ASSESSMENT OF MICROBIAL CONTAMINATION OF RAW COW MILK AND ANTIMICROBIAL RESISTANCE OF *SALMONELLA* SPP ISOLATED IN ILALA DISTRICT, DAR ES SALAAM, TANZANIA

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ABSTRACT

The current cross sectional study was conducted to determine factors influencing microbial contamination, proportion and antimicrobial susceptibility profiles of *Salmonella* spp isolated from raw cow milk in Ilala district, Dar Es Salaam, Tanzania. A total of 138 smallholder dairy farmers were randomly selected and interviewed, and subsequently, milk samples were aseptically collected from Kivule, Kitunda, Magole and Ukonga between July and October 2020. Identification was done by conventional culture method, biochemical tests and serotyping. Disc diffusion method was used for antimicrobial sensitivity testing. Reference organisms used in the study included; *Salmonella typhimurium* (ATCC 14028) and *E. coli* (ATCC 25922). Results showed that, majority of smallholder dairy farmers were males with primary education, 8% of respondents consume milk from animals under medication and 23.9% did not adhere to withdrawal periods. Furthermore, results indicated that, 34.8% and 57.1% reported not to wash hands before milking and between milking different cows and 30.4% reported to milk sick cows practices which were found to significantly predispose milk to microbial contamination ($p=0.000$; $p=0.001$ and $p=0.042$) respectively. Out of 138 samples, 8 (5.8%) samples confirmed to be *Salmonella* whereby 3 were *S. typhimurium*, 3 were *S. enteriditis* and 2 were *S. typhi*. Kivule ward showed high prevalence (14.6%) of *Salmonella* than the other wards with no statistical difference ($P>0.05$) between them. Antimicrobial susceptibility results showed all isolates were resistant to ampicillin, amoxicillin/clavulanic acid and penicillin but susceptible to gentamycin, tetracycline, chloramphenicol, ciprofloxacin and trimethoprim-sulfamethoxazole and 100% of isolates showed multi-drug resistant against three antibiotics. This study revealed the presence of *Salmonella* in apparently healthy dairy cows in Ilala district with antimicrobial resistances. Improvement in animal husbandry practices and public education on general milk hygiene

are recommended. Additionally, extension officers, veterinarians and all other stakeholders should play a part in ensuring that consumers receive safe, high-quality milk.

DECLARATION

I, Agnes Jonathan, do declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor concurrently being submitted in any other institution.

_____ _____

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The declaration is hereby confirmed by:

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DEDICATION

This work is dedicated to my parents, Mr. and Mrs. Jonathan James, for their unconditional love and support which created the foundation of my education and my precious daughter Aaliyah. May God continue to bless them.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATION AND SYMBOLS

%	Percentage
°C	Degree Celsius
AMR	Antimicrobial Resistance
MDR	Multidrug Resistance
CLSI	Clinical and Laboratory Standards Institute
FBDs	Foodborne diseases
GDP	gross domestic product
km^2	Square kilometers
MCA	MacConkey Agar
ml	Milliliter
Mm	Millimeter
NA	Nutrient Agar
No	Number
pH	Hydrogen iron concentration
ppm	Parts per million
spp	Species
SSA	Salmonella Shigella Agar
SUA	Sokoine University of Agriculture
TSI	Triple Sugar Iron agar
µl	Microliter

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Cow milk is the most often consumed milk among dairy animals, and it provides the human body with animal proteins, lipids, minerals and vitamins (Tamba *et al.*, 2016). More than 150 million farm households around the world rely on smallholder dairy farming, which is characterized by small herds of 2 - 3 milking cows (Hemme and Otte, 2010). Total annual milk output in Tanzania is currently estimated to be 1.65 billion liters, with 70% of the milk coming from indigenous cattle kept in rural regions and 30% coming from improved cattle primarily kept by smallholder farmers (Njombe *et al.*, 2011; Lubote *et al.*, 2014). Smallholder dairy producers sell 70% of their milk whereas 30% consumed at home. The dairy industry has a tremendous deal of potential to improve people's living conditions and contribute to poverty reduction through increased nutrition resulting from milk intake and income generated from the sale of milk and milk products (Joseph, 2015). Tanzania's dairy industry accounts for 30% of the livestock GDP (Njombe *et al.*, 2011). Smallholder dairy producers produce around 90% of the milk consumed in Dar es Salaam, with 74% of all milk sold as raw milk through informal markets (Kivaria *et al.*, 2006a).

Raw milk and its products have been discovered as a major source of food-borne illness in people over the years. Milk that is intended for human consumption must be free of any contaminants to eliminate the danger of foodborne illness (Mensah *et al.*, 2018). Microbial contamination in milk has been linked to human milk-borne disorders, while others have been linked to milk deterioration. Primary microbial contamination in milk comes from an infected or sick lactating animal. Secondary sources of microbial contamination can occur

anywhere along the milk value chain, including contamination during milking by milkers, milk handlers, filthy milking equipment and water supplies utilized in sanitary activities, soils, feeds or air (Parekh and Subhash, 2008; Kanyeka, 2014; Gwandu *et al.*, 2018). Tertiary microbial contamination occurs when milk is re-contaminated after processing due to unsanitary circumstances and/or poor milk handling and storage during consumption (Parekh and Subhash, 2008; Bukuku, 2013; Kanyeka, 2014; Gwandu *et al.*, 2018; Mpatswenumugabo *et al.,* 2019). Generally, the level of microbial contamination in raw milk can be affected by cow health, equipment cleaning, milking practices and the environment including water and employees (Adzitey *et al.,* 2020).

Humans can become infected with milk-borne diseases by consuming raw or unpasteurized milk and milk products that have been contaminated (Bertu *et al.,* 2010). Studies in Tanzania have reported presence of milk-borne pathogens including *Salmonella*, *Brucella*, *Mycobacterium*, E. *coli* O157: H7, Staphylococcus *aureus*, *Listeria*, *Pseudomonas aeruginosa* and *Proteus* (Karimuribo *et al.,* 2005; Bukuku, 2013; Schoder *et al.,* 2013; Kanyeka, 2014; Lubote *et al.,* 2014). The existence of these harmful bacteria in milk has raised serious public health concerns, particularly among those who still consume raw milk (Kivaria *et al.,* 2006a; Lubote *et al.,* 2014). Salmonellosis, campylobacteriosis, tuberculosis, mastisis, listeriosis, Q-fever, brucellosis and yersinoses are diseases that can be transmitted to humans via milk (Shirima *et al.,* 2003; Kivaria *et al.,* 2006a; Hyera, 2015; Joseph, 2015). Among the most frequent bacterial foodborne infections, salmonellosis is a major public health concern around the world (Ketema *et al.,* 2018).

Salmonella causes approximately 93.8 million cases of gastroenteritis and 155 000 deaths in humans each year, with 80.3 million cases being linked to foodborne contamination

(Majowicz *et al.,* 2010). Salmonellosis in humans has been linked to contaminated food product such as dairy products as well as direct contact with sick animals (Ketema *et al.,* 2018). Animals become infected after eating contaminated feed, coming into contact with the feces of infected animals or direct nose-to-nose contact (Eines, 2009).

Overuse of antibiotics in veterinary treatment is thought to promote antimicrobial resistance in bacteria found in animal facilities (Addis *et al.,* 2011). In many parts of the world, resistance to routinely used antibiotics for the treatment of *Salmonella* infection in animals and humans has been studied and reported (Mengistu *et al.,* 2014; Muthumbi *et al.,* 2015; Manyi-Loh *et al.,* 2018). Antibiotics used as prophylaxis, treatments or growth promoters in animal farming have been related to the development and spread of antibiotic-resistant bacteria in animals, including zoonotic pathogens like *Salmonella typhimurium*, *Salmonella infantis* and *Salmonella enteritidis* (Hamada *et al.,* 2003; Van *et al.,* 2007; Andino and Hanning, 2015). Bacteria in tissues and products from these animals that have been subjected to frequent low doses of these antibiotics may be less sensitive to medications and when these bacteria enter the human body through contaminated food, they may cause diseases that are resistant to many drugs (Wang *et al.,* 2011).

In Dar es Salaam, information on *Salmonella* in milk, as well as the risk of contamination, the efficacy of hygienic measures and antimicrobial resistance is lacking. Thus this study aimed at establishing proportion, serotypes and antimicrobial resistance profile of *Salmonella* in Ilala district in Dar es Saaam, Tanzania.

1.2 Problem Statement and Justification of the Study

Raw milk, which is easily contaminated during milking and handling, is a significant vehicle for the transfer of milk-borne diseases to people (Kanyeka, 2014). There is evidence of incorrect milking and inadequate milk handling in the dairy sector, which puts milk at risk of microbial contamination. In addition, because tropical diseases are more common among livestock in the dairy sector, lactating and milking animals may have inborn infections in their blood. These may release hazardous bacteria into milk, posing a health risk to milk or milk product consumers (Hyera, 2015). Brucellosis caused by *Brucella* spp as well as tuberculosis caused by *Mycobacterium tuberculosis* and *Mycobacterium bovis* are the main health risks associated with milk (Kanyeka, 2014). Milk is still a major cause of these infections and other FBDs in several parts of the world especially developing countries like Tanzania (Shirima *et al.,* 2003).

There are few studies on the occurrence of *Salmonella* spp in Tanzania's milk industry. Antimicrobials are also commonly used in the dairy industry at various levels to combat various diseases. It is unclear the effect of these in selecting antimicrobial resistant *Salmonella* spp. Furthermore, due to the rising possibility of antimicrobial resistance, treatment for both humans and animals has become a difficulty, posing a hazard to human and animal health (Mwambete and Stephen, 2015; Britto *et al.,* 2018). However, due to the study scope and limitations of the analytical methodologies used, the majority of the existing studies have concentrated on culture and sensitivity, with little or no information on serovars of isolated *Salmonella* spp (Schoder *et al.,* 2013; Kanyeka, 2014).

Therefore, it was worthy to conduct a study to fill in these knowledge gaps. In this work, a serotyping technique was used to identify *Salmonella* at the species level in raw cow milk. Antibacterial susceptibility testing was also done on the *Salmonella* spp isolates to

determine their antimicrobial profile. The goal of this study was to provide information about the circulating *Salmonella* serovars in the Ilala district in Dar es Salaam, Tanzania, as well as the antibiotic resistance pattern. Furthermore, a greater understanding of the prevalence and types of *Salmonella*, as well as antibiotic resistance patterns in raw milk, would result in better recommendations for *Salmonella* spp. control and antimicrobial stewardship in the country.

1.3 Objectives of the Study

1.3.1 General objective

To determine proportion, serotypes and antimicrobial resistance profiles of *Salmonella* spp isolated from raw cow's milk in Ilala district in Dar es Salaam.

1.3.2 Specific objectives

i. To determine proportion of *Salmonella* spp contaminating raw cow milk in Ilala district in Dar es Salaam.
ii. To determine the serotypes of the isolated *Salmonella* spp.
iii. To determine antimicrobial susceptibility of *Salmonella* spp isolated from raw cow milk in the study area.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Composition of Milk

Milk comprises 87.2% of water, 12.8% total solid, 4.5% lactose, 4% fat, 3.4% protein, and 0.7% ash/minerals (Pandey and Voskuil, 2011). It also contains gases, enzymes and vitamins (Hyera, 2015). Other milk component includes immunoglobulins which protect newborns from a variety of illnesses (Pandey and Voskuil, 2011; Bukuku, 2013; Kanyeka, 2014). Milk's composition isn't constant and there's a lot of variance. It varies across species and between breeds and subspecies within a species, within a breed between individual animals, feeds, lactation stage, season, health and physiological status of a given animal. It is even possible that the composition may vary from day to day, depending on nutrition and weather, however, the first milk drop differs from the last milk drop during milking (Pandey and Voskuil, 2011; Bukuku, 2013).

2.2 Source of Microbial Contamination in Milk

Microbial contamination in milk comes from sick cow, infected udder and/or teats, improper milking practices, animal skin, unsanitary milking and storage equipment, soil, food handlers, feed, faeces and grasses (Parekh and Subhash, 2008; Swai and Schoonman, 2011; Lubote *et al.,* 2014; Hyera, 2015; Ndahetuye *et al.,* 2020). Other bacterial sources include milkers, handlers, medications or chemicals used during animal treatment, air and water used for adulteration which could be polluted and cause extra health issues

(Karimuribo *et al.,* 2005; Swai and Schoonman, 2011; Kanyeka, 2014; Hyera, 2015). When milk is exposed to these sources, it may become more contaminated and lowering its quality.

2.3 Prevention and Control of Microbial Contamination of Milk

Microbial quality of milk can be prevented and controlled by removing organisms from human carriers through public education, improvements in water supplies and personal and environmental cleanliness. In addition adequate pasteurization or boiling of milk before processing and consumption can be accomplished (Kanyeka, 2014). To prevent contamination of milk by air, outdoor milking should be done in dusty yards. Prevention can also be achieved by thoroughly cleaning and sanitizing milking utensils and/or equipment, controlling flies and insects to prevent the introduction of microorganisms into milk, and removing dung on a regular basis (Mosalagae *et al.,* 2011).

Microorganisms from lactating animals can be controlled by maintaining excellent animal practices and improving animal husbandry while microorganisms from equipment and environment can be avoided by following general hygiene procedures and maintaining environmental sanitation (Kanyeka, 2014). To avoid being a source of infectious diseases, it is also critical for all individuals involved in production of milk to be in healthy condition (Hyera, 2015).

2.4 *Salmonella* spp

Salmonella is a genus of Enterobacteriaceae rod bacteria that are aerobic and facultative anaerobic, catalase positive, oxidase negative, and gram negative (Umeh and Enwuru, 2014). On Salmonella-Shigella agar, *Salmonella* produce colorless colonies with black centers (Eines, 2009). *Salmonella* thrives best at 35-37ºC, water activity of 0.84-0.94 and pH of 6.5- 7.5 (Adzitey *et al.,* 2020). The genus contains two species which

are *Salmonella enterica* and *Salmonella bongori*. Based on biochemical characteristics and genomic relatedness, *S. enterica* is divided into six subspecies (I, II, IIIa, IIIb, IV and VI) (Reeves *et al.*,1989; Eng *et al.*, 2015).

Salmonella enterica subsp. *enterica* is responsible for nearly all Salmonella infections in warm-blooded animals, such as animals and humans. Other *S. enterica* subspecies and *S. bongori* are more widespread in cold-blooded animals and the environment, with reduced human and livestock pathogenicity (Brenner *et al.*, 2000; Eng *et al.*, 2015; Wibisono *et al.*, 2020). According to the Kauffmann–White scheme, there are presently about 2700 *Salmonella* serovars, which are serologically characterized by antigenic variation in the Lipopolysaccharide (O), Flagella (H) and Capsular (Vi) antigens (Ketema *et al.*, 2018). Only the serovars *typhi*, *paratyphi* C and *dublin* express the Vi antigen, which is linked to virulence (Grimont *et al.*, 2000).

Typhoidal Salmonella and non-typhoidal Salmonella (NTS) are the two types of *Salmonella* serovars that cause sickness in humans (Ngogo *et al.*, 2020). Non-typhoidal serovars include host generalist serovars like *S. enteritidis* and *S. typhimurium* (Varma *et al.*, 2005) that cause acute gastroenteritis without requiring antibiotic therapy (Nyabundi *et al.*, 2017). Antimicrobial medicines, on the other hand, are frequently prescribed for patients with salmonellosis, especially those who are at high risk of extraintestinal infection, such as the very old, those with immune suppression and the very young (Nyabundi *et al.*, 2017). Typhoidal serotypes such as *S. typhi* and *S. paratyphi*, may only be transmitted from person to person, causing food-borne illness, typhoid fever and paratyphoid fever that can be fatal (Ryan and Ray, 2004). *Salmonella* can be transferred to people by the ingestion of contaminated food and its products, as well as direct contact with animals and their surroundings. Animals become infected by eating contaminated

feed, direct contact with the feces of infected animals or direct nose-to-nose contact (Eines, 2009).

2.5 Prevalence of *Salmonella* in Raw Cow Milk

Salmonella is one of the most common causes of foodborne illness in both developing and developed countries (Adzitey *et al.,* 2020). *Salmonella* has been found in raw cow milk in a number of researches conducted in Tanzania and other parts of the world. For instance Addis *et al.* (2011) in Addis Ababa reported prevalence of 10.7% from lactating cows, no *Salmonella* serovars were reported. Rwanda there were a prevalence of 16.4% from raw milk, no *Salmonella* serovars were reported (Mpatswenumugabo *et al.,* 2019). In India was 7.61% from milk and milk product, no *Salmonella* serovars were reported (Singh *et al.,* 2018).

In South Punjab-Pakistan the prevalence was 25.89% from milk and environment samples whereby *S. typhi*, *S. paratyphi A*, *S. paratyphi B* and *S. typhimurium* were identified (Qamar *et al.,* 2020) and Pakistan 28% from raw milk whereby *S. typhi* was identified (Jalbani *et al.,* 2019). Eastern Ethiopia had a prevalence of 3.3% from raw milk, no *Salmonella* serovars were reported (Reta *et al.,* 2016). In Bangladesh the prevalence was 25.71% from milk and milk based products with no *Salmonella* serovars reported (Yasmin *et al.,* 2015). In Egypt 22% from milk and dairy products whereby *S. enteritidis*, *S. typhimurium* and *S. infantis* serovars were reported (Omar *et al.,* 2018). In Nigeria was 4% from raw and fermented milk with no *Salmonella* serovars reported (Tamba *et al.,* 2016).

In Tanzania, Kanyeka (2014) reported prevalence of 2.04% in Kilosa and Mvomero districts from raw cow milk with no *Salmonella* serovars reported. Also a study conducted by Lubote *et al.* (2014) in Arusha on milk value chain showed *Salmonella* prevalence of 37.33% whereby *S. arizonae* was identified. A study by Schoder *et al.* (2013) showed a

prevalence of 10.1% in Dar es Salaam and Lake Victoria whereby *Salmonella* serovars were not identified. Isolated *Salmonella* spp. prevalence varies depending on type of sample taken, sample size and analytical procedures used.

2.6 Antimicrobials Commonly used in Dairy Cattle

Antimicrobial agents used at the farm level for the treatment or prevention of cattle illnesses fall into several groups whereby tetracyclines, aminoglycosides, sulphonamides, beta-lactams, macrolides and chloramphenicol are the most commonly used groups (Omore *et al.,* 2002; Movassagh and Karami, 2010; Bukuku, 2013). When treating dairy cattle, these antibiotics can be used separately or in combination. According to researches done by (Kivaria *et al.,* 2006b; Katakweba *et al.,* 2012) these antibiotics are widely utilized to treat a variety of cattle illnesses.

2.7 Antimicrobial Resistance

AMR occurs when a bacterium acquires the ability to survive exposure to antimicrobials that are intended to kill or stop it from growing, and it has been a global health issue that has put human and animal health at risk (Balamurugan *et al.,* 2018). AMR develops naturally as a result of acquisition of foreign resistance genes or bacterial gene mutations via horizontal gene transfer between bacteria (ECDC, 2015). By using mobile genetic elements such as naked DNA, plasmids, transposons or bacteriophages resistance genes can be transferred between bacteria from various ecological and taxonomic groups Although several genes with a single drug resistance feature might accumulate in the same organism, these genes are usually directed against a particular family or kind of antibiotic (Levy and Marshall 2004). The use of antimicrobial agents and the transmission of antimicrobial resistant microbes between animals; humans and humans, animals and the environment are the key causes behind the incidence and spread of AMR (ECDC, 2015). In developing countries, health services for both humans and animals have been

suboptimal, with an increased tendency for animal owners to stock drugs and treat their animals with unskilled people such as farmers and animal attendants, as well as a human tendency to take medicine based on previous disease history rather than relying on medical diagnosis (Karimuribo *et al.,* 2005; Katakweba *et al.,* 2012). People in Tanzania have free access to antimicrobials from agro-veterinary shops without prescriptions, as is the case in every other African country (Tagoe and Attah, 2010; Katakweba *et al.,* 2012). Antimicrobials are used in animal production, which produces selection pressure that favors antibiotic-resistant bacteria' survival. Antimicrobial resistance in Salmonella strains has become widespread, posing a severe public health threat (Chiu *et al.,* 2002).

Recent studies have reported strains of *Salmonella* resistant to antimicrobials such as ampicilin, amoxicillin/clavulanic acid, penicillin, chloramphenicol, ciprofloxacin, tetracycline and gentamycin (Kanyeka, 2014; Yasmin *et al.,* 2015; Beyene *et al.,* 2016; Tamba *et al.,* 2016; Jalbani *et al.,* 2019). The Study conducted in Addis Ababa (Addis *et al.,* 2011) showed that 83% of *Salmonella* isolates were resistant to more than two antimicrobial agents. In Tanzania (Kanyeka, 2014) reported resistance rate of 100% to isolated *Salmonella* spp against ampicillin and amoxicillin. AMR has a number of consequences, including a loss of effectiveness and therapeutic efficacy, as well as the treatment of infectious illnesses becoming less successful resulting in productivity losses, lower livelihoods and food security and higher mortality (FAO, 2016).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was conducted in Ilala District in Dar es Salaam, Tanzania. Ilala District is categorized into three administrative divisions: Ukonga, Kariakoo and Ilala. The divisions of the district are subdivided into wards, which are further subdivided into mitaa. There are 36 wards and 159 localities in the Ilala District. It has a population of 1 220 611 people and a 365 km^2 area with 300 674 households (PHCT, 2012). It is situated between longitude 39°17' East and latitude 6º48' South at the centre of Dar es Salaam. It is bordered by Indian Ocean on the East, on the South by Temeke and Kigamboni Municipality, on the West by Kisarawe district and on the North by Kinondoni and Ubungo Municipality. The district is characterized by hot and humid climate with extended rain seasons in April and May and short rains in November to December with an annual rainfall of approximately 1100 mm.

3.2 Study Design

A cross sectional study was conducted from July 2020 to October 2020. Multistage random sampling technique was used (Jain and Hausman, 2006). First stage involved selection of four wards within Ilala District council; Kitunda, Kivule, Magole and Ukonga. Second stage involved the selection of streets whereby in each ward streets were purposively selected based on the accessibility and availability of smallholder dairy farmers. 138 households with lactating dairy cow were randomly selected from the

purposively selected wards based on inclusion and exclusion criteria using a simple random selection procedure. The inclusion criteria were; Smallholder dairy farmers who had one to five dairy cows, willingness to engage and provide essential information and availability of milk during the study period. The exclusion criteria included; Unwillingness to engage and inability to provide essential information, and the absence of raw cow milk during the study. Those who did not have time for interviews were also removed. List of all dairy farms within the wards were used as a sampling frame. Raw cow milk was obtained and questionnaires were administered simultaneously to smallholder dairy farmers with lactating cows as study units.

3.3 Study Animals

The study animals were cross breed lactating dairy cows from smallholder dairy farmers in the four wards. Farmers practice zero grazing, which entails completely confining and feeding dairy animals indoors. In other cases, dairy cattle were managed using a semi-intensive management system, in which they were grazed on natural pasture and then supplemented with cut grasses and concentrates when they returned home.

3.4 Sample Size Determination

Sample size was estimated using a formula by (Kothari, 2004).

$$n = (z^2pq)/e^2$$

Whereby n= required sample size, Z= estimated standard variation for a given confidence interval, p = expected prevalence, q = (1 - p) and e =acceptable error (the precision).

The confidence level was assumed 95% with an acceptable error of 5% and Z was 1.96. Prevalence of 10% from a previous study by (Schoder *et al.*, 2013) on microbiological quality of milk was used in the calculation, which resulted into n = 138 as sample size.

3.5 Data Collection

3.5.1 Questionnaire survey

To collect information from 138 smallholder dairy farmers with lactating cows, a structured questionnaire was presented via face-to-face interview. The questionnaire was used to collect data on demographic characteristics, possible risk factors for microbial contamination in milk including hygiene of milking cows' udders and milk handlers, utensils used for milking, type of milk storage containers, milk storage conditions, frequency of cleaning of the storage containers, water source, milk consumption behaviors and awareness of the risk of diseases associated with consumption of raw milk. Furthermore, animal treatment, antibiotic residues and compliance to drug withdrawal period were also assessed (Appendix 1). Direct observations on overall cleanliness and hygienic circumstances as well as practices related to milk were made and recorded while administering questionnaires. After the questionnaires were completed, milk samples were collected for laboratory analysis.

3.5.2 Sampling and handling of milk

Milk samples were taken from the storage containers used by farmers in the visited households early in the morning, between 6:00 and 8:00 a.m. To avoid contaminated water leaking into the teat cups, a clean washing cloth was used to properly wash the udders with clean water and then dried with paper towels before milking. A sterile syringe was used to aseptically collect approximately 10ml of pooled raw cow milk from the milk container into sterile labeled universal bottles. To prevent microbial proliferation, the obtained samples were put in a cool box with an ice pack (4°C). Following that, the samples were taken to the Department of Veterinary Medicine and Public Health's laboratory for further analysis.

3.6 Inoculation and Cultivation

1 ml of milk sample was added to 5 ml of Selenite F broth and incubated at 37°C for 24 hours. A loopfull of culture was sub-cultured into SSA plates from incubated Selenite F broth and incubated at 37°C for 24 hours and then examined for characteristic *Salmonella* colonies. For Enterobacteriaceae differentiation, colonies from SSA were streaked onto MCA plates. The plates were incubated at 37°C for 24 hours. Following that, MCA plates were examined for lactose fermentation and results were recorded (Wallace *et al.,* 2020).

3.6.1 Isolation of *Salmonella* spp

Salmonella spp. were isolated from milk samples using conventional and standard microbiological methods (Wallace *et al.,* 2020). All the media used in this study were prepared aseptically following manufacturer’s instructions. The media used in this study included; Selenite F Broth (HiMedia, Lot 0000364831-India), Salmonella-Shigella Agar (HiMedia, Lot 0000318146-India), MacConkey Agar (HiMedia, Lot 0000246514-India),

Nutrient agar (Liofilchem, Lot 120116502-Italy) and Mueller-Hinton Agar (Oxoid® Ltd., Lot 744451-England). The sterility of the non-inoculated medium plates was checked by incubating them at 37°C for 24 hours. Until culture time, all ready-to-use media were kept at 4°C.

3.7 Identification of Suspected *Salmonella* Colonies

Colonial morphology, cultural characteristics, Gram staining reaction and biochemical assays such as methyl red (MR), indole test, simmons citrate agar test, triple sugar iron agar (TSI) test and catalase test were used to identify suspected *Salmonella* colonies from the inoculated media. To identify species, a serotyping test was performed (Macfaddin, 2000; Wallace *et al.*, 2020).

3.7.1 Morphological identification

Salmonella isolates were morphologically identified using a variety of culture medium including MCA as a differential media and SSA as a selective media. Color, size and the presence of black-centred colonies on SSA, which indicates the presence of hydrogen sulphide (H_2S), as well as the presence of colorless colonies on MCA, were used to identify suspected colonies. In each media, colony characteristics such as size and color were recorded (Allen, 2005; Jalbani *et al.*, 2019).

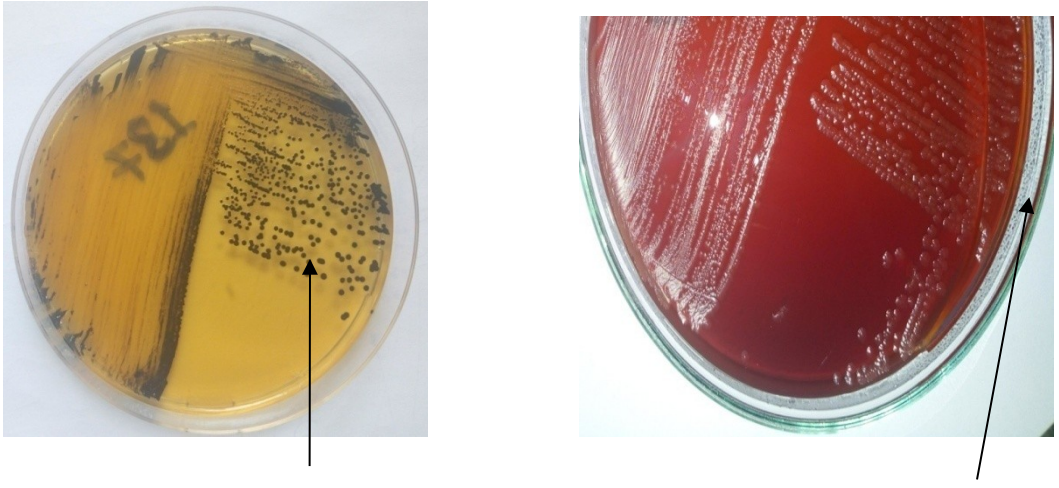

Figure 1: *Salmonella* colonies on

Figure 2: *Salmonella* colonies on MacConkey Agar

3.7.2 Microscopic identification

The suspected colonies were smeared on microscopic glass slides with a sterile wire loop and normal saline, then fixed and stained using the standard gram staining procedure and viewed under a light microscope with a 100X objective lens with immersion oil (Cheesbrough, 2006; Kanyeka, 2014). Biochemical tests were used to identify all Gram-negative isolates that appeared rod-shaped and red in color.

3.7.3 Biochemical identification

Presumptive colonies were inoculated on TSI, tryptophan broth (indole test), Simmons citrate agar, and methyl red broth and then incubated for 24 to 48 hours at 37°C for biochemical identification of *Salmonella*. Colonies that produced alkaline slant, acid butt, with/without gas production and/or blackening of the medium on TSI, blue purple color on Simmons citrate agar, pink to cherry red color for indole test, red coloration for MR test were considered to be *Salmonella.* The ability to create catalase was examined in all suspected *Salmonella* spp. and those that produced gas bubbles were considered to be *Salmonella* (Macfaddin, 2000).

3.7.4 Serotyping of isolates

Commercial somatic O antisera and flagellar H antisera were used to serotype identified *Salmonella* isolates, with commercial *Salmonella* spp to the antiserum serving as a positive control and an organism in saline only serving as a negative control. A portion of a *Salmonella* colony grown on sheep blood agar was picked using a sterilized wire loop. A drop of physiological saline was used to emulsify the colony on a slide and it was

completely mixed. After adding a small drop of polyvalent O (poly A-S) antisera, the slide was tilted back and forth to look for agglutination. Within 1 minute of a positive reaction, noticeable agglutination (clumping) emerges. Following agglutination by the polyvalent group O, the isolates were tested against monovalent O (O:4,5 (B); O:1,2 (A); O:6,7,8 (C1-C4); O:7 (C1, C4); O:8 (C1, C3); O:9 (D1)) using the same techniques. The isolate is positive for that group if it agglutinates.

A slide agglutination method was also used with flagellar H antisera. Phase I involved applying adding a small drop of polyvalent H (HMA-HG) antisera to an emulsified colony on a slide and tilting the slide back and forth to check for agglutination. Within 1 minute of a positive reaction, noticeable agglutination (clumping) emerges. The isolates were tested against monovalent H (2; 5; 6; 7; a; b: c; d; g,m; g,p; h; I k; r) after the polyvalent group H was positive for agglutination. Then, using Sven Gard medium, a culture was obtained near the edge of the invasion zone of the Sven Gard agar and analyzed with polyvalent H antisera; if there was no agglutination, the serotype contained just one phase. If the isolate agglutinated, the agglutination phase was repeated by testing it in each monovalent H. Antigenic formulae based on the White–Kauffmann–Le Minor scheme were used to define the serovars of isolates. The outcomes were observed and documented. These procedures were carried out in accordance with the Standard Operating Procedure for Isolation and Identification of *Salmonella* spp. provided by Muhimbili University of Health and Allied Sciences (MUHAS) (Appendix 2).

3.8 Antimicrobial Susceptibility testing of *Salmonella* Isolates

Antimicrobial susceptibility test was performed using disc diffusion method on Mueller-Hinton Agar plates according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2011). Antibiotic selection criteria were based on how frequently antimicrobials

were used in animal and human therapy. Eight antibiotics from different classes were used including, tetracycline (30µg), gentamycin (10µg), ciprofloxacin (5µg), penicilin (10µg), chloramphenicol (30µg), ampicillin (10µg), trimethoprim-sulfamethoxazole (25µg) and amoxicillin/clavulanic acid (30µg).

Salmonella isolates stored in 15% glycerol were sub cultured on Nutrient Agar and incubated at 37°C for 24 hours. Then, using a sterile wire loop, pure colonies from distinct colonies on NA were picked up and emulsified in 200µl sterile normal saline solution to make bacterial suspension. The turbidity of the bacterial suspension was then adjusted to match that of a 0.5 McFarland tube, which equals 10^8cfu/ml (CLSI, 2011). A standard reference strain of *E. coli* (ATCC 25922) was also prepared and utilized as a quality control for the antimicrobial susceptibility test (Hendriksen, 2002; Addis *et al.*, 2011).

Using sterile cotton swabs, the suspensions of each isolate and the positive control (*E. coli* ATCC 25922) were dispersed across the whole surface of the Mueller-Hinton agar plate. Using sterile forceps, antimicrobial discs were then placed on the surface of the inoculation plates. The plates were then incubated for 24 hours at 37°C. Finally, using a ruler, the zones of inhibition were measured in millimeters and the diameter of the clear zone was used to determine if the antibiotic disc inhibited the growth of bacteria in the media (CLSI, 2011). The chart (Appendix 4) was used as a quality control (QC) for test procedures to evaluate results, where zones of inhibition of *Salmonella* spp were interpreted by comparing with those provided in the chart and recorded as Sensitive (S/≥mm), Intermediate (I/≤mm) and Resistant (R/<).

3.9 Data Management and Analysis

All data from questionnaires and laboratory analysis were recorded and kept in Microsoft Office Excel 2007 spreadsheets, which were subsequently analyzed with a statistical software for social sciences (SPSS version 20). Survey data was described using descriptive statistics such as frequencies and percentages, which were presented in tables. Continuous and proportional categorical variables were generated and Chi square analysis was used to examine relationships between *Salmonella* in milk and possible risk factors. The results were reported as significant for $p < 0.05$.

3.10 Ethical Consideration

The permission to carry out this study was granted by Regional Administration Secretary, District Administrative Secretary with Ref. No: AB.60/87/01 and Municipal Director with Ref. No: IMC/QR.3/VOL.1/88 (Appendix 7, 8 and 9) while ethical approval for the study was given by the Ethical Committees of Sokoine University of Agriculture, Tanzania with Ref. No: SUA/DPRTC/R/5. Moreover, verbal consent was obtained from each household representative after being informed of the study's purpose and importance prior to commencement of interviews and sampling and participation was entirely voluntary. All of the information gathered from the participants as well as the laboratory results obtained following milk sample analysis was kept as confidential. The study participants were also anonymized.

CHAPTER FOUR

4.0 RESULTS

This chapter comprises results from sociological survey employing questionnaires and laboratory analysis which are based on study's objectives. Tables and graphs are used to summarize the data. Sociological survey presents the findings for demographic characteristics of the respondent, animal housing, animal health, health risks related with consumption of raw milk, use of sick and treated animals' milk and factors influencing microbial contamination of milk at farm level. Laboratory based part presents the finding of isolation and identification of *Salmonella* spp, prevalence of *Salmonella*, serotypes of the *Salmonella* isolates and antimicrobial profile test.

4.1 Sociology

4.1.1 Demographic characteristics

According to the findings, 82.6% of the total household respondents in the research were males and 17.4% were females. 58.5% of the respondents were above the age of 40 while 41.5% were under the age of 40. In terms of educational attainment, the majority (56.5%) had barely completed primary school. Characteristics of household respondents are presented in Table 1.

Table 1: Respondents' demographic characteristics

Demographic information	Category	(N=138) n	Percentage %
Sex	Males	114	82.6
	Females	24	17.4
Age	15-20 years	3	2.2
	21-30 years	22	15.9
	31-40 years	32	23.2
	41-50 years	64	46.2
	>50 years	17	12.3
Education	Primary	78	56.5
	Secondary	39	28.3
	University	21	15.2
Position in the household	Head	96	69.6
	Spouse	17	12.3
	Daughter	4	2.9

	Son	8	5.8
	Employee	13	9.4

4.1.2 Animal housing

The results revealed that 90.6% of cow houses were made of trees/bomas, 3.6% of blocks and 5.8% had no house at all. Floor materials were; 81.2% of mud or earthen while 18.2% were of concrete. Animal houses were found to be filthy, full of cow manure or dust posing a risk of microbial contamination in the milk.

4.1.3 Animal health

The findings revealed that veterinarians are mostly responsible for animal therapy and medicine (79%). Common diseases that affect animals, such as respiratory disease, foot and mouth disease (FMD), mastitis and helminthiosis have been described as driving reasons for veterinary medicine use. Animals were treated with a variety of veterinary medications in both wards including tetracycline, penicillin, albendazole, tylosin, gentamycin, streptomycin and sulphonamide to mention a few.

4.1.4 Health risks related with consumption of raw milk

According to the findings, 100% of respondents were aware that consumption of raw un-boiled milk could have negative health consequences. According to the respondents, tuberculosis (97.1%), brucellosis (25.4%) and typhoid (5.2%) are among the milk-borne diseases transmitted through consumption of raw milk. Milk-borne infections related with raw milk consumption can be avoided by boiling milk, according to all smallholder dairy farmers (100%).

4.1.5 Use of sick and treated animals' milk

The findings revealed that udder disease (mastitis) was one of the most common diseases impacting their herds, according to all respondents. 30.4% of smallholder dairy farmers reported to milk sick animals while 69.6% do not milk sick animals. Respondents stated that milk from animals with udder problems was utilized to feed calves (71.4%). 7.1% of respondents said they consume and sell the milk while 14.3% discard the milk. Milk from animals on medicine, on the other hand, was mostly discarded (46.7%). 29.2% of respondents reported to give milk to pet, 16.1% sell the milk while 8% consume the milk.

According to the findings, 100% of smallholder dairy farmers were aware of the likelihood of drug residues in milk following animal medication or treatment, and 76.1% reported to comply with withdrawal periods. However, 23.9% of the respondents reported not complying with withdrawal period by selling milk right after the final dose. 57.2% of the respondents were unaware of potential health effects to consumers from veterinary medication residues in milk. The results are summarized in Table 2.

Table 2: Use of milk from sick animals, under medication and habit of milking sick animals

Parameter assessed	Category	(N=138) n	Percentage %
Milking sick animal	Yes	42	30.4
	No	96	69.6
Milk from sick animals	Feed calves	30	71.4
	Consume	3	7.1
	Discard	6	14.3
	Sale	3	7.1
Milk from treated animals	Discard	64	46.7
	Give pets	40	29.2
	Sale	22	16.1
	Consume	11	8
Use of antibiotics and adhering to	Adhering	105	76.1
	Not adhering	33	23.9

withdrawal period			
Health effects due to consumption of milk containing drugs	Yes	59	42.8
	No	79	57.2

4.1.6 Factors influencing microbial contamination of milk at farm level

Results indicated that 34.8% of farmers do not clean their hands before milking (Table 3), in this study hand washing before milking was found to significantly influence microbial contamination in milk ($\chi2$= 15.923, df=1, p=0.000) (Table 4). 57.1% reported not to wash hands between milking different cows. In this study, hand washing between milking different cows was found to significantly predispose milk to microbial contamination ($\chi2$= 11.714, df=1, p=0.001) (Table 4). Those who reported to wash hands were using water only for washing hands and the cleaning agent for hand washing was found not predispose milk to microbial contamination ($\chi2$= 3.345, df=1, p=0.067). 30.4% of smallholder dairy farmers reported to milk sick animals (Table 3), a practice that led to microbial contamination in milk ($\chi2$= 4.124, df=1, p=0.042) (Table 4). In this study the main source of water for animals and sanitary activities including washing hands, udder and equipment was wells (89.1%) and was always used during milking in non-portable form (Table 3). Source of water did not influences microbial contamination in milk ($\chi2$= 1.036, df=1, p=0.309) (Table 4).

The most common type of containers used during milking and storage were plastic containers. The storage containers were cleaned on daily basis using cold water and soap (71%) (Table 3).

Table 3: Factors influencing microbial contamination of milk at farm level

Parameter assessed	Category	(N=138) n	Percentage %
Source of water	Wells	123	89.1
	Tap	15	10.9
Type of storage container	Plastics	138	100
Cleanliness of storage containers	Cold water with soap	98	71
	Hot water with soap	40	29
Milk storage	At room temperature	122	88.4
	Refrigerator	16	11.6
Covering of milk during storage	Covered	104	75.4
	Not covered	34	24.6
Washing hands before milking	Yes	90	65.2
	No	48	34.8
Hand washing between milking different cows	Yes	80	58
	No	58	42

Cleaning agent of milking and storage containers did not pose a risk for microbial contamination in milk ($\chi 2= 3.466$, df=1, p=0.063) (Table 4). There were no cold storage facilities as milk was being stored under room temperature (88.4%) while few respondents reported to store milk in refrigerator (11.6%) before selling or other home uses (Table 4). Cold storage facilities did not influence microbial contamination in milk ($\chi 2= 1.114$, df=1, p=0.291) (Table 4). 23.9% of respondents reported not to cover milk after milking (Table 3), not covering milk did not influence microbial contamination of milk ($\chi 2= 0.661$, df=1, p=0.416) (Table 4). In general, there was an association between bacterial contamination of milk with hand washing before milking, hand washing between milking different cows and milking of sick cows.

Table 4: Chi Square analysis for factors influencing microbial contamination of milk

Selected factors	$\chi 2$	P-value	df
Water source	1.036	0.309	1
Milking sick cow	4.124	0.042	1
Hand washing before milking	15.923	0.000	1
Hand washing between milking different cows	11.714	0.001	1
Cleaning agent for hand wash	3.345	0.067	1
Cleaning agent for milking and storage utensils	3.466	0.063	1
Milk handling at household	0.661	0.416	1
Milk storage	1.114	0.291	1

*** $\chi 2$= chi square, df=degree of freedom***

4.2 Isolation and Identification of *Salmonella* spp

Results revealed that 8 isolates of *Salmonella* were recovered from 138 milk samples collected from Kitunda, Kivule, Magole and Ukonga. Primary identification of *Salmonella* was based on cultural and morphological growth characteristics, as well as biochemical assays, as shown in Tables 5 and 6. Gram stain smears from suspected colonies revealed Gram negative rods in scattered arrangement.

Table 5: Results of cultural and morphological growth characteristics of Salmonella spp

Culture media	SSA	MCA	TSI
Colony characteristics	Colourless, transparent with black centre, medium size colonies	Colourless, transparent, smooth, medium size colonies	Alkaline slant/acid butt with hydrogen sulfide production and gas

				formation

Table 6: Results of biochemical characteristics of Salmonella isolates

Sample ID	Indole production	Methyl red	Citrate utilization	Catalase
71	-	+	+	+
94	-	+	+	+
101	-	+	+	+
103	-	+	-	+
110	-	+	+	+
111	-	+	+	+
123	-	+	-	+
137	-	+	+	+

*+ = **Positive reaction, - = Negative reaction***

4.3 Prevalence of *Salmonella* Isolates in Various Wards

The findings revealed that the prevalence of *Salmonella* is higher in Kivule (14.6%) than in Magole (3.8%), with no significant difference between them (Table 7). All 36 and 9 milk samples from Kitunda and Ukonga, respectively, were found to be free of *Salmonella*.

Table 7: Prevalence of salmonella isolates among selected wards within Ilala district

Ward	No. of samples examined	No. of positive samples	Percentage (%) of isolation	Alpha	P-value	$\chi 2$
Magole	52	2/52	3.8	0.05	0.065	3.3935
Kivule	41	6/41	14.6			
Kitunda	36	0	0			
Ukonga	9	0	0			
Total	138	8/138	5.8			

4.4 Serotyping of the Isolated *Salmonella* spp

A total of 8 isolates were serotyped and identified as *S. typhimurium* (3/8; 2.2%), *S. enteritidis* (3/8; 2.2%) and *S. typhi* (2/8; 1.4%) (Table 8, Figure 3 and Appendix 3).

Table 8: Serotyping test results of the isolated Salmonella spp

ID	Poly "O" antigen	Mono "O" Antigens		Phase I Poly "H" Antigens		Phase I mono "H" Antigens			Phase II poly "H" Antigen	Phase II mono "H" Antigens		
	Poly A	O:4(B)	O:9(D1)	HMA	HG	i	g,m	d	H1	2	7	Serovar
71	+	-	+	-	+	-	+	-	+	-	+	*S. enteritidis*
94	+	+	-	+	-	+	-	-	+	+	-	*S. typhimurium*
101	+	-	+	-	+	-	+	-	+	-	+	*S. enteritidis*
103	+	-	+	-	+	-	+	-	+	-	+	*S. enteritidis*
110	+	-	+	+	-	-	-	+	+	+	-	*S. typhi*
111	+	+	-	+	-	+	-	-	+	+	-	*S. typhimurium*
123	+	-	+	+	-	-	-	+	+	+	-	*S. typhi*
137	+	+	-	+	-	+	-	-	+	+	-	*S. typhimurium*

+ = Positive reaction, - = Negative reaction

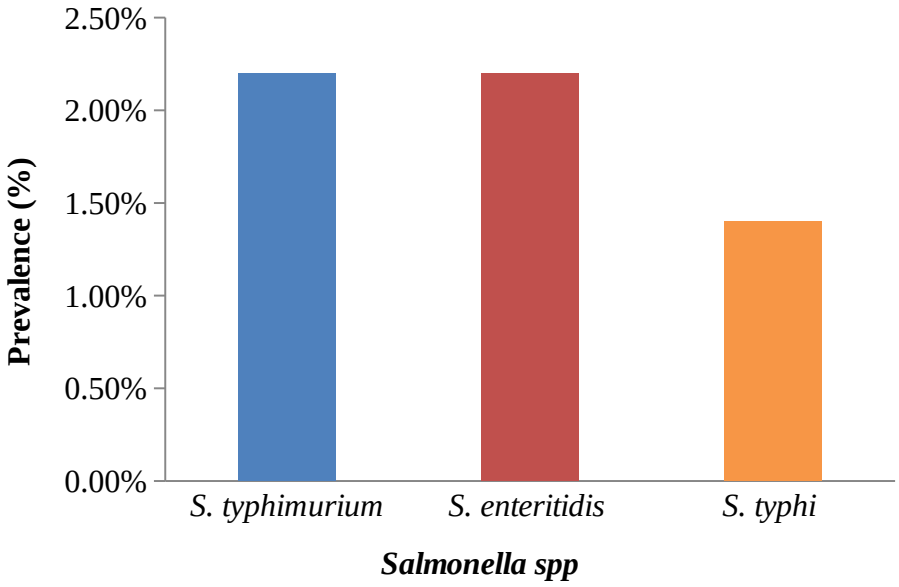

Figure 3: Prevalence rate of the detected serotypes of isolated *Salmonella* spp

4.5 Antimicrobial Profile test of isolated *Salmonella* spp

Salmonella isolates were shown to be completely susceptible to gentamycin, tetracycline, chloramphenicol, ciprofloxacin and trimethoprim-sulfamethoxazole but completely resistant to ampicillin, amoxicillin/clavulanic acid and penicilin (Table 9 and Figure 4). All 8 (100%) isolates had displayed multidrug resistance (MDR) against 3 antibiotics (ampicillin, amoxicillin/clavulanic acid, penicilin) (Table 10).

Table 9: Antimicrobial susceptibility results from the isolated Salmonella spp

Antimicrobials	Sensitivity profiles	*Salmonella* spp *S. enteritidis* n=3 Frequency (%)	*S. typhimurium* n=3 Frequency (%)	*S. typhi* n=2 Frequency (%)	Overall n=8 Frequency (%)
Penicilin	R	3 (100)	3 (100)	2 (100)	8 (100)
	I	0 (0)	0 (0)	0 (0)	0 (0)
	S	0 (0)	0 (0)	0 (0)	0 (0)
Tetracycline	R	0 (0)	0 (0)	0 (0)	0 (0)
	I	0 (0)	0 (0)	0 (0)	0 (0)
	S	3 (100)	3 (100)	2(100)	8 (100)
Gentamycin	R	0 (0)	0 (0)	0 (0)	0 (0)
	I	0 (0)	0 (0)	0 (0)	0 (0)
	S	3 (100)	3 (100)	2 (100)	8 (100)
Ciprofloxacin	R	0 (0)	0 (0)	0 (0)	0 (0)
	I	0 (0)	0 (0)	0 (0)	0 (0)
	S	3 (100)	3 (100)	2 (100)	8 (100)
Trimethoprim-Sulfamethoxazole	R	0 (0)	0 (0)	0 (0)	0 (0)
	I	0 (0)	0 (0)	0 (0)	0 (0)
	S	3 (100)	3 (100)	2 (100)	8 (100)
Amoxicillin/ Clavulanic acid	R	3 (100)	3 (100)	2(100)	8 (100)
	I	0 (0)	0 (0)	0 (0)	0 (0)
	S	0 (0)	0 (0)	0 (0)	0 (0)
Ampicillin	R	3 (100)	3 (100)	2 (100)	8 (100)
	I	0 (0)	0 (0)	0 (0)	0 (0)
	S	0 (0)	0 (0)	0 (0)	0 (0)
Chloramphenicol	R	0 (0)	0 (0)	0 (0)	0 (0)
	I	0 (0)	0 (0)	0 (0)	0 (0)
	S	3 (100)	3 (100)	3 (100)	3 (100)

Table 10: Multiple drug resistance (MDR) patterns of the isolated *Salmonella* spp

Antimicrobial	*S. typhimurium*	*S. enteritidis*	*S. typhi*	Overall MDR profie
Penicillin	3/3 (100%)	3/3 (100%)	2/2 (100%)	8/8 (100%)
Amox/Clavulanic acid	3/3 (100%)	3/3 (100%)	2/2 (100%)	8/8 (100%)
Ampicillin	3/3 (100%)	3/3 (100%)	2/2 (100%)	8/8 (100%)

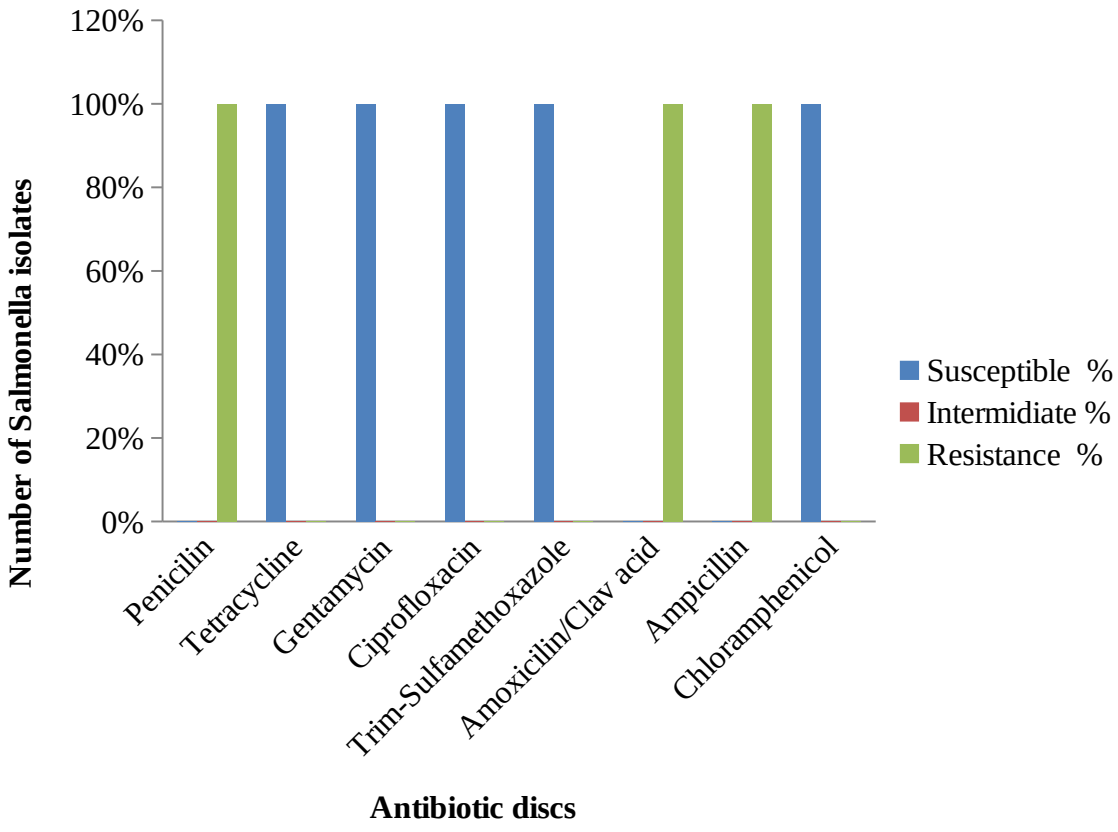

Figure 4: Overall Antimicrobial susceptibility profile of *Salmonella i*solates from raw cow milk

CHAPTER FIVE

5.0 DISCUSSION

According to the findings of the current study, various farm practices such as milkers not washing their hands before and between milking different cows, milking sick animals, and those with udder problems predispose raw milk to microbial contamination. *Salmonella* spp isolated in this study included *S. typhimurium*, *S. typhi* and *S. enteriditis* with an overall prevalence of 5.8%. The isolated *Salmonella* spp showed resistance to penicillin (100%), ampicilin (100%) and amoxicillin/clavulanic acid (100%).

During the current study it was observed that most smallholder dairy farmers kept their animals in filthy animal houses that are full of cow manure which could have ramifications for pathogen origins for various animal diseases. Meanwhile, milk contamination is likely to occur in such filthy environments. Similar observations have been reported in Tanzania (Bukuku, 2013; Kanyeka, 2014) and (Mosalagae *et al.,* 2011) in Zimbabwe. The majority of farmers fail to follow excellent milking protocols by skipping or failing to perform some of the most important procedures during milking. The amount of microorganisms in the milk is also known to be affected by general cleanliness at milking time. In general, animal attendants' unsanitary behaviors may lead to microbial contamination of the cow's milk. Previous studies in Tanzania had similar findings (Karimuribo *et al.,* 2005; Mdegela *et al.,* 2009; Swai and Schoonman 2011; Shija, 2013; Kanyeka, 2014).

Hand washing before milking cows was found to be about 65.2% which is insufficient for sustaining milk quality. This result is lower than the reports in Mvomero and Njombe districts Tanzania by (Mdegela *et al.,* 2009) and that in Jimma (>94%) by (Yilma, 2012).

Furthermore, all farmers reported udder washing before milking using either bare hands or a piece of cloth. In this study, the sole cleaning agent used for cleaning the udder was water with no detergents. This result has an agreement with the study in Tanzania (Mdegela *et al.,* 2009; Gwandu *et al.,* 2018) and Ethiopia (Tegegne and Tesfaye, 2017) in which farmers did not use detergents for udder cleaning.

Considering water is a known cause of microbiological contamination in milk, hypochlorite should be added at a rate of 50 ppm to the cleaning water or it should be boiled if an unauthorized piped supply is available (Hyera, 2015). In this study, the majority of smallholder dairy farmers (89.1%) reported using well water for their cows and sanitation. When using water from sources other than the tap, it is essential that the handlers filter and heat treat the water before using it for cleaning (Yilma, 2012).

Plastic containers were the most commonly used utensils for collecting and storing milk during the current study which is consistent with findings of (Schoder *et al.,* 2013; Gwandu *et al.,* 2018) in Tanzania which revealed that farmers used plastic containers for milk collection. 100% of the dairy cow owners utilized water and soap for cleaning milk handling equipment which is in agreement with the reports of (Tegegne and Tesfaye, 2017). Cleaning the equipment with soap and good quality water is likely to eliminate milk residue, including microorganisms, thereby affecting the milk's microbiological quality. In addition, it was observed in the current study that milk was stored at room temperature for a long time, prompting growth of microorganisms over time. Similarly, (Kivaria *et al.,* 2006a) reported that the high microbial load in milk is due to a lack of cold chain. In general, unhygienic milk handling may have contributed to microbial contamination in milk. However, there was no statistical significant association between microbial contamination in milk and most of the unhygienic practices that were observed

in this study (P>0.05) (Table 4). Based on the Chi square analysis, the statistical significant associations ($\chi2=15.923$, df=1, p=0.000; $\chi2=11.714$, df=1, p=0.001 and $\chi2= 4.124$, df=1, p=0.042) were observed in hand washing before milking, hand washing between milking different cow and milking sick cow respectively. This is in agreement with the study on bacteriological milk quality by Tegegne and Tesfaye (2017) in Ethiopia who showed that there was a significant effect on hand washing prior to milking in total bacterial count. The findings are in contrast with those from (Kivaria *et al.*, 2006a) who reported that cleaning frequency of milk container, milk storage time, milk storage containment and mixing fresh with previous milk significantly influenced the microbial quality of marketed milk.

Furthermore, farmers reported animal diseases such as mastitis, which in addition to causing a high microbial load in milk increases the use of veterinary medicine potentially leading to veterinary drug residues in milk and as a result antimicrobial resistance due to the development of resistant bacteria. Surprisingly, some farmers reported to use raw cow milk from sick or treated animals. Others to sell the milk or gave it to their pets or calves. In contrast to the current study, a study by (Mosalagae *et al.*, 2011) in Zimbabwe in Zimbabwe found that (84.9%) of farmers interviewed discard milk from sick cows. Different levels of knowledge about animal diseases and the potential consequences of consuming contaminated milk could explain the variations in outcomes. As a result, farm-level animal disease prevention measures as well as community-wide public health education, should be implemented to reduce infections like mastitis in lactating cows.

Furthermore, customers should avoid drinking milk from sick animals because it could be contaminated with a range of agents including harmful pathogens, putting their health at risk. Milk from animals on antibiotics should be discarded for the duration of the

medication since it may include antibiotics and antibiotic residues, affecting milk quality as well as consumers' health (Hyera, 2015).

Furthermore, the majority of farmers stated that they follow drug withdrawal periods after treating or medicating their animals however sometimes they do not do so. Non-compliance with withdrawal periods was linked to concern of losses from milk disposal which was contributed by the majority of respondents in this study having a poor educational level. Other studies in Tanzania (Katakweba *et al.,* 2012; Bukuku, 2013) found that farmers are generally aware of medication withdrawal periods, however they do not always adhere to them. In contrast to this study, Kanyeka (2014) reported majority of the farmers not to adhere to drug withdrawal periods after treating or medicating animals due to a lack of knowledge about drug residues and the associated health effects such as allergic reactions, toxicity and carcinogenic effects. Furthermore, all farmers were aware of the negative health effects of drinking raw milk. In contrast a study by (Karimuribo *et al.,* 2005) reported only 20.8% were aware that consumption of raw milk could be harmful to their health. The efforts of livestock extension officers, who were reported to contact frequently with smallholder dairy farmers, should be credited for the high degree of awareness exhibited in this study. More research is needed to assess microbial contamination in milk along the milk value chain as well as to assess the impact/safety implications of poor milk quality on human health upon consumption.

Serotyping confirmed the presence of *S. typhimurium, S. enteritidis* and *S. typhi* with an overall prevalence of 5.8% in apparently healthy cows. This implies that raw cow milk in Ilala district was contaminated with lactating cows being the carriers of the *Salmonella* spp which could be potential sources of *Salmonella* illness to dairy farm workers and the general population. The prevalence in this study is in line with 4% from raw and

fermented milk in Nigeria (Tamba *et al.*, 2016), 6.5% from dairy farms and abattoir and 3.3% from raw milk in Ethiopia (Beyene *et al.*, 2016; Reta *et al.*, 2016) respectively, 7.61% from milk and milk products in India (Singh *et al.,* 2018) and 4.4% from raw cow milk in Ghana (Mensah *et al.*, 2018).

The prevalence was relatively lower compared to 10.1% and 37.33% from raw milk in Tanzania (Schoder *et al.,* 2013; Lubote *et al.,* 2014) respectively, 16.4% and 14% from milk in Rwanda (Mpatswenumugabo *et al.*, 2019; Ndahetuye *et al.*, 2020) respectively, 28% from raw milk and 25.89% from raw milk and environment samples in Pakistan (Jalbani *et al.,* 2019; Qamar *et al.*, 2020), 10.7% from lactating cows and in contact humans in Ethiopia (Addis *et al.*, 2011) and 25.71% from milk and milk based products in Bangladesh (Yasmin *et al.*, 2015). The prevalence observed in Ilala district was relatively higher compared to 2.04% from raw cow milk in Tanzania (Kanyeka, 2014). (Ekici *et al.,* 2004) in Turkey reported that *Salmonella* was not isolated in all milk samples collected from individual cows. The differences in prevalence of *Salmonella* spp observed in various studies could be attributed to sample size, farm size, bacterial isolation method, farming system, milking technique, milking equipment, hygienic conditions, location and ecology (Soomro *et al.,* 2002). The prevalence of *Salmonella* in raw milk has been reported to range from 0.17 to 28.6% depending on the method used and the frequency of detection (Lubote *et al.,* 2014).

Comparing the ward wise prevalence of *Salmonella* spp in Kivule ward was shown to indicate higher positive samples of *Salmonella* spp (Table 7), the variation in prevalence rate in wards can be attributed by unhygienic milking practices. *Salmonella* are enteric bacteria present in the intestine of animals and their presence in milk could indicate that the animal is a carrier or infected with them (McGuirk and Peek, 2003). Furthermore, the

absence of *Salmonella* in all samples from Kitunda and Ukonga might be linked to the health of the cows whose milk was examined. *Salmonella* can only be shed through milk when an animal has acute clinical salmonellosis, although it can also be shed by carrier animals (McGuirk and Peek, 2003). As a result, the absence of *Salmonella* in milk samples from Kitunda and Ukonga wards indicates that the milk is clear of bacteria in the udder's interior.

The current study found that *S. typhimurium*, *S. enteritidis* and *S. typhi* were most common isolates from raw cow milk. The results are in line with (Qamar *et al.,* 2020) in South Punjab-Pakistan who found *S. typhi*, and *S. typhimurium* from milk and environment samples and (Jalbani *et al.,* 2019) who reported *S. typhi* from raw milk. In Egypt (Omar *et al.,* 2018) reported *S. enteritidis* and *S. typhimurium* from milk and dairy products. The findings are in contrast with those from (Lubote *et al.,* 2014) who reported *S. arizonae* from milk samples. More researches are recommended for better establishment of prevalence such as using a multiplex polymerase chain reaction (m-PCR) assay which is specific and fast alternative method for identifying *Salmonella* spp as compared to this study which employed culture based technique (colony isolation). Sampling at various units such as milkers' hands, milking and storage containers, cattle feed samples and cattle drinking water as well as that used for sanitary activities is also recommended. The current study sampled on pooled milk, so this should be taken into consideration while studying this prevalence as it does not directly reflect the status of individual cows or herds.

Antimicrobial sensitivity results showed that all the isolated *Salmonella* were 100% resistant to penicillin, ampicilin and amoxicillin/clavulanic acid. This finding is in line with report from Tanzania (Kanyeka, 2014) and from Addis Ababa (Addis *et al.,* 2011)

who reported that 100% of the isolates were resistant to ampicilin. Other reports from Nigeria (Tamba *et al.,* 2016), from India (Kanyeka, 2014), from Bangladesh (Yasmin *et al.,* 2015) and from Ethiopia (Beyene *et al.,* 2016) reported 85.7%, 56.2%, 88.89%, 58.3% respectively of the *Salmonella* isolates were resistance to ampicillin. The findings are in contrast to Singh *et al.* (2018) and Yasmin *et al.* (2015) who reported 68.7% and 77.78% resistance of *Salmonella* to amoxicillin/clavulanic acid. The high resistance of penicillin, ampicilin and amoxicillin/clavulanic acid can be explained by the fact that they are extensively used antibiotics in Tanzania livestock agriculture as growth enhancers, prophylaxis and disease therapy. Antibiotic misuse on farms contributes to resistance, this misuse of antibiotics could be linked to a lack of knowledge about animal husbandry and health (Kanyeka, 2014) as evidenced in the present study 52.2% of the dairy farmers kept no records of any health interventions performed on their animals. Other factors that contribute to the development of antibiotic-resistant bacteria include, the occurrence of resistant clonal strains that have successfully disseminated within populations, limited extension services and uncontrolled antibiotic availability even in livestock markets in Tanzania, where antibiotics are sometimes sold without a prescription (Katakweba *et al.,* 2012; Kanyeka, 2014). However, due to small sample size, caution should be used in interpretation because no indication of antimicrobial usage was established.

In the present study all of the isolated *Salmonella* spp 100% were sensitive to gentamycin, tetracycline, chloramphenicol, ciprofloxacin and trimethoprim-sulfamethoxazole. The findings are in line with reports from Kanyeka (2014) in Tanzania who reported that *Salmonella* spp. isolates were sensitive to gentamycin. (Tamba *et al.,* 2016) reported *Salmonella* isolates sensitivity to gentamycin (100%), ciprofloxacin (100%), chloramphenicol (93%) and tetracycline (64.3%). (Beyene *et al.,* 2016) reported sensitivity to gentamycin (100%), ciprofloxacin (100%), trimethoprim-sulfamethoxazole

(91.7%) and chloramphenicol (75%). Addis *et al.* (2011) reported sensitivity of (100%) isolates to ciprofloxacin and chloramphenicol. Singh *et al.* (2018) reported sensitivity of 62.5% of the isolates to ciprofloxacin. The findings are in contrast to Yasmin *et al.* (2015) who reported 22.2% resistance of isolates to chloramphenicol and 11.11% resistant to ciprofloxacin. (Jalbani *et al.,* 2019) found 73.68%, 68.42% and 36.84% of the isolates were resistant to tetracycline, gentamycin and ciprofloxacin respectively. This could be related to different serovars obtained in their studies, antimicrobial usage and overuse, geographic variation and livestock management practices. The antimicrobial sensitivity profile also shows that the antibiotic can be used to treat *Salmonella* spp. found in the study area, as shown in the data.

Furthermore, all (100%) *Salmonella* isolates showed multi-drug resistant to penicillin, ampicilin and amoxicillin/clavulanic acid. There are several factors that could explain for the reported multi-drug resistance, this include antibiotic type, organism type, long-term exposure, antibiotic concentration and the immunological condition of the host. Furthermore, the multi-drug resistance pattern observed could be the consequence of plasmids accumulating resistance genes, each coding for resistance to a specific antibiotic or multi-drug efflux pumps pumping out multiple antibiotics (Nikaido, 2009). Multidrug-resistant bacteria are a public-health problem because they lead to inadequate infection treatment and poor patient recovery (Levy and Marshall, 2004). Therefore, more research is needed to identify the extent of antimicrobial resistance and antimicrobial residues in milk from farm to Table.

This study had a number of limitations that need to be considered. First, this study sampled only pooled milk samples, sampling at different units such as milkers' hands, milking and storage containers, cattle feed samples and cattle drinking water as well as

that used for sanitary activities, would have resulted to good establishment of prevalence and antimicrobial susceptibility of *Salmonella* spp. Second, the sample size used was small, larger sample size is recommended.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Overall, the findings revealed that raw cow's milk was contaminated with *Salmonella* spp with a prevalence of 5.8%. Hand washing before and between milking different cows as well as milking sick cows has a stronger impact on raw milk microbial contamination and contributes to the transmission of zoonotic infections. Consumption of raw milk may cause health issues. This is supported by evidence of *Salmonella* spp isolated in this study. This raises a public health concern regarding safety of milk to consumers. The high levels of antimicrobial resistance to antibiotics revealed in this study are a major public health problem for both animals and humans. This is a worrisome indication that requires quick public health attention since it may impede the treatment of human diseases. Furthermore, given the high prevalence of antibiotic-resistant *Salmonella* isolates, antimicrobial usage in the veterinary and public health sectors must be judicious and sensible.

6.2 Recommendations

i. This study therefore recommended that animal husbandry techniques should be improved in order to limit the occurrence of infections and the need for excessive antimicrobial drugs.

ii. Smallholder dairy farmers should be taught the importance of personal hygiene, such as washing their hands with soap and water before milking the cows, wearing clean clothing and maintaining a clean environment in which the animals are kept.

iii. It is critical to discourage the consumption of raw milk and raw milk-derived products. Milk industry stakeholders must play a role in informing the general public about the dangers of such behavior to public health.

iv. Public health education should be given to the public about the proper use of antibiotics in order to avoid the problem of antimicrobial resistance.

v. In order to prevent the transmission of resistance genes from animals to humans and vice versa, legislation is essential to assure correct animal and human medical use.

vi. Antibiotics should be selected entirely based on their antibiogram pattern.

vii. It is advised that veterinarians, extension officials, and all stakeholders play their parts in ensuring that consumers receive safe, high-quality milk.

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APPENDICES

Appendix 1: Questionnaires survey for respondents in the study area

This questionnaire is designed to collect information related to smallholder dairy farmer's knowledge on microbial contamination and antibiotics. It will take less than 45 minutes to complete. Please note that your answer is absolutely confidential and your name will not be discussed in any report. Also, your individual answer will not be shared with anyone.

Questionnaire number:……

1. Date of interview:……/……/20…….....
2. Village/ Street……
3. Ward……..
4. District……
5. Region……

PART A: Respondent particulars

6. Age of Respondent…….
7. Gender

   ☐ Male ☐ Female

8. Marital Status

   ☐ Single

   ☐ Married

   ☐ Divorced

   ☐ Widowed

☐ Others (Specify)……..

9. Respondent's highest level of education

☐ Primary education

☐ Secondary education

☐ College education/University education

☐ None

10. Respondent's position in the household

☐ Head of the household (Father)

☐ Wife of head of household

☐ Child

☐ Employee

☐ Others (Specify)…….

Part B-1: Microbial contamination and Farm management

11. Are there any sources of microbial contamination in milk that you are aware of?

☐ Yes

☐ No

12. If Yes mention (Multiple choice)

☐ Diseased cows

☐ Unclean udder and teats

☐ Unhygienic milking procedures

☐ Hands and arms of the milker and dairy workers

☐ Unclean milking utensils/equipments

☐ Water used at the farm for adulteration and sanitary activities

☐ Others (specify)…….

13. What kind of animal housing do you have?

- ☐ Trees/boma
- ☐ Block house/mud
- ☐ Grass
- ☐ No house

14. What kind of flooring/bedding do they have? (single choice)

- ☐ Natural earth/mud
- ☐ Concrete/cement
- ☐ Others (Specify)…….

15. What are your animal's water source, as well as hygienic procedures such as hand washing, utensils and/or equipment? (single choice)

- ☐ Drilled wells
- ☐ Tap water
- ☐ River/streams
- ☐ Other (Specify)……

16. Is illness screening and prevention done on a regular basis?

- ☐ Yes
- ☐ No

17. If Yes, for what diseases? (multiple choice)

- ☐ Mastitis
- ☐ Foot and Mouth Disease
- ☐ Anthrax
- ☐ Brucellosis
- ☐ Helminthiosis
- ☐ Tuberculosis

☐ Other (Specify)……

18. What is the practice when a cow becomes ill?

☐ Milking

☐ Not milking

19. What do you do with the milk of a sick cow? (single choice)

☐ Family consumption

☐ Sale the milk

☐ Leave for calves

☐ Discard

☐ Other (Specify)…….

20. Do you consume raw milk?

☐ Never

☐ Sometimes

☐ Always

21. If so, what are the most prevalent disorders induced by raw milk consumption? (multiple choice)

☐ Tuberculosis

☐ Brucellosis

☐ Diarrhoea

☐ Typhoid

22. How do you usually remove or minimize germs in milk?

☐ Sieving/filtering

☐ Boiling

☐ Letting it to settle down

☐ Fermenting it

☐ Other (specify)……..

Part B-2: Milk handling practices

23. Is the udder washed before milking?

☐ Never

☐ Sometimes

☐ Always

24. What do you use to clean the udder? (single choice)

☐ Water

☐ Water with soap

☐ Water with a disinfectant

25. What is washed?

☐ Teat

☐ Whole udder

26. Does the milker wash hands before milking?

☐ Yes

☐ No

27. Does the milker wash hands between milking different cows?

☐ Yes

☐ No

28. How does the milker wash hands before milking?

☐ Water only

☐ Water with soap

☐ Water with a disinfectant and soap

29. What utensils and/or equipment are utilized in the milking and handling process? (single choice)

- ☐ Plastic containers
- ☐ Aluminium/Stainless steel containers
- ☐ Wooden containers
- ☐ Traditional pots
- ☐ Other (Specify)……

30. How often do you wash the utensils and equipment you use for milking? (single choice)

- ☐ Daily
- ☐ Weekly
- ☐ Monthly
- ☐ Others (Specify)…….....

31. How do you clean your milking utensils? (single choice)

- ☐ By cold water only
- ☐ By Soap and cold water
- ☐ By soap and hot water
- ☐ Others (Specify)……

32. How do you handle the milk at household?

- ☐ Always covered soon after milking
- ☐ Not covered at all

33. How do you store your milk, including storage conditions? (single choice)

- ☐ Refrigerator
- ☐ In bucket/can at room temperature
- ☐ Chiller

☐ Others (Specify)……

34. Do you know that consumption of raw milk can cause human illness?

☐ Yes

☐ No

Part B-3: Knowledge about Antibiotics

35. Who usually diagnoses sickness in your cattle?

☐ Self

☐ Veterinarian

36. Who is in charge of administering medication to your cattle on a regular basis?

☐ Myself

☐ Veterinarian

☐ Farm employee

☐ Neighbor

☐ Others (specify)……

37. When a cow is being treated with antibiotics, she is:

☐ Visibly marked

☐ Milked last

38. Do you maintain written records for antibiotic treatments including medicated feeds?

☐ Never

☐ Sometimes

☐ Always

39. Do you follow the prescriber's instructions regarding the dosage or number of treatments?

☐ Never

☐ Sometimes

☐ Always

40. If not, why?

☐ Not enough money

☐ The cow appears healed

☐ The Treatment doesn't work

☐ Because the milk production decreases

☐ Because of side effects

41. Do you know what drug withdrawal/withholding period is?

☐ Yes

☐ No

42. If answered **yes**, do you follow it?

☐ Never

☐ Sometimes

☐ Always

43. Do you immediately sell milk following the last dose of cattle treatment?

☐ Yes

☐ No

44. If the answer in above question is **No**, why not selling milk immediately after last dose of treatment?

☐ Observe veterinary drugs withdrawal periods

☐ Milk contains veterinary drugs

☐ Others (specify) ……..

45. How long do you wait before selling milk from a cow that is undergoing treatment?

- ☐ Less than one week
- ☐ More than one week
- ☐ Stop from selling as per drug manufacturer's recommendations
- ☐ Other (specify)…….

46. What do you do with milk from a recently treated animal?

- ☐ Sale the milk
- ☐ Family consumption
- ☐ Give them to pet animals like dogs and cats
- ☐ Discard
- ☐ Other (specify)……..

47. Is an antibiotic residue detection test used to screen calves after freshening for antibiotics?

- ☐ Never
- ☐ Sometimes
- ☐ Always

48. What type of antibiotic is given to your dairy cattle? (Interviewer to observe if there are any empty bottles/packs)

- ☐ Penicilin
- ☐ Streptomycin
- ☐ Tetracycline (OTC & CTC)
- ☐ Sulphonamide
- ☐ Kanamycin intrammamary infusion

☐ Tyrosine

☐ Gentamycin

☐ Other (specify)……..

49. Is there any risk to a person's health if they drink milk that has antibiotic residues?

☐ Yes

☐ No

50. If yes above, mention the effects;

☐ Allergic reactions to some sensitive individuals

☐ Toxicity

☐ Bacterial resistance to antibiotics

☐ Cancer

☐ Other (specify)……

Thank you* very *much for devoting time to participate in this study

Appendix 2: Laboratory standard operating procedure for isolation and serotyping of Salmonella spp extracted from Muhimbili University of Health and Allied Sciences

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DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
9 United Nations Road, Upanga P. O. Box 65001
Dar es Salaam, Tanzania

Standard Operating Procedure: # MI – 0112 Standard operating procedure for Salmonella Isolation and Stereotyping V3.0

Prepared by	**Date Adopted**	**Effective Date**	**Supersedes Procedure #**
Norah Massawe	02/12/2009	29/04/2020	V1.5

Review Date	**Revision Date**	**Name**	**Signature**
16/11/2009		Fidelis Charles Bugoye	On file
18/11/2010		Michel W. Alexandre, BS, MT(ASCP)	
21/11/2011		Michel W. Alexandre, BS, MT(ASCP)	
28/08/2012		Moshi Bilingo	
April 28, 2020	April 28, 2020	Lilian Nkinda	

Distributed to	**# of Copies**	**Distributed to**	**# of Copies**
MUHAS Clinical Research Lab	1	Makuti Clinic	1
Microbiology and Immunology Laboratory	1		

Laboratory Director/Coordinator	**Date Approved**	**Signature**
Mabula J. Kasubi, MD, PhD	02/12/2009	On file

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DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
9 United Nations Road, Upanga P. O. Box 65001
Dar es Salaam, Tanzania

Document History

Version Number	Reason for Changes	Date
V2.0	Centralization of all SOPs at the Department of Microbiology and Immunology, change in SOP number	29/04/2020

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DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
9 United Nations Road, Upanga P. O. Box 65001
Dar es Salaam, Tanzania

1. Principle of the Procedure

Salmonella O antigens are somatic (O) heat-stable antigens and are identified first. The Vi antigen is a heat-labile envelope antigen that may surround a cell wall and mask somatic antigen activity. Microorganisms having the Vi Antigen will not agglutinate in O antisera. In order to determine the O antigen of these cultures, a suspension of the organism must be boiled to destroy the heat-labile envelope antigen and then tested with O antisera. The flagellar (H) antigens are heat labile and are usually associated with motility.

Complete serological characterization of *Salmonella* is not required for successful detection of the microorganism when it occurs as a pathogen. The use of adequate isolation procedures and differential biochemical tests is of primary importance. Because antigenic relationships exist between genera of the family *Enterobacteriaceae*, it is recommended that the isolate be biochemically identified as *Salmonella* prior to Serology testing. Possible *Salmonella* isolates can be presumptively identified with a minimum of serological identification.

Identification of *Salmonella* species includes both biochemical and serological identification. Serological confirmation involves the procedure in which the microorganism (antigen) reacts with its corresponding antibody. This *in vitro* reaction produces macroscopic clumping called agglutination. The desired homologous reaction is rapid, does not dissociate (high avidity) and bonds strongly (high affinity). Because a microorganism (antigen) may agglutinate with an antibody produced in response to another species, heterologous reactions are possible. Such unexpected and perhaps unpredictable

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reactions may lead to some confusion in serological identification. Therefore, a positive homologous agglutination reaction should support the morphological and biochemical identification of the microorganism.

Agglutination of the somatic antigen in the slide test appears as a firm granular clumping. Homologous reactions are rapid and strong (3+). Heterologous reactions are slow and weak. Agglutination of flagellar antigens in the tube test appears as a loose flocculation that can easily be resuspended.

2. Specimen

Pure culture of microorganism, that biochemical test reactions are consistent with the identification of the organism as a *Salmonella* species. The isolate for serological testing should be subcultured from selective media to a nonselective media.

3. Materials

Applicator sticks

Slides

5ml tubes

Shaker

PPE

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4. Reagents/Test Kits

1. 0.85% NaCl solution.
2. *Salmonella* somatic O Antisera: Polyvalent A-S, Monovalent O:2 (A), O:4 (B), O:7 (C1), O:8 (C2C3), O:9 (D).
3. *Salmonella* H Antisera: Polyvalent HMA-HG, Monovalent 2, 5, 6, 7, a, b, c, d, g,m, g,p, h, i, k, m
4. Biochemical identification kit (API 20E).
5. Culture media: MacConkey Agar with CV and salt (MAC), Sheep Blood Agar (SBA), Xylose Lysine Deoxycholate (XLD) and Selenite Broth (SEL).

5. Quality Control Organisms

a. Positive control: Known Commercial *Salmonella* spp to the antiserum

b. Negative control: Organism in saline only

6. Procedure

A. Primary Inoculation

1. Inoculate samples on MAC and XLD plates and SEL broth
2. For referred isolates, subculture to a fresh SBA and MAC plate.
3. Incubate plates overnight at 35 ± 1°C. Note: Maximal recovery of Salmonella from fecal specimen is obtained by using an enrrichment broth (e.g., SEL), although

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isolation from acutely ill persons is usually possible by direct plating of specimens. Subculture SEL to XLD after 16 – 18 hours incubation; incubate plate overnight at 35 ± 1°C.

B. Culture Examination

1. Examine plates for characteristic colonies of *Salmonella*.
2. Select one of each type of suspect colony from the plates.
3. Inoculate API 20E mini test tubes with the saline suspensions of the cultures according to manufacturer's directions. For each colony type, use a single colony to inoculate all mini tubes.
4. Incubate the test set up at 35 ± 1°C for 18-24 hrs in a humidity chamber.

C. Identification

1. Check growth on SBA and MAC for purity and colonial morphology. Repeat test if mixed.
2. Perform oxidase test on colonies from SBA. All *Salmonella* are oxidase negative.
3. Read and interpret colour reactions on the API 20E set up.
4. Observe for reactions typical of *Salmonella*. Consult table of reactions below.
5. Once the biochemical reactions are done, perform serotyping using colonies grown in SBA as per described serotyping SOP.

Note:

Typical Colonial morphology on Primary Isolation Media:

MAC: Transparent or colorless opaque; 2-3 mm

XLD: Red (with or without black centers), or yellow with black centers; 1-2 mm

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HE: Blue or green with or without black centers) or yellow with black centers

DCA: Colorless colonies 2-3 mm

SBA: Most Enterobacteriaceae are indistinguishable on SBA

Biochemical reactions involved in API 20E test kit and typical *Salmonella* reaction

Tests	**Substrate**	**Reaction**	**(-) Results**	**(+) Results**	***Salmonella spp.***
ONPG	ONPG	Betagalactosidase	Colorless	Yellow	-
ADH	Arginine	Arginine dihydrolase	Yellow	Red/Orange	-
LDC	Lysine	Lysine decarboxylase	Yellow	Red/Orange	+
ODC	Ornithine	Ornithine decarboxylase	Yellow	Red/Orange	+
CIT	Citrate	Citrate Utilization	Pale to green/Yellow	Blue-green/ Blue	-
H2S	Na thiosulfate	H2S production	Colorless/Gray	Black deposit	+
URE	Urea	Urea hydrolysis	Yellow	Red/Orange	-
TDA	Tryptophan	Deaminase	Yellow	Brown-Red	-
IND	Tryptophan	Indole production	Yellow	Red (in 2 min)	-
VP	Na-pyruvate	Acetoin production	Colorless	Pink/Red (in 10 min)	-
GEL	Charcoal gelatin	Gelatinase	No diffusion of black	Black diffusion	-

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GLU	Glucose	Fermentation/Oxidation	Blue/ Blue-green	Yellow	+
MAN	Mannitol	Fermentation/Oxidation	Blue/ Blue-green	Yellow	+
INO	Inositol	Fermentation/Oxidation	Blue/ Blue-green	Yellow	-
SOR	Sorbitol	Fermentation/Oxidation	Blue/ Blue-green	Yellow	+
RHA	Rhamnose	Fermentation/Oxidation	Blue/ Blue-green	Yellow	+
SAC	Sucrose	Fermentation/Oxidation	Blue/ Blue-green	Yellow	-
MEL	Melibiose	Fermentation/Oxidation	Blue/ Blue-green	Yellow	+
AMY	Amygdalin	Fermentation/Oxidation	Blue/ Blue-green	Yellow	-
ARA	Arabinose	Fermentation/Oxidation	Blue/ Blue-green	Yellow	+

Serotyping

Test Isolate for Autoagglutination: From the test culture on nonselective media, transfer a loopful of growth to a drop of sterile 0.85% saline on a clean slide and emulsify the organism then rotate the slide for 1 min and observe for agglutination. If agglutination (autoagglutination) occurs, the culture is rough and cannot be tested. Subculture to nonselective agar, incubate and test the organism again. If no agglutination occurs, proceed with testing the organism.

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Determination of O antigens: First test the isolate with polyvalent O antisera (poly A-S) by dispensing 1 drop (35 µL) of antiserum to be tested on an agglutination slide. **Negative control:** Dispense 1 drop of 0.85% sterile NaCl solution on an agglutination slide then transfer a loopful of an isolated colony to each reaction area and mix thoroughly. **Positive control:** Dispense 1 drop of each *Salmonella* O Antiserum to be tested on an agglutination slide followed by addition of stock culture (*Salmonella*) of known serological identification. Once the polyvalent group O is positive for agglutination, test the isolate with monovalent O antisera against O groups 2, 4, 7, 8, 9.

When a strain does not agglutinate the polyvalent sera, it is recommended to test this strain with Vi serum and the other polyvalent O sera. If a Vi positive reaction is observed, the bacterial suspension must then be heated to 100 ^{0}C for 30 minutes, before repeating the test with polyvalent O sera and the corresponding monovalent sera to define the O antigen.

Determination of H antigens (Tube agglutination method)

Phase 1: Fist test the isolate with polyvalent H antiserum (HMA-HG) by dispensing 0.5 mL in each tube. **Test isolate:** Add 0.5 mL to the appropriate tube containing the polyvalent sera. **Positive control:** Add 0.5 mL of antigen positive control to a tube containing 0.5 mL of antiserum. **Negative control:** Add 0.5 mL of 0.85% NaCl solution to a tube containing 0.5 mL of test isolate. Thereafter, incubate all tubes in a water bath at 50 ± 2 °C for 1 h then read for flocculation (agglutination). Once the polyvalent H is positive for agglutination, test the isolate with monovalent H antisera (2, 5, 6, 7,

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a, b, c, d, g,m, g,p, h, i, k, m). Once the first H antigen is identified, a phase inversion on the isolate was performed to force the organism to repress its dominant H phase and grow in the second phase.

Phase invasion: Sven Gard medium is used during serotyping of *Salmonella* to demonstrate the inapparent H antigen phase of biphasic Salmonella (Sven Gard method). Sven Gard agar should be used with the following antisera: SG 1 to SG 6.

Phase 2: A culture at the periphery of the invasion zone of the Sven Gard agar should be taken. Start testing by using the H polyvalent antisera (HMA-HG). If there is no agglutination, this serotype contains only one phase. If one of these groups shows agglutination, define the specific H phase by using the relevant H monovalent antisera. As the antigenic formula with O, H-phase 1 and 2 are identified the serotype is now specified by referring to a reference catalog.

7. **Identification Problems:** Several potential problems may prevent accurate serotype determination.
    - The strain may express the Vi antigen, which can block the binding of antibodies against the O antigens
    - The strain may be rough, i.e., fails to make complete O antigens. Rough strains have a tendency to weakly agglutinate in multiple O grouping antisera.
    - The strain may be mucoid and not agglutinate in any O antisera.
    - Isolates can be nonmotile and not express any flagellar antigens
    - Salmonella Paratyphi C may express the Vi antigen.

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- Salmonella Paratyphi A may be overlooked because it is not routinely screened with group O:2 (A) antiserum, or because it is H_2S negative and lysine negative.

1. **Interpretation and Reporting of Results**
   a. A preliminary report of *Salmonella spp.* may be issued when an isolate shows typical reactions in the biochemical kit and is positive with *Salmonella* polyvalent O antisera.
   b. An isolate is confirmed as *Salmonella* when the specific O serogroup (2, 4, 7, 8, 9) has been determined and biochemical identification has been completed (i.e., API 20E for this lab)
   c. Report confirmed *Salmonella* isolates by group (O:2, O:4, O:7, O:8, O:9).
   d. Report to the serotype level if biochemically and serogically confirmed.
      "*Salmonella* Paratyphi A"
      "*Salmonella* Paratyphi B"
      "*Salmonella* Paratyphi C"
      "*Salmonella* Typhi"
   e. Report isolates that are serologically A, B, C, or D but are not biochemically/ serologically serotype Paratyphi A, B, C, Typhi:
      "Salmonella group O:2 (A) – not Paratyphi A"
      "Salmonella group O:4 (B) – not Paratyphi B"
      "Salmonella group O:7 (C) – not Paratyphi C"
      "Salmonella group O:9 (D) – not Typhi"

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Source:

a. Clinical Microbiology Procedures Handbook. American Society for Microbiology. Washington D.C., USA, 2nd edition, 2007.
b. Manual of Clinical Microbiology. American Society for Microbiology (ASM), Washington D.C., USA. 9th edition, 2007.
c. Antigenic Formulae of the Salmonella Serovars. WHO Collaborating Centre for Reference and Research on Salmonella and Institute Pasteur. Paris, France. 2007.

Appendix 3: Results of Salmonella serotyping from Muhimbili University of Health and Allied Sciences (MUHAS)

Final Report

Friday,February 5, 2021

MUHIMBILI UNIVERSITYOF HEALTH AND ALLIED SCIENCES
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
MUHAS Clinical Research laboratory
9 United Nations Road, Upanga P. O. Box 65001
Dar es Salaam, Tanzania

Patient ID :N/A

Order ID:N/A

RABORATORY REPORT

	ID:NUMBER	RESULT
Salmonela Serotyping	123	S.Typhi
	110	S.Typhi
	101	Entereditis
	137	Typhimurium
	94	Typhimurium
	71	Enteriditis
	103	Enteriditis
	111	Typhimurium

MUHIMBILI CLINICAL RESEARCH LABORATORY
P.O. BOX 65001 DAR ES SALAAM
5/2/2021

Appendix 4: Antimicrobial susceptibility profile interpretation chart for Salmonella spp

Antibiotics	Code	Conc	S/ ≥ mm	I/mm	R/≤ mm
Penicillin	P	10µg	15	-	14
Tetracycline	TE	30µg	15	12-14	11
Gentamycin	GE	10µg	15	13-14	12
Ciprofloxacin	CPR	5µg	21	16-20	15
Sulfamethazole-Trim.	SXT	25µg	16	11-15	10
Amoxicillin/ Clav. acid	AUG	30µg	18	14-17	13
Ampicillin	AMP	10µg	17	14-16	13
Chloramphenicol	C	25µg	18	13-17	12

***S=Sensitive I=Intermediate R=Resistant, Conc=Concentration** *
Source(CLSI, 2011; Liofilchem, 2017)

Appendix 5: Antimicrobial Susceptibility results profiles based on zones of inhibition (mm)

Sample ID	P/10	Te/30	Ge/10	CPR/ 5	SXT/ 25	AUG/ 30	AMP/ 10	C/25
71	0	22	22	30	23	0	0	20
94	0	22	20	30	21	0	0	20
101	10	20	20	30	21	13	8	26
103	12	20	19	30	21	10	6	25
110	0	22	21	30	18	0	0	20
111	0	22	20	30	21	0	0	23
123	0	19	19	30	20	0	0	20
137	0	20	20	30	18	0	0	22

Appendix 6: The antibiotics susceptibility patterns for isolated Salmonella spp

Sample ID	P/10	Te/30	Ge/10	CPR/ 5	SXT/ 25	AUG/ 30	AMP/ 10	C/25
71	R	S	S	S	S	R	R	S
94	R	S	S	S	S	R	R	S
101	R	S	S	S	S	R	R	S
103	R	S	S	S	S	R	R	S
110	R	S	S	S	S	R	R	S
111	R	S	S	S	S	R	R	S
123	R	S	S	S	S	R	R	S
137	R	S	S	S	S	R	R	S

Key: S: Sensitive; I: Intermidiate; R: Resistance

Appendix 7: Regional administration secretary permit

THE UNITED REPUBLIC OF TANZANIA
President's Office
REGIONAL ADMINISTRATION AND LOCAL GOVERNMENT

DAR ES SALAAM REGION
Phone Number: 2203158
Fax number: 2203158
email: ras@dsm.go.tz
website: www.dsm.go.tz

REGIONAL COMMISSIONER'S OFFICE,
3 RASHID KAWAWA ROAD,
P.O. BOX 5429,
12880 DAR ES SALAAM

In reply please quote:
Ref. No. .....

30/6/ 2020

District Administrative Secretary,
Ilala Municipal Council,
P. O. Box .....,
DAR ES SALAAM.

RE: RESEARCH PERMIT

~~Prof~~/~~Dr~~/~~Mrs~~./~~Ms~~/Miss ..... Agnes Jonathan ..... is student/**Research** from ..... Sokoine University of Agriculture ..... has been permitted to undertake research on ..... Assessment of Microbial Contamination of raw cow milk and Antimicrobial resistance of Salmonella spp. in Ilala district, Dsm. .....

From..... July ..... 2020 to ..... October .....2020.

I Kindly request your good assistance to enable her/his research.

.....
For; **REGIONAL ADMINISTRATION SECRERTARY**
DAR ES SALAAM

Copy: Municipal Director,
Ilala
.....
DAR ES SALAAM.

" Principal/Vice Chancellor
Sokoine University of Agriculture.

Appendix 8: District administrative secretary permit

The United Republic of Tanzania

Prime Ministers' Office

REGIONAL ADMINISTRATION AND LOCAL GOVERNMENT

ILALA DISTRICT
Phone Address:

Phone No: 2203185/2203182

In reply quote: **Ref. No:** AB.60/87/01/

DISTRICT COMMISSIONER'S OFFICE
ILALA DISTRICT
P. O. Box 15486,
DAR ES SALAAM

Date: 30/6/20.20.

Municipal Director,
P. O. Box 20950,
Ilala,
DAR ES SALAAM.

RE: RESEARCH PERMIT

~~Prof./Dr./Mr./Mrs./Ms.~~/Miss: Agnes Jonathan,
from The Sokoine University of Agriculture, she/~~he~~ has been permitted to undertake a field work research on "Assessment of Microbial contamination of raw cow milk and Antimicrobial resistance of Salmonella spp. in Ilala district, Dsm." The case study at Ilala District from July 20.20 to October, 20.20.

Therefore, you are asked to give the said researchers necessary assistance and Cooperation.

District Administrative Secretary
ILALA

Copy: .....

.....

Principal/Vice Chancellor,
Sokoine University of Agriculture,

.....

Appendix 9: Municipal director permit

ILALA MUNICIPAL COUNCIL

PHONE NO: 2128800
2128805

Municipal Director's Office,
1 Mission Street,
P.O. Box 20950,
11883 – DAR ES SALAAM.

Our Ref. IMC/QR.3/VOL.1/88

13/07/2020

Ms. Agness Jonathan,
Sokoine University of Agriculture,
Office of the Deputy Vice-Chancellor (Academic),
P.O. Box 3000 Chuo Kikuu,
MOROGORO.

RE: THE PERMISSION TO CONDUCT RESEARCH ON ASSESSMENT OF MICROBIAL C0NTAMINATION OF RAW COW MILK AND ANTIMICROBIAL RESISTANCE OF SALMONELLA spp. IN ILALA DISTRICT, DAR ES SALAAM

The reference is made to above subject and letter with Ref. no. SUA/ADM/R.1/8A/643 of 22/06/2020.

2. The permission is granted to you to conduct research in Ilala District from July to October, 2020. The title of the research in question is " Assessment of Microbial Contamination of Raw Cow Milk and Antimicrobial Resistance of Salmonella spp. in Ilala District, Dar Es Salaam"

3. Looking forward to work with you,

4. Yours sincerely,

Majaliwa M. Andrea
For: MUNICIPAL DIRECTOR

C.C:
Municipal Director,
Ilala Municipal Council.

Appendix 10: Turnitin Originality Report

Turnitin Originality Report

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Disertation By Agnes Jonathan

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