ISOLATION, IDENTIFICATION AND ANTIBIOGRAM OF *SALMONELLA ENTERICA* FROM CHICKEN EGGS COLLECTED FROM SELECTED WARDS IN MOROGORO MUNICIPALITY, TANZANIA

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ABSTRACT

Eggs provide protein to human. However, consumption of contaminated eggs with pathogenic *Salmonellae* (*Salmonella enterica*) imposes negative impacts to human health causing salmonellosis. The present study aimed at isolation, identification and antibiogram testing of S*almonella enterica* from chicken eggs in Morogoro Municipality. A total of 570 raw, boiled, cracked, clean, and faecal soaked chicken eggs (120 local and 450 exotic) were collected from selected areas and examined from November to May 2016. Analysis was done by pooling samples using bacteriological standard methods where eleven (11) pathogenic *Salmonellae* (*Salmonella. enterica*) were confirmed to genus level by PCR using *Spec* primers pair for *Salmonella invA* gene amplified 284 bp of DNA fragment. Out of eleven (11) recovered *Salmonellae* 6 (20.0%) from egg contents and 5 (16.7%) from eggshell surfaces. The overall prevalence of isolated *Salmonella enterica* in eggs was 36.7% (95% CI: 0.199 – 0.561). It was worth noting that, local eggs were highly contaminated (20.0%) than exotic eggs (16.7%) whereas raw eggs were highly contaminated (26.7%) than boiled eggs (10%). Interestingly clean eggs were highly contaminated (20.0%) than faecal socked (13.3%). Statistical analysis between raw and boiled eggs showed significant differences. Moreover, eggs from Mlimani ward were highly contaminated 4 (13.33%) compared with eggs from other wards. Antibiogram test results showed sensitivity to: Ciprofloxacin, Chloramphenicol, Gentamicin, Tetracycline, Sulfamethaxole-Trimethoprim, Imepenem, Cefotaxime, Ceftriaxone and Caftazidime, and resistant only to Kanamycin. This study reports for the first time presence of pathogenic *Salmonellae* (*Salmonella enterica*) in chicken eggs and their antibiogram patterns, indicating that eggs are unwholesome for human consumption and population in Municipality is at risk for salmonellosis. Therefore, it is recommended that control

measures from eggs production to table chain should be implemented by health sectors to prevent human/animal salmonellosis.

DECLARATION

I, Selestina Amos Mrope, do hereby 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 being concurrently submitted in any other institution.

Selestina Amos Mrope
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Date

The declaration is hereby confirmed by:

19/10/2017

Dr. Athumani M. Lupindu
(Supervisor)

Date

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LIST OF ABBREVIATIONS AND SYMBOLS

%	percentage
AIDS	Acquire Immunodeficiency Disease Syndrome
°C	degree Celsius
µg	microgram
µl	microliter
µM	micromole
BA	Blood Agar
Bp	Base pair
BSA	Bismuth Sulphite Agar
CDC	Centre for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards institution
Conc.	Concentration
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
E- test	Epsilometer test
ESBL	Extended Spectrum of Beta-Lactamase
FDA	Food and Drugs Authority
$FESO_4$	Ferric Sulphate
g	gravitational force
HIV	Human Infectious Viruses
IMVIC	Indole, Methyl Red, Voges Proskauer, Citrate
ISO	International System Organization
IU	International Unit
K	Kilo

LD	Ladder
LIA	Lysine iron Agar
M	Marker
MCA	MacConkey Agar
mg	milligram
MIC	Minimum Inhibition Concentration
MKTn	Muller Kauffman Tetrathionate broth
mL	Millilitre
mM	millimore
NA	Nutrient Agar
NC	Negative control
NLF	Non Lactose Fermenters bacteria
NRI	National Research Institution
PC	Positive control
PCR	Polymerase Chain Reaction
pH	hydrogen iron concentration
PHE	Public Health of England
GIS	Geographic Information System
RV	Rappaport Vasilliadis
RVS	Rappaport Vasilliadis Soy broth
S. E	*Salmonella enteritidis*
S. T	*Salmonella typhimurium*
SB	Selenite Broth
SUA	Sokoine University of Agriculture
TSB	Tripticase Soya Broth
TSI	Triple sugar Iron agar

TT	TetraThionate broth
USA	United States of America
USDA	United State Department of Agriculture
UV	Ultraviolet rays
XLD	Xylose Lysine Dextrose agar
XLT4	Xylose Lysine Tergitol agar

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Foodborne illness caused by pathogenic *Salmonell*a species is a major problem worldwide (Majowicz *et al*., 2010; PHE, 2014). Food safety is the conditions that preserve the quality of food and prevent it from contamination and food-borne illness (Jalali *et al*., 2012). The magnitude and impacts of salmonellosis in humans and animals especially chickens has been studied in Tanzania by Mdegela *et al*. (2000) and Msoffe *et al*. (2006). However, these were not similar to the present study since they isolated *Salmonella enterica* serovar Gallinarum from local and commercial chickens indicating that Fowl typhoid caused by this serovar is the major problem of animal salmonellosis in poultry especially in developing world such as Tanzania. *Salmonella* bacterium belong to the Enterobacteriaceae family and is classified into two main species *Enterica* and *Bongoli* where only *enterica* species with serovars *Enteritidis* and *Typhimurium* which are common foodborne species found in contaminated eggs and cause humans salmonellosis. The group contain more than 2 500 serovars (Malorny *et al*., 2003a; Bahness *et al*., 2015). People acquire illness via consumption of contaminated foods such as eggs with incubation period ranging from 7-72 hours after ingestion with clinical signs of abdominal pain, cramps, diarrhoea, fever, vomiting, headache and nausea (Jamshindi *et al*., 2009). Infections are fatal in immunosuppressed individuals such as HIV/AIDS, elders, infants and pregnant women, and also cause economic loss in poultry industry (Meremo *et al*., 2012; Montvile *et al*., 2001; Doorduyn *et al*., 2006; Rahman *et al*., 2009).

Many risk factors have been identified to contribute the transfer of pathogenic *Salmonella* species in the world such as; consumption of contaminated raw for the sake of people local

beliefs or under cooked, cracked, and faecal soaked eggs in which eventually will infect consumers. Other transmission factors include poor hygienic practices in farms and catering services, improper storage temperatures in egg retails, and contaminated environmental factors such as dust and soil also may play part (Green *et al*., 2006; Shanmugasamy *et al*., 2011; Mughini- Gras *et al.,* 2014). Additionally, HIV victim also is an important risk factor for salmonellosis transmission Meremo *et al.,* 2012). Conventional and standard methods have been developed and used in diagnosis of *Salmonellae* from eggs such as; cultivation of samples by pooling of estimated number of samples (eggs) such as 10, 15 and 20 according to nature and their status (Wallace *et al.,* 2009; Betancor *et al*., 2010; Maha and Ashmawy, 2013; PHE, 2014).

Several methods have been recommended for antibiogram testing of *Salmonellae* however, the Kirby-Bauer disc diffusion method is the common method used in the world in accordance to Clinical and Laboratory Standards Institute (CLSI, 2014) (Bauer *et al.,* 1966; Tsegaye *et al.,* 2016). Antibiotic sensitivity testing against a pathogen such as bacteria is often necessary in order to determine the effective drug of choice to kill pathogens (bacteria). This art is crucial because antimicrobials susceptibility patterns cannot be predicted and the emergence of drug resistance is being reported frequently in the world (Yemisi *et al.,* 2014; Akind *et al*., 2012). However, *Salmonella* infections are self-limiting, but if illness persists to infected individuals and may cause life-threatening they should be treated using the drug of choice obtained after performing antimicrobial susceptibility test using recommended first line and current drugs (Bauer *et al*., 1966; Nchawa *et al*., 2015).

1.2 Problem Statement and Justification

Currently, food poisoning caused by bacteria and an increased trend of antibiotic resistance are major problems all over the world where the later problem is high in developing countries (Shanmugasamy *et al.,* 2011; Bayu *et al*., 2013). Studies to isolate and antibiogram testing of foodborne pathogens from foods such as eggs and egg products has been done in many countries worldwide such as African countries like Ethiopia, and Nigeria, European countries, and United States. Despite of their nutrition value, eggs can also harbour pathogenic *Salmonella*e and impose negative impacts to both veterinary and public health sectors if mishandled (Maha and Ashmawy, 2013; Pires *et al*., 2014). *Salmonella enterica* has been identified in the world as the principal source of human and animal salmonellosis whereby human salmonellosis is mainly caused by *Salmonella typhimurium* (Betancor *et al*., 2010; Phagoo and Neetoo, 2015). People acquire salmonellosis via consumption of eggs contaminated with *Salmonella enterica* as raw, undercooked, cracked or faecal soaked (Edema and Atyese, 2006; Yhiler and Bassey, 2015). Moreover, *Salmonella enterica* infection occurred when people eat raw eggs with a believes that it cures cough and softens singer`s voice, and addition of raw eggs into child meal such as porridge also increase nutritional values. In fact, these traditional beliefs increase the risk of acquiring salmonellosis. Poultry has been noted to be major principal host of animal salmonellosis caused by *Salmonella enterica* serovar *Gallinarum* (Mdegela *et al*., 2000; Msoffe *et al.,* 2006).

The misuse of antimicrobial agents to treat bacterial infections without susceptibility testing and prescription for use may lead into emergence of drug resistance. The emergence of antimicrobial resistance due to drug misuse and development of gene mutation of pathogens as reported now days in many studies in the world is the greatest challenge to be addressed (Akind *et al*., 2012) in both poultry husbandry as well as public

health sectors. Additionally, the use of animal products after the animal been treated with antibiotics without observing the withdraw periods indicated of that drug also might cause multiple drug resistance in human beings.

Currently, no documentation about safety status of chicken eggs consumed in the country as far as *Salmonella* infection is concerned. Therefore, this study was first designed in Morogoro Municipality in order to assess bacteriological quality of chicken eggs against *Salmonella enterica*, and determine the antibiogram patterns using antimicrobials commonly used in animals and human disease. Results from this study will create awareness to stakeholders and hence help in planning and implementation of proper control measures to prevent salmonellosis and antimicrobial resistance in human and animals.

1.3 Objectives of the Study

1.3.1 The overall objective

The present study aimed at Isolation, identification and antibiogram testing of *Salmonella enterica* from chicken eggs in Morogoro Municipality.

1.3.2 Specific objectives

i) To determine occurrence of Salmonella enterica in chicken eggs for human consumption in Morogoro municipality.

ii) To assess the antimicrobial susceptibility patterns of *Salmonella enterica* from eggs for human consumption in Morogoro Municipality.

1.3.3 Research questions

i) Are local and exotic chicken eggs contaminated with *Salmonella species* in Morogoro municipality?

ii) Which of the isolated *Salmonella* are likely to be pathogenic?

iii) What are the antibiogram patterns of isolated pathogenic *Salmonellae* from chicken eggs?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Background Information

Salmonellosis is one of the leading foodborne disease in the world causing an estimated of 93.8 million illness and 15 500 deaths each year (Majowicz *et al*., 2010; Al-Ledeni *et al*., 2014). The major causative agent of this disease is from *Salmonella enterica* serovars which contain more than 2,500 closely related bacteria that cause illness by reproducing in the digestive tract. Poultry products such eggs and meat has been identified as major sources of both animal and human salmonellosis in the world (Mdegela *et al*., 2000; Msoffe *et al*., 2006; Paiao *et al*., 2012; PHE, 2014). Additionally, HIV/AIDS victims also play part in transmission of salmonellosis as indicated by the study by Meremo *et al*., (2012) at Bugando Hospital in Mwanza where of 346 febrile adult patients had blood stream infections where the common isolates were *Salmonella* spp 13 (39.4%), and a total of 156 (45.1%) patients were HIV infected; of whom 12 (7.6%) were infected by *Salmonella enterica* (Non-typhoid *Salmonella* spp).

Salmonellae belong to the *Enterobacteriaceae* family. They contain two main species *Enterica* and *Bongoli* where only *enterica* is pathogenic. Transmission of infections to humans is through consumption of contaminated raw or under cooked, cracked and faecal contaminated foods such as chicken eggs (Doorduyn *et al.*, 2006; Hasan *et al.*, 2009; Bayu *et al.*, 2013; PHE, 2014). The serovars *Enteritdis* and *Typhimurium* have been reported to be the common cause of food borne illness where *Enteritidis* serovar is mainly found in chicken eggs. The serovars G*allinarum* and P*ullorum* commonly cause major threats to chickens where these threats can be loss or reduction in eggs production and meat leading into economic loss and poverty to farmers and worldwide, however cause no

illness to humans (PHE, 2014; Bahness *et al.*, 2015). *Salmonella* infection in human is characterized by sudden onset of abdominal pain or cramps, diarrhoea, vomiting, nausea and invasive properties which occur within 72hrs incubation period after ingestion. The common mode of infection of *Salmonella* in eggs is via vertical and horizontal transmission routes. Additionally, cross contamination during storage, and worker`s hand washing practices also play part in *Salmonella* transmission to foods (Shanmugasamy *et al.,* 2011). Although infections caused by *Salmonella* bacteria are self-limiting in healthy individuals some become serious infections in immune compromised individuals such as HIV/AIDS victims, elders, pregnant women and children where care must be under taken (Meremo *et al*., 2012). However, if illness persists the drug of choice should be obtained after conducting antibiotic sensitivity test using both first line drugs such as Chloramphenicol, Sulfamethaxole and Ciprofloxacin and third generations like Ceftriaxone, Caftazidime (Yhiler and Bassey, 2015). Also antibiogram testing is performed in order to determine presence of exogenous resistance genes which acquired routinely by the pathogen which will help them to resist antimicrobial susceptibility effect and make them difficult to be killed. Several studies have been done in the world and indicated the transfer of these resistance genes from environmental bacteria to pathogenic ones which facilitate emergence of Extended Spectrum Beta-Lactamases (ESBL) pathogens such as bacteria. The emergence of (ESBL) genes encourage antibiogram susceptibility test against isolates so as to determine the drug of choice to treat *Salmonellae* infections (Phagoo and Neetoo, 2015).

2.2 Public and Veterinary Health Importance of Chicken Eggs

Chicken eggs are good source of protein. They are eaten by all ages of people either separate or mixed with other dishes such as fried potatoes (chips). Despite of their nutritional values they can as well cause health problems through consumption of

contaminated eggs with pathogenic *Salmonella* spp. In developing countries cracked and faecal contaminated eggs are being sold at half price and consumed in which they can act as risk factor to acquire *Salmonella* illness (Edema and Atyese, 2006). Eggs are of economic importance based on poultry industry production which can rise farmer`s income worldwide. The intact egg has natural physical and chemical barriers which inhibit pathogen from entering into egg contents. However, eggs contain antimicrobial factors yet, they can be infected by many bacteria such as *Salmonella* imposing negative impacts to both animals and humans' sectors (Pires *et al.,* 2014; Mughini- Gras *et al.,* 2014). Chicken are believed to be the major source of poultry and human salmonellosis in which is a zoonotic infection (Sasaki *et al*., 2011).

2.3 Contamination and Transmission Risk Factors of *Salmonella* spp

Many risk factors of salmonellosis have been identified in many studies done in the world. Such as cracks and faecal materials on egg shell surfaces. Additionally, vertical and horizontal routes are the common transmission factors followed by used trays, water, and other environmental factors such as dust, litter materials, and soil which also play part in transmission of salmonellosis in eggs **(**Rahman *et al.,* 2009; Sasaki *et al*., 2011; Bayu *et al.,* 2013).

In humans the common risk factors noted are contaminated clean, cracked, faecal soaked eggs when consumed as raw or undercooked. Additionally, cross contamination between foods, or knife to food, un-use of hand gloves, workers hand washing practices all act as a vehicle of transmission of human salmonellosis (Hasan *et al.,* 2009). A contaminated egg might go to retail or institutional users. It might or might not be pooled with other eggs, and it can be contaminated by a handler during breakage (Green *et al.*, 2006). Actually, food service establishments, eating food outside home and food workers` poor personal

hygiene are important contributors to foodborne diseases **(**Green *et al*., 2006). Many studies reported salmonellosis outbreaks from eggs or egg-containing foods which originated from food service kitchens as the result of inadequate refrigeration, handling and insufficient cooking. Additionally, some studies reported that poor personal hygiene of food workers also play part as contributing risk factor for transmission of food borne illness outbreaks (Green *et al*., 2006; Mughini-Gras *et al*., 2014).

2.4 Epidemiology and Pathogenesis of *Salmonella* Species

Worldwide spread of salmonellosis which account for major veterinary and public health threats affect both animals and humans (Majowicz *et al*., 2010; PHE, 2014). Human salmonellosis is commonly caused by consumption of contaminated eggs with *Salmonella enterica* serovars, *S. enteritidis*, *S. heideberg* and S. *typhurium* serogroups which associated by *iroB* and *invA* genes with virulence factors *flagellin C* 15 and *protective 5-6E*. These genes have been used to detect *Salmonella* specific species (Malorny *et al*, 2003a; Jamshindi *et al*., 2009) where *invA* gene detects pathogenic *Salmonella* at genus level and *flagellin C15* and *protective 5-6E* are for serovars. The *invA* gene is found in their chromosomes (Shanmugasamy *et al.,* 2011; Abdullahi *et al.,* 2014). These serovars infects a wide range of hosts and are isolated from chicken and frequently reach the human food chain. *Salmonella enterica* serovars *Pullorum* (*Salmonella*. *pullorum*), and *Gallinarum* (*Salmonella*. *gallinarum*) are host specific and represent major concern for poultry industry, but have no impact on public health (Betancor *et al.*, 2010).

2.5 Reported *Salmonella* Outbreaks

Salmonellosis has been included among the emerging foodborne diseases recognized by the national and international countries been mainly isolated from food and environmental samples (PHE, 2014). The data provided from studies done in England and Ethiopia they

isolated pathogenic *Salmonella* species from chicken eggs, water, and environment indicating that *S. enteritidis* was the most common serotype from chicken eggs and was the major cause of zoonotic infections **(**Bayu *et al*., 2013; PHE, 2014**)**. Also the egg industry, public health community and government agencies have been working diligently to deal with *Salmonella* spp (Doorduyn *et al*., 2006; Hassan *et al*., 2009; Shanmugasamy *et al*., 2011). Also the study done in Kuwait has reported that approximately 48 million of Americans get sick, 128,000 are hospitalized and 3 000 die each year from foodborne illness (Rahman *et al*., 2009).

Many research has been conducted in order to determine the prevalence of *Salmonella* species in eggs in many countries in the world which showed different results for example; Japan the prevalence was 0.25% (Yemisi *et al*., 20140), Ethiopia 2.5% (Bayu *et al.,* 2013) and, Iran was 1.6% (Jamshindi *et al.,* 2009). In East Africa countries such as Tanzania some studies on isolation of pathogenic *Salmonella* from local and commercial chicken has been conducted by Mdegela *et al*., 2000 and Msoffe *et al.,* 2006 where the study from Mdegela *et al*., 2000 isolated *Salmonella enterica* serovar *Gallinarum* with the prevalence of 2.6%. Of the *Salmonella* outbreaks that occurred from 1985 through 1999 in India, (62%) out of 522 outbreaks of *S. enteritidis* infection were associated with foods prepared at restaurants/hotels, caterers, bakeries, cafeteria, or markets (Suresh *et al.,* 2006). In 2006 forty-six (46) food related outbreaks were reported in Finland in which among bacteria isolated *Salmonella enterica* serovar *Enteritidis,* and *Typhmur*ium and Fowl typhoid were included (Sasaki *et al.,* 2011).

2.6 Laboratory Diagnosis of *Salmonella* Species from Eggs

Many protocols have been developed and employed for isolation of *Salmonella* spp from food, faces and environmental swabs (PHE, 2014). These include both traditional and

modern methods such as cultures and molecular techniques like PCR respectively (Schoeni *et al.,* 1995; Wattiau *et al.,* 2011). In culture method, liquid and solid media are used which are regarded as ``Gold standards. ``Even though it is tedious and time consuming as compared to the modern techniques such as PCR (Rahn *et al*., 1992; Makind *et al*., 1999; Paiao *et al.,* 2012). In eggs, *Salmonella*e are present in low numbers in addition to a lot of other micro-organisms, and may be injured during breakage and mixing of egg contents. Therefore, to recover these non-viable cells and diminish the risk of obtaining false negative results; the pooling method of eggs and use of both pre enrichment broth such as Buffered Peptone Water (BPW) and two enrichment broths such as Selenite Broth (SB) / Tetrathionate broth (TT), and Rappaport Vasiliadis (RV) / Rappaport Vasiliadis Soy (RVS) are recommended (Vassiliadis *et al*., 1983). The use of non- selective plating media such as Blood Agar (BA) and MacConkey Agar (MCA), moderate selective agar such as Xylose Lysine Dextrose Agar (XLD) and Bismuth Sulphite agar (BS), and highly selective agar such as Xylose Lysine Tergitol (XLT4) agars also are recommended. The isolation of *Salmonella* spp in faeces or food samples use a large amount of sample such as 25g or 25mls and incubation is done according to protocol used such as at 35℃, 41℃ and 41.5℃ **(**Wallace *et al*., 2009; PHE, 2014).

2.6.1 Physical examination of individual shelleggs

Individual shelleggs are examined macroscopically before processing to observe any abnormal colour on egg shells surfaces such as black or green which indicate bacterial or fungal infection respectively. Although is not necessarily to be *Salmonella* bacterium.

2.6.2 Identification of *Salmonella* spp.

2.6.2.1 Macroscopic Identification

This is based on examination of growth appearance of the colony on solid media which is done macroscopically using naked eyes and record the characteristic features such as

colour, shape, size, growth percentages and other features depending on the type of agar used with or without production of a unique colour identified as black due to formation of Hydrogen Sulphide (H_2S) gas produced by some species of *Salmonella*. In addition, indicators are been added in the media in order to enhance visualization of colonies accordingly. The colour of *Salmonella* colonies in various solid media differs according to indicator used. The colours may be colourless, yellow- pink or pink/red with or without black centre (Wallace *et al*., 2009; PHE, 2014).

2.6.2.2 Microscopic identification

Salmonella species are examined microscopically based on morphological appearance after been stained by Gram staining techniques which show gram reaction as negative rods, non- sporing, motile with peritrichous flagella for most species except *Salmonella. pullorum* and *Salmonella*. *galllinarum* which revealed after grown in motility agar medium such as SIM or by using hanging drop test (Bayu *et al.*, 2013; Betancor *et al*., 2010; Tsegaye *et al.*, 2016).

2.6.2.3 Biochemical identification

Different types of sugars either in-cooperated in agar media such as TSI, KIA and LIA or single prepared have been recommended as biochemical tests for identification of *Salmonella* species from foods such as eggs. These are such D–glucose is fermented to produce acid and usually gas. Other carbohydrates usually fermented are L- arabinose, maltose, D-Mannose, D mannitol, L-rhamnose, D-sorbitol (except spp. VI), trehalose, D-xylose, Simmons citrate, lysine and dulcitol (Bayu *et al*., 2013; PHE, 2014). Also catalase and urease enzymes tests, IMVIC test are used, where all *Salmonella* species are catalase positive., urease negative, Indole negative, Methyl red positive and Voges Proskauer

negative and Citrate utilization positive/negative (PHE, 2014). Additionally, motility tests are used such as hanging drop method and growing in semi- solid motility medium.

2.6.2.4 Serological identification

Serological confirmation of suspected *Salmonella* isolates can be done using specific antisera (Omnivalent/Polyvalent) based on which *Salmonella* spp are intended to be isolated. Usually antisera of the groups I- IV are the common used to diagnose pathogenic *Salmonella* of the public and veterinary health importance (PHE, 2014).

2.6.2.5 Molecular identification methods

In addition to serological confirmation many molecular techniques have been developed and used as an alternative of traditional methods. These techniques are based on amplification of DNA products of tested organism. They are highly recommended as quick detection method as compared to conventional methods which takes about 5-7 days (Malorny *et al.,* 2003a; Shanmugasany *et al.,* 2011). However, some of them have limitations such as PCR due to lack of standardization among laboratories because of factors variations (Rahn *et al.,* 1992; Rahman *et al.,* 2009; Wattiau *et al.,* 2011). Some of the DNA amplification assays include conventional and Multiplex PCR where extraction of DNA is done from 5-20 suspected colonies of the bacterium (PHE, 2014). In PCR specific primers are used to amplify DNA products of the suspected colonies using specific temperatures, time and cycles in a thermal cycler. There after electrophoresis is run in stained agarose gel to enhance visibility of amplified DNA bands (Makind *et al.,* 1999; Jamshindi *et al.,* 2009; Paiao *et al.,* 2012).

2.7 Antimicrobial Susceptibility Testing

The greatest challenge of antibiotics therapy is the development of resistance in which mostly is associated with people via drug misuse and gene mutations of microbes (Akind

et al., 2012). Drug resistant is the ability of a pathogen to resist killing effect of the drugs (Yemis *et al*., 2014). Testing of individual pathogens against appropriate antimicrobial agent is often necessary since susceptibility of many pathogens such as bacteria to antimicrobial agents cannot be predicted and is done for the sake of preventing emergence of drug resistance to these strains (Yhiler and Bassey, 2015; Tsegaye *et al.,* 2016). Nowadays there is a problem of Expanded Spectrum Beta-Lactamase bacteria (ESBL) in the world especially in developing countries (Akind *et al.*, 2012; Abdullahi *et al.,* 2014). Various methods of antimicrobial susceptibility testing against pathogen (bacteria) have been employed such as agar disc diffusion, dilution method and Epsilometer test (E-Test). The Kirby-Bauer method using single disk agar diffusion determines the diameter of inhibition zones of drugs against tested pathogens (Bauer *et al*., 1999; CLSI, 2014).

The dilution method is done by Minimum Inhibition Concentration (MIC) test to determine the lowest concentration of drug which will inhibit growth or kill pathogen. Interpretation of results of the diameter of inhibition zones are measured in millimetre (mm) and recorded as sensitive (S), intermediate sensitive (I) and resistant (R) according to guidelines recommended such as Clinical and Laboratory Standards Institute (CLSI) (Nchawa and Bassey, 2015).

Many studies have indicated that, there are transfers of genes from environmental bacteria to pathogens via gene mutations which can cause emergence of multiple resistances against antimicrobial agents (Yemisi *et al*., 2014). The dynamics of trend of transmission of zoonotic infections and environmental components which obviously need to be addressed in order to control the escalating spread of these infections. The environmental strains of *Enterobacteriaceae* are usually susceptible to aminoglycosides, Imipenem, Quinolones and third generation Cephalosporins **(**Phagoo and Neetoo, 2015).

2.8 Control Measures of *Salmonella* Infections

Many control measures have been recommended in the world by WHO research agencies, companies and other research institutions such as FDA, CDC and USDA. These measures should start from the farm level to table, since the major sources of salmonellosis are from poultry farms due to poor farm management (Hassan *et al.*, 2009; Al- Ledeni *et al.,* 2014). Faecal contaminated and cracked eggs in developed countries are destroyed and farmers are refunded by their governments, but in developing countries these eggs are sold and consumed. Some studies conducted in the world have advised to use crack eggs for baking purposes and faecal soaked eggs after washed before and steaming for 30 minutes (Edema and Atyese, 2006; Hasan *et al.*, 2009). Other prevention measures to be used are such as thoroughly cooking of vulnerable foods and avoid eating raw or under cooked eggs. Practicing good personal hygiene for workers in food establishments such as wearing gloves during handing and preparation of foods, washing hands after handling of foods such as eggs and meat, visiting of toilets with soaps under running water by friction of hands for 20 seconds, socking of food serving plates in hot water before and after use, proper storage of foods under cold chain and separation of foods types during preservation t in the fridge to avoid cross contamination with food pathogen (Shanmugasamy *et al*., 2011; PHE, 2014).

The use of antibiotics to treat infections without testing their sensitivity effect, and prescription should be avoided because they can promote emergence and spread of drug resistance. Also excessive use of antibiotics should be avoided because will enhance existence and survival advantage of *Salmonella* pathogen in site of infection via reduction of colonizing normal flora/agents which act as a host protection.

Therefore, proper handling of eggs as food and antimicrobial usage should abide to local guidelines and needs to be audited. However, mishandling of eggs and inappropriate use of antibiotics as under dosing or multiple or excessive duration of dosage might lead into emergence and spread of multiple drug resistance in both man and animals (Akind *et al*., 2012; Phagoo and Neetoo, 2015).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

Morogoro Municipality was a selected study area within Morogoro region which is located about 200km West of Dar Es Salaam. It has 531.6 square kilometres of total land area and the population growth rate is 4.4% per annum, projecting a total population of 316 603. About 33% of the population is engaged in subsistence farming and livestock keeping. The Municipal Council has one division, which is subdivided into 29 Administrative Wards where out of these, 4 Wards were purposively selected as sampling areas based on availability of chicken eggs as study material. Justification of selected study area was based on the rate of growing population of people, where it is high in this area compared to other parts of Morogoro region. Hence, the requirement of animal protein such as eggs also will be increased. Additionally, many exotic and local chicken eggs are consumed much in this Municipality due to population growth of people present. Availability of study material in selected wards based on the number of chicken farmers found in this place, and vicinity to the research centre also were considered. Selected wards in the Municipality are shown (Fig. 1).

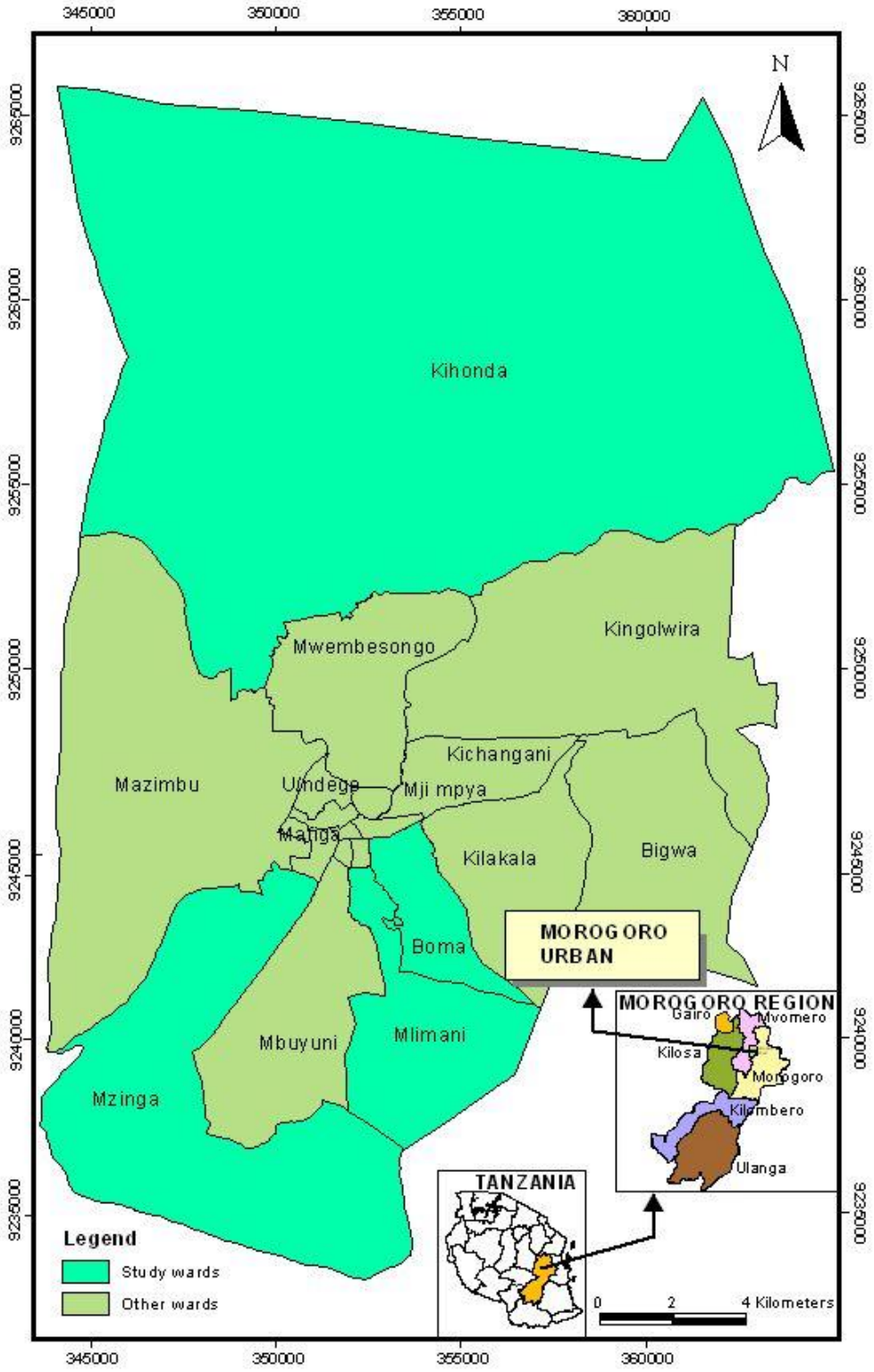

Figure 1: The map of Morogoro Municipality wards where samples were collected as indicated in the map by green colour (Map constructed using Arc View 3.2 GIS Software).

3.2 Study Materials

Local and exotic chicken eggs including egg parameters: boiled and raw: clean, faecal soaked and cracked were used in this study utilizing both eggshell surfaces and egg contents. Interpretation of egg parameters was done as follows clean eggs with no dirty material on shells surfaces; cracked eggs with cracks on shells surfaces; faecal soaked eggs with faecal material on shell surfaces; raw eggs; fresh eggs, and boiled eggs; cooked eggs. Selection justification of these materials was based on the fact that pathogenic *Salmonella* (*Salmonella enterica*) have been commonly isolated from these egg parameters and was a source of human infection as indicated in many studies done in Bangladesh by Hasan *et al*. (2009) and in Ethiopia by Bayu *et al.* (2013). In addition, eggs are consumed much in urban areas by people of all ages as source of protein.

3.3 Research Design

A cross-sectional design was employed in this study where eggs were collected randomly from chicken farms and food catering services within Municipality.

3.4 Sample Size and Sample Collection

Sample size was determined based on Thrusfield (2007) whereby expected prevalence of *Salmonella*, confidence level and precision were set at 50%, 95% and 5% respectively. Correlation between egg sources was accounted for by using intra-cluster correlation coefficient of 0.18 and design effect of 1.89 (Otte and Gumm, 1997). The resultant sample size of 720 eggs was adjusted to 570 eggs by considering the proportion of sample clusters in the population according to Thrusfield (1997). The sample was composed of 120 raw local, 360 raw and 90 boiled exotic eggs. The eggs were categorized as clean (330), faecal soaked (120), and cracked eggs (120) collected aseptically and placed into new trays then transported to SUA Microbiology laboratory for analysis (Appendix 1). The sample

chicken eggs were collected from four wards: Mlimani which contributed a total of 400 local eggs and exotic eggs (160 clean, 120 faecal soaked and 120 cracked). Mzinga contributed 70 clean eggs, Boma contributed 60 clean eggs, and Kihonda ward contributed 40 clean eggs.

3.5 Laboratory Analysis of Samples

Isolation of pathogenic *Salmonella* (*Salmonella enterica*) from egg samples was done by using conventional and standard microbiological protocols as described by Wallace *at al.* (2009) and PHE, (2014) utilising both basic, selective solid media (Fig. 2), and enrichment broths (Fig. 3) for observation after 24hrs and 48hrs of incubation (Appendices 2 and 3).

3.5.1 Media preparation

Six types of solid culture media (Himedea, India) and three enrichment broths (Difco, USA) were prepared aseptically and following manufacturer's instructions and used in this study. Enrichment broths used were such as Buffered Peptone Water (BPW), Tripticase Soy Broth (TSB) with or without $FeSo_4$ as pre enrichment broths, Rappaport Vasilliadis (RV) and Rappaport Vasilliadis Soy (RVS) as selective enrichment broths (Vassiliadis *et al.*, 1983). Solid culture media used were MacConkey Agar (MCA, Himedia, LOT 1089136, Expiry 2016/09), Nutrient Agar (NA, Himedia, LOT, 0000290922, Expiry 2022/2) and Blood Agar (BA, Himedia, LOT 0000289885, Expiry 2022/01) as non-selective media, Xylose Lysine Dextrose Agar (XLD, LOT 0000126494, Expiry, 2015/10) and Bismuth Sulphite Agar (BS) from Difco, USA as moderate selective media , and Xylose Lysine Tergitol 4 (XLT4, Difco, USA, LOT 3315270, Expiry 2018/10) as highly selective media were used (Fig. 2 and 3). Sterility check-up was done by incubating fresh non inoculated media of different batches prepared at 37℃ for 24hrs.

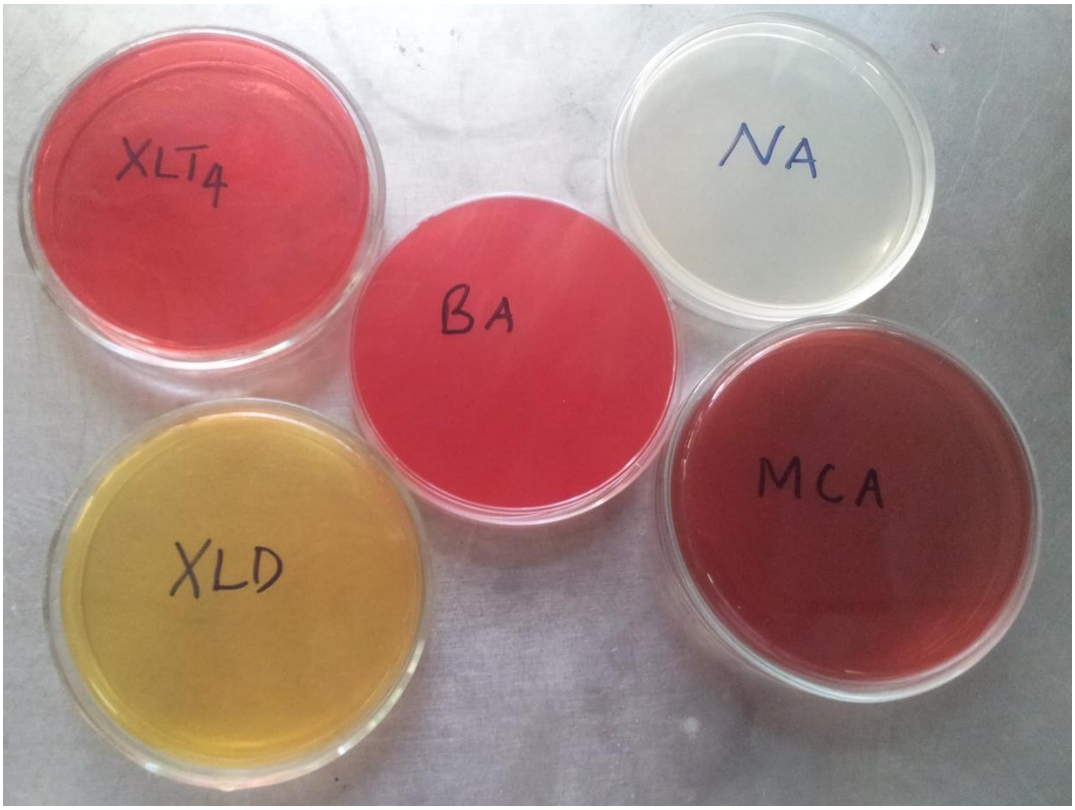

Figure 2: Different types of solid media prepared and used in the study

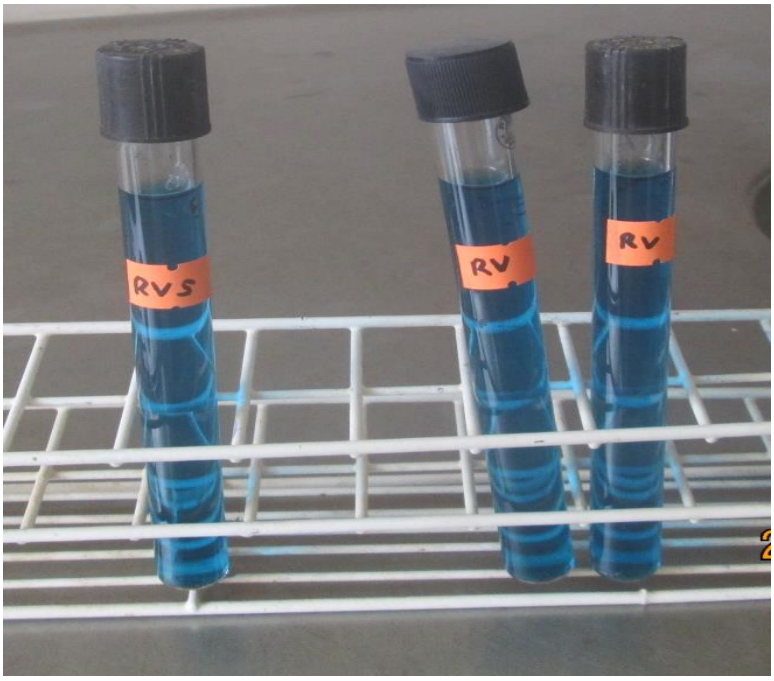

Figure 3: Different types of enrichment broths prepared and used in the study

3.5.2 Physical examination of individual chicken shelleggs

All individual shelleggs were physically examined before inoculation to detect the presence of black or green colour on the egg shell surfaces. The presence of these colours on shells indicates either bacterial or fungal infections. Although is not necessarily to be *Salmonellae* infections.

3.5.3 Isolation of *Salmonella* from samples

Isolation was done carefully with consideration of aseptic conditions especially when using more than one enrichment broths and selective solid media at a time. Justification of using more than one media was to maintain purity as quality control of the procedure in order to assure reproducible results of isolated *Salmonella* pathogen throughout this study.

A total of 570 chicken eggs were examined by pooling of samples (Appendix 1) using protocol described by Wallace *et al*. (2009) and PHE, (2014) using both egg contents and eggshell surfaces which formed a total number of 30 pools contributed by 24 pooled samples (18 from raw exotic and 6 from local eggs pools), and 6 pooled sample from boiled eggs (Appendix 1).

Analysis of 480 raw eggs was done by pooling randomly selected eggs into two (2) pools of each 20 eggs contents obtained from each 40 eggs collected from each house/farm whereas a total of 90 boiled eggs collected also were pooled into two (2) pools of each 15 eggs contents obtained from each 30 eggs collected from each farm/house then inoculated into respective media and incubation time (Appendix 2). Analysis of eggshell surfaces was done by swabbing the surface of each six eggs of same status /parameters as raw and boiled as a pool (PHE, 2014) (Appendix 3).

According to methodology used (Wallace *et al.*, 2009; PHE, 2014) each pooled sample analysed was regarded as one composite sample and justification of interpretation results of isolates was based on qualitative results of either pathogenic *Salmonella* (*Salmonell*a *enterica*) is present or absent from examined eggs. However, the presence of more than one colonies of *Salmonella* pathogen in one pool was regarded as one *Salmonella*, this is due to pooling method used and based on the main objective of this study which intended to check bacteriological quality/status of consumed eggs by isolating pathogenic *Salmonella* to genus level (*Salmonella enterica).* Isolation steps has been described and shown in series below.

3.5.3.1 Preparation of samples

Shell surface samples were collected aseptically by using a swabbing technique (PHE, 2014; Tsegaye *et al.*, 2016) then dipped in sterile BPW (buffered peptone water) and inoculated into 10 ml BPW in screw capped bottles. After swabbing, unbroken eggs were washed by normal saline (8.5g/l) and submerged into disinfection solution consisting of 3 parts 70% ethanol to 1 part iodine solution (250 ml iodine/potassium iodide solution to 750ml 70% ethanol) for exactly 10 seconds then air dried (Wallace *et al.,* 2009).

3.5.3.2 Inoculation of shell surface swabs

Pools of shells surface swabs in 10ml Buffered Peptone Water (BPW) were incubated at 37°C for 24hrs. Thereafter, were transferred in two selective enrichment broths (2 way steps) with slight modifications using 1:10 dilution (1ml sample + 9ml Selenite Broth). Selenite broth was a modification of Muller Kauffman Tetrathionate Novobiocin (MKTTn), and the second broth were added at 1:100 (1ml sample + 99ml Rappaport Vasiliadis Soy Broth) (Vasilliadis *et al.,* 1983). Selenite Broth (SB) was incubated at 37°C

for 24hrs whiles Rappaport Vasiliadis Soy (RVS) were incubated at 42°C for 24hrs (Appendix 2).

3.5.3.3 Sub-culturing of shell surface swabs

From Inoculated enrichment broths (Selenite Broth and Rappaport Vasilliadis Soy broth) were sub cultured into basic solid media; Blood Agar (BA) and Mac Conkey Agar (MCA), and in moderate selective agars; Xylose Lysine Dextrose (XLD) and Bismuth Sulphite agar (BS), and then in highly selective agar; Xylose Lysine Tergitol (XLT4) using a sterile 10µl loopful of proper mixed cultures. All inoculated agars were incubated at 37°C for 24hrs (Wallace *et al.*, 2009; PHE, 2014). Negative growth culture results were re-incubated for another 24hrs (Appendix 2) and results recorded.

3.5.3.4 Inoculation of egg content pools

3.5.3.4.1 Room temperature incubation of content cools

Sterilized eggs were broken aseptically and the contents were mixed properly by blending for 2 minutes, ending up with a homogeneous solution (PHE, 2014) then dispensed into sterile 1000 ml capacity Schott bottles. Finally, the contents were mixed properly by inverting the bottles for 25 times as described by ISO (International Organization for Standardization) stipulated by Wallace *et al.* (2009). Then the egg contents pools were incubated at room temperature for 96hrs in order to recover injured or non-viable cells which may occur during egg processing (Appendix 3).

3.5.3.4.2 Inoculation of eggs content pools in pre enrichment broth

After incubation, egg content pools were mixed well by swirling and 25ml were transferred into 500ml Schott bottle containing 225ml Tripticase Soya Broth (TSB) with

$FeSO_4$ (35mg) for raw eggs and without $FeSO_4$ for boiled eggs , then incubated at 35℃ for 24hrs (Wallace *et al*., 2009) (Appendix 3).

3.5.3.4.3 Sub -culturing of egg content pools in enrichments and solid media

After incubation, the enrichment broth cultures of egg contents were sub cultured in (two way steps); in Selenite Broth (SB) as a modification of Tetrathionate broth (TT) (PHE, 2014) and in RRV, by adding 1ml of cultures in 9ml Selenite Broth (SB), and 0.1ml of the culture in 1ml of RV broth. The inoculated Selenite Broth was incubated at 35℃ for 24hrs (PHE, 2014) whereas RV was incubated at 42℃ for 24hrs (FDA, BAM 8, 2009). After incubation, proper mixed broth cultures were inoculated on non-selective agars (BA and MCA), moderate selective agars (XLD and BS), and highly selective agar (XLT4), then they were incubated at 35℃ for 24hrs. The Negative cultures were re-incubated for another 24hrs (Wallace *et al*., 2009) (Appendix 2).

3.6 Identification of Suspected *Salmonella* Colonies

Identification of suspected *Salmonella* colonies from different inoculated media was done phenotypically by using Gram stain method and biochemical testing, and genetically confirmed by using PCR technique.

3.6.1 Macroscopic identification

Phenotypic identification of *Salmonella* isolates was done macroscopically by using more than one type of routine and selective media in order to assure reproducibility of results (similar results) based on morphological appearance of suspected different colonies of *Salmonella* after cultured on routine (BA and MCA), selective agar media (XLD, XLT4 and BS), and biochemical test appearance on tested substrates such as urea agar and citrate. Characteristic features of suspected *Salmonella* isolates were compared with

typical features shown by *Salmonella typhimurium* (Surrey University, USA) and *Escherichia coli* (Sokoine University, Tanzania) as positive and negative controls respectively in this study. Some of these features were such as colour, shape, unique smell, formation of special colour as indication of some gas production such as black centred colonies as production of hydrogen sulphide gas, and presence of brown metallic sheen appearance on selective agar like XLT4 and BS respectively was indication for *Salmonella* isolation, sizes, and colonial formation such as moist, raised, colour of colonies and specific colours formed by certain *Salmonella* species on certain media were observed and recorded (Table 1).

Table 1: Macroscopic Identification of Suspected Pathogenic *Salmonella* on used Solid Media

Media	**Colonial Morphology**
MCA	NLF colonies (pale pink colonies)
BA	Greyish, small, raised moist non haemolytic colonies
XLD	Large glossy, pink colonies with or without black centre or completely black colonies
XLT4	Black colonies or black cantered with a yellow periphery, yellow to red (*Salmonella enterica*)
BSA	Brown, grey or black colonies, some time they have metallic sheen. Surrounding medium which is usually brown at first turns black after 48hrs incubation, in what is the so called *halo effect.*

3.6.2 Microscopic identification

Smears of the suspected colonies were prepared on microscopic slides by using sterile wire loop, fixed and stained by using Gram staining technique. The stained smears were observed under microscope using 100x objective lens in order to identify typical

characteristics of the bacterium based on gram stain reaction, cell morphological appearance, and cell arrangement (Fig. 6).

3.6.3 Biochemical tests identification

Prior to biochemical identification, several purification steps for the isolates were employed in order to use pure discrete colonies through sub culturing them into a series of non-selective, moderate and highly selective agar media such as MacConkey agar (MCA), Xylose Lysine Dextrose agar (XLD) and Bismuth Sulphite (BS) agar and Xylose Lysine Tergitol (XLT4) agar respectively so as to ensure purity of isolates. The biochemical tests of suspected *Salmonella* species were performed and recorded using several tests: TSI and LIA as differential media, enzyme production tests: Urease and catalase, IMVIC tests: (Indole, Methyl Red, Voges Proskauer and Citrate) (Table 2).

Table 2: Typical (Phenotypic) Biochemical Characteristics Shown by Suspected Pathogenic *Salmonella* Isolates on Tested Biochemical Test/substrates

Test or substrate	**Positive results based on colour, bubbles, fuzzy growth appearance**
Glucose (TSI)	yellow butt
Lysine decarboxylase (LIA)	purple butt
Production of H_2S from (TSI and LIA)	Blackening
Indole test	violet colour at surface
Voges-Proskauer test	no colour change
Methyl red test	diffuse red colour
Simmon`s Citrate	growth, blue purple
Urea for urease enzyme production	no colour change (not hydrolysed)
Catalase enzyme test	formation of bubbles
Motility test on motility test medium	fuzzy and non- fuzzy growth (Motil*e* and non-motile) *Salmonella enterica*

3.6.3.1 Triple Sugar Iron (TSI) and Lysine Iron Agar (LIA) biochemical tests

Suspected *Salmonell*a isolates were subjected to different biochemical tests using differential media such as Triple Sugar Iron agar (TSI) and Lysine Iron Agar (LIA) to test fermentation of different sugars and lysine decarboxylation processes. Inoculation of isolates in TSI and LIA was done aseptically using McFarland tubes with a straight wire and pure cultures of the isolates. The cultures were incubated at 37°C for 24hrs where their growth profiles were read and recorded based on appearance of the Slant, butt with/ without production of H_2S (Tseyage *et al.,* 2016).

3.6.3.2 Urease enzyme test

The isolates were tested for their ability to produce this enzyme by inoculating them into urea agar medium using sterile straight wire and incubated at 37°C for 24hrs. After incubation time the change of colour of the medium were examined and recorded.

3.6.3.3 IMVIC biochemical test

Suspected isolates were tested for their ability to produce some indication profile from this combination of four substrates (I: Indole; M: methyl; V: Voges Proskauer and C: Citrate). The test was done by using sterile wire loop to pick few pure colonies and inoculated in respective substrates such as Peptone water for indole production, Methyl-Red-Voges Proskauer (MR-VP medium for production of Methyl red, and Voges Proskauer reactions, and Simmon`s Citrate for utilization of citrate as source of energy. The inoculated substrates were incubated at 37°C for 24-48 hrs. Then the results were read and recorded.

3.6.3.4 Motility test

The suspected *Salmonella* colonies were done in order to test their ability to move from one place to another. This test was done by using the motility medium agar prepared in

McFarland tubes as semi–solid and incubated at 37℃ for 24 hrs. After incubation the results were read and recorded.

3.6.3.5 Catalase enzyme test

All suspected *Salmonell*a spp were tested for the ability to produce this enzyme. Sterile tooth picks were used to pick up the pure colonies and transferred on clean microscopic slides. Sterile dropper was used to take few millilitres of 3% Hydrogen peroxide (H_2O_2) and mixed up with the test organism on the same slide, then observed for the formation of gas bubbles. The results were read and recorded.

3.7 PCR for Detection of Pathogenic *Salmonella* spp

3.7.1 DNA extraction

Salmonella bacterial genomic DNA was extracted by using the boiling method. Frozen bacteria stored at 45℃ in 15% glycerol nutrient broth were activated in Nutrient broth (NB) and inoculated into MCA then NA and incubated at 37°C for 24 hrs for visible discrete pure colonies. Rapid DNA extraction was done using the protocol (Paiao *et al*., 2013; Wattiau *et al.,* 2013) where 5-10 pure colonies (PHE, 2014) were scooped using sterilized wire loop and added into 1.5ml eppendorf tubes containing 200µl of distilled water. The tube caps were secured by sealing with clean masking tapes to avoid contamination during boiling process. Finally, the tubes were placed in boiling water bath at 95 -100°C for 10 minutes. Centrifugation was done at 14 000 x g for 1 minute and the supernatant was kept to be used as template DNA (Rahn *et al.,* 1992; Jamshindi *et al.*, 2009;Wattiau *et al*., 2011).

3.7.2 DNA gene amplification of suspected *Salmonella* spp

The DNA amplification of the *invA* gene was carried out using *Spec* primer pair (Appendix 9) which was obtained from (Macrogen Inc. Seoul, South Korea)

(Shanmugasamy *et al*., 2011). The PCR was run in a total volume of 20µl with the initial concentration of 0.5µM of primers. The PCR reaction mixture comprises of 2µl of suspected isolates DNA template, and 18µl of prepared Master mix reaction made by adding required 1 µl volumes of primers, 10 µl Dream Taq Green PCR Master Mix (2x), and 7 µl Nuclease Free Water (NFW) in which the latter two reagents were obtained from Thermo Fisher Scientific Invitrogen Company, Westburg). The PCR mixture were run for DNA amplification in TAKARA Thermal Cycler Dice Gradient TP600 (Takara Bio, Tokyo, Japan) by using the amplification conditions of 35 cycles, initial denaturation at 95℃ for 5 minutes, denaturation at 95℃ for 1minute, annealing temperature at 62℃ for 30 seconds, extension at 72°C for 30 seconds, final extension at 72°C for7 minutes, and holding time at 4℃ with the expected amplicon size of 284 bp (Jamshindi *et al*., 2009; Paiao *et al.,* 2012).

3.7.3 Agarose gel electrophoresis and visualization of PCR DNA products

The PCR amplification of products were analysed by Agarose gel electrophoresis using 1.5% agar rose gels prepared by dissolving 1.5 g of agarose (Paiao *et al.*, 2013) mixed with 100 ml of 10 x Tris - Borate EDTA (TBE) buffer (10ml TBE+90ml sterile distilled water) heated to boil on hot plate. The agarose gel was cooled down to 45℃ where 3µl of GelRed nucleic acid stain (Phenix Research Product, Condler, NC) was added to stain the gel and poured into gel casting tray fixed with a combs for solidification process. Ten µl of each PCR products were run on a 1.5% agarose gel electrophoresis in 10x TBE buffer with 100 bp DNA ladder (BioLabs, New England, USA) as molecular marker, and added with 6x loading dye (Excellgen, Rockville, MD, USA). Electrophoresis were run either at 80 / 100 voltages for 30/ 40 minutes respectively using Mupid- One Electrophoresis system (Advance, Tokyo, Japan). The agar rose gel was visualized under UV trans-illuminator in gel Doc. EZ Imager machine (Bio Rad, California, USA).

3.8 Antibiotics Susceptibility Testing of Pathogenic *Salmonella* Isolates

Agar disc diffusion method was used to obtain antimicrobial susceptibility profile of *Salmonella* isolates by using protocol previously described (Bauer *et al*., 1966; Yemisi *et al.,* 2007; Tsegaye *et al*., 2016) with some modifications. *Salmonella* isolates stored in 15% glycerol/ nutrient broth were sub cultured on Nutrient Agar and incubated at 37°C for 24 hrs. The inoculum of isolates and standard *S. Typhimurium* (USA, Suresh University) used as positive and quality control bacterium for the procedures were employed and aseptically prepared by taking one pure colony from discrete colonies on NA using sterile wire loop and emulsified in 200µl sterile normal saline solution. Turbidity of bacterial suspension was adjusted to 0.5 Standard McFarland solution. The inoculums suspension of isolates and the control were spread on dried surface of Muller Hinton agar using the L-shape glass rod by rotating the hand at 90° angle. Inoculated plates were dried for 30 minutes before applying drugs to avoid dilution of the drugs. Thereafter, selected first line and the current drugs such as third generation antibiotics impregnated discs obtained from (Oxoid, England and Liofilchem-Italy) were applied on the agar surface and incubated at 37°C for 18hrs.

Antibiogram profile were determined based on diameter of inhibition zones showed by respective drugs and in (mm) using a ruler and interpreted CLSI (2014) which recorded as Sensitive (S), Resistant (R), and Intermediate sensitive (I). Isolated pathogenic *Salmonella* were tested against first line agents such as Fluoroquinolone, Aminoglycosides, and third generation Cephalosporins such as Sulfamethaxazole-Trimethoprim (SXT25µg), Chloramphenicol (C10µg), Tetracycline (TE30µg), Gentamicin (Cn10µg), Ciprofloxacin (CIP5µg), Imipenem (IMI10µg), Cefotaxime (CTX30µg), Ceftazidime (CAZ30µg), Ceftriaxone (CRO30µg) and other antibiotics such as Kanamycin (K30µg) (Appendix 4). The chart also used as quality control (QC) for test

procedures in order to ascertain these results where inhibition zones of *Salmonella enterica* were interpreted by comparing with those provided by the chart provide and recorded as Sensitive (S/≥mm), Intermediate (I/≤mm), and Resistant (R/<) has been shown in Appendix 4 and 5.

3.9 Data Analysis

Data analysis in the present study was done by using GenStat software (v.14). Descriptive statistics such as proportions was computed to check the magnitude of *Salmonella enterica* isolation from the samples. Chi-square test was used to test for significance difference of *Salmonella enterica* isolates between egg categories at 5% significance level.

3.10 Ethical Issues

Ethical clearance and permission to conduct the present study was obtained based on University guidelines stipulated after proposal presentation and evaluation. Additionally, the title of this research also justified both economic and public health threats of chicken eggs in Tanzania. Hence, acceptance and approval of this study from relevant authorities lead into release of permission to conduct research (Appendix 10).

3.11 Limitations

Failure to determine *Salmonella enterica* serovars as well as Phylogenetic tree was considered as limitations of the study.

Failure to conduct questionnaires in order to assess risk factors contributed to contamination of these eggs.

Failure to collect large sample size of local chicken eggs from selected areas due to nature and management systems experienced in this type of chicken.

CHAPTER FOUR

4.0 RESULTS

The results from the present study has been described and showed stepwise/ chronologically below based on objectives of the study.

4.1 Physical Examination of Individual Shell Surfaces of Eggs Types

Out of 570 individual eggs examined physically, 1(0.57%) egg was identified green in colour indicating that an egg could possibly be contaminated with bacteria. However, is not necessarily to be *Salmonella* infection (Fig. 4). Chicken eggs examined were from four Morogoro Municipality wards in which Mlimani ward contributed large number of eggs (400) from raw egg parameters; clean (160), cracked (120), and faecal soaked eggs (120) compared to Magadu which contributed (40) raw clean eggs and (30) boiled eggs whereas Boma ward contributed (60) boiled eggs and Kihonda contributed (40) raw Clean eggs.

Figure 4: Physically observed green egg (the first egg from right side) among eggs.

4.2 Bacteria Isolation

Results from the present study showed that, eleven (11) *Salmonella enterica* were recovered from eleven pooled samples out of 30 pools examined. Six of the positive samples (20%) were egg content while five positive samples (16.7%) were from eggshell surfaces. The total prevalence of Salmonella enterica in eggs was 36.7% (95% Confidence interval: (19.4% – 53.9%). The occurrence of *Salmonella enterica* from eleven (11) pools of chicken eggs examined in Morogoro Municipality and ward is shown in Table 3 and Appendices 7, and their prevalence (Appendix 8).

The results showed that local eggs were highly contaminated (20.0%) than exotic eggs (16.7%) whereas raw eggs were highly contaminated (26.7%) than boiled eggs (10%). Interestingly clean eggs were highly contaminated (20.0%) than faecal socked (13.3%).Furthermore it was revealed that there was significant difference on occurrence of *Salmonella enterica* between the boiled and raw eggs examined ($P<0.05$) (Table 4).

Table 3: Occurrence of *Salmonella enterica* isolated from eleven pooled samples of chicken eggs examined in Municipality wards.

Wards	***Salmonella enterica* in examined pools of eggs parameters**		**Total *Salmonella***	**% of *Salmonella***
	Pool 1	**Pool 2**		
Mlimani	1	3	4	13.33
Boma	1	2	3	10.00
Mzinga	1	1	2	6.67
Kihonda	1	1	2	6.67
Total	**4**	**7**	**11**	**36.67**

Table 4: Occurrence of *Salmonella enterica* and egg category

Eggs categories	Occurrence (n)	Occurrence (%)	χ^2	df	P value
Local	6	20.0	0.37	1	0.54
Exotic	5	16.67			
Raw	8	26.67	9.28	1	0.00
Boiled	3	10.0			
Faecal socked	4	13.3	1.62	1	0.20
Clean	6	20.0			

Statistical results showed significant difference between Raw and Boiled eggs (*P*-value=0.00)

4.3 Identification of Suspected Pathogenic *Salmonella* Isolates

4.3.1 Macroscopic identification

Macroscopic features of isolated pathogenic *Salmonellae* (S*almonella enterica*) showed colonial morphological differences on different media inoculated: routine media such as MCA (colonies were non lactose fermenters), on BA some *Salmonellae* colonies were (greyish/whitish, medium size, raised, non-haemolytic colonies) whereas from selective media such as XLD colonies of some species showed blackish colonies which have the same colonial features with (*S. typhimurium* used as control), and on XLT4 media colonies of some *Salmonellae* were black cantered with a yellow periphery, yellow to red medium size, on selective agar media (Fig. 5).

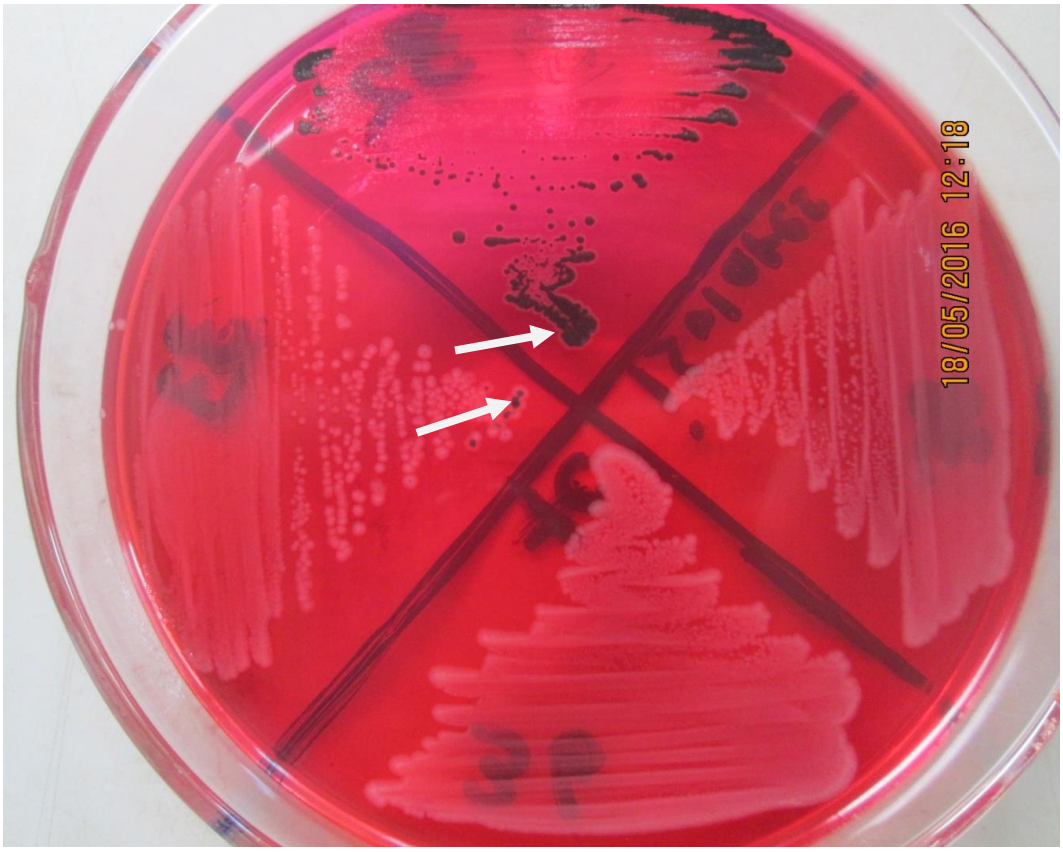

Figure 5: Typical bacteria colonies of isolated *Salmonella enterica* on Xylose Lysine Tergitol (XLT4) shown by the white arrows

4.3.2 Microscopic identification

All eleven (11) isolated pathogenic *Salmonellae* (*Salmonella enterica*) were microscopically Gram negative, non sporing, rods in scattered arrangement which obtained after staining of pure colonies of suspected *Salmonellae* and used positive control organism (*S.T*) as shown in Fig. 6 below;

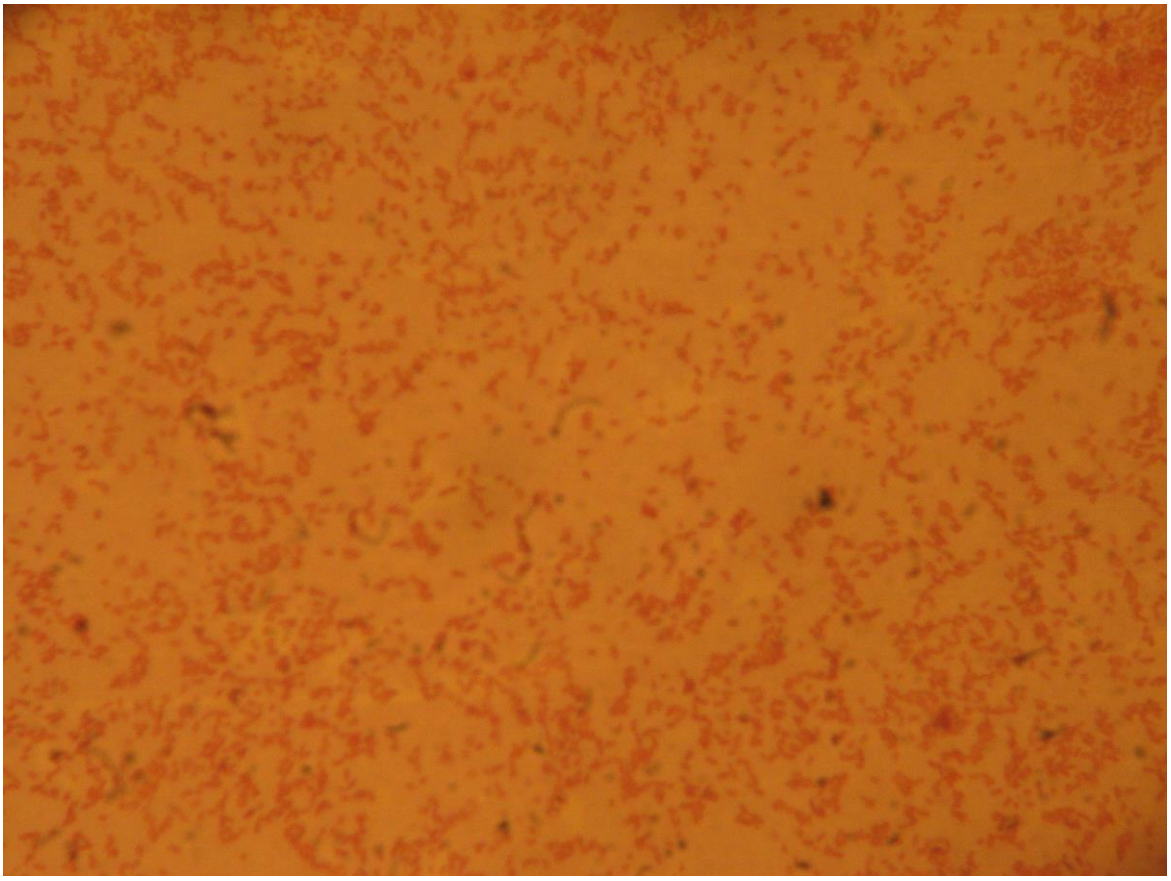

Figure 6: **A picture showing Gram negative non sporing rods bacteria (*Salmonella enterica* isolates)**

4.3.3 Biochemical identification tests of suspected pathogenic *Salmonella* isolates

Biochemical tests done for identification of isolated *Salmonella enterica* as pathogenic *Salmonellae* included; TSI and LIA tests (Fig. 7) and IMVIC tests (Fig. 8) and motility test. The enzymes production tested were for Catalase and Urease. The results of all 11(36.67%) *Salmonella* isolates tested with these biochemical tests together with the control (*S.T*) both revealed typical characteristics of positivity reactions indicated that they were *Salmonella enterica* been isolated from examined eggs. In addition, results from catalase enzyme test showed positive reactions with the formation of air bubbles when mixed with 10% hydrogen peroxide on microscopic slide. For Citrate test all tested *Salmonella* reacted positive with colour change as dark blue whereas for urease enzyme

test, all *Salmonella enterica* isolates and the control were urease negative with no colour change. Results for motility test, some of isolated *Salmonella enterica* were motile whiles others were non motile *Salmonellae*. TSI, LIA and IMVIC test results for tested *Salmonella enterica* and the control organism gave positive reactions accordingly (Fig. 7) and (Fig. 8).

Figure 7: Triple Sugar Iron agar (TSI) and Lysine Iron agar (LIA) test results for suspected pathogenic *Salmonellae* isolates

Note: From left side; tube 1, 3 and 6 show positive TSI test result indicated by Slant: red; Butt: yellow with production of H_2S gas (black in medium). Tubes 2, 4 and 5 show positive LIA test results indicated by Slant: Purple; Butt: Purple with production of H_2S gas (black in medium). Additionally, the IMVIC test results are shown in (Fig. 8).

Figure 8: Indole, Methyl Red, Voges Proskaue, Citrate (IMVIC) test showing positive results for suspected pathogenic *Salmonellae* isolates

Note: From left sides; bottle 1: Indole (I) negative (no colour changed); bottle 2: Methyl red (MR) positive (colour changed); bottle 3: Voges- Proskauer negative (no colour changed); bottle 4: Simmons Citrate (C): positive (colour changed).

4.4 PCR Detection of the *invA* Gene of Isolated *Salmonella enterica*

The PCR test results confirmed that, all 11(36.67%) presumed *Salmonella*e isolates were pathogenic when tested with using the specific primer (284 kb) for pathogenic Salmonella. Therefore, the results indicated that *Salmonella enterica* have been isolated among 30 pooled samples of eggs examined in this study (Fig. 9).

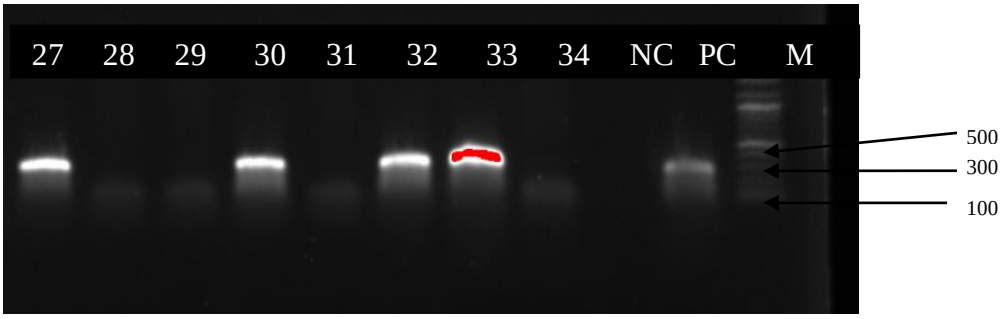

Figure 9: Polymerase Chain reaction (PCR) detection for *Salmonella enterica* isolates: PCR products Visualized under UV trans illuminator

Note: Lane 27, 30, 32 and 33 are positive amplified pathogenic *Salmonella* at 284 bp DNA fragment of the gene, lanes: 28, 29, 31, and 34 are negative amplified bacteria, NC is negative control, PC: positive control and M: I Kb DNA ladder

Results of this study have revealed that *Salmonella enterica* has been recovered from 11 pools out of thirty pooled samples with a total prevalence of 11 (36.7%) contributed by both egg contents 6 (20.00%) and eggshell surfaces 5 (16.67%). Additionally, the occurrence of *Salmonella enterica* from other parameters was; 6 (20.0%) from local eggs and 5 (16.7%) from exotic eggs whereas from boiled eggs were 3 (10.0%) and 8 (26.7%) from raw eggs. Also 1 (3.3%) *Salmonella enterica* were from cracked eggs while 4(13.3%) were from faecal contaminated eggs, and 6 (20.00%) *Salmonella enterica* were from clean eggs (Appendix 8). Among the wards involved Mlimani had many pools of eggs and isolated high prevalence (13.33%) of *Salmonella enterica* compared to other wards in Municipality (Table 3).

4.5 The Antimicrobial Susceptibility Profile of Isolated *Salmonella enterica*

Ten types of antibiotics test results are shown in tables 4, 5 and Appendices 5 and 6.

Antibiogram test results according to the number of *Salmonella enterica* tested against drugs revealed that; all 11 (100%) *Salmonella enterica* were resistant to Kanamycin only. All 11 (100%) *Salmonella enterica* exhibited sensitivity to Imepenem (IMI), Chloramphenicol (C), Ciprofloxacin (CIP), and Sulfamethaxole-Trimethoprim (SXT). 10(91%) *Salmonella enterica* were sensitive to Ceftazidime (CAZ), Gentamycin (Gn), Tetracycline (Te) whereas 9(82%) *Salmonella* isolates were sensitive to Cefotaxime (CTX) and Ceftriaxone (CRO). The patterns (Table 5) and the percentages (Table 6) of antibiotics susceptibility results for isolated *Salmonella enterica*.

Table 5: The patterns of antibiotic susceptibility profiles based on number of *Salmonella enterica*

Inhibition zone	K30	Gn10	CIP5	CTX 30	C10	Te	CAZ	IMI	SXT	CRO
S	0	10	11	9	11	10	10	11	11	9
I	0	1	0	2	0	1	1	0	0	2
R	11	0	0	0	0	0	0	0	0	0
Total number of isolates	11	11	11	11	11	11	11	11	11	11

KEY: Gn: Gentamycin; CIP: Ciprofloxacin; C: Chloramphenicol; Te: Tetracycline; IMI: Imepenem; K: Kanamycin; CXT: Cefotaxime; SXT: Sulfamethaxole-Trimethoprim; CAZ: Ceftazidime; CRO: Ceftriaxone.

Table 6: The percentages of antibiotics susceptibility results for isolated *Salmonella enterica*

Inhibition zone	K30	Gn10	CIP5	CTX 30	C10	Te	CAZ	IMI	SXT	CRO
S	0.0	90.9	100.0	81.8	100.0	90.9	90.9	100.0	100.0	81.8
I	0.0	9.1	0.0	18.2	0.0	9.1	9.1	0.0	0.0	18.2
R	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

KEY: Gn: Gentamycin; CIP: Ciprofloxacin; C: Chloramphenicol; Te: Tetracycline; IMI: Imepenem; K: Kanamycin; CXT: Cefotaxime; SXT: Sulfamethaxole; CAZ: Ceftazidime; CRO: Ceftriaxone.

CHAPTER FIVE

5.0 DISCUSSION

The present study assessed bacteriological quality of local and commercial chicken eggs against pathogenic *Salmonella* (*Salmonella enterica*) and their antibiogram profile. Eggs are delicious and most nutritious foods with high values and eaten by all ages of people. However, nutritious they are also vulnerable to food borne pathogens such as *Salmonella enterica* which can survive in under cooked eggs or raw egg dishes and cause human salmonellosis (Bayu *et al*., 2013; PHE, 2014; Peres *et al*., 2014).

According to this study, *Salmonella enterica* was recorded in egg shells surfaces with prevalence (16.7%) and egg contents (20%). The prevalence levels of *Salmonella enterica* contamination of eggs shell surfaces and egg contents in other countries were reported to vary from 0% in egg surfaces (Jamshindi *et al*., 2009) to 5.9% of eggshells surfaces, and 1.8% of egg contents in India (Suresh *et al*., 2006). The prevalence of *Salmonella enterica* in the current study is higher than those reports, with an overall prevalence of 36.7% for both egg surfaces and contents. This could be due to different protocols used among studies whereby in the present study the pooling of samples were employed based on Wallace *et al*., (2009) and PHE (2014), and the use of pre and enrichments media which increased chances of *Salmonella* recovery from eggs as described by Vassiliadis (1983).

In the present study both conventional and standard methods utilizing both egg shell surfaces and egg contents of raw and boiled chicken eggs were used for isolation and identification of (pathogenic) *Salmonella enterica* which were confirmed by using PCR and *Spec* primer which detected *Salmonella invA* gene of *Salmonella* genus. All PCR products of *Salmonella enterica* obtained had an amplicon size of 284 bp which was

similar to that obtained from studies done by Jamshindi *et al*. (2009), Shanmugasamy *et al*. (2011), Paiao *et al.* (20012).

Invitro amplification of DNA by the PCR method is a powerful tool in microbiological diagnostics (Malorny *et al*., 2003a) where several *Salmonella* genes are used to detect *Salmonella* virulence genes and serotypes (Shanmugasamy *et al*., 2011) where these virulence genes includes chromosomal genes like the one detected from the present study the Invasive A gene (*invA* gene) which coded for invasion of *Salmonella* into host cells and acts as indication of detection of all pathogenic *Salmonella* to genus level.

In developing countries such as Bangladesh, crack eggs are consumed especially by poor people because of their cheap prices (Hasan *et al*., 2009). However, their consumption can impose health problems if they are contaminated with *Salmonella enterica* and are consumed as raw or undercooked (Green *et al*., 2006; Edema and Atyese, 2009). Many studies have been conducted in Africa to determine the prevalence of pathogenic *Salmonella* species from chicken and eggs in many countries and indicated different results. For example in East Africa, in Tanzania a study by Mdegela *et al*. (2000) reported isolation of *Salmonella enterica* serovar *Gallinarum* from local chicken at a prevalence of 2.6%. Similar studies to isolate *Salmonella* from clean and cracked eggs was done in Ethiopia by Bayu *et al*. (2013) in clean eggs and by Jamshindi *et al*. (2009) in cracked eggs where Bayu obtained the prevalence of 2.5% and Jamshindi obtained the prevalence of 1.6% respectively. These results are with disagreement with that obtained by the present study where from clean eggs the prevalence of *Salmonella* isolates was 20% and from cracked eggs was 3.3%. The reason of this difference could be due to protocol used and difference in chicken management systems among chicken farms and between countries.

Additionally, many studies have been done in many countries in the world such as in Japan where the prevalence of *Salmonella* in eggs was 0.25% which is low than that obtained from this study. In United States from 1985 to 1995 the occurrence *Salmonella* outbreaks has indicated that (62%) out of 522 outbreaks of *S. enteritidis* infections were associated with foods prepared at restaurants/hotels, caterers, bakeries, cafeteria, or markets (Schoeni *et al*., 1995). The United State finding of *Salmonella* species in food prepared at restaurants and other catering services is slightly comply with results from this study due to the fact that even in this study *Salmonella enterica* has been isolated from boiled eggs collected from catering services such as cafeteria and restaurants from Boma and Mzinga wards.

The presence of faecal materials on egg shells can contaminate the egg contents through cracks present on the shells or via destroyed egg membranes due to temperature variations during storage. The apparently looking clean eggs can be contaminated with *Salmonellae* as shown by results of this study and many studies conducted in many countries in the world such as Ethiopia by Bayu *et al*. (2013). Also *Salmonella* can infect the reproductive tract of a chicken hen and contaminate forming eggs as ovaries where these infections are usually indicated by *Salmonella Enteritidis* which also persists after eggs being laid (Jamshindi *et al*., 2009).

The overall prevalence of isolated *Salmonell*a *enterica* in 30 pooled samples of chicken eggs examined in the present study was 36.7%. Statistical results of isolated *Salmonella enterica* shows that there was significance difference on occurrence of *Salmonella enterica* between raw and boiled eggs. The interpretation of these statistical results is that raw had high occurrence of pathogenic *Salmonellae* (*Salmonella enterica*) and be the

source of salmonellosis and if somebody consume a contaminated egg is likely to get the disease.

Additionally, the presence of pathogenic *Salmonellae* (*Salmonella enterica*) does not depend on egg types or locations as indicated from results of this study which isolated at 36.7% as an overall prevalence where the prevalence of *Salmonella enterica* from egg shell surfaces was low (16.7%) compared to that from egg contents (20%).

The study results have revealed that *Salmonella enterica* is from pathogenic serovars has been detected from each egg types of local and commercial chicken eggs examined for both shells surfaces and egg contents. These results call for concern to implement suitable measures by all stake holders from the farm to table chain in order to prevent animal and human salmonellosis as zoonotic infection caused by *S. enteritidis* and *S. typhimurium*. in Morogoro Municipality and country as a whole.

Antimicrobial testing of isolated pathogens such as bacteria is crucial to be done in order to determine the drug of choice to treat infections caused by microorganism such as *Salmonella enterica* and rule out any emergence of multiple drug resistance. Sensitivity results of the present study are similar with that of described by Abdullahi *et al.* (2013) who tested antibiotics susceptibility and conclude that *Salmonella enterica* can be killed by first line drugs such as Chloramphenicol, and Sulfamethaxole-Trimethoprim and the current used drugs such as Ciprofloxacin as drugs of choice. Sensitivity results of this study are disagreement with that done by Yemisi *et al.* (2014), Tseyage *et al.* (2016) and Abdullahi *et al.* (2013) in which tested *Salmonellae* were highly resistance to Chloramphenicol, Caftazidime, Ciprofloxacin, Tetracycline and Sulfamethaxole-Trimeyhoprim while from this study the isolates were sensitive to these antibiotics.

Similar sensitivity results of antibiogarm testing have shown between results of this study and that found by Al-Ledeni *et al*. (2014) where all *Salmonella* isolates were sensitive to current used drugs; third generation Cephalosporins; (CAZ, CTX and CRO) together with Ciprofloxacin and Imipenem and hence justifying their continual use in treating *Salmonellae* infections.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Salmonella enterica as pathogenic *Salmonellae* are present *i*n both egg contents and eggshell surfaces of local and exotic chicken eggs examined indicating that the products are unwholesome for human consumption, and the population of Morogoro Municipality is at high risk to acquire *Salmonellae* infections.

From statistical results of this study it shows that *Salmonella enterica* can be found in any type of eggs regardless of their nature/status such as apparent clean, raw or boiled, and consumption of these eggs if are contaminated they can be a source to get salmonellosis.

The study has revealed that eggs from Mlimani ward were highly contaminated 4 (13.33%) than other wards. The recovery of *Salmonella enterica* from shells surface swabs 5 (16.67%) and egg contents 6 (20%) indicates that horizontal (trans-shells and vertical (trans- ovarian) transmission routes might be the source of contamination of these eggs.

The presence of *Salmonella enterica* in boiled eggs 3 (10%) indicates that either eggs were undercooked while contaminated or were contaminated by a handler during processing or via contaminated water to wash plates in restaurant before consumption as expected. Raw eggs were highly contaminated 8 (26.67%) with *Salmonella enterica* than boiled eggs 3 (10%). Also the recovery of *Salmonella enterica* from local chicken eggs were high (6 (20%) than that from exotic chicken eggs 5 (16.67%).

Antibiogram results showed that all isolated *Salmonella enterica* were sensitive to majority of antibiotics tested. However, their usage for treatment of animal and human

salmonellosis/typhoid should be continued under justification, and if illness persists especially in immune-compromised individuals (HIV/AIDS) treatment is warranted.

6.2 Recommendations

Based on results of the present study, it would be pertinent to carry out routine surveillance on vulnerable foods such as eggs and further Molecular studies to determine *Salmonella enterica* serovars and phylogenetic trees existing within Morogoro Municipality.

Improvement of hygienic conditions from the farm level to table should be implemented by all stake holders respectively such as producers, consumers, food processers and farmers to minimize *Salmonellae* infections. Proper disposal of wastes especially those from animal materials/ litters should be exercised to avoid environmental contamination.

Highly recommended to initiate preparation of *Salmonella* vaccine as control measure in the country rather than importing from abroad because it will be very expensive.

Antibiogram results from this study showed that, isolated *Salmonella enterica* were sensitive to majority of drugs tested which included both first line drugs such as Chloramphenicol and the current used drugs such as Ciprofloxacin. However, their use to treat *Salmonellae* infections should be justified, and compliance with local guidelines needs to be audited to avoid emergence of drug resistance.

Recommendation should go to the Public health sectors to conduct various training/workshop/seminars in order to educate health stake holders from veterinary and medical sectors such as nurses, catering officers, veterinarians, farm workers about safe

handling of eggs for human consumption and other risk factors which contribute to acquire salmonellosis such as eating of raw, under cooked, faecal soaked and cracked eggs.

Highly recommended to people not implement some local believes such as addition of raw eggs in a child meal such as poragge and eating raw eggs for the sake of soften voices to singers where these increase chances to acquire *Salmonellae* infections.

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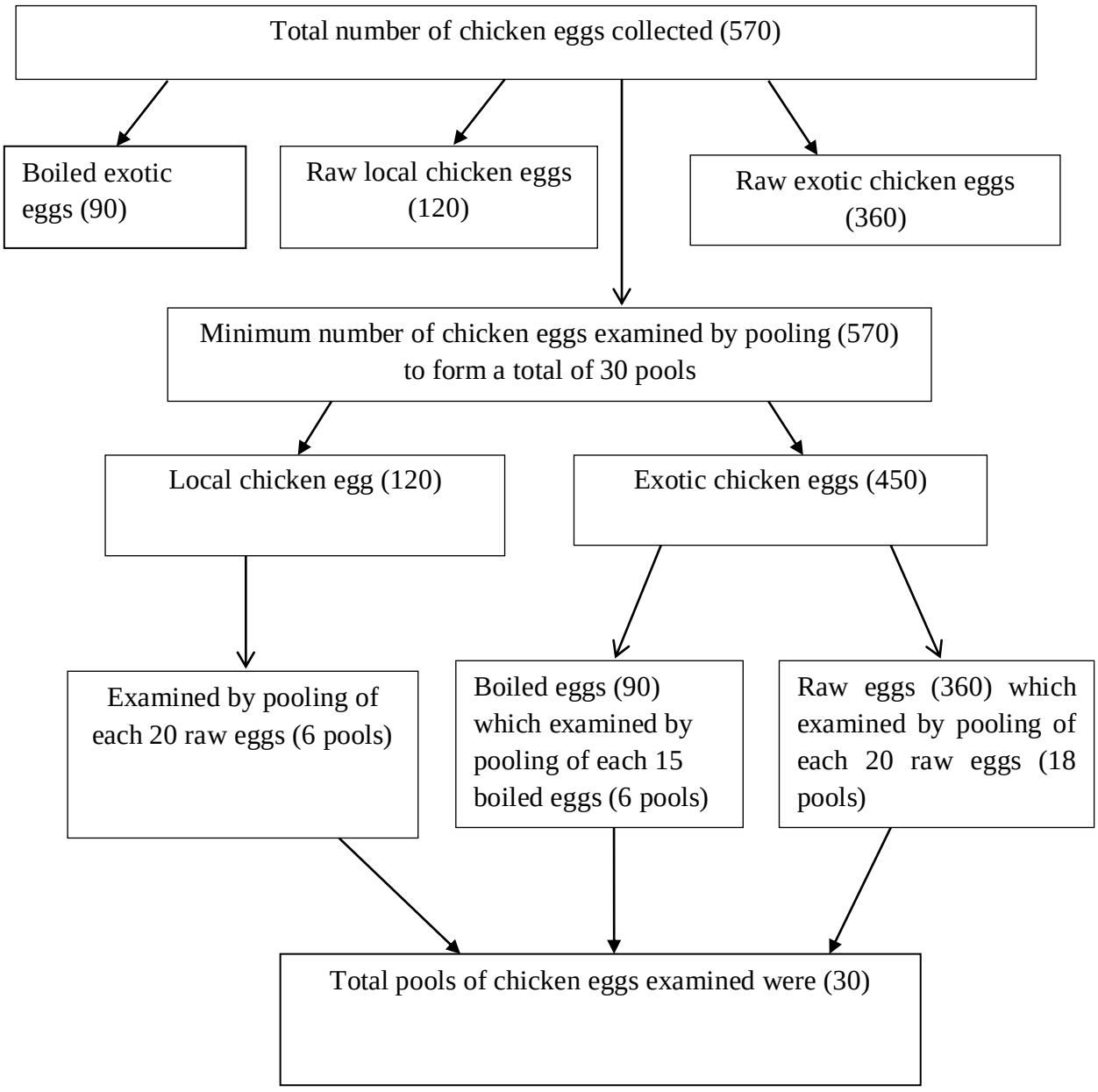
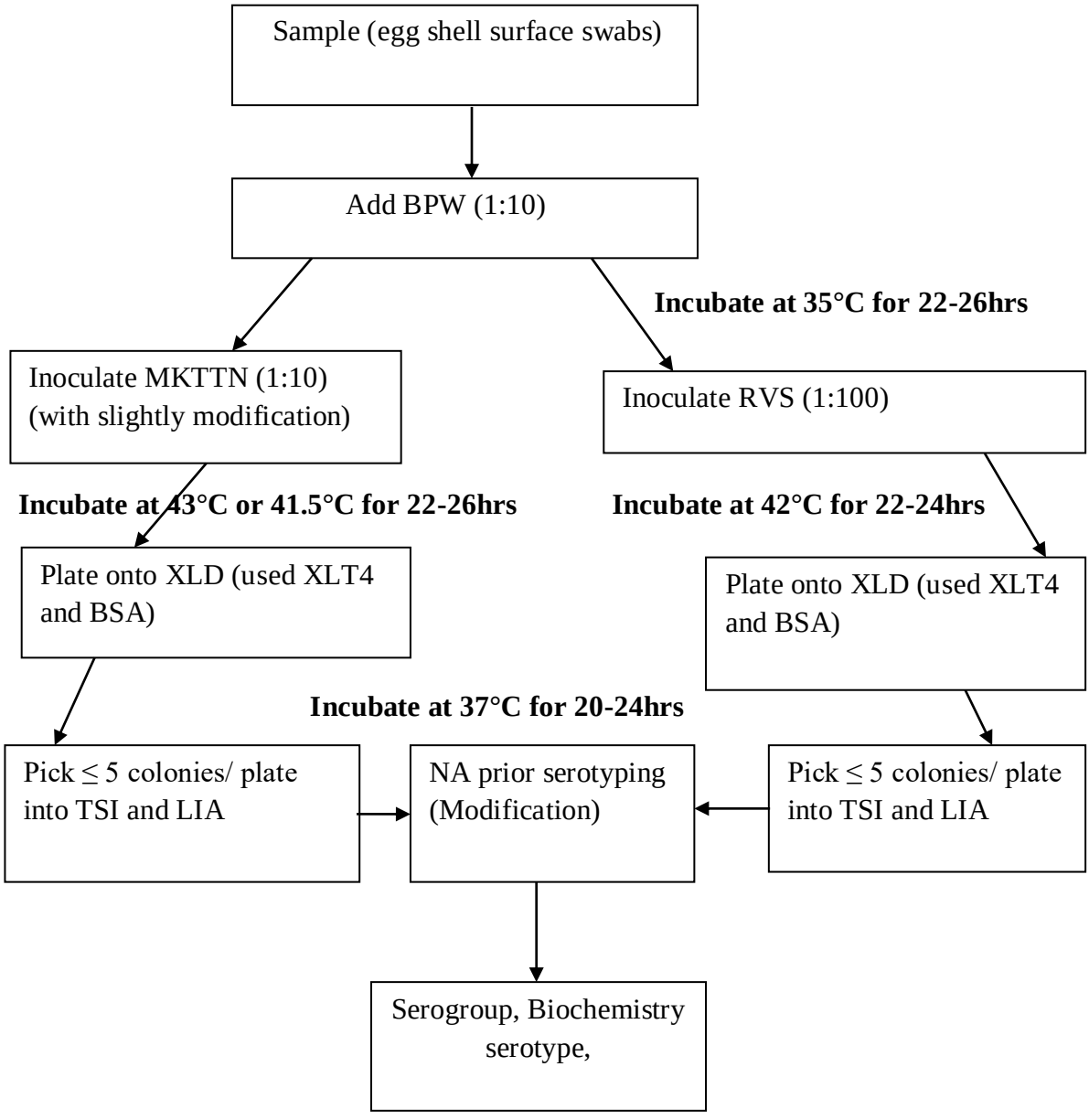
APPENDICES

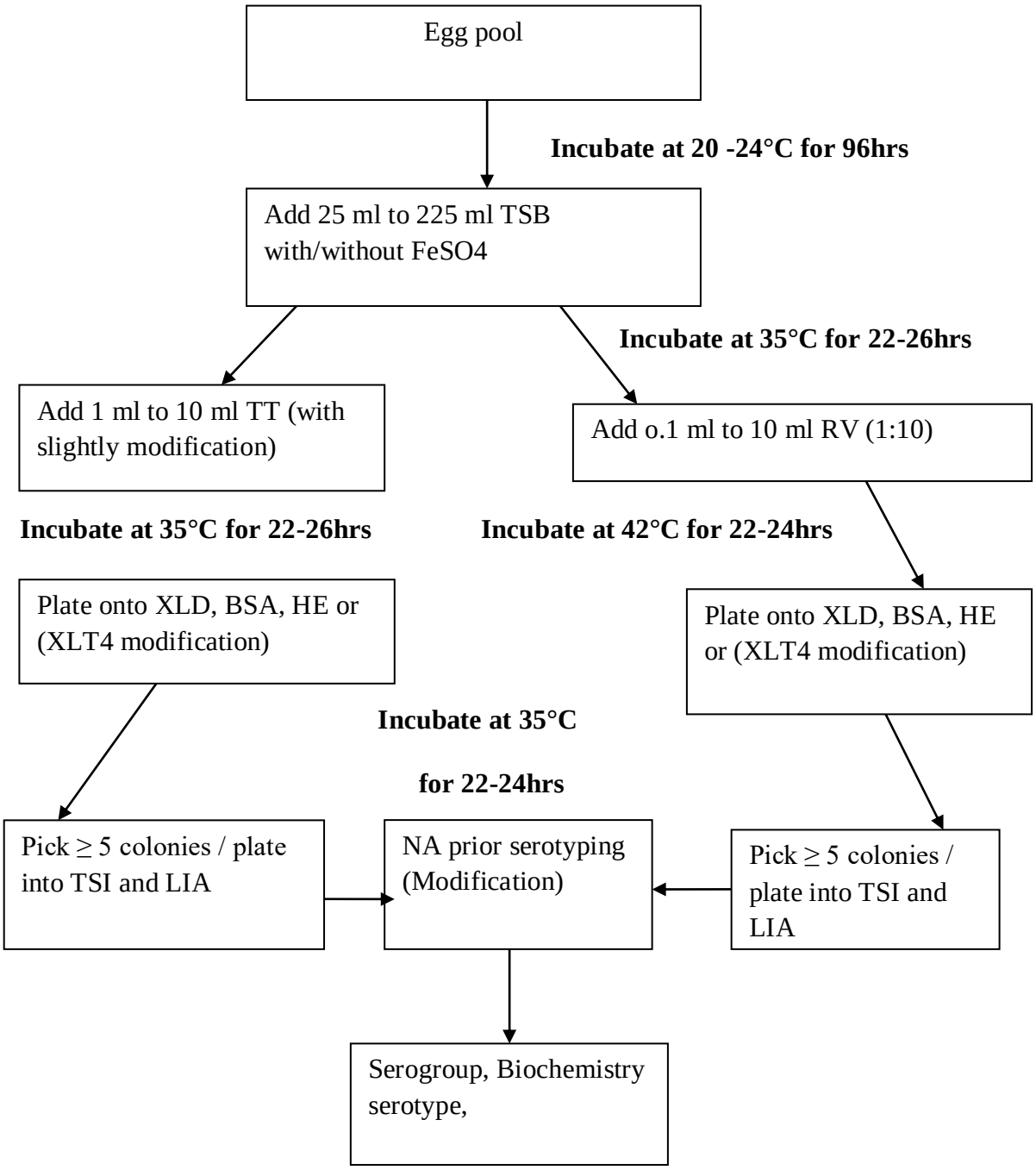
Appendix 1: The flow chart of total number of chicken eggs collected and examined by Wallace *et al.* (2009) and Thrusfield, M. (2007).



Appendix 2: The protocol flow chart for isolation of *Salmonella* spp from Environmental/egg surface swabs (PHE, 2014).



Appendix 3: The protocol flow chart for the isolation of *Salmonella* spp from eggs content pools (Wallace *et al*., 2009: PHE, 2014).



Appendix 4: Antimicrobial susceptibility profile interpretation chart (CLSI, 2014) for pathogenic *Salmonella* spp (used as quality control for the test).

Antibiotic Disc	**Code**	**S / ≥ mm**	**I /mm**	**R /≤ mm**
Amoxicillin	$Amoly_{25\mu g}$	17	14-16	13
Cefotaxime	$CTX_{30\mu g}$	26	23-25	22
Ceftazidime	$CAZ_{30\mu g}$	21	18-20	17
Ceftriaxone	$CRO_{30\mu g}$	23	20-22	19
Gentamycin	$GN_{10\mu g}$	15	13-14	12
Ciprofloxacin	$CIP_{30\mu g}$	21	16- 20	15
Trimethoprim	$SXT_{25\mu g}$	16	11-15	10
Imepenem	$IMI10_{\mu g}$	16	14-15	13
Chloramphenicol	$C_{10\ \mu g}$	25	24-23	22
Tetracycline	$Te_{30\mu g}$	25	24-23	22
Kanamycin	$K_{30\mu g}$	25	24 -20	19
Cephalothin	KF	16	6-20	15

Appendix 5: Interpretation results of antimicrobial susceptibility profile for tested *Salmonella enterica* based on diameter zones observed after comparison with interpretation chart (CLSI, 2014)

Antibiotic Disc	**Code**	**S / ≥ mm**	**I /mm**	**R /≤ mm**
Cefotaxime	$CTX_{30\mu g}$	26	0	0
Ceftazidime	$CAZ_{30\mu g}$	21	0	0
Ceftriaxone	$CRO_{30\mu g}$	23	21	0
Gentamycin	$GN_{10\mu g}$	15	0	0
Ciprofloxacin	$CIP_{30\mu g}$	21	0	0
Trimethoprim	$SXT_{25\mu g}$	16	0	0
Imepenem	$IMI10_{\mu g}$	16	0	0
Chloramphenicol	$C_{10\mu g}$	25	0	0
Tetracycline	$Te_{30\mu g}$	25	0	0
Kanamycin	$K_{30\mu g}$	0	0	19

Appendix 6: The antibiotics susceptibility patterns for isolated *Salmonella* spp

Sample	antibiotics used in µg											
ID	**K/ 30**	**GN/ 10**	**CIP/ 5**	**CTX/ 30**	**C/ 10**	**AMOLY/ 25**	**TE/ 30**	**CAZ/ 30**	**IMI/ 10**	**KF/ 30**	**SXT/ 25**	**CRO 30**
23	R	S	S	S	S	S	S	I	S	I	S	S
24	R	S	S	S	S	S	S	S	S	S	S	S
27	R	S	S	S	S	S	S	S	S	S	S	S
30	R	S	S	S	S	S	S	S	S	S	S	S
32	R	S	S	S	S	S	S	S	S	S	S	S
33	R	S	S	S	S	S	S	S	S	S	S	S
36	R	S	S	I	S	I	I	S	S	I	S	I
38	R	S	S	S	S	S	S	S	S	S	S	S
40	R	S	S	S	S	S	S	S	S	S	S	S
42	R	S	S	S	S	S	S	S	S	S	S	S
44	R	S	S	I	S	S	S	S	S	S	S	I

KEY; S: Susceptible; I: Intermediate; R: Resistant

Appendix 7: Occurrence of *Salmonella enterica* isolated from 30 pools of eggs examined in Municipality wards.

Wards	**Number of pools examined**	**Number of *Salmonella* isolates**	**% of *Salmonella***
Mlimani	20	4	13.33
Boma	4	3	10.00
Magadu	4	2	6.67
Kihonda	2	2	6.67
Total	**30**	**11**	**36.67**

The table show an overall prevalence 11(36.67%) of pathogenic *Salmonella enatreica* isolated from chicken eggs. One pool is regarded as one composite sample (Wallace *et al.*, 2009).

Appendix 8: Occurrence of *Salmonella enterica* isolated from chicken egg parameters in Morogoro Municipality Wards

Wards	Local	Exotic	Raw	Boiled	Cracked	Faecal soaked	Clean	Shells	Contents
Mlimani	2	2	4	-	1	2	1	1	3
Boma	-	3	-	3	-	-	3	2	1
Magadu	2	-	2	-	-	1	1	1	1
Kihonda	2	-	2	-	-	1	1	1	1
Total *Salmonella*	**6**	**5**	**8**	**3**	**1**	**4**	**6**	**5**	**6**
% of *Salmonella*	**20.00**	**16.67**	**26.67**	**10**	**3.33**	**13.33**	**20.00**	**16.67**	**20.00**

The table show variation of an overall prevalence of *Salmonella* isolates from egg parameters.

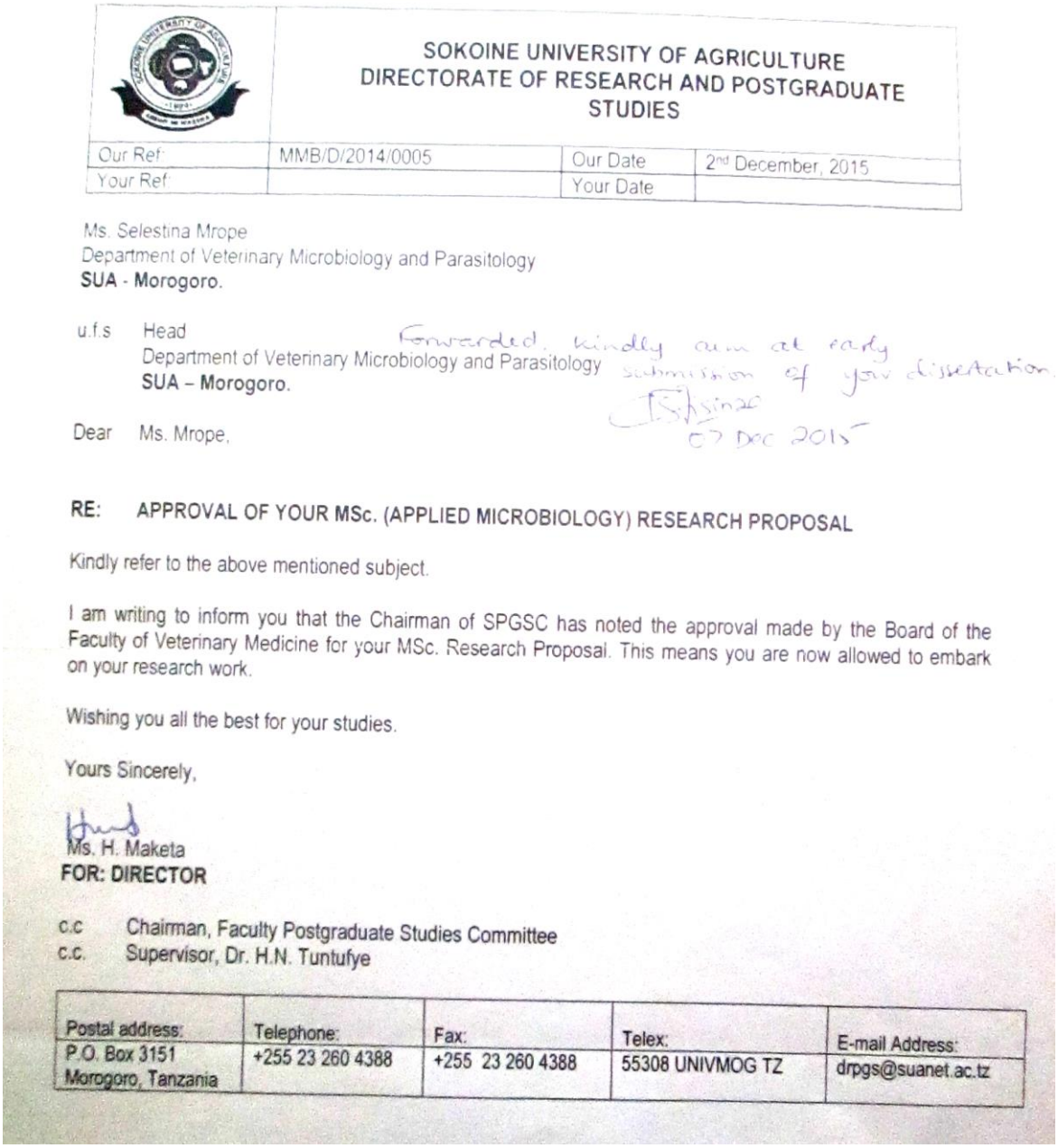
KEY: - (dash): eggs were not collected; Cracked: eggs with cracks on shell; Clean: egg without any dirt on shells; Faecal soaked: eggs with faecal material on shells; Clean: eggs without any dirty material on shells; Shells: external surfaces of eggs; Contents: internal sites of eggs.

Appendix 9: List of the primer set used in this study

Primer code	Oligonucleotides sequence (5' - 3')	Conc. (nM)	Amplicon size (bp)	Reference
S139- F	5' GTG AAA TTA TCC CCA TCG GGC AA-3'	24.0	284	Malorny *et al.* (2003a)
S 141 - R	5' TCA TCG CAC CGT CAA AAC C-3'	21.0	284	

A set of Spec *invA* gene primers used for detection of *Salmonella* spp (genus level)

Appendix 10: Sokoine University of Agriculture research permit



SOKOINE UNIVERSITY OF AGRICULTURE
DIRECTORATE OF RESEARCH AND POSTGRADUATE STUDIES

Our Ref:	MMB/D/2014/0005	Our Date	2nd December, 2015
Your Ref:		Your Date	

Ms. Selestina Mrope
Department of Veterinary Microbiology and Parasitology
SUA - Morogoro.

u.f.s Head
Department of Veterinary Microbiology and Parasitology
SUA – Morogoro.

Forwarded. kindly aim at early submission of your dissertation.
07 Dec 2015

Dear Ms. Mrope,

RE: APPROVAL OF YOUR MSc. (APPLIED MICROBIOLOGY) RESEARCH PROPOSAL

Kindly refer to the above mentioned subject.

I am writing to inform you that the Chairman of SPGSC has noted the approval made by the Board of the Faculty of Veterinary Medicine for your MSc. Research Proposal. This means you are now allowed to embark on your research work.

Wishing you all the best for your studies.

Yours Sincerely,

Ms. H. Maketa
FOR: DIRECTOR

c.c Chairman, Faculty Postgraduate Studies Committee
c.c. Supervisor, Dr. H.N. Tuntufye

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