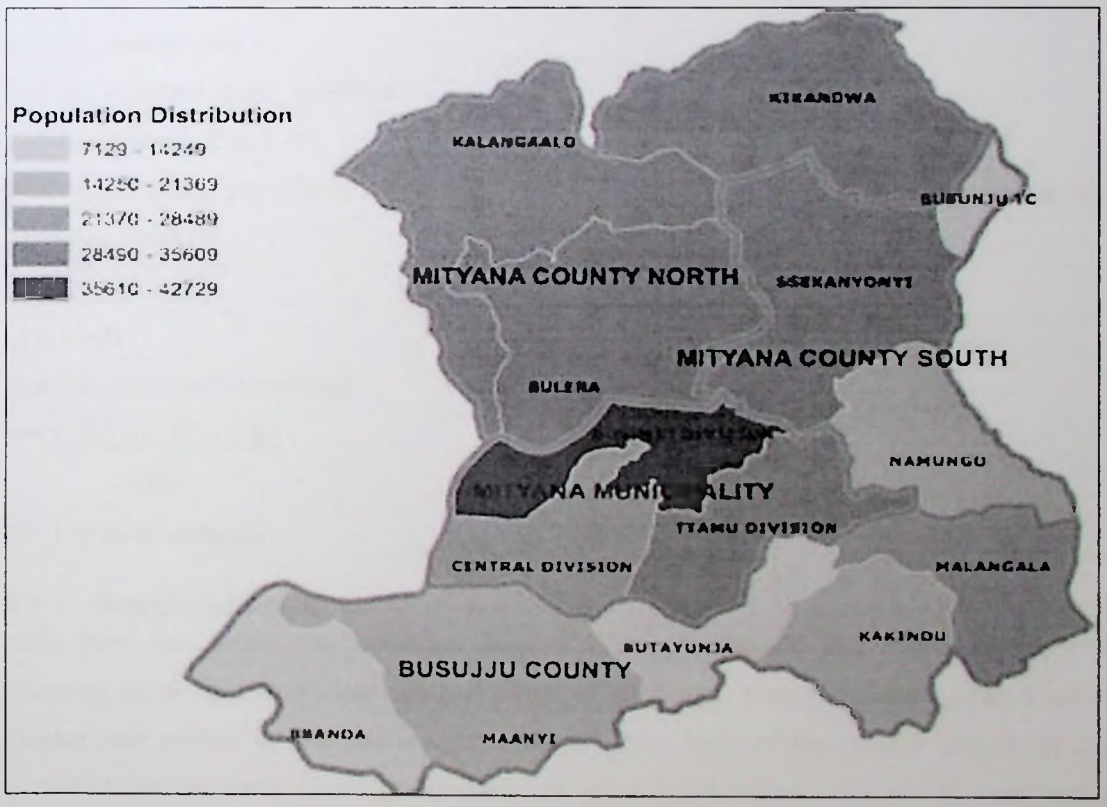

Figure 1 Map of Mityana district and its population distribution

Source: Uganda Bureau of Statistics (UBOS) 2017 Mityana area specific report

3.2 Study area

Mityana district is 73kms from Kampala in the North western direction with a population of 328634 people of which 49% are male. The district is divided into 14 sub-counties and among which Kakindu Subcounty where this study was based has a population of 21,567 people. The people of Kakindu are farmer engaged in crop production and livestock keeping. Farmers who engage in livestock keeping have each an average of Six (6) heads of cattle. Though, Livestock keeping is not on a large scale, the sub-county has an average cattle population of 4,287 with an average production of 280,000 litres of milk monthly (UBOS, 2017). Some of this milk is consumed locally but 75% is delivered to milk collection centres and after transported to Kampala where there is high demand.

3.3 Sample size

Sample size was calculated according to Martin et al., 1987

$$n = \frac{Z^2 PQ}{E^2}$$ where;

n is the sample size

Z is the standard score, at 95% c

onfidence level, Z is 1.96

P is the estimated prevalence of *E.coli* in milk, taken at 15% according to a previous study by Majalija, 2009.

Q is (1-P)

E is the statistical error (5%)

$$n = \frac{1.96^2 \times 0.15 \times 0.85}{0.05^2}$$

n = 195 milk samples

3.4 Sample collection

Milk from the farms was collected directly from pre-cleaned and dried udder by first stripping three times; to clear the teat canal of each teat, then the fourth strip from each quarter was pooled into sterile universal bottle. Only 3mls of the pooled sample in sterile universal bottles were carefully labelled and immediately placed on ice in cool box and transported within 12 hours to the Microbiology Laboratory at the National Livestock Resources Research Institute (NaLIRRI) at Tororo. The samples were cultured within 8 hours after reaching the laboratory.

At the selected farms from which fresh milk samples were collected, variables relating to the kraal environment, milking practices, lactating cow health (age, parity, physiological status, udder cleanliness, and mastitis control) and other farm management practices were collected. This data was captured using an interviewer administered structured questionnaire (Appendix 1) administered to farm attendant/milking personnel. All quantitative data was captured in *EpiData* (Version 3.1) and analysed using STATA. The existence of significant relationships between variables was tested at 95% level of confidence.

3.5 Ethical Consideration

Participating farmers were given full and adequate oral and written information about the nature, purpose, possible risks, and benefits of the study in both English and Luganda languages. They were given adequate opportunity to ask questions and allowed time to consider the information provided. The participants signed and dated informed consent that was obtained before conducting this study. The study data were entered in *Epidata* (Version 3.1) while maintaining confidentiality. Participating farms in this study were identified by the unique enrolment number only. Ethical clearance to carry out this study was sought from the Research and Ethics Committee of the National Agricultural Research Organisation (NARO) and Mityana District Administration.

3.6 Culturing and identification of *E.coli*

The milk samples were subjected to a two fold dilution up to 2^{-5} (1mL of concentrated milk in 31mLs of PBS) with 1χ PBS. From each of the serially diluted sample, 10µl was poured on freshly prepared Chromogenic agar plate (Merck) and spread evenly using a sterile glass rod. The inoculated plates were then placed upside down in an incubator at 37°C for 18-24 hours for the growth of the organisms. Chromogenic medium is a selective medium for the detection of *E.coli* and other Coliforms in waters and foods. The interaction of ingredients in the medium, such as peptone, sorbitol and others, grants a quick colony growth, including infectious Coliforms. Tergitol-7 inhibits Gram-positive bacteria. Sodium chloride maintains the osmotic balance and the Phosphate is the buffer. Bacteriological agar is the solidifying agent. The chromogenic mixture contains chromogenic substrates as Salmon-GAL and X-glucuronide. Coliform enzymes produced, such as galactosidase and glucuronidase, cleave these substrates, resulting in the different coloration of certain bacteria colonies. The ß-D-galactosidase cleaves Salmon-GAL substrate, and gives a salmon to red colour to the coliform colonies. The ß-D-glucuronidase cleaves both substrates Salmon-Gal and X-

glucuronide, giving a dark blue to violet colour to the colonies, easily distinguishable from other coliform colonies that have a salmon to red colour.

3.7 Determination of level of contamination

The level of *Escherichia coli* contamination was estimated by quantification of Colony Forming Units (CFUs) in the serially diluted samples cultured on chromogenic agar using established procedures (Hirth, 2004).

3.8 DNA extraction

DNA was extracted using the DNeasy^{R} Blood & Tissue Kit (Quiagen, Germany), following the Quick-Start protocol. Five distinct blue (*E.coli*) colonies on the freshly grown overnight cultures on Chromogenic agar were swept using a wire loop and suspended into 200µl of PBS, and then 20µl of proteinase K was added to digest contaminating proteins. To this mixture, 200µl of buffer AL (Lysis buffer) was added to break the cell membrane and this was mixed thoroughly by vortexing for 15sec, after which, 200µl absolute ethanol (96-100%) was added to the mixture to precipitate the nucleic acid out of the solution and vortexed for another 15sec. A volume of 620µl of the homogenate from the previous step was then pipetted into the DNeasy mini spin column placed in a 2ml collection tube and centrifuged at 8,000 rpm for 1 min. The DNeasy mini spin column was then placed into another clean 2ml collection tube and the filtrate discarded. A volume of 500µl of buffer AW1 (Wash buffer) was added to the DNeasy mini spin column and the mini column was centrifuged at 8,000 rpm for 1 min to wash out all the cell debris while the required DNA is attached on the membrane. The DNeasy min spin column was placed in another clean 2ml collection tube and the tube that contained the filtrate was discarded. A volume of 500µl of buffer AW2 (Wash buffer) was added to the DNeasy mini spin column and the column was centrifuged at 14,000 rpm for 3 min to wash out any cell debris that could have not been washed out by buffer AW1. The DNeasy mini column was then placed in a clean 1.5ml micro centrifuge tube and the tube that contained the filtrate was discarded. To the DNeasy mini spin column, 200µl of AE buffer (Elusion buffer) was carefully added to the centre of the spin column and incubated at room temperature for 1 min. After which, the column was centrifuged at 8,000rpm for 1 min to wash out the DNA that had adsorbed on the mini spin column. The mini column was then discarded and the filtrate (extracted DNA) was stored at -20^{0}C awaiting use for PCR reactions.

3.9 DNA Multiplex PCR amplification to detect Shiga toxigenic *E.coli*

DNA extracted from five *E.coli* single colonies from each plate was used to run Multiplex PCR using the specific oligonucleotide primers for each STEC. Different pathotypes were detected using specific sets of primers. Multiplex PCR was performed using *PxE2.0* Thermal cycler (Hybaid, Midland, ON, Canada) carried out in a 25µl reaction mixture containing 2µl of the template DNA, 12.5µl of 2χ Dream Taq green Master mix and 4 µmoles of each of the primer pair. The cycling conditions were; initial denaturation at 95°C for 4 minutes, denaturation at 95°C for 45 seconds, annealing at 58°C for 60 seconds, extension at 72°C for 60 seconds for 35 cycles and the final extension for 72°C for 7 minutes and subsequent holding temperature of 4°C. The multiplex targeting three genes of EHEC (*Stx_1*, *Stx_2* and *EHEC-hly*) was carried out in the reaction. All the 80 *E.coli* isolates that were positive on Chromogenic agar were each run with different gene markers,

Table 1: **Primers for detection of *E.coli* pathotypes**

Pathotype	*Target gene*	*Primer sequence (5'-3')*	*Size of genes (bp)*	*Reference*
EHEC	*stx2*	*F:GTTTTGACCATCTTCGTCTGATTATTGAG* *R: AGCGTAAGGCTTCTGCTGTGAC*	*324*	*(Muller et al., 2007)*
	EHE-hly	*F. TTCTGGGAAACAGTGACGCACATA* *R. TCACCGATCTTCTCATCCCAATG*	*688*	
	Stx_1	*F: CGATGTTACGGTTTGTTACTGTGACAGC* *R: AATGCCACGCTTCCCAGAATTG*	*244*	

3.10 Separation and visualisation of PCR products

Electrophoresis of PCR products was carried out on a 1.5% agarose gel at 100V for 90 minutes, by loading10µl of product. A molecular marker (O'GeneRuler 100bp plus, DNA ladder) was run concurrently. Tri-borate-EDTA (1χTBE) containing ethidium bromide (10mg/ml, Sigma Aldrich) was used as the buffer in the electrophoresis chamber and in the agarose gel. The fragments of DNA in the gel was visualized by exposing the gel to UV light using U.V Trans illuminator equipment and converted into digital photographic image.

3.11 Interpretation of the multiplex data

The sample was considered positive for the STEC if the result of the gel electrophoresis showed the bands of the right size as determined by the positive control and negative if it gave no right band. The different *E.coli* isolates from different milk samples positive for a given strain were tabulated for further use on antibiotic resistance profiles.

3.12 Antibiotic susceptibility test

Escherichia coli pathotypes isolated from the milk samples were used to establish their response on selected common antibiotic drugs. This was done using Bauer-Kirby agar disc diffusion method on Mueller-Hinton (MH) agar (Oxoid) according to the recommendations and reported by the Clinical and Laboratory Standards Institute (CLSI 2014). Isolates were at first cultured on Chromogenic agar to obtain fresh colonies. Five colonies from the subculture of the isolated *E.coli* pathotype were then picked using a sterile wire loop and inoculated onto Mueller-Hinton broth. The broth was then incubated at 37°C until turbid, and turbidity adjusted to a proper density by comparing with 0.5Mcfarland standard. Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the suspension and rotated to remove excess liquid. The swab was then streaked over the entire surface of the Mueller-Hinton agar (prepared according to manufacturers instructions) three times, rotating the plate approximately at 60° after each application to ensure an even distribution of the inoculum.

Antimicrobial discs were then individually applied to the plates using sterile forceps and they were gently pressed down on to the agar and after incubated at 37°C for 16 to 18 hours, after which zones of clearance around the antibiotic discs were measured using an inhibition zone ruler and results interpreted. The antibiotic discs used for testing susceptibility included; Sulfisoxasole 30µg (ST), Tetracycline 30µg (TE), Penicillin 10µg (P), Gentamycin 10µg (CN), Kanamycin 30µg (K), Cloxacillin 10µg (CX), Nalidixic acid 30µg (NA), Neomycin 30µg (N), Ciprofloxacin 30µg (CIP), Erythromycin 15µg (E), Griseofulvin 10µg (GRS), Ampicillin 10µg (AM) and Chloramphenicol 30µg (C) (Himedia Laboratories Ltd, Mumbai India).

3.13 Data analysis, management and presentation

Factors that were suspected to result into milk contamination with *E.coli* were collected using an interviewer administered questionnaire and 17 risk factors were examined (Appendix 1). Data on these 17 variables relating to the lactating cow, herd, environmental conditions and farm management practices were entered and stored in *EpiData* (Version 3.1). Existence of statistic relationships between predictor and outcome variables was done by determining the Chi-square (χ^2) values, while the strength of association was established by computing for odds ratio (OR) at a 95% confidence internal (CI).

For the antibiotics susceptibility, diameters of the zones of clearance were measured using Inhibition zone ruler to determine whether the isolates were resistant, intermediate or

susceptible to the commonly used antibiotics. The ranges for which a drug can be interpreted as Susceptible, Intermediate or Resistant are as in table 2.

Table 2. Growth Inhibition zones around the antibiotic discs to guide interpretation

Antibiotic type	**Symbol**	**Antibiotic Concentration**	**Intermediate ranges**
Ciprofloxacin	CIP	30	15-16
Penicillin	P	10	21-28
Ampicillin	AM	10	13-15
Cloxacillin	CX	10	21-28
Chloramphenicol	C	30	13-17
Sulfisoxasole	ST	30	13-16
Gentamicin	CN	10	13-17
Tetracycline	TE	30	15-18
Neomycin	N	30	13-18
Nalidixic acid	NA	30	14-18
Griseofulvin	GRS	10	13-17
Kanamycin	K	30	14-17
Erythromycin	E	15	14-17
Interpretation	> Susceptible	= Intermediate	< Resistant

CHAPTER FOUR

RESULTS

4.1 Level of contamination

The study observed that of the 200 fresh milk samples cultured, 40.0% (80/200) were contaminated with *E. coli*. This included 46% (46/100) and 34% (34/100) samples from Kakindu sub county farms and that from Mityana Town council small holder farms respectively. The average contamination of milk was (1.60-1.68) $x10^5$ CFUs/mL for samples collected from Kakindu Sub county farms with a standard deviation of 5.79 x 10^{-1}, while for Mityana Town council small holder units was 1.69 x 10^5 CFUs/mL with a standard deviation of 4.77 x 10^{-1}. The growth of E.coli (Blue colonies) and other coliforms (red) on chromogenic agar is shown in **Figure.2.**

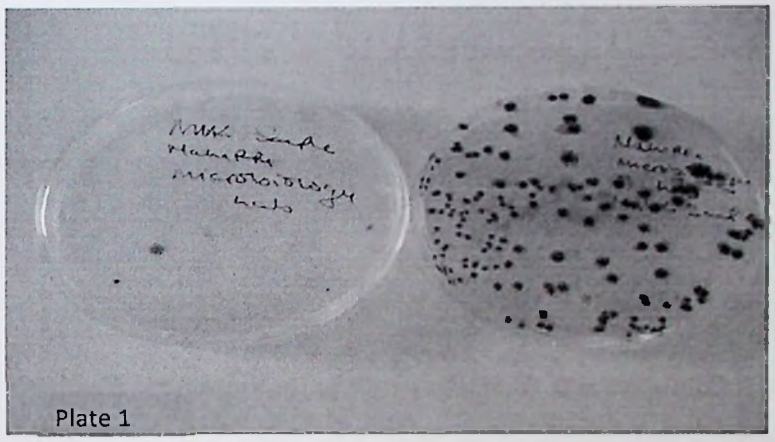

Figure 2 Milk cultures on Chromogenic media showing different colonies

The average count of E.coli in different sample at different dilution is as shown in Table 3.

Table 3 E.coli load in milk obtained from Kakindu S/County and Mityana T/Council

Source of sample	Dilution	Contamination (Average CFUs/ML)
Kakindu S/county Farms	2^{-3}	1.63 $x10^5$
	2^{-4}	1.68 $x10^5$
	2^{-5}	1.67 x 10^5
Mityana T/Council Zero grazing units	2^{-3}	1.69 x 10^5
	2^{-4}	1.67 x 10^5
	2^{-5}	1.69 $x10^5$

4.2 Presence of Shiga Toxigenic *E.coli* in Milk

Only four samples (S2, S5, S11 and S15) had a combination of *EHE-hly* (688bp), Stx_2 (324bp) and Stx_1 (244bp) genes, one sample (S4) had Stx_1 (244bp) and Stx_2 (324bp) and another sample (S12) had Stx_1 (244bp) and *EHEC-hly* (688bp) only as in the gel picture in **Figure. 3.**

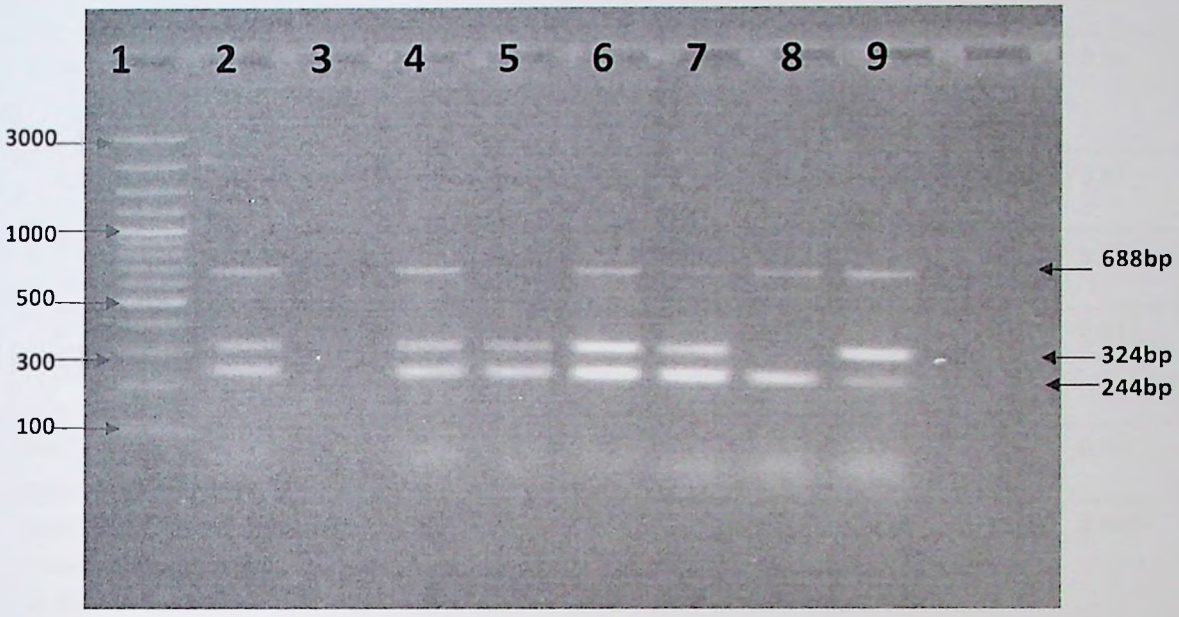

Figure 3 Gel picture of Shiga toxigenic E.coli isolated from bovine milk in Mityana.

Lane 1 is the DNA ladder, lane 2 is the positive control (RH 4270) containing the 3 bands (688bp for *EHEC-hly*, 324bp for Stx_2 and 224bp for Stx_1). Lane 3 is the negative control (Nuclease free water), lane 4: S2, lane 5: S4, lane 6: S5, lane 7: S11 and lane 8: S12 and lane 9: S15. Isolates S2, S5, S11 and S15 have all the 3 genes, while isolate S4 has genes Stx_1 and Stx_2, and isolate S12 has *EHEC-hly* and Stx_1 only.

4.3 Risk factors for E.coli contamination

These included factors that were related to the lactating cow, environment and the different practices on the farm. The explanatory variables were tested for their relation with *E.coli* contamination using chi square test and logistic regression analysis as indicated in **Table 4.**

Table 4 Factors significantly leading to contamination of milk collected from Kakindu Subcounty and Mityana Town council.

Variable	*Category*	*Sample(+)*	*Sample (-)*	*Prevalence*	χ^2	*P-Value*
Herd size	1 milking cow	8	4	66.7	1.54	0.46
	2-3 milking cows	40	34	54.1		
	4-7milking cows	56	58	49.1		
Physiological status	Milked pregnant	41	33	55.4	0.55	0.46
	Milked not pregnant	63	63	50.0		
Parity	1st Calving	24	26	48	3.61	0.16
	2-3 Calvings	62	62	50		
	4 or more Calvings	18	8	69.2		
Use antiseptic	Yes	47	49	49	0.68	0.41
	No	57	47	54.8		
Strip cup use	Yes	6	8	42.9	0.5	0.48
	No	98	88	52.7		
Washing milking Utensils	Yes	102	94	52	0.01	0.94
	No	2	2	50		
Sun dry milking cans	Yes	102	94	52	0.01	0.94
	No	2	2	50		
Source of water used at the farm	Bore hole	6	0	100	18.27	**0.000**
	Well	69	86	44.5		
	Tap	13	7	65.0		
	Stream	16	3	84.2		
Breed	Cross	102	94	52	0.01	**0.001**
	Exotic	2	2	50		
Dry clean environment	Yes	13	14	48.1	0.19	0.67
	No	91	82	52.6		
Muddy environment	Yes	80	71	53	0.24	**0.002**
	No	24	25	49		
Wet clean environment	Yes	12	14	46.2	0.41	0.52
	No	92	82	52.9		
Udder hygiene	Yes	28	36	43.8	2.6	0.12
	No	76	60	55.9		
Source of sample	udder	60	50	54.5	0.64	0.43
	pooled	44	46	48.9		
Feeding method	Intensive	24	13	64.9	3.01	0.08
	Semi Intensive	80	83	49.1		
Chicken present	Yes	82	64	56.2	3.76	0.07
	No	22	32	40.7		
Breeding method	Bull	57	45	55.9	1.26	0.26
	AI	47	51	48		

The identified risk factors were subjected to a multivariate logistic regression and tested for their strength of association with contamination of *E.coli* **(Table 5).**

Table 5 Multivariable logistic regression of risk factors associated with *E.coli*

Variable	**Category**	**Odds Ratio (CI 95%)**	**P-Value**
Parity	1st calving	1.96(0.67-5.70)	0.22
	2-3 calvings	2.5(0.96-6.52)	0.06
	>3calvings	Reference	
Source of water	borehole	0.00	1.00
	well	7.42(1.98-27.85)	**0.00**
	Tap	2.62(0.53-13.0)	0.24
	stream	Reference	
Muddy environment	Yes	1.55(0.81-2.97)	**0.02**
	No	Reference	
Breed	cross	0.87(0.38-2.01)	**0.01**
	Exotic	Reference	

4.4 Antibiotic susceptibility

The growth inhibition zones around each antibiotic disc was assessed as in Figure 4

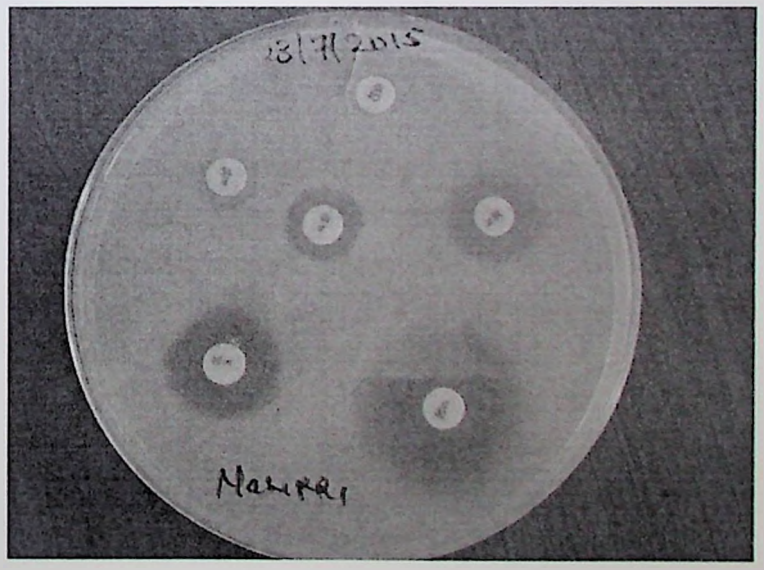

Figure 4 Plate showing Zones of clearance around Antibiotic discs on Mueller Hinton agar

The susceptibility of the six (6) STEC isolates to the different antibiotics was as represented in **Table 6**. All the six STEC isolates were susceptible to Ciprofloxacin and Kanamycin. No resistance was exhibited in Nalidixic acid, Erythromycin or Tetracycline but it was high in Chloramphenicol, Cloxacillin, Ampicillin, Penicillin and Sulfisoxasole.

Table 6 Susceptibility of the six (6) STEC isolates to commonly used antibiotics

Antibiotic	*S*	*I*	*R*
Ciprofloxacin	6	0	0
Kanamycin	6	0	0
Erythromycin	5	1	0
Nalidixic Acid	4	2	0
Tetracycline	4	2	0
Griseofluvin	3	2	1
Neomycin	1	3	2
Gentamycin	3	1	2
Sulfisoxasole	1	0	5
Ampicillin	1	0	5
Chloramphenical	0	1	5
Penicillin	0	0	6
Cloxacillin	0	0	6

S=Susceptible, I=Intermediate, R= Resistant

Eighty (80) samples which had *E.coli* were each subjected to antibiotic susceptibility testing and the profiles indicate that 100% (80) were susceptible to Ciprofloxacin and 100% were resistant to Cloxacillin as in Table. 7

Table 7 Antibiotic Susceptibility profiles of E.coli isolates from milk collected from Mityana

	Antibiotics												
Susceptibility profile		*CIP*	*NA*	*C*	*E*	*CN*	*N*	*K*	*TE*	*P*	*AM*	*CX*	*ST*	*GRS*
	S	**80**	50	59	04	51	13	68	30	00	09	00	05	36
	I	00	30	21	22	22	56	12	35	02	18	00	20	25
	R	00	00	00	54	07	11	00	15	78	53	**80**	55	19
	Total	80	80	80	80	80	80	80	80	80	80	80	80	80

Penicillin(p), Ampicillin(AM), Cloxacillin(CX), Ciprofloxacin(CIP), Nalidixic Acid(NA), Erthromycin(E), Chloramphenical(C), Neomycin(N), Kanamycin(K), Tetracycline(TE), Gentamycin(CN), Sulfisaxole(ST) and Griseofluvin(GRS) S=Susceptible, I=Intermediate, R= Resistant

The *E.coli* isolates susceptibility profiles were also categorised depending on the class of antibiotic susceptible, intermediate or resistant to and this is represented in the graph **Fig.5**

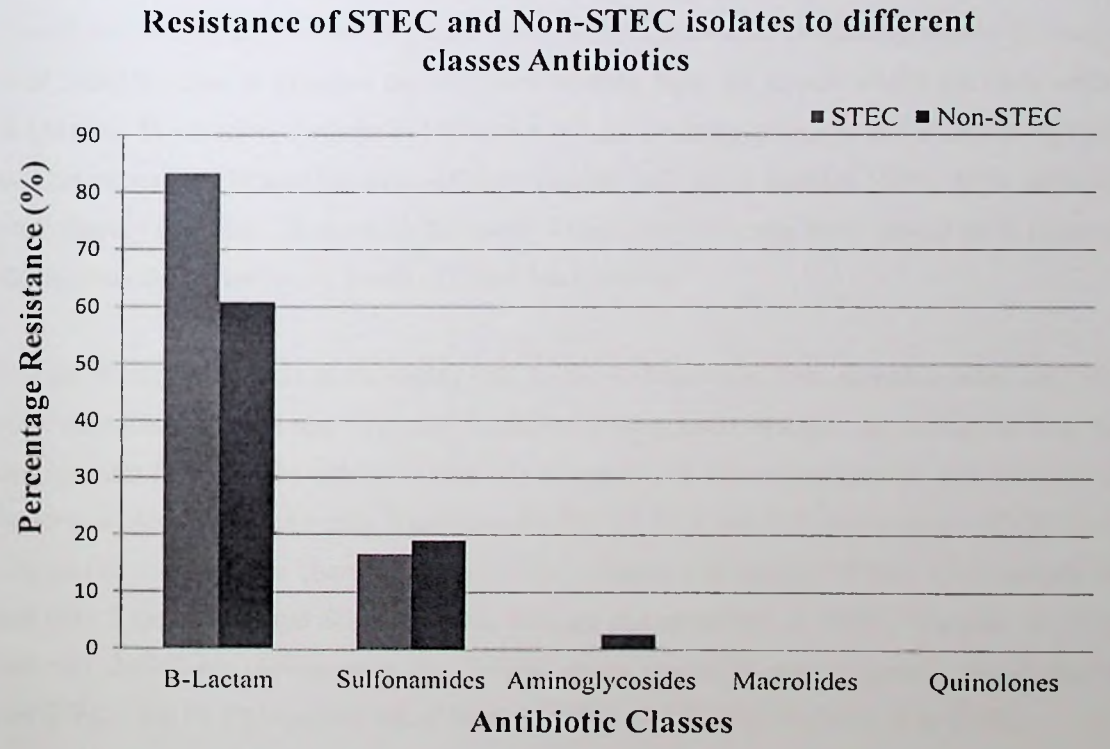

Figure 5 Graph showing multi-drug resistance of STEC and Non-STEC isolates obtained from Milk collected from Mityana district

CHAPTER FIVE

DISCUSSION

Contamination with non pathogenic *E.coli* is a common finding in milk samples as it was reported by Grimaud *et al.*, 2006 in a research carried out in Mbarara .STEC one of the pathotypes of *E.coli* was isolated and characterised from cattle faecal samples, water sources, and diarrhoeagenic children in the pastoral community of Nyabushozi County in Kiruhura district by Majalija 2009. This study is the first to establish the presence of STEC in milk

The *E.coli* load contaminating milk collected from Kakindu Subcounty was 1.68 $x10^5$ Cfus/mL. This contamination is attributed to unhygienic practices at the farm like not washing milking utensils, use of dirty water from streams or wells, improper washing of the udder before milking, lack of antiseptic use after milking as established by(Kato., 2007). The contamination level in Mityana Town council was 1.69 x 10^5 CFUs/mL which is the same as contamination at the farm in Kakindu Sub County and this could be attributed to the same practise of milking, and animal management. The level of contamination of milk with *E.coli* was low (1.69 $x10^5$ cfus/mL) compared to the findings (2.0 $x10^6$ cfus/mL) in a research carried out in Mbarara western Uganda which is the major milk producing region (Grimaud *et al* 2006) because in Mbarara farmers were milking from the Kraals which are dirty unlike in Mityana. In addition, farmers in Mbarara were not cleaning properly and disinfecting their milking containers before the next milking. Further still, most farmers (60%) from Mbarara were sharing the water source with the cattle which was not to the same extent as in mityana district thus the variations in levels of *E.coli* load in milk.

The six (6) STEC isolates of the eighty (80) *E.coli* isolates were from samples collected from three zero grazing units and two semi intensive management systems an indication that the environment in which the animal is kept has an impact on the contamination and the type of bacteria contaminating the milk. Four samples S2, S5, S11 and S15 had 3 genes (*EHEC-hly, Stx_2 and Stx_1*) which are characteristic of STEC (Nataro and Kaper, 1998a). One sample S4 had only 2 genes Stx_2 and Stx_1 also genes that are characteristic of STEC. Sample 12 (S12) had only *EHEC-hly* (688bp) and Stx_1 (244bp) genes amplified which equally shows that it had STEC. Six (7.5%) isolates out of 80 were STEC and 74 (92.5%) were non-STEC.

The farms whose water comes from the streams or wells had a higher contamination with *E.coli* than those whose water source was either boreholes or tap water. This difference is

attributed to the level of protection and treatment for the bore hole water and tap water respectively compared to that from streams and wells in which it is contaminated with urine and faecal samples from animal as established by Majalija (2009) in his work done in southwestern Uganda.

The environment (Muddy) around the animal and the breed of the animal significantly influenced the level of contamination of milk with *E.coli*. Sometimes the milking personnel don't clean the udder and this predisposes the milk from these animals to contamination (Kato., 2007). Three of the milk samples from which STEC isolates were recovered were from zero grazing units which indicate that the zero grazing management system is a predisposing factor to contamination of the milk with *E.coli*.

The isolated *E.coli* was subjected to antibiotic sensitivity testing using 13 antibiotic discs with different antibiotics concentrations. It was established that all the 80 *E.coli* isolates, were resistant to Cloxacillin (100%), and Penicillin (97.5%) while 100% susceptible to Ciprofloxacin and 85% susceptible to Kanamycin. All the STEC isolates (7.5%) were 100% susceptible to Ciprofloxacin and 100% resistant to Cloxacillin while the non-STEC isolates had multi drug resistance to antibiotics from the β-lactam class (60.8%), Sulfonamide class (19%) and Aminoglycoside class (2.7%). The resistance of STEC isolates to β-lactam drugs may be attributed to possibly transfer of resistance genes like β-lactamase gene from Non-STEC isolates by either conjugation, transduction, transposition or transformation because they are in the same environment

There was no multi drug resistance to Macrolides (E & C) and Quinolones (CIP & NA) among the non-STEC isolates though individually, different isolates were selectively resistant to either Erythromycin or Chloramphenicol and this is attributed to the mode of action of these new antibiotic classes which affect the bacterial DNA synthesis unlike the β-lactam which are effective for gram positive bacteria.

STEC isolates were more resistant to β-lactams (83.3%) compared to the Non-STEC isolates whose resistance to β-lactams was 60.8%.The reverse was seen with sulfonamides where Non-STEC were more resistant (19%) compared to STEC isolates (16.6%). None of the STEC isolates was resistant to Aminoglycosides, Macrolides or Quinolones but although some Non-STEC isolates were resistant to Aminoglycosides resistance to Macrolides and

Quinolones has no yet been acquired. This could be attributed to acquisition of resistant genes from the bacteria in the environment by lateral transfer of resistant genes.

CHAPTER SIX
CONCLUSION AND RECOMMENDATION

6.1 Conclusion

There is high contamination of milk at the farms attributed to use of water from contaminated water sources and unhygienic milk collection practices done at the farm.

Some of the *E.coli* contaminating milk from Kakindu Sub County and Mityana Town Council contains pathogenic strains of STEC ascertained by the Stx_1, Stx_2 and *EHEC-hly* genes.

The source of water used at the farm for washing milking utensils and general equipment cleaning is responsible for the contamination of the milk with *E.coli* because the contamination was high in areas where water used on the farms is either from wells or streams

The environment in which the animal stays or milked from has a serious bearing on the chances of possible contamination of the milk with different bacteria including *E.coli.*

The Shiga toxigenic *E.coli* pathotype has acquired resistance to a number of commonly used antibiotics and this poses a very big problem when individuals who may get infected with this pathotype are treated with the commonly available antibiotics.

6.2 Recommendations

- Since some samples of milk were contaminated with very deadly strains of *E.coli,* milk collection centers should be equipped with *E.coli* testing strip which when dipped into the sample could easily differentiate the samples heavily contaminated and those that are not. This will rid the market or protect the consumers from the highly contaminated milk samples. The consumers are also advised to refrain from taking raw or under cooked milk as this will predispose them to these pathogenic *E.coli* strains.

- Water used at the farm for all activities should come from protected water sources and the milking shed, milking cans, transportation cans should be washed and sun dried before the next milking. Animals under the zero-grazing management system should be

cleaned and their pen cleaned of the wastes like urine and dung as these can be a source of contamination to the milk harvested from the animal.

- Further still, in cases of infections due to consumption of milk contaminated with Shiga Toxin producing *Escherichia coli*, the drugs of choice in treatment would be Ciprofloxacin, Nalidixic acid, Tetracycline, Kanamycin, Gentamycin and Chloramphenicol but not β–lactam drugs

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APPENDIX

Appendix I: Questionnaire for data collection from sampled farms

1. Name of respondent.....
2. Village.....Sub county.....District.....
3. Season 1. Dry..... 2. Wet.....
4. Herd size.....
   1. 1-2 animals 2.3- 4 animals 3. >5 animals
5. Animal Physiological Status

1. Milking/ Pregnant 2. Milking/ Not pregnant

6. Parity
   1. First Calving 2. 2-3 Calvings 3. > 4 Calvings
7. Method of Milking
   1. Hand Milking 2. Machine milking

8. Preparation before milking
   a) Washing udder with antiseptic
      1. Yes 2. No
   b) Using a strip Cup to test for Mastitis
      1. Yes 2. No
   c) Application of milking salve before and after milking
      1. Yes 2. No
   d) Hand washing before milking
      1. Yes 2. No
   e) Washing of Milking utensils before milking.
      1. Yes 2. No
9. Washing and sun drying of milk transporting cans
   1. Yes 2. No
10. Source of water for washing the milking utensils.
    1. Boreholes 2. Wells 3.Taps 4. Stream
11. Animal Breed
    1. Cross 2. Local 3. Exotic
12. Kraal Hygiene
    1. Dry clean 2. Muddy 3. Wet clean
13. Udder Hygiene

1. Clean 2. Dirty

14 Source of milk sample

1. Udder 2. Pooled sample

15 Temperature

1. Hot 2. Moderate 3. Low

16 Feeding Method

1. Intensive 2.Semi Intensive 3. Extensive

17 Presence of Pigs

1. Yes 2. No

18. Presence of Chicken

1. Yes 2. No

19, Breeding Method

1. Bull 2. A/I

20. Presence of Flies

1. Yes 2. No