2021-02-24

OCCURRENCE AND ANTIMICROBIAL RESISTANCE PATTERNSOF ESCHERICHIA COLI O157 AND SALMONELLAISOLATESFROM RAW COW MILK IN AND AROUND BAHIR DAR CITY, AMHARA REGION, ETHIOPIA

Asamrew Adino

BAHIR DAR UNIVERSITY

COLLEGE OF AGRICULTURE AND ENVIRONMENTAL SCIENCE

SCHOOL OF ANIMAL SCIENCE AND VETERINARY MEDICINE

MASTER OF SCIENCE IN VETERINARY PUBLIC HEALTH

OCCURRENCE AND ANTIMICROBIAL RESISTANCE PATTERNSOF *ESCHERICHIA COLI* O157 AND *SALMONELLA*ISOLATESFROM RAW COW MILK IN AND AROUND BAHIR DAR CITY, AMHARA REGION, ETHIOPIA

M.Sc. Thesis

By

Asamrew Adino Gezahegn

July, 2020

Bahir Dar, Ethiopia

BAHIR DAR UNIVERSITY

COLLEGE OF AGRICULTURE AND ENVIRONMENTAL SCIENCE

SCHOOL OF ANIMAL SCIENCE AND VETERINARY MEDICINE

MASTER OF SCIENCE IN VETERINARY PUBLIC HEALTH

OCCURRENCE AND ANTIMICROBIAL RESISTANCE PATTERNS OF *ESCHERICHIA COLI* O157 AND *SALMONELLA*ISOLATES FROM RAW COW MILK IN AND AROUND BAHIR DAR CITY, AMHARA REGION, ETHIOPIA

M.Sc. Thesis

By

Asamrew Adino Gezahegn

A THESIS SUBMITTED TO THE COLLEGE OF AGRICULTURE AND ENVIRONMENTAL SCIENCE OF BAHIR DAR UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIRMENTS OF THE DEGREE OF MASTER SCIENCE IN VETERINARY PUBLIC HEALTH

July, 2020

Bahir Dar, Ethiopia

THESIS APPROVAL SHEET

As a member of the Board of Examiners of the Master of Sciences thesis open defense Examination, certifying that we have read and evaluated the thesis prepared by**Mr.Asamrew Adino Gezahegn**entitled "**OCCURRENCE AND ANTIMICROBIAL RESISTANCE PATTERNS OF *ESCHERICHIA COLI* O157 AND *SALMONELLA* ISOLATES FROM RAW COW MILK IN AND AROUND BAHIR DAR CITY, AMHARA REGION, ETHIOPIA,"**We here by certify that the thesis be accepted for fulfilling the thesis requirementfor the award of the Degree of Master of Science in**Veterinary Public Health.**

Board Examiner

Sefinew Alemu(PhD)		8/12/2012
Name of External Examiner	Signature	Date
Tiliksew Bialfew (DVM, MVSc)		11/12/2012
Name of Internal Examiner	Signature	Date
Sam Laiju (PhD)		
Name of Chairman	Signature	Date

DECLARATION

This is to certify that this thesis entitled **"OCCURRENCE AND ANTIMICROBIAL RESISTANCE PATTERNS OF *ESCHERICHIA COLI* O157 AND *SALMONELLA* ISOLATES FROM RAW COW MILK IN AND AROUND BAHIR DAR CITY, AMHARA REGION, ETHIOPIA,"**submitted in partial fulfillment of the requirements for the award of the degree of Master of Science in **"Veterinary Public Health"** to the Graduate Program of College of Agriculture and Environmental Sciences, Bahir Dar University by Mr. **Asamrew Adino** (ID. No. BDU1100539) is an authentic work carried out by him under our guidance. The matter embodied in this project work has not been submitted earlier for the award of any degree or diploma to the best of our knowledge and belief.

Name of the Student

Asamrew Adino Gezahegn

Signatureand Date _____

Name of the Supervisors

Biruhtesfa Asrade (DVM, MVSc, Associate Professor) (Major Supervisor)

Signatureand Date: _____

Takele Ayanaw (DVM, MVSc, Assistant Professor) (Co-Supervisor)

SignatureandDate: _____

STATEMENT OF THE AUTHOR

I declare that this thesis is my original work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for MVSc degree at Bahir Dar University, Agriculture and Environmental science and is deposited at the University or College library to be made available to borrowers under the rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

Name: **Asamrew Adino Gezahegn**

Signature: _____

Date of Submission: _____

College of Agriculture and Environmental science, Bahir Dar University, Bahir Dar, Ethiopia

ACKNOWLEDGEMENTS

Firstly, my thanks go to God for giving me the strength and opportunity to conduct this research in good health. Next to this, I would like to express my profound appreciation and sincere thanks to Dr. Bruhtesfa Asrade, my major advisor, and Dr. Takele Ayanaw, Co-adviser for their consistent guidance, valuable comments and encouragement at all stages of the research. I would like to express my deepest and sincere gratitude to Dr. Mussie H/melekot for their encouragement, insight, valuable guidance, and professional expertise during the thesis write-up.

Special thanks and appreciations also goes to Bahir Dar University, Institute of Technology, Food Microbiology Laboratory staff, particularly Dr. Takele Ayanaw and Bekalu Abay for permitting me to carry out the laboratory in their laboratory and tireless involvement that enabled me for timely completion of the study.

I wish to express my thanks to the Bahir Dar University College of Agriculture and Environmental Sciences in general and School of Animal Science and Veterinary Medicine, Department of Veterinary Science in particular for providing allowing me to pursue my MSc study and availing essential research materials and support during my study.

I am endebted to staff members of Bair Dar city agriculture office and Bahir Dar *zuriaworeda* agricultural offices, development agents, Amhara livestock promotion and development center, Amhara regional livestock diagnostic center and smallholder milk producer, dairy farm, milk collection centers and cafeterias owner/workers for their cooperation and generous response to requested information during survey data collection and milk samplings.

I need to express deepest gratitude tothank my beloved father Adino Gezahegn and mother Beteha Adal, all my family members for support and encouragement in every step of my life.

Finally, I would like to extend my special thanks to all my friends for their all.

DEDICATION

This piece of work is dedicated to my beloved father **ADINO GEZAHEGN** and mother **BETEHA ADAL** for all love they have given me and their dedicated partnership in the success of my life.

LIST OF ABBRIVATIONS

BCOARD	Bahir Dar City Office of Agriculture and Rural Development
BOARD	Bureau of Agriculture and Rural Development
BZOARD	Bahir Dar *Zuria* Office of Agriculture and Rural Development
CDC	Center for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CSA	Central Statistical Agency
EHEC	Enterohemorrhagic *Escherichia coli*
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
ISO	International Organization for Standardization
MDR	Multi-Drug Resistant
NSF	Non-Sorbitol Fermenting
SMAC	Sorbitol MacConkey Agar
STEC	Shiga Toxigenic Producing *Escherichia coli*
WHO	World Health Organization

OCCURRENCE AND ANTIMICROBIAL RESISTANCE PATTERNS OF *ESCHERICHIA COLI* O157 AND *SALMONELLA* ISOLATES FROM RAW COW MILK IN AND AROUND BAHIR DAR CITY, AMHARA REGION, ETHIOPIA

Asamrew Adino [1], Biruhtesfa Asrade[2], Takele Ayanaw[2]
[2]Bahir Dar University College of Agriculture and Environmental Sciences
School of Animal Science and Veterinary Medicine, Department of Veterinary Science

ABSTRACT

A cross-sectional study was conducted from November, 2019 to June, 2020 to assess the occurrence and antimicrobial susceptibility patterns of E. coli O157 and Salmonella in raw cow milk collected from smallholder milk producers, dairy farms, milk collection centers and cafeterias in and around Bahir Dar city, Ethiopia. Samples were collected using a simple random sampling technique and analyzed using the recommended standard procedures to isolate and identify the pathogen. Additionally, a structured questionnaire survey was conducted to assess the status of hygienic practices in the smallholder milk producers, dairy farms, milk collection centers and cafeterias. The data was processed and analyzed by using SPSS version 20.0 software. Descriptive statistical analysis such as percentage, chi-square and fishery exact test of various risk factor and dependent variables. Out of 150 raw milk samples, 70 smallholder milk producers, 29 dairy farms, 35 milk collection centers and 16 cafeterias examined.The occurrence rate E. coli O157 and Salmonella was 9 (6%) and 7(4.7%), respectively. The occurrence of E. coli O157 was highest in smallholder milk producers (7.15). While, lowest in dairy farms (3.4). Similarly, the occurrence of Salmonella was highest in cafeteria (6.3%). While, lowest in dairy farms (3.4%). The antimicrobial susceptibility profile showed that all isolates were 100% susceptible to Gentamicin and Ciprofloxacin, and resistant to Ampicillin. While,11% of E. coli O157 and 42.9 % of Salmonella isolates were found to be multidrug resistance. Physicians within the area should consider Ciprofloxacin and Gentamicin as first choice drugs within the treatment of clinical diseases associated with Escherichia coli O157 and Salmonella. It is recommended training should be provided on hygienic practices for the stakeholders involved in the milk value chain, and therefore the proper pasteurization of milk to maintain the safety and quality of milk for consumers in the area.

Keywords: *Antimicrobial resistance, Milk, Occurrence, Escherichia coli O157, Salmonella, H ygienic practice.*

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF TABLES

Tables **Pages**

LIST OF TABLES (Continued)

LIST OF FIGURES

Figures **Pages**

LIST OF LIST OF APPENDICES

Appendices **Pages**

Chapter 1. INTRODUCTION

1.1. Background and Justification

Raw milk is a highly nutritious (nearly perfect food since it contains vital nutrients) and wholesome food; it forms an important component of the human diet (Msolo, 2016). It is also considered an excellent growth medium for several microorganisms (Zelalem Yilma and Bernared Faye, 2006; Rundasa Megersa*et al.,* 2019).The consumption of raw milk and milk products is common in Ethiopia (Dessalegn Alehegn, 2018).

The most widely used livestock for milk production could be cows (Abebe Bereda *et al.,* 2014). Cattle milk constitutes the foremost important share of the wholeworld milk production (FAO, 2020). Similarly, in Ethiopiancows contribute to about 94.58% of the overall annual milk produced at the national level (CSA, 2017). Similarly, around Bahir Dar area, milk is produced from cows only, while goat and camel milk are not used for human consumption (Adebabay Kebede, 2009; Asaminew Tassew and Eyassu Seifu, 2009).

Consumers need clean, wholesome and nutritious food that is produced and processed in a sound sanitary manner and free from pathogens. Hence, quality milk production is necessary for fulfilling consumers demand (Khan *et al.,* 2008). Within the milk value chain, milk producers hygienic practice can influence the occurrence of harmful pathogens in milk (Jordan, 2007; Ali *et al.,* 2010; Segni Bedasa *et al.,* 2018; Rundasa Megersa*et al.,* 2019). The presence of pathogenic bacteria in milk not only degrades the milk quality and shelf life of milk or milk products but also poses a serious health threat to consumers (Yuen et *al.,* 2012; Solomon Dadi*et al.,* 2020).

Milk contamination by Zoonotic pathogens is usually natural.Howevers,also can also occur through unhygienic conditions of milk production (Rundasa Megersa*et al.,* 2019).

Over the last 20 years, the emergence of major food borne pathogens has persisted as a significant public health concern (Newell *et al.,* 2010). Among them, *Enterohaemorrhagic strains of Escherichia coli, especially E. coli O157 and Salmonella* are the major ones (Brown *et al.,* 2000; Hoffmann *et al.,* 2012; Ayalew Assefa and Amare Bihon, 2018), In Ethiopia, thayhave been identified in raw milk and milk products (Zelalem Yilma*et al.,* 2011; Diriba Hunduma, 2018; Solomon Dadi*et al.,* 2020).

1.2. Statement of the Problem

Raw milk isa complete nutritious fluid and common diet in and around Bahir Darand provides a fast way of supplying nutrients.It is an ideal medium for the growth of varied sorts of microbes. It would be a vehicle for the transmission of a wide range of pathogenic microorganisms. Common bacterial diseases can be transmitted through the consumption of raw milk produced from diseased cows and poor hygienic practices.

Various authors (Nkya *et al.,* 2007; McDermott *et al.,* 2010; Mekonnen Tola *et al.,* 2010; Chagunda *et al.,* 2015) indicated that lack of appropriate technology, training and finance services constrained top quality milk production in Eastern African countries.

In Ethiopia, Earlier reports by Zelalem Yilma (2006); Mogessie Ashenafi (2006); Asaminew Tassew and Eyasu Seifu (2011); Alganesh Tola and Fekadu Beyene (2012) and Amistu Kuma *et al.* (2015) indicated the hygienic practices during production, processing and handling of milk and milk products because of lack of ordinary and appropriate facilities in several parts of Ethiopia were the standards and safety of milk products is questionable. Such safety problems in extreme cases can have a negative impact on the food security status of the country.

Interms of the economic point of view because of raw milk is highly perishable in our country, its short shelf life and should cause wastage and poor quality product (Fernandes, 2008; Pandey and Voskuil, 2011). These unhygienic milking practices and daily demand for the consumption of dairy products necessitate the investigation of pathogenic microorganisms like *E. coli* O157 and *Salmonella* in raw cow milk in and around Bahir Dar.The indiscriminate use of antibiotics in the treatment of infections in animals and humans has led to increasing antibiotic resistance by *E. coli* O157 and *Salmonella*and its transmission through the food chain. Therefore, the determination of antimicrobial resistance patters is required for the identification of the right antibiotics for effective treatment and continuous monitoring of resistance of pathogenic organisms to commonly used antibiotics.

Therefore, this study was launched with the aim of isolation of *Escherichia coli O157* and *Salmonella* in smallholder milk producers, dairy farms, milk collection centers and cafeteria in the study area.

1.3. Objectives of the Study

1.3.1. General objective

- To determine the occurrence and antimicrobial resistance patternsof *Escherichia coli* O157 and *Salmonella*isolates from raw cow milk value chain in and around Bahir Dar city, Ethiopia.

1.3.2. Specific objectives

- ✓ To determine the occurrence of *E coli* O157 and *Salmonella* in raw cow milk value chain in the study area.
- ✓ To identify the major constraints of hygienic practices of the raw milk value chain in the study area.
- ✓ To determine the antimicrobial resistance pattern of the isolated pathogens.

1.4. Research Questions

- What would be the occurrence rate of*Escherichia coli*O157 and*Salmonella*from the raw milk sample collected samples?
- What would be the associated risk factors with the occurrence of *Escherichia coli* O157 and *Salmonella?*
- What wouldbe the antimicrobial resistant patterns of *Escherichia coli* O157 and *salmo nela*isolates from raw cow milk?

Chapter 2. LITERATURE REVIEW

2.1. Taxonomy, Nomenclature and Characteristics

2.1.1. Taxonomy and nomenclature of *Escherichia coli*O157

Escherichia coli (*E. coli*) was first described in 1885 by Theodor Escherich. Escherichia, a Bavarian pediatrician, had performed studies on the intestinal flora of infants and had discovered a normal microbial inhabitant in healthy individuals, which he named Bacterium commune. In 1919, the bacterium was renamed in his honor to *Escherichia coli*. In 1892, *E. coli* was suggested as an indicatormicroorganism to monitor the quality of water and foods (Purohit and Kapley, 2002). *E. coli* is transmitted by food and water, directly from one person to another, and occasionally through occupational exposure. Most foodborne outbreaks have been traced to foods derived from cattle, especially ground beef and raw milk (Gyles, 2007). In the genus *Escherichia* there are hundreds of serotypes of *Escherichia coli,* which are classified on the bases of various surface antigens, referred to as Somatic (O), Capsular (K), Flagellar (H) and Fimbrial (F) (Zhang *et al*., 2006). Thus, there are approximately 174 O antigens, 56 H antigens, and 103 K antigens that have been identified. Additionally, there are several strains of the pathogen that have been isolated (Frenzen *et al.,* 2005). Moreover, the pathogenic groups of *E. coli* are divided into five groups on the basis of their virulence properties such as enterohemorrhagic (EHEC, found in human, cattle and goats), enteroinvasive (EIEC, found only in humans), enteropathogenic (EPEC, the causative agent of diarrhea in humans, rabbits, dogs, cats and horses), enterotoxigenic (ETEC, the causative agent of diarrhea in humans, pigs, sheeps, goats, cattle, dogs and horses) and enteroaggregative (EAggEC, which found only in human) *E. coli* (Biswas *et al.,* 2006; Xia *et al.,* 2010). Hence, regarding zoonosis, the most important category is enterohemorrhagic (EHEC), which is also the most severe (Nguyen and Sperandio, 2012).

Shiga toxin-producing *Escherichia coli* (STEC)species are classified into two subtypes: O157 and non-O157, with cases involving O157strains more frequently associated with more severe diseases (Oporto *et al*., 2008). *E.coli* O157 is the most predominant and most virulent

serotype in a pathogenic subset of EHEC. *E. coli* O157 is so named because it expresses the 157th O antigen identified (Chapman *et al*., 2001).

2.1.2. General characteristics of *Escherichia coli*

Escherichia coli are gram-negative, facultatively anaerobic, rod-shaped, highly mobile and nonsporulating bacteria. (Oliver *et al.,* 2009; Farrokh *et al*., 2012; CDC, 2015). All Shiga toxin-producing *E. coli* including serotype O157:H7 have the same morphology. They are Gram-negative, facultative anaerobic bacteria that belong to the family *Enterobacteriaceae* and the genus Escherichia. They are commonly motile in liquid media by means of peritrichous flagella. Some *E. coli* strain like STEC O157 have acquired Virulence factors that have allowed them to adapt to new niches and in some cases, to cause severe disease (Farrokh *et al.,* 2012).The growth range for *E. coli* O157 is thought to be between 7 and 45°C, with an optimum of approximately 37°C. It is not notably heat resistant and effectively killed by standard pasteurization processes (>60°C). A near neutral pH is optimal for growth, but growth is possible down to pH 4.4. It is unusually acid-tolerant and survives well in foods with low pH values (3.6 - 4.0), especially at chill temperatures. The minimum water activity required for growth is 0.95 (Adams and Moss, 2008; Fernands, 2008). Theviability and growth of bacteria depend primarily on the availability of essential nutrients including organic carbon, phosphate, and nitrogen (Persson *et al*., 2015).

Unlike any other non-pathogenic strains of *Escherichia coli,E. coli* O157 is non-sorbitol fermenting (NSF). It has no ability to ferment sorbitol, unable to produce β- glucuronidase, has an attaching and effacing gene (eae) and, produces Shiga toxins (Stxs) that inhibit host protein synthesis (Pennington, 2010; Wilson *et al.,* 2018). It is catalase positive, indole positive, oxidase negative, Urease negative, VogesProskauer negative, and citrate negative (Rosser *et al*., 2008).

2.1.3. Taxonomy and nomenclature of *Salmonella*

Theobald Smith in 1885 discovered *Salmonella* (Scott, 2012) but has its name after a veterinarian called Daniel E. Salmon, who first isolated *Bacillus choleraesuis* from porcine intestines and later in 1900 Lignieres changed it into *Salmonella choleraesuis*. Nowadays the

Salmonella genus is divided into two species: *Salmonella enterica* and *Salmonella bongori*, with *S. enteric* further subdivided into 6 additional subspecies (Ryan and Ray,2017).The species *S. enterica* is further subdivided into six subspecies named (or numbered) as follows*: S. enterica subsp. enterica* (I), *S. enterica subsp. salamae* (II), *S. enterica subsp. arizonae* (IIIa), *S. enterica subsp. diarizonae* (IIIb), *S. enterica subsp. houtenae* (IV), and *S. enterica subsp. indica* (VI) (Popoff and Minor, 2001).

The nomenclature for the genus *Salmonella* is complicated because of the ever-changing nomenclature system, newly detected species and different systems used to refer to this genus (Brenner *et al.,* 2000). The *Salmonella* nomenclature has evolved over time and at the early stages, each *Salmonella* serovar was considered a separate species (Kauff mann, 1966). However, this one serovar-one species concept was found to be misleading since most serovars cannot be distinguished by biochemical tests (Nataro *et al.,* 2007).

2.1.4. General characteristics of *Salmonella*

*Salmonella*is generally considered as a facultative anaerobe, Gram-negative, motile (chicken adapted serovars Gallinarum and Pullorum are an exception), non-lactose fermenting, oxidase negative, urease negative, citrate positive and potassium cyanide negative, rod-shaped bacteria which are about 2-5 x 0.7-1.5 μm in size (Montville and Matthews, 2008; Markey *et al*., 2013; Moxley, 2017; Carr, 2017). Almost all *Salmonella* species possess flagella, which areuseful for motility except for *S. Pullorum* and *S. Gallinarum* (Scott, 2012). *Salmonella* species can adapt to extreme environmental conditions. For example, *Salmonella* can grow at a temperature between 5-47°C with an optimum temperature of 35-37°C (Gray and Fedorka-Cray, 2002). They are sensitive to heat and killed at a temperature of 70°C or higher. *Salmonella* grow in a pH range of 4.5 to 9.5 with an optimum between pH 6.5 and 7.5 (Montville and Matthews, 2008).

Brilliant Green agar, Brilliant Green Sulfa agar, *Salmonella- Shigella* agar, Xylose Lysine Deoxycholate agar and Hektoen Enteric agar are selective media used to culture *Salmonella*. On Brilliant Green agar, colonies and the medium are red indicating alkalinity, and on Xylose Lysine Deoxycholate agar, colonies are red (alkaline) with a black center, indicating Hydrogen Sulphide production (Quinn *et al*., 2011;Markey *et al*., 2013; Moxley, 2017). Upon

inoculation of triple sugar iron agar, they give an acid butt or alkaline slant with Hydrogen Sulphide (Quinn *et al*., 2011), and alkaline or purple after inoculating to Lysine iron agar (Markey *et al*., 2013). They are indole negative, which forms a yellow ring after the addition of Kovacs reagent on the top of *Salmonella* inoculated and incubated SIM medium. In addition to this, they are urea negative and citrate positive (Mikoleit, 2015).

2.2. Global Occurrence

2.2.1. Global occurrence of *Escherichia coli*O157 in milk

The incidence of *E. coli* O157 in humans is difficult to determine, because cases of uncomplicated diarrhea is not be tested for these organisms. In 2004, the estimated annual incidence of *E. coli* O157 reported in Scotland, the U.S., Germany, Australia, Japan and the Republic of Korea ranged from 0.08 to 4.1 per 100,000 population, with the highest incidence in Scotland. In the USA, estimates indicate that *E. coli* O157 causes approximately 73,000 illnesses, 2,000 hospitalizations, and 50-60 deaths each year (Schroeder *et al*., 2002: Dulo, 2014).

Annual incidence rates of 0.4 to 2.74 per 100,000 inhabitants per year have been reported in the region of Australia, USA, Mexico and Japan by Sakuma *et al.* (2006), Vally *et al.* (2012), Canizalez-Roman *et al.* (2013) and Sodha *et al.* (2015) Also, reports from Africa have shown that the rates of infections by the pathogen but in countries lacking diagnostic capabilities might be underestimated (Tarr *et al.,* 2005).

The overall prevalence of *E. coli* O157 in cattle at the global level seems to be 5.68% even though a wide range of prevalence estimates ranging from 0.1% to 62% in cattle was reported worldwide (Pennington, 2010; Fox *et al.,* 2008; Hussein and Sakuma, 2005). Furthermore, a prevalence of 5.68% of *E. coli* O157:H7 in cattle at the global level (Islam *et al.*, 2014).

The random effects pooled 5 prevalence estimates of it in Africa, Northern America, Oceania, Europe, Asia and Latin AmericaCaribbean are likely to be 31.20%, 7.35%, 6.85%, 5.15%, 4.69% and 1.65%, respectively. The highest prevalence estimate (31.20%) was in African cattle and the forecasts from each of the four studies from Africa were comparably high, although each of two of them was based on the investigation of a sample size of only 120 cattle (Akanbi *et al.,* 2011; Ateba and Mbewe, 2011).There are a number of studies from

different countries of the world concerning the prevalence of *E. coli* O157 in raw milk. Arafa and Soliman (2013) reported that of raw milk and fresh cream examined in Egypt 2.6% and 1% were contaminated with *E. coli* O157, respectively. Allerberger *et al.* (2001) reported 3% of the milk samples tested in Austria to be positive for *E. coli* O157 and Klie *et al.* (1997) found that 3.9% of the raw milk analyzed in Germany was contaminated with *E. coli* O157. Despite the greater burden caused by foodborne infections in developing countries than developed countries, there is a great scarcity of information on their occurrences (Havelaar, 2013).

2.2.2.Global occurrence of *Salmonella*in milk

Salmonella infections are a significant public health concern around the world(Majowicz *et al*., 2014). *Salmonella* is the leading cause of food borne illness in worldwide causing diarrhea, cramps, vomiting, and often fever (Majowicz *et al*., 2010). It is a modifiable disease in all Australian states and territories, with a notification rate in 2012 of 49.8 cases per 100,000 populations (11,273 cases). This was an increase on the previous 5-year mean of 46.9 cases per 100,000 populations per year (ranging from 38.6–54.2 cases per 100,000 populations per year) (NNDSS, 2013). The salmonellosis notification rate varied between jurisdictions from 40.5 cases per 100,000 populations in New South Wales to 180.1 cases per 100,000 populations in the Northern Territory in 2012. The notification rate for salmonellosis in New Zealand in 2011 was 24 cases per 100,000 populations (1,056 cases). This was a slight decrease from the 2010 rate of 26.2 cases per 100,000 populations (Lim *et al.,* 2012). In the United States (US) 17.73 cases of salmonellosis were notified per 100,000 population in 2010. This was a slight increase from the 2009 rate of 16.18 cases per 100,000 populations (CDC, 2016). In the European Union, the notification rate for salmonellosis was 20.7 cases per 100,000 populations in 2011 (ranging from 1.6 to 80.7 cases per 100,000 populations between countries). This was a 5.4% decrease in the number of cases from 2010 (EFSA, 2013). Outbreaks attributed to *Salmonella spp*. have predominantly been associated with animal products such as eggs, poultry, raw meat, milk and dairy products, but also include fresh produce, salad dressing, fruit juice, peanut butter and chocolate (Jay *et al.,* 2003; Montville and Matthews 2005).

In Africa, *S. enteritidis* and *S. typhimurium* represented 26% and 25% of the isolates, respectively. In Asia, Europe and Latin America/Caribbean, *S. Enteritidis* was the most frequent isolate (38%, 87% and 31%, respectively). In North America *S. typhimurium* was the most frequentes reported (29%) followed by *S. enteritidis* (21%) and other *Salmonella* species (21%) (Majowicz *et al.,* 2010).

2.3. Occurrence in Ethiopia

2.3.1. Occurrence of *Escherichia coli*O157from milk

Considerable number of studies have reported occurrence of *E. coli andE. coli* O157 from food of animal origin (mainly meat and milk). Epidemiology of foodborne pathogens especially that of *E. coli* O157: H7 was not well studied in Ethiopia in the past years. Though some studies were reported from the central Ethiopia, and reports from southern, eastern, western and northern parts of the country.

There were few studies conducted by some researchers to determine the prevalence of *E. coli* O157 in raw milk of cow in different areas of the country. Nigatu Disassa *et al.* (2017) reported a prevalence of 2.9% of *E. coli* O157:H7 from traditionally marketed raw cow milk in and around Asosa town, western Ethiopia. Similarly, a prevalence of 6.9% was reported from raw milk in selected commercial dairy farms of Holeta district (Alemu Ayano*et al.,* 2013). On the other hand, Segni Bedasa *et al.* (2018) reported 12% from raw milk sample collected from open markets in Bishoftu town.

However, there were few studies conducted on the prevalence and antimicrobial susceptibility patterns of *E. coli* O157:H7 isolated from traditionally marketed raw cow milk in and around Asosa town showed that out of 380 raw milk samples examined, 129 (33.9%) and 11 (2.9%) were contaminated with *E. coli* and *E. coli* O157:H7 respectively. The highest prevalence *E. coli* was recorded in samples obtained from vendors (39.1%) compared with samples from farmers (28.1%) with significant differences (Nigatu Disassa *et al.,* 2017). The prevalence from raw milk is also reported as 44.4% from Mekelle town (Shunda *et al.,* 2013), 33.5% from Malaysia (Chye *et al.,* 2004) and 38.0% from India (Thaker *et al.,* 2012). It is also reported that the prevalence of *E. coli* O157:H7 in the fecal samples is as low as 2% from feces and 0.8% from intestinal mucosa (Rosa Abdissa*et al.,* 2017).

Table 2.1: Studies conducted on *Escherichia coli* O157 from milk in Ethiopia

Author (year)	P	SA	MD
Zelalem Addis *et al.* (2011)	3.08%	Central Ethiopia	Culture and biochemical test
Abebe Bereda*et al*. (2014)	10.4%	Tigray region	Culture and biochemical test
Nigatu Disassa *et al*. (2017)	2.9%,	Asosa town	Serological
Diriba Hunduma (2018)	4.67%	Borana pastoral area	Culture and biochemical test
Frehiwot Mesele (2018)	4.54%	Kombolcha district	Culture and biochemical test
Haileyesus Dejene (2018)	4.08%	Central Ethiopia	Culture and biochemical test
Segn Bedasa *et al*. (2018)	3.5%,	Bishoftu town	Culture and biochemical test

SA=study area, MD= methods of detection, P=prevalence.

2.3.2. Occurrence of *Salmonella*from milk

In Ethiopia, like other developing countries, Studies indicated the widespread occurrence and distribution of Salmonella.The number of out breaks of Salmonella in humans has increased considerably in the country. *Salmonella* isolates in Ethiopia may have similar phenotypic and genotypic characteristics with isolates elsewhere in the world (Misganaw Birhaneselassie and Williams, 2013). In addition, under reporting of cases and the presence of other diseases considered to be of high priority may have over shadowed the problem of Salmonellosis in some countries, including Ethiopia (Gizachew Yismaw *et al.,* 2007; Gashaw Andargie *et al.,* 2008; Bayeh Abera *et al.,* 2010). This is mainly because of the very limited scope of studies, lack of coordinated epidemiological surveillance system and inadequacy of laboratory facilities for culture (Misganaw Birhaneselassie and Williams, 2013). However, Studies indicated the widespread occurrence and distribution of *Salmonella*.

For example, a study conducted on prevalence of *Salmonella* isolates from dairy products in Addis Ababa shows that the overall prevalence of *Salmonella* was 1.6% (6 out of the total 384 samples of cheese, butter, yogurt and milk). *Salmonella* was detected from cheese, butter, and milk with prevalence of 3 (3.1%), 1 (1.04%), and 2 (2.1%), respectively (Liyuwork Tesfaw*et al*., 2013). Additionally, 47.8 % and 7.7 % reported by Zelalem Addis*et al*., (2011) formAddis

Ababa has shown farm level prevalence of and animal level occurrence of *Salmonella,* respectively.

Table 2.2: Studies conducted on *Salmonella*from milk in Ethiopia

Author (year)	P	SA	MD
Teshome Tadesse and Anbessa Dabassa (2011)	12.1%	Kersa District	Culture and biochemical test
Zelalem Addis *et al*. (2011)	7.7%	Addis Ababa	Culture and biochemical test
Deresse Hailu *et al.* (2015)	12.5%,	Gondar Town	Culture and biochemical test
Takele Beyene*et al*. (2016)	14.3%	Asella Town	Culture and biochemical test
Fufa Abuna *et al.* (2017)	10.5	Modjo Town	Culture and biochemical test
Fufa Abuna *et al.* (2017)	3.2%	Holeta Town	Culture and biochemical test
Diriba Hunduma (2018)	4%	Borana Pastoral	Culture and biochemical test

SA=study area, MD= methods of detection, P=prevalence.

2.4. Isolation and Identification

2.4.1. Isolation and identification*Escherichia coli*O157

Development of a rapid microbial detection methods with high sensitivity and specificity for pathogen identification allows the prompt notification of outbreaks and prevents more cases (Baker *et al.,* 2016). The first step for STEC detection is to enrich the sample to be analyzed. Enrichment media vary in composition but generally provide an environment appropriate to increase a bacterial cell population. Such constituents provide a supportive nutritional matrix for growth of microorganisms (Sanath Kumar *et al.,* 2001).

Conventional culture methods

Enrichment, colony isolation and confirmation are the three general phases of the standard method recommended by the US Department of Agriculture for detection and identification of *E. coli* O157:H7. The first phase is a 24h enrichment, which provides conditions that promote

growth of *E. coli* but are inhibitory to other species. The USDA enrichment culture medium includes lactose as a carbohydrate source, bile salts for suppression of certain Grampositive species, and novobiocin to suppress Gram-negative species other than *E. coli*. In the second phase, the enrichment culture is plated onto a selective medium to obtain isolated colonies. The medium is a modification of MacConkey agar, in which the lactose is replaced by sorbitol (Sorbitol MacConkey agar, SMAC) and a chromogenic indicator for 13-glucuronidase activity is included. Sorbitol-fermenting colonies appear red, but *E.coli* O157:H7 colonies are color less owing to lack of sorbitol fermentation (Smith *et al.,* 2014). The other recommended confirmatory tests be H_2S production and carbohydrate fermentation in triple-sugar-iron agar, indole production, methyl red-Voges-Proskauer test, citrate utilization, lysine decarboxylase, and antigen agglutination (Hunt, 2010; Bryan *et al.,* 2015; Zelyas *et al.,* 2016).

Detection by latex agglutination techniques

Latex agglutination test kit is another common method used for the rapid identification of *E. coli* serotype O157. The non-sorbitol fermenting (NSF) colonies will be subjected to slide agglutination with the *E. coli* O157 Latex test kit (Oxoid). The latex beads are coated with antibodies which bind to any O157 or H7 antigens on the test organisms enabling to form a visible antigen antibody precipitate. Colonies giving a precipitation reaction were confirmed as *E. coli* O157 positive (De Boer and Heuvelink, 2000).

Molecular detection methods

Molecular approaches involving the isolation, detection, and in some cases quantitation of either DNA or RNA are instrumental in the emergence of rapid detection systems for*E. coli* O157. Primers have been developed to detect virulence genes such as *stx1, stx2* and *eae*, and distinguish *E. coli* pathotypes, as well as common STEC serotypes. In theory, with proper DNA extraction techniques and sufficient DNA purity level, PCR methods can detect a single DNA molecule, which can be amplified to obtain a greater amount of DNA for further analysis (Clermont *et al.*, 2000).

2.4.2. Isolation and identification of *Salmonella*

Culture method

The transfer of microorganism from its natural habitat to artificial growth-permitting laboratory medium is referred to as culturing (Jasson *et al*., 2010). Infections that are clinically suspected are ultimately confirmed by isolation and identification of the causative agent. To provide effective and efficient anti-microbial therapy, appropriate and accurate identification of the microorganism and antibiotic susceptibility tests is required (Han, 2013). Their main limitation is that they are labor-intensive and time consuming (Jasson *et al*., 2010). In culture based method the targeted pathogen is isolated from enrichment after inoculation and incubation of selective and differential media with specified pathogen, and then confirmed depending on biochemical properties (Han, 2013).

Culture based methods are commonly used techniques and remain the gold standard for the detection of *Salmonella* due to their selectivity and sensitivity (Garrido-Maestu *et al*., 2019). A series of steps are employed including nonselective enrichment, selective enrichment, and selective or differential plating biochemical and serological confirmations (Lee and Choi, 2015).

Non-selective pre-enrichment media, such as Buffered Peptone Water (BPW) are used to increase the number of target cells as these are generally not uniformly distributed in foods, typically occur in low numbers, and may be present in a mixed microbial population. Next, primary enrichment cultures are typically inoculated into secondary selective enrichment broths, such as Selenite Cystine broth (SC), Rappaport Vasiliadis Soya broth (RVS), Muller Kauffmann Tetrathionate- Novobiocin broth (MKTTn) and incubated at elevated temperatures (37/42°C for 18-24hrs) before being struck onto selective agars such as on Xylose Lysine Desoxycholate agar and Brilliant Green Sulfa agar (ISO-6579, 2017).

Detection of antibodies by enzyme immunoassay (EIA)

The detection of antibodies to *Salmonella* by EIA offers a sensitive and cost effective method for mass screening of animal herds for indications of a past/present *Salmonella* infection(Clermont *et al.*, 2000). The advantage of this method is that

it can be automated and no incubation is required to increase the numbers of bacterial cells(De Boer and Heuvelink, 2000).

The well-established technique for assaying antigens is EIA. Antibodies labeled with an enzyme are bound to *Salmonella* antigens, and the level of antigen present is determined by enzymatic conversion of a substrate, usually resulting in a color change, which can be read visually, or by a spectrophotometer. Serological test, such as ELISA, serum agglutination and complement fixation can be used for the retrospective diagnosis of salmonellosis or the detection of carriers(Jasson *et al*., 2010).

Enzyme-linked Immunosorbent Assay: is also used to detect the presence of an antibodyor an antigen in a *Salmonella* suspect samples. Accordingly, a sample with an unknown amount of antigen is immobilized on a solid support either non-specifically or specifically. Between each step, the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. Following the final wash, enzymatic substrate (ABTS or 3, 3', 5, 5'-tetramethylbenzidine) is added to produce a visible signal (colorimetric or fluorescent product) due to the enzymatic cleavage of the substrate. The presence of target antigen in the sample can be measured by indicating colorimetric equipment (Odumeru and León-Velarde, 2012).

Chapter 3. MATERIALS AND METHODS

3.1. Description of the Study Area

3.1.1. Bahir Darcity

The study was conducted in and around Bahir Darcity, which is the capital city of Amhara National Regional State. It's located in north western part of Ethiopia, in latitude 11°35'37.10" N and Longitude 37°23'26.77" E on the South of Lake Tana where Abay River starts. Ata distance of 565 km away from Addis Ababa(BoARD, 2016).

The area coverage, 42,160 hectare (ha) and elevation,1801 meter above sea levelin an mean reported for the city and The area receives anannualaverage rainfall of 1,224 mm and a mean annual minimum daily temperature 10.3°C and maximum of 26.3°C(BoARD, 2016).

The region covers a total area of 152,600 km^2;the city has 10.6 million cattle, 5.7 million sheep, 4 million goats and 2.1 million equines managed under extensive management system (BCOARD, 2017).

Bahir Dar city divided 9 sub cities and 24 urban *kebeles*, 11 peri-urban *kebeles,*. In Bahir Dar, there are seven organized and private milk collection center (Bahir Darcity administration livestock office,2019).

3.1.2. Bahir Dar*zuria woreda*

Bahar Dar *zuria woreda* is one of the *woredas* found in West Gojjam administrative zone. The *woreda* is located at an altitude of 1500-1800 meter above sea level with mean annual minimum and maximum rainfall of 800-1250 mm. The area receives an annual average rainfall of 1,224 mm and a mean annual minimum daily temperature 10°C and maximum of 32°C(BZOARD, 2016). There is around 31, 271 km2 uncultivated lands. The livestock population found in Bahir Dar*zuria woreda* is estimated to be 206,865 cattle, 39,537 sheep, 39,369 goats, 27,790 donkeys, 770 mule 56 horse and 151,944 poultry. Furthermore, there are about 18,850 honeybee colonies (Bahir Dar*zuria woreda* livestock office, 2017).

Both Bahir Bar city administration and *zuriaworeda* is suitable for dairy production and have a total cattle population of 237,550 out of which 6036 are exotic cross-bred (Bahir Darcity administration and Bahir Dar *zuria woreda* livestock offices, 2017).

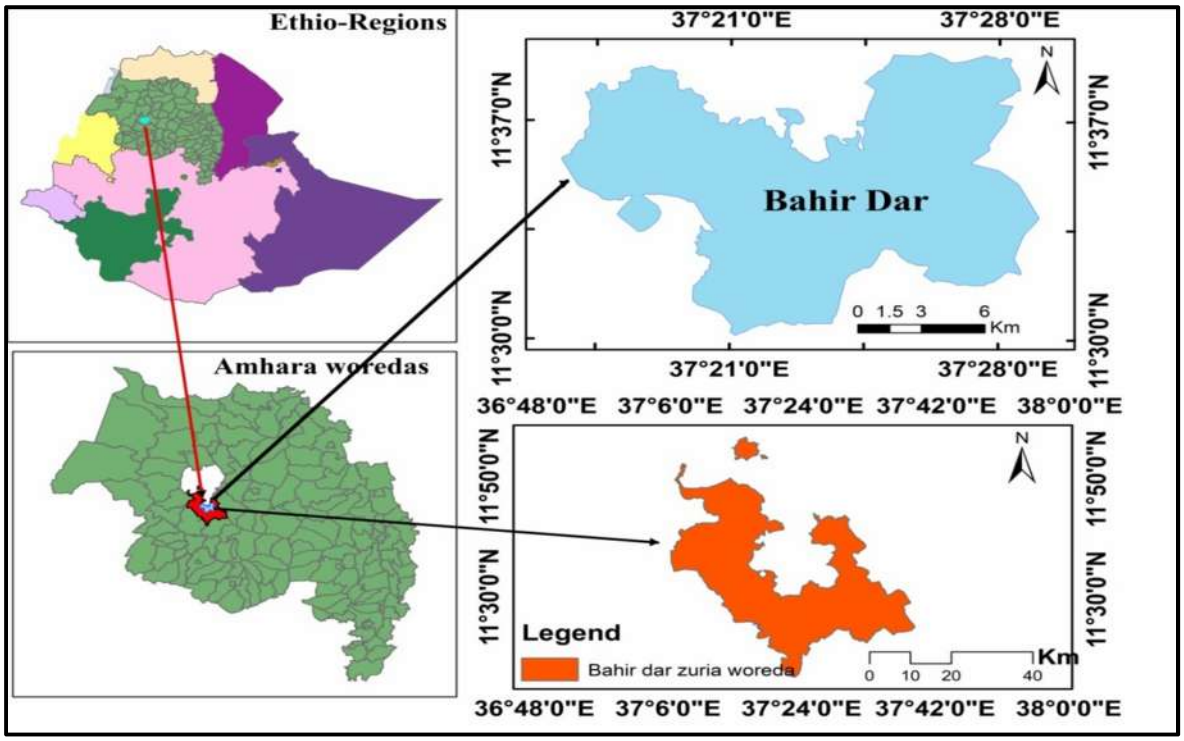

Figure 3.1: Map of the study area

3.2. Study Design

A cross sectional study was conducted from November, 2019 to June, 2020 to assess the occurrence and antimicrobial susceptibility patterns of *E. coli*O157 and *Salmonella*from raw cow milk value chaines in and around Bahir Dar city, Ethiopia.

3.3. Sample Size Determination

The sample size was calculated by using Thrusfield (2005). The required sample size was calculated considering a previously published a pooled prevalence of 7.47% by Getachew Tadesse and Tesfaye Tesema (2014) reported from studies ameta-analysisof *Salmonella* from animal origin food in Ethiopia;.

$$n = \frac{1.96^2 P_{exp}(1 - P_{exp})}{d^2}$$

Where:n = required sample size, d^2 = desired absolute precision, P_{exp} = expected prevalence

Therefore $n = \frac{1.96^2 0.0747(1-0.0747)}{0.05^2} = 106$, sample

The estimated sample size was 106, but 150 samples were taken in the study that increases the precision and equal sample size were used for both *Escherichia coli* O157 and *Salmonella* species.

A total of 150 samples were proportionally collected from smallholder milk producers (70), dairy farms(29, milk collection centers (35) and cafeterias (16) based the availability on the data taken from both Bahir dar city agriculture office and Bahir dar zuria woreda agriculture office (2019) .

3.4. Study Population

The study population was comprised of smallholder milk producer, dairy farms, and milk collection center and cafeterias in the study area.

3.5. Sampling Methods

Sampling frame included smallholder milk producers, dairy farms, milk collection centers and cafeterias from urban, peri-urban and rural areas. Bahir Dar city has 9 sub cities and 24 urban *kebeles*, 11 peri-urban *kebeles,*while Bahir Dar *zuria woreda*has 36 rural *kebeles.*

According to the data obtained fromBahir Dar city agriculture office,*kebeles* from each area were selected purposively based on their potential for diary production. From the total areas, the 8*kebeles* (2 urban, 3 peri-urban and 3rural production system) were selected. From Bahir Dar city, *Kebele* 13 and *Kebele* 11*,*from peri-urban, Woreb, Tiss Abay and Zenzelima, and from Bahir dar *zuria woreda*rural *Kebeles*Robit, Sebatmit and Yigoma Huletuwere selected. Then, simple random sampling was used to find smallholder milk producer, dairy farms, milk collection centers and cafeterias of raw milk sample and at the same time, respondents were parallel selected by simple random sampling in the selected *Kebeles*.

The majority of the farms were at the smallholder milk producer with herd size not more than 5 cows per farm. Milk collection centers site at the main road were identified as main sources of milk for consumers and processing center and included in the study. Simple random sampling technique was applied to collect raw milk samples from each group of site (dairy farms, milk collection center and cafeteria bulk tank milk). Milk collection center and cafeteria were selected active milk producer members and sell milk and milk products to consumers.

Further elaboration of the sampling procedure of the study site is presented in Figure 3.2 bellow.

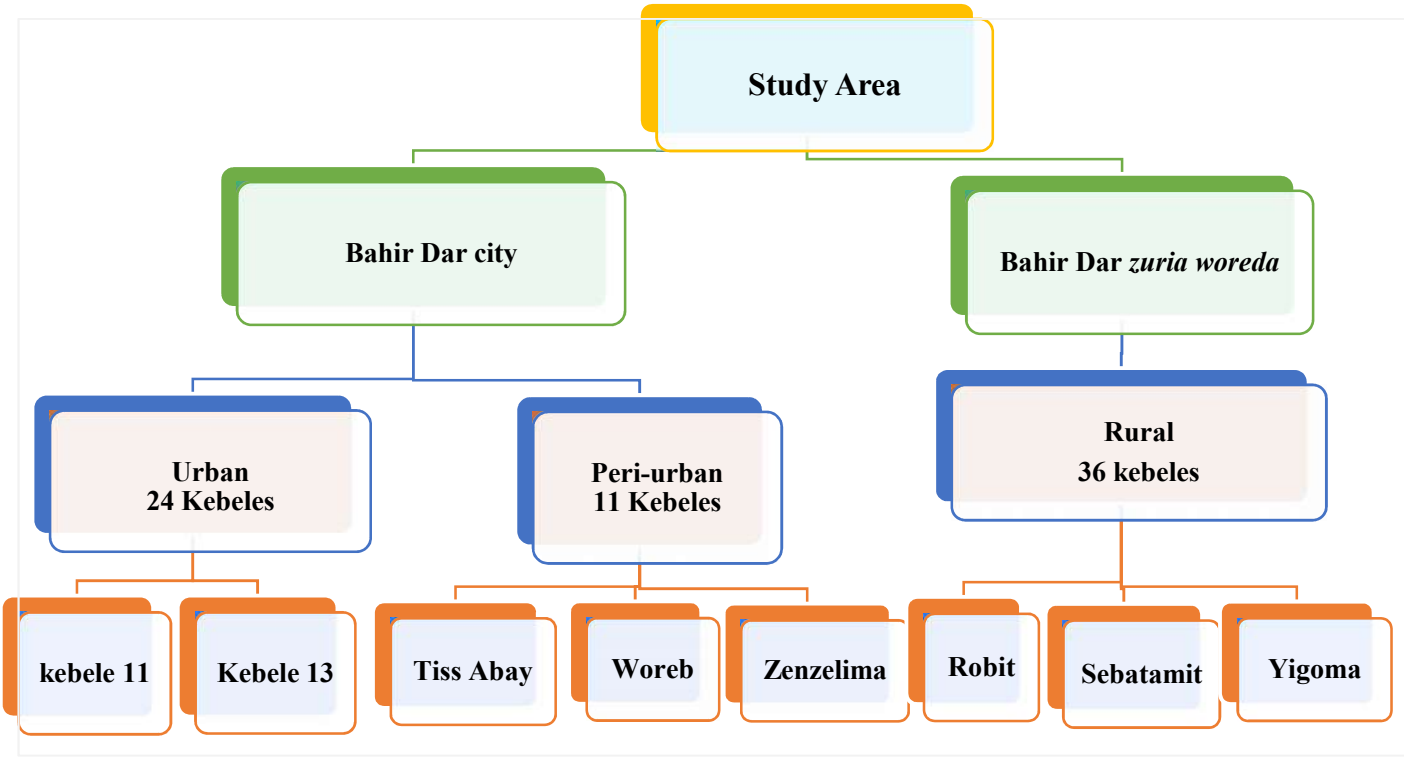

Figure 3.2: Illustration of sampling procedure of the study

[1]*kebele* is the smallest administrative unit of Ethiopia, similar to a ward, a neighborhood or a localized and delimited group of people.

Inclusion criteria

Those owners of lactating cows, owners of cafeteria and milk collection centers who were willing to participate in this study were included.

Exclusion criteria

Unwilling smallholder milk producer and dairy farm, cafeteria and milk collection center owners and dairy farmers who hold only dry of cows were excluded from this study.

Table 3.1: Number and sources of sample collected and interviewed in the study areas

Source	Bahir Dar city		Bahir Dar *zuria woreda*	Total
	Urban	Peri-urban	Rural	
Smallholder milk producer	10	30	30	70
Dairy farm	20	6	3	29
Milk collection center	20	9	6	35
Cafeteria	10	3	3	16
Total	60	48	42	150

3.6. Data Collection Methods

Prior to the sample collection, smallholder milk producer, dairy farm, milk collection center and cafeterias were visited to facilitate research collaboration.

3.6.1. Questionnaire survey

Verbal consent was obtained, and the objective of the survey was also explained to the respondents. A total of 150respondents including smallholder milk producer, dairy farms, milk collection centers and cafeteria owners or workers were interviewedparallel tomilk samples collection using structured questionnaire. The questionnaire was preparedthrough Kobo Tool Box Softwarethere isfour types of questioners for smallholder milk producer, dairy farms, milk collection centers and cafeteria, separately (Annex 1). Translation was done in to

the local language (Amharic). Data obtained from smallholder milk producer and dairy farms were about management system, milking hygienic practices (washing of milkers hand, udder and teats) and sources of water. Also, milk collection center and cafeteria owners were interviewed regarding the sources of water and type of storage container of milk, source of milk and cooling methods were collected through Kobo Tool Box. This data collection tool was used as it saves printing cost and time, and increased data quality and simple to operating.

3.6.2. Milk sample collection and transportation

Approximatelly, 100ml of raw milk samples with universal bottle was aseptically collected in morning time, Because, most of smallholder milk producers and dairy farms milking of the cow at morning time.Similarly, most of dairy farms submit their milk to milk collectors in the morning time soon after milking and handle with in universal bottles and placed in ice box with ice or ice packs within 20 minutes. Consequently, samples was labeled with identification code such as household and date of collection, and put in ice box with ice to restrict microbial multiplication until transported to laboratory.

3.7. Isolation and Identification of *Escherichia coli* O157 and *Salmonella*

Laboratory investigations were carried out at Bahir Dar University, Institute of Technology, Faculty of Chemical and Food Engineering, Department of Food Microbiology Laboratory.

3.7.1. Isolation and identification of *Escherichia coli* O157

The isolation and identification involves foursteps (Figure 3.3).

Escherichia coli O157isolation and identification was carried out in line with the guidelines of theInternational Organization for Standardization (ISO-16654:2001). Within the protocol are steps that include enrichment in selective liquid medium (selective-enrichment), plating out on selective media, biochemical test and finally, serological tests of*Escherichia coli* O157latex kit.

Step 1: Selective Enrichment

Twenty-five milliliters of samples were added to 225ml of modified Trypton Soy Broth in a ratio of 1:9 and homogenized. The homogenate was incubated overnight at 41±5°Cfor 24hr increase the recovery rate of stressed cells. After 24hrsthe broth was observed for turbidity and growth (ISO-16654:2001).

Step 2: Selective Differential Plating:

A loopful from TSB was streaked on Sorbitol MacConkey agar supplemented with Cefixime Tellurite (CT-SMAC)(Oxide, England) and incubated at 37°C for 24hrs. Following incubation, Sorbitol negative (colorless) colonieswere identified by their color and further streaked onto sorbitol MacConkey agar plates againto get a clear colorless typical *E. coli* O157 isolates(ISO-16654:2001).

Step 3: Colony Selection and Purification:

After the incubation period, From the pure culture, isolates two to three typical sorbitol negative colorless colonies were inoculated to nutrient agar for further preservation, additional biochemical and serological confirmation(ISO-16654:2001).

Step 4: Biochemical Tests:

For the confirmation of *E. coli* O157 by biochemical tests, suspectedcolonies of thebacteria were selected from nutrient agar, streaked ontothe surface of tryptone soaya agar (TSA) plates andincubated at 37°C for 24hrs. *E. coli* O157 suspected colonies picked from tryptosayaagar and inoculated to killgler iron (KIA) agar slant andtryptophan broth (indole test) was incubated for 24hrs at 37°C. The colonyconsidered positive if acid butt and acid slant (yellow) on KIA andproduction of indole in tryptophan broth indicate the presence of *E. coli* O157 withformation of pink red rings up on addition of Kovac regents. Thekillgler iron and indole tests were conducted according to Quinn *et al* (2002). Thebacteriological media used for the study were prepared following the instructions of themanufacturers (Annex 3).

Step 4: Latex Agglutination Test:

Pure cultures from the nutrient agar plates for serological tests by using *E. coli* O157 latex kit,*E. coli* O157 latex agglutination test were performed to the well-isolated colony from

nutrient agar plates to confirm the presence of *Escherichia coli*O157 in the test samples. Rapid Latex Test kit is a rapid latex agglutination testintended for confirmatory identification of *E. coli* serogroup O157 (Non-SorbiotlFermenting isolates). This test allows the rapid differentiation of *E. coli* O157 fromother *E. coli* serotypes (OXOID, Hampshire, UK). The test was conducted by addingone drop of latex suspension and dispensing near the edge of the circle on the reactioncard. Then a portion of a typical 2 to 5 colony to be tested was emulsified using a loopin a drop of sterile saline solution near the drop of test latex on the test card. Afterensuring a smooth suspension of the bacteria and saline, the test latex was mixed withthe suspension and spread to cover the reaction area over the loop. Then, the card wasrocked in a circular motion for one minute and examined for agglutination by naked eye. Agglutination of the test latex within one minute was considered as positive result(Annex 6).

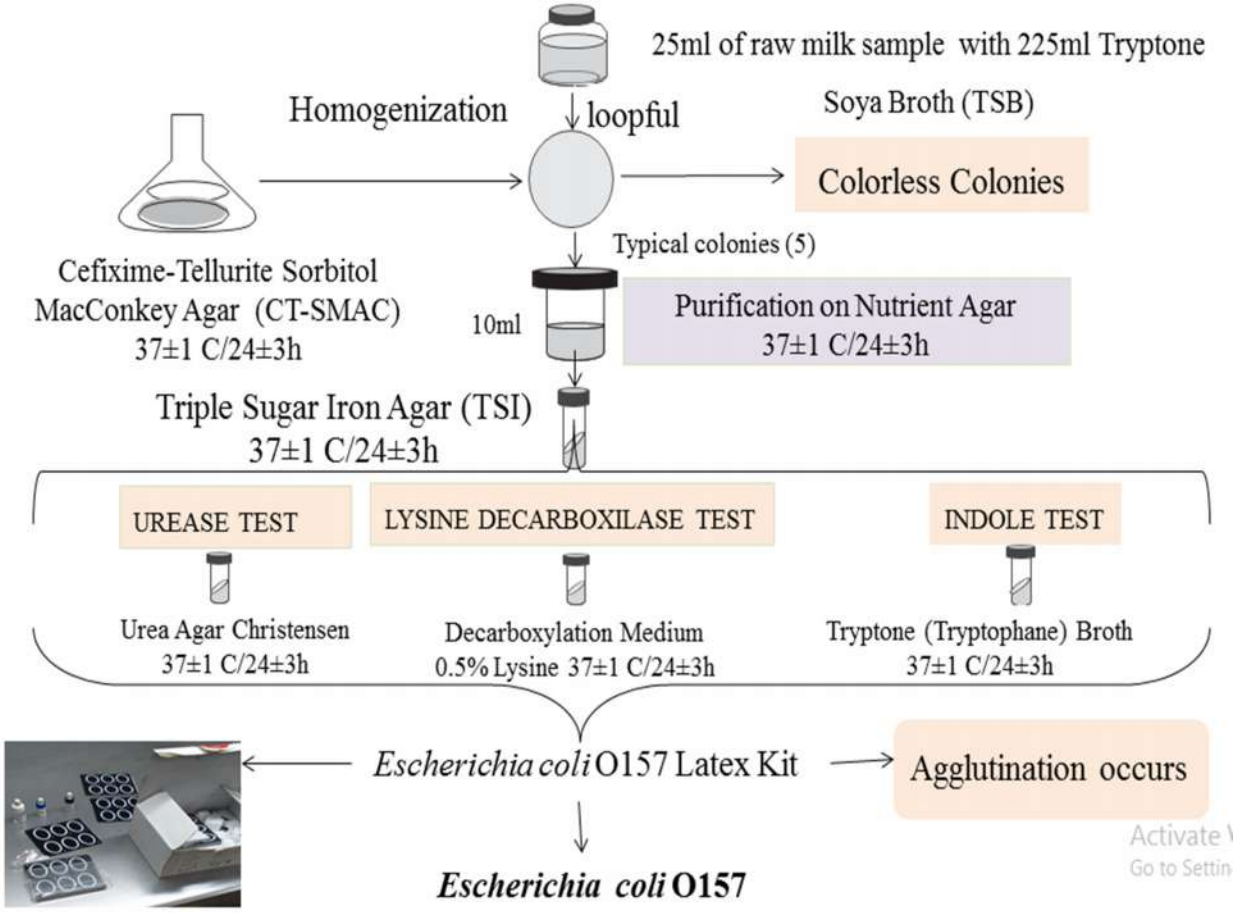

Figure 3.3: Schematic illustration of analysis for detection of *E. coli* O157 (Source: ISO-16654:2001 andISO-6579, 2002).

3.7.2. Isolation andidentification of *Salmonella*

The isolation and identification involves five steps(Figure 3.4).

*Salmonella*isolation and identification was carried out in line with the guidelines of theInternational Organization for Standardization, (ISO-6579, 2017). Within the protocol are steps that include primary enrichment in non-selective liquid medium (pre-enrichment), secondary enrichment in selective liquid media, plating out on selective and non-selective media and finally, confirmation by biochemical methods.

Step 1: Pre Enrichment:

Twenty-five milliliters of raw milk sample were homogenized with 225ml of Buffered Peptone Water (BPW) (Oxoid CM509, Basingstoke, England). Incubate at 37±1°C/18 ± 2hrs(ISO-6579, 2017).

Step 2: Selective Enrichment:

After incubation, a portion (0.1 ml) of the pre-enriched cultured was transferred to 10 ml of Selenite Cysteine (SC) broth (Himedia M025, Mumbi) broth and another portion were transferred to10 ml of Rappaport-Vassiliadis soya broth (RVS) (Merk, Darmstadt, Germany) broth and incubated at 37 ± 1°C and 41.5 ± 1°C for 24 ± 3hrs,respectively (ISO-6579, 2017).

Step3: SelectiveDifferential Plating:

From each, Selenite Cysteine broth and Rappaport-Vassiliadis soya broth cultures were used for streak a loopful on Xylose Lysine Desoxycholate agar. Proceed in the same manner with a second *Salmonella* selective isolation medium on brilliant green sulfa agar. Both Xylose lysine desoxycholate and Brilliant green sulfa agar plates wereincubate at 37 ± 1°C/24 ± 3hrs(ISO-6579, 2017).

Step 4: Colony Selection and Purification:

After the incubation period, the plates were examine for typical *Salmonella* colonies onxylose lysine desoxycholate, which are pinkwithout black centers. Many *Salmonella* cultures may produce colonies with large black centers or may appear as almost completely black colonies. Atypically, a few lactose positive *Salmonella* cultures produce yellow colonies with or

without black centers. Proceed in the same manner on brilliant green sulfa agar, which are Pink and surrounded by a red color in the medium. For purification, the plates werestreak the selected colonies onto the surface of nutrient agar plates. Incubate the plates at 37 ± 1°C/24 ± 3hrs(ISO-6579, 2017).

Step 5: BiochemicalTest:

Colonies suspected to be *Salmonella* were picked from nutrient agar and inoculated to triple sugar iron (TSI) agar slants (OXOID, Basingstoke,England), L-lysine decarboxylation medium (DIFCO, Becton, Dicknson, USA) andtryptophan broth and incubated for 24hrs at 37°C. A Colony was considered *Sallmonella* ifan alkaline slant (Red), with acid butt (yellow) on TSI with hydrogen sulfideproduction, positive for lysine (purple) color formation, negative for tryptophanutilization or indole production (yellow-brown ring) up on addition of Kovac reagents(ISO-6579, 2017).

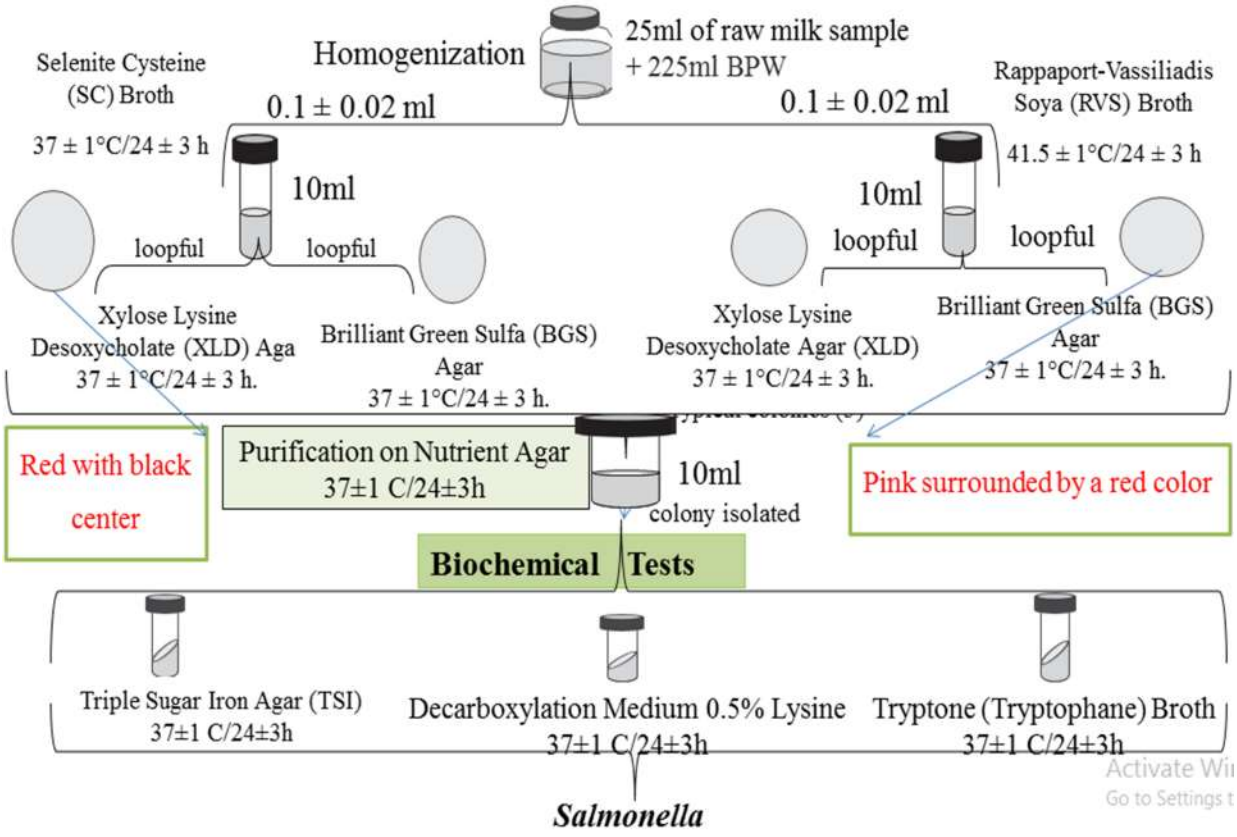

Figure 3.4: Schematic illustration of analysis for detection of *Salmonella.*

*(*Source:ISO-6579, 2017).

3.8. Antimicrobial Susceptibility Test

Antimicrobial susceptibility test were done based on the criteria of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2018)for all isolated of *E. coli* O157 and *Salmonella* using seven commercially available antimicrobial disks which are commonly used for treatment of *E. coli O157 and Salmonella* in animal and human was selected.Antimicrobial susceptibility test of the isolates were performed using the Kirby–Bauer disc diffusion method on Mueller-Hinton agar

The bacterial suspension were prepared by adding 2-4 colonies to a 5 ml tube containing 0.9% normal saline, to achieve absorbance of 0.17 – 0.18 at wavelength of 600 nm (equivalent to 0.5 McFarland standards (Walker, 2000), and spread onto Mueller-Hinton agar media using a sterile cotton swab, and the antibiotic disc were laid on the top of the agar plate. Inoculated plates were incubated aerobically at 37°C for 18–24 hrs, after which zone of inhibition results were measured with caliper meter in millimeter (mm) and interpreted according to the standard of CLSI guideline and manufacturers recommendation as susceptible, intermediate or resistant (CLSI, 2018). The list of panel of antimicrobial utilized,their symbols and concentrations and break points are shown in (Table 3.2).

Table 3.2: CLSI breakpoints for Enterobacteriaceae available for these antimicrobial

Antimicrobial Agent	Disk Content	Susceptible(mm)	Resistant(mm)	Intermediate(mm)
Ampicillin (AMP)	10 µg	≥17	≤13	14-16
Chloramphenicol (CHL)	30 µg	≥18	≤ 12	13-17
Ciprofloxacin (CIP)	5 µg	≥21	≤15	16-20
Gentamicin (GEN)	10 µg	≥15	≤12	13-14
Streptomycin (STR)	10 µg	≥15	≤11	12-14
Tetracycline (TE)	30 µg	≥15	≤11	12-14
Kanamycin (K)	30 µg	≥18	≤13	14-17

Source: CLSI, (2018)

3.9. Ethical Clearance

Ethical clearance was obtained from Bahir Dar University. informed written consent was also obtained from all study participants and confidentiality was assured by the use of codes in records.

3.10. Statistical Analysis

After collecting the data by using Kobo Tool Box Software automatically the data convert to SPSSform then arranged and managed. Processed data was analyzed by using Statistical Package for Social Science (SPSS) version 20.0 software. Then, descriptive statistical analysis such as percentage and chi-square test of various risk factor and dependent variables were presented in tables using percentage. The total prevalence was calculated by dividing the number of positive samples by the total number of samples tested. The Fisher's exact test was used to measure the association of *E. coli* O157 and *Salmonella* occurrence with incriminated categorical risk factors. The data was interpreted A p-value < 0.05 was considered as indicative of a statistical significance difference. For antimicrobial susceptibility test, the results were interpreted according to Clinical and Laboratory Standards Institute (CLSI, 2018) interpretive criteria for Enterobacteriaceae (Table 3.2).

Chapter 4. RESULTS AND DISCUSSION

4.1. Occurrences of *E .coli* O157 and *Salmonella* in Raw Cow Milk

Out of the total 150 raw milk samples examined, 9 (6%) and 7 (4.7%) were found to be contaminated with *Escherichia coli* O157 and *Salmonella,* respectively, as indicated in Table

4.1.1. Occurrences of *E .coli* O157 in raw cow milk

The overall occurrence rate of *E. coli* O1579 (6%) on raw milk sample the current result was slightly comparable with 4.08% , 4.54%, 4.67% and 6.9% and reported by Haileyesus Dejene (2018) from central Ethiopia, Frehiwot Mesele (2018) from Kombolcha district, Diriba Hunduma (2018) from Borana pastoral area and Alemu Ayano*et al.* (2013) from Holeta town, respectively.

The current result showed a slightly higher rate of raw milk contamination by *E. coli* O157 compared with 2.9%, 3.08% and 3.5% reported by Nigatu Disassa *et al*. (2017) from Asosa town, Zelalem Addis *et al.* (2011) from central Ethiopia and Segn Bedasa *et al*. (2018) in Bishoftu town, Ethiopia, respectively.

Similarly, the current finding from raw cow milk was higher when compared with the reports from abroad, 3.6% in Iran reported by Rahimi *et al*., (2012), 3.5% in Libya reported by Garbaj *et al*. (2016) and 2% in Nigeria reported by Ivbade *et al*. (2014), 0.74% by Solomakos *et al*. (2009) in Greece and 0.5% by Ahmed and Shimamoto (2014) in Egypt,

Virtually, the current finding was lower when compared with the reported, 12% by Segni Bedasa (2018) in Bishoftu towns and 10.4% by Abebe Bereda*et al*. (2014) in Tigray region and also the current result was lower when compared with the reports from abroad, 8.75% by Lye *et al*. (2013) from Malaysia, 33.5% by Chye *et al*. (2004) in Malaysia and 55% by Msolo *et al*. (2016) in South Africa. The difference occurrence of*E. coli* O157 might be due to the different dairy farming system, milking techniques and hygienic practices.

4.1.2. Occurrences of S*almonella* inraw cow milk

The overall occurrence rate of S*almonella* 7 (4.7%) on raw milk sample was comparable with 4% reported by Diriba Hunduma (2018) from Borana pastoral area, 3.2% reported by Fufa Abuna *et al.* (2017)from Holeta town and 3.08% reported by Zelalem Addis *et al.* (2011) in Addis Ababa.

Virtually, the current result was lower when compared to the reported 23.6% by Tesfa Mossie and Assefa Dires (2016) in and around Debre Zeit, 14.3% by Takele Beyene *et al.* (2016) from Asella town, 12.5% by Deresse Hailu *et al.* (2015) from Gondar town, 12.1% by Teshome Tadesse and Anbessa Dabassa (2011) from Kersa district,10.5% byFufa Abunna *et al.* (2017) from Modjo town and 7.7% byZelalem Addis *et al*. (2011) from Addis Ababa, Ethiopia.

However, it is higher than the previous study by Liyuwork Tesfaw*et al*. (2013) reported 1.6% on a dairy product in Addis Ababa, Ethiopia.The difference in the occurrence of *Salmonella*could be associated with different risk factors are related to hygienic and management practice, type and amounts of feed, accessible water supplies, usage of contaminated utensils and production facilities in different areas play a role for *Salmonella* occurrence (Karin, 2011).

4.2. The occurrence of *E. coli* O157 and *Salmonella* from Different Source of Milk

By taking of occurrence of *Escherichia coli* O15 and *Salmonella* association to different risk factors were also assessed, described and presented in below listed.

4.2.1. The occurrence of *E. coli* O157 related tothe source of milk sample

From the total raw milk samples examined at the different source, a relatively higher occurrence of *Escherichia coli* O157 was observed in smallholder milk producer (7.1%) compared with the occurrence ina cafeteria (6.3%), milk collection center (5.7%) and dairy farm (3.4%) (Table 4.2).

The current findings from the cafeteria (6.3%) were found comparable with the report of Geremew Zemenu (2017) in Bishoftu town reported 4.17%, in addition to this, Haileyesus Dejene (2018) have reported a higher occurrence of *Escherichia coli* O157 from the cafeteria in central Ethiopia (34.62%).

On the other hand, the current result from a dairy farm (3.4%) was comparable with the report of Haileyesus Dejene (2018)reported 1.33% in central Ethiopia and Nigatu Disassa*et al.* (2017) reported 0.6% in and around Asosa town. In contrast to this, Alemu Ayano*et al.* (2013) have reported a higher occurrence of *Escherichia coli* O157 from dairy farms in Holeta district (6.9%).

Also, the finding from milk collection centers (5.7%) in the current study and compared with the report of Nigatu Disassa*et al.* (2017) reported 5% in and around Asosa towns and Nigatu Disassa*et al.* (2017) reported 15.38% in Bishoftu town and lower than with report of Haileyesus Dejene (2018) reported 0.00% in central Ethiopia.The difference might be due to management system of animals, farm hygiene practice and milk hygienic practice and difference in milking and milk storage equipment.

4.2.2. The occurrence of *Salmonella*related tothe source of milk sample

A higher occuerence of*Salmonella* was observed in the milk samples collected from cafeterias (6.3%) than milk collection center (5.7%), smallholder milk producer (4.3%) and dairy farm (3.4%) (Table 4.2).

The current findings from the milk collection center (5.7%) were found lower than with the report of Almaz Kehase (2014)reported 20% in Mekelle city. On the other hand, the current result from the dairy farm (3.4%) was lower than with the report of Almaz Kehase (2014)reported 13.3% in Mekelle city.

The occurrence rate of *Salmonella* from the dairy farm in this study is lower than previous studies conducted from a dairy farm in Ethiopia with Teshome Tadesse and Anbessa Dabassa (2011) reported 12.1% in raw milk from Kersa district and Takele Beyene *et al.* (2016) reported 14.3% from Asella town.

The variation may be due to the difference in the source of the sample, different hygienic practices and agro-ecological factors, as well as accessible water supplies and usage of contaminated utensils in different areas play a role in the occurrence rate of *Salmonella*.

Table 4.1: Occurrence of *E. coli*O157and*Salmonella*in raw cow milk derived froma different source of milk sample from Bahir Dar city and Bahir Dar *zuria woreda*by using Pearson's Chi-square test.

Source of milk sample	**NoofTested (%)**	***E. coli* O157**		***Salmonella***	
		Positive (%)	X^2(p-value)	Positive (%)	X^2(p-value)
Smallholder milk producer	70(47)	5(7.1)	0.50 (0.918)	3(4.3)	0.04 (0.956)
Dairy farm	29(19.3)	1(3.4)		1(3.4)	
Milk collection center	35(23.3)	2(5.7)		2(5.7)	
Cafeteria	16(10.7)	1(6.3)		1(6.3)	
Total	150(100)	9(6)		7(4.7)	

No= Number, X^2 = Chi-square.

4.3. The occurrence of *E. coli O157* and *Salmonella* from different production system

The occurrence of *E. coli* O157 was numerically higher in rural areas (56%) than peri-urban (33%) and urban (11%) areas respectively. However, the occurrence of *Salmonella* was numerically higher in rural areas (57%) than urban (43%) and peri-urban (0%) areas respectively. However, these differences were not found statistically significant(0.11) for both *E. coli* O157 and *Salmonella*(Table 4.3).

Table 4.2: Occurrence of *E.coli O157*and*Salmonella*in raw cow's milk derived from different production system of milk sample from Bahir Dar city and Bahir Dar *zuria woreda*by using Pearson's Chi-square test.

Area	***E. coli* O157**		***Salmonella***		**No of Tested(No=150)**
	Positive (%)	X^2(p-value)	Positive (%)	X^2(p-value)	
Urban	1(11.11)		3(42.86)		60(40)
Peri-urban	3(33.33)	4.36	0(0)	4.45	48(32)
Rural	5(55.55)	(0.11)	4(57.14)	(0.11)	42(28)

No= Number, X^2 = Chi-square.

4.4. The occurrence of *E. coli O157* and *Salmonella* in Smallholder Milk Producer

4.4.1. Occurrence related to feeding and feeding system

Based on the survey data analysis, feeding system methods of smallholder milk producers in the study area practiced grazing (12.86%), stall feeding (44.29%) and both system (42.86%) (Table 4.4).

The current study in line with the findings, Birhanu Yeserah (2018) reported was grazing (15.6%), stall-feeding (34.9%) and both system (49.5%) from in and around Bahir Dar city, Kiros Abebe (2019) reported grazing and stall feeding were the main feeding systems in peri-urban Sululta (80%) and urban Holetta (50%) areas, Dessalegn Genzebu*et al*. (2016) reported stall feeding and stall feeding with limited grazing feeding systems in Bishoftu (74.6%) and Akaki town (25.4%), Adebabay Kebede (2009) reported stall feeding were the common feeding system method in Bure district (84%).

This study also indicated the type of feed provided were concentrate (62.86%), roughage (5.17%) and both alternatively (roughage and concentrate) (31.43%) feed were provided for dairy cow (Table 4.4). This result, also in line with Birhanu Yeserah (2018) reported were concentrate (21.3%), roughage (32%) and both alternatively (24%) from in and around Bahir Dar city. This difference was depending on the availability of feeds and management systems among smallholder milk producer farmers.

Table 4.3: Occurrence of *E. coli* O157 and *Salmonella* and the associated feeding practice in smallholder milk producers from Bahir Dar city and Bahir Dar *zuriaworeda*by using Pearson's Chi-square test.

Risk factors	**Overall (No=70)**	***E. coli* O157**		***Salmonella***	
		Positive (%)	X^2 (p-value)	Positive (%)	X^2 (p-value)
Feeding system					
Grazing	9(12.86)	1(20)	0.246 (0.884)	1(33.3)	1.173 (0.556)
Stall feeding	31(44.29)	2(40)		1(33.3)	
Both	30(42.86)	2(40)		1(3.33)	
Total	70(100)	5(100)		3(100)	
Feeds provided					
Roughages	4(5.17)	0(0)	0.441 (0.802)	1(33.3)	0.441 (0.802)
Concentrates	44(62.86)	3(60)		0(0)	
Both	22(31.43)	2(40)		2(66.7)	
Total	70(100)	5(100)		3(100)	

No= Number, X^2 = Chi-square.

4.4.2. Occurrence related with source of water

Water is anessential element for milk production and different operations in dairying (Mboya Neema John, 2016). Therefore, the source and type of water used for washing the hand and milking utensils have a profound effect on microbial contamination of the milk.

In this regard, the majority of smallholder milk producer farmers in the study area used tape water (52.86%) followed by pond water (40.71%) for dairy cows to drink. There was a statistically significant difference in the occurrence of *E coli.* O157 (X^2=11.523, P=0.003) and *Salmonella* (X^2=17.239, P= 0.000) among the source of water for a dairy cow was observed (Table 4.5).

The current result was comparable with the reports of Assaminew Shewangizaw (2014) reported, 76.7% of milk producer around Holetta town used tap water for of dairy cow and Azage Tegegne *et al.* (2013) reported the majority (71.8%) in Hawassa, Shashemene, Yirgalem and Dilla used tap water for the dairy cow.

In the present study, the majority of smallholder milk producer farmers in the study area used pond water (47.14%) followed by tape water (38.57%) for sanitation (Table 4.5). This result comparable with the reports of Asfawwossen Asrat *et al.* (2016) in and around Wolaita Sodo and Nigatu Disassa *et al.* (2017) in and around Asosa towns of Ethiopia reported 56.6% and 45% of the producers, respectively, used pipe water.

In virtually, the currents study lower than with Haileyesus Dejene (2018) reported, 75.9% of the producers in central Ethiopia used pipe water sources, Amistu Kuma *et al.* (2015) in Sebeta town reported 76.7% of the producers used pipe water, Abera Jabessa (2018) stated, 100% dairy farmers in Bishoftu and Asella obtained water from Tape water and Solomon Mebrahtu (2014) reported, 81.3 % dairy farmers in Mekelle city got water from Tape. The variation observed in the different studies may be due to the difference in the availability of water between the various study areas.

Table 4.4: Occurrence of *E. coli* O157 and *Salmonella* and the associated source of water in smallholder milk producers from Bahir Dar city and Bahir Dar *zuriaworeda*by using Pearson's Chi-square test.

Risk factors	**Overall**	***E. coli* O157**		***Salmonella***	
	(No=70)	Positive (%)	X^2 (p-value)	Positive (%)	X^2 (p-value)
Sources of water for dairy cow					
Pond water	28(40)	3(60)	11.523 (0.003)*	1(33.33)	17.239 (0.00)*
River/stream water	5(7.14)	2(40)		2(66.66)	
Tape/pipe water	37(52.9)	0(0)		0(0)	
Total	70(100)	5(100)		3(100)	Total
Sources of water for sanitation					
Pond water	33(47.14)	3(60)	4.758 (0.093)	1(33.33)	7.356 (0.025) *
River/stream water	10(14.29)	2(40)		2(66.66)	
Tape/pipe water	27(38.57)	0(0)		0(0)	
Total	70(100)	5(100)		3(100)	

No=number, X^2 = Chi-square, * =statistically significant.

4.4.3. Occurrence related to milking hygienic practice

In the present study, the majority 80% of the respondents washed their hand before milking, while the remainder 20% did not wash their hands before milking. There was a statistically significant difference in the occurrence of *E coli.* O157 (X^2=21.538, P=0.000) and *Salmonella* (X^2=12.537, P= 0.000) in handwashing were observed (Table 4.6). Comparable result reported by Birhanu Yeserah (2018), 54.2% dairy farmers in and around Bahir Dar city, Saba Haile *et al.* (2015), 69.4% dairy farmers in Adea Berga and Ejerie districts of west Shoa zone and Asaminew Tassew and Eyassu Seifu (2009), 100% of dairy farmers in Bahir Dar *Zuria* and Mecha district washed there hand before milking. On the contrary, Abeygunawardana *et al.* (2017)reported only 26% of milk producershas practiced hand washing in Sri Lanka.

Washing of udder and teatsbefore milking was not a common practice in the study area, as there is a belief that calves could wash it with saliva when they are allowed to have few suckling before and after milking. However, practices washing of udder and teats before milking happened only in the absence (death) of the calf to remove dirt from udder and teats.

About 14.29% of the respondents were washed while the remaining 85.71% do not wash udder and teats of their dairy cow (Table 4.6). This result agreed with the report of Birhanu Yeserah (2018), only 14.1% of respondents in and around Bahir Dar city, Ketema Worku(2014) only 5.7% of respondents in Kersa Malima *woreda*, Lemma Fita (2004) 5.6% of respondents inEastShewa zone of Oromia regionand Yien Deng (2014) only 4.8 % of respondents in Jikawo district of Nuer zone dairy farmerswere washed udder and teats.

The current result lower than with the report of Saba Haile *et al.* (2015) reported 62.2% of the milk producers washed their cowudder and teatsin Adea Berga and Ejerie districts of west Shoa zone and Almaz Kehase (2014) reported 83% of the dairy farm respondents washed the cow udder and teats in Mekelle city.On the contrary with Abebe Bereda *et al.* (2012) reported there was no udder and teats washing practice.

About 100% of the smallholder milk producer in the study area have washed the udder and teats of the cow only before milking(Table 4.6). In agreement with this finding, Haileyesus Dejene (2018) in central Ethiopia reported 74.1% of the producers have washed the udder and teats of the cow only before milking, Shewangzaw Addisu*et al.* (2016) in Gonder town and Wangalwa *et al.* (2016) in Uganda have reported that majority of the producers washed the udder and teats of the cows only before milking.

About 100% 0f the used towel after washing the udder and teats of the cow. Contrary to the current study, Lencho Getechew and Seblewongel Ayichew (2018) reported, 54% in Bishoftu town, Birhanu Yeserah (2018) reported, 14.1% in and around Bahir Dar city, Tsadkan Zegeye (2012) reported, 51.6% in Enderta district and Saba Haile *et al,* (2015) reported 6.7% in Adea Berga and Ejerie districts of west Shoa zone of the the respondents practiced udder and teats dryingafter washing.

Also, results of the current findings revealed that 80% of the smallholder milk producers in study areas used a common towel and the remaining 20% used to massage with a bare hand for drying udder and teats after washing (Table 4.6). Comparable to this, Belay Duguma (2016) have reported 61.1% of the producers in Jimma town used a common towel, Lencho Getechew and Seblewongel Ayichew (2018) showed that 28% of milk producer used common towel in Bishoftutown, Saba Haile *et al.* (2015) reported, about 15.6% of the smallholder used common towel in Adea Berga and Ejerie Districts of West Shoa Zone and Birhanu Yeserah (2018) indicated 9.4% of respondents were common towel used in and around Bahir Dar city.

The variation observed in the different studies may be due to lack of training for the smallholder milk producers on the washing of their hands, udder and teats, milk utensils and use of towels that prevent the growth of microorganisms and maintaining the safety of the products. Allover, the production of good hygienic quality of milk for consumers requires good sanitary practices, such as washing milkers' hands, cleaning udder and teats and use of individual towels during milking is imperative (Oliver *et al.,* 2009).

Table 4.5: Occurrence of *E. coli* O157 and *Salmonella* and the associated milking hygienic practice in smallholder milk producers from Bahir Dar city and Bahir Dar *zuriaworeda*by using Pearson's Chi-square test.

Risk factors	Overall (No=70)	*E. coli* O157		*Salmonella*	
		Positive (%)	X^2(pvalue)	Positive (%)	X^2(p-value)
Hand washing before milking					
Yes	56(80)	0(0)	21.538 (0.000)	0(0)	12.537 (0.000)*
No	14(20)	5(100)		3(100)	
Udder and teats washing					
Yes	10(14.29)	0(0)	0.897 (0.343)	0(0)	0.522 (0.470)
No	60(85.71)	5(100)		3(100)	
Frequency of udder and teats washing					
Before milking	10(100)	0(0)	0.897(0.343)	0(0)	0.522 (470)
Used towel for udder and teats drying					
Yes	10(100)	0(0)	0.897(0.343)	0(0)	0.522(0.470)
Types of towel used					
Common towel	8(80)	0(0)	0.897 (0.638)	0(0)	0.522 (0.770)
Massage with bar	2(20)	0(0)		0(0)	

No=number, X^2=Chi-Square,* =Statistically significant.

4.4.4. Occurrence related to milk utensils hygienic practice

Producers should pay particular attention to the type as well as the cleanliness of milk equipment. The use of plastic containers for milking and milk storage can compromise milk quality since plastic can easily crack and these cracks harbor spoilage bacteria and are difficult to clean. Aluminumutensil cans are advised in milk storage as they are easily cleaned. As indicated in Table 4.7, about91.43% of the interviewed smallholder milk producer has used plastic-made containerswhile the rest 8.57% were used calabash (local name; *Geryera*), which are made of traditionally called *Kell*during milking.

Invirtually, the currents study agreed with Mesfin Zewdu (2015) reported 82.5% of interviewed households were plastic jars used, Birhanu Yeserah (2018) reported 34.9% of the respondent used plastic made container in and around Bahir Dar city and the result is in agreement with Teshome Gemechu *et al.* (2014) reported the majority (84.62%) of milk producers used plastic buckets in Shashemene town, Almaz Kehase (2014) 66.7% of the respondents have used plastic equipment in Mekelle city and Teklemichael Tesfaye *et al.* (2013) reported that 75% were plastic utensils used in Dire Dawa town. The current result also agreed with Abebe Bereda*et al*. (2012) in Ezha district of Gurage Zone and Saba Haile *et al.* (2015) in Adea Berga and Ejerie districts of west Shoa zone reported similar result where all of the interviewed milk producer farmers were using plastic made milk containers.

Similarly, upon evaluation of the types containers in which samples were collected, the contamination frequency of *E. coli* O157, was detected in the samples collected from Calabash (16.7%) compared with samples collected from plastic (6.25%) containers (Table 4.7).

Meanwhile, the finding, level of contamination, from plastic containers was higher than the finding of Haileyesus Dejene (2018) in central Ethiopia reported contamination frequency of 8.76% plastic containers and Nigatu Disassa*et al.* (2017) in and around Asosa town, western Ethiopia have reported contamination frequency of 3.5% from plastic containers compared to Aluminumcontainer.The difference may be due to the difference in the level and awareness of hygienic methods adopted for milk and milking containers.

The current survey result showed that cleaning of milking equipment is common among most of the respondents. The majority (85.71%) of smallholder farmers practice regular cleaning of their milking utensils (Table 4.7).

The smallholder milk producers responded with 36.67%, 26.67%, 25% and 11.67%proportions that they used cold water with detergents, warm water with detergents, cold water and warm water, respectively, for cleaning milking equipment (Table 4.7). They use cold and warm water to clean milking utensils without detergents, which was insufficient to remove all the dirt on the milking equipment, which is also similar to the finding of Yien Deng (2014), and Alehegne Wubet (2004). Hence, the possibility of consuming contaminated milk resulting from unsanitary cleaning practices is high. This result comparable with the finding of Yien Deng (2014), reported, 48.3%, 43.4%, and 8.3% of the respondents used cold water, both cold and warm water and warm water, respectively, in Jikawo Woreda, Gambella Region.

The current finding lower than with the report of Haile Welearegay *et al.* (2012) and Saba Haile *et al.* (2015) reported about 85.6% and 77% of the smallholder milk producer washed their milk container with warm water with soap and cold water and soap respectively.

The cleaning frequency of milk handling containers using either cold or warm water depends upon the cleanliness of containers and types of dairy products that were kept on the containers.The majority of the respondents (80%) practiced both washing and smoking as cleaning methods of milking utensils while (20%) clean by using only washing without smoking (Table 4.7). In virtually the current result,ultimately agreed with Birhanu Yeserah (2018), reported Almost all (92.9%) of respondents performed both washing and smoking as cleaning methods while the rest 7.1% of dairy producers were cleaning their utensils only by washing.

Table 4.6: Occurrence of *E. coli* O157 and *Salmonella* and the milk utensils hygienic practice in smallholder milk producers from Bahir Dar city and Bahir Dar *zuriaworeda*by using Pearson's Chi-square test.

Risk factors	**Overall (No=70)**	***E. coli* O157**		***Salmonella***	
		Positive (%)	**X^2(p-value)**	**Positive (%)**	**X^2(p-value)**
Milking utensils					
Plastic container	64(91.43)	4(80)	0.897 (0.343)	2(66.7)	2.452 (1.117)
Calabash	6(8.57)	1(20)		1(33.3)	
Regularly cleaning of utensil					
Yes	60(85.71)	3(60)	32.308 (0.000) *	0(0)	18.806 (0.000) *
No	10(14.29)	2(40)		3(100)	
Type of water used					
Cold water	15(25)	2(66.7)	21.538 (0.000) *	0(0)	12.537 (0.014) *
Warm water	7(11.67)	1(33.3)		0(0)	
Frequency of utensil cleaning					
After each usage using cold water	28(46.66)	0(0)	32.308 (0.000) *	0(0)	18.806 (0.000) *
After each usage using warm water	16(26.66)	0(0)		0(0)	
Both alternatively	16(26.66)	0(0)		0(0)	
Methods of utensil cleaning					
Washing	12(20)	0(0)	32.308 (0.000) *	0(0)	18.806 (0.000) *
Smoking	0(0)	0(0)		0(0)	
Both alternatively	48(80)	0(0)		0(0)	

No=number, X^2=Chi-Square,* =Statistically significant.

4.4.5. Occurrence related to public health aspects

In the study area, about 37.14% of the smallholder milk producer was the habit of raw milk consumption. This result, contrary toKassu Tsegaye (2016)reported23.3% of the respondents to have a habit of raw milk consumption in Bona Zuria district of Sidama Zone, Southern Ethiopia.

In the current study,About 31.42% of the respondents are aware of the health risk associated with the consumption of raw milk and also all dairy cattle owners (84.3%) do mix fresh milk with leftover milk from the previous milking, and milk of various cows of an equivalent farm is mixed together before consumption.

Additionally, 84.3% of the respondents reported they have suffered from foodborne infections of unknown origin (Table 4.8).

Table 4.7: Public health aspects associated with consumption of milk in smallholder milk producer from Bahir Dar city and Bahir Dar *zuriaworeda*by using Pearson's Chi-square test.

Risk factors	Overall (No=70)	*E. coli* O157		*Salmonella*	
		Positive (%)	X^2(p-value)	Positive (%)	X^2(p-value)
Habit of milk consumption					
Yes	26(37.14)	1(20)	0.678	1(33.3)	0.019
No	44(62.86)	4(80)	(0.410)	2(66.6)	(0.889)
Knowledge associated consumption of raw milk health risk					
Yes	22(31.42)	2(40)	0.184	0(0)	1.437
No	48(68.6)	3(60)	(0.668)	3(100)	(0.231)
Suffered from foodborne infections					
Yes	59(84.3)	5(100)	1.004	2(66.6)	0.735
No	11(15.7)	0(0)	(0.316)	1(33.3)	(0.391)
Mixed fresh milk with milk left					
Yes	59(84.3)	3(60)	2.398	3(100)	0.584
No	11(15.7)	2(40)	(0.12)	0(0)	(0.45)
Total	70(100)	5(100)		3(100)	

No=number, X^2 = Chi-square.

4.5. The occurrence of *E. coli* O157 and *Salmonella* in Dairy Farm

4.5.1. Occurrence related to milking hygienic practice

The study made by Getachew Felleke (2003b) indicated that milking producers should follow hygienic practices (clean utensils, washing milker's hands, washing the udder and teats, use of individual towels) during milkingbefore delivery to consumers or processors.

Most loses of the dairy products occurs as a result of contamination of poor production or handling practices and lack of technical knowledge on clean milk production, use of unclean milking equipment, lack of potable water for cleaning purpose contributed to the poor hygienic quality of dairy products produced in central Ethiopia (Zelalem Yilma and Bernared Faye, 2006). Similarly, Gran *et al.* (2002) reported that insufficient cleaning of the udder and teats might result in the contamination of milk. The use of detergent and good-quality water for cleaning could be expected to remove milk remains, including microorganisms that affect the microbial quality of milk(Bruktawit Shimeles, 2016).

Therefore as Murphy (1996) pointed out, cleaning and disinfection of equipment are after each milking is essential to reduce contamination of milk by microorganisms from the equipment and with rinsing, about 10% of the number of bacteria found in milk can be reduced (Bramley and McKinnon, 2004) also found out that milk residue left on equipment contact surfaces supports the growth of a variety of microorganisms (Bruktawit Shimeles, 2016). Maintaining the sanitary condition of the milking area is essential for the production of good quality milk (Bruktawit Shimeles, 2016). The drainage condition of the milking area is one of the determinant factors (Zelalem Yilma, 2003).

As observed in this study,the majority of dairy farmers (75.86%) milk their cows in the milking home(Table 4.9).

A proper and clean housing environment is a prerequisite to produce milk and milk products of acceptable quality (Asaminew Tassew, 2007).

In the current study, 41.38% and 37.93% % respondents on the study area of the dairy farmers clean their milking shed once and twice daily, respectively (Table 4.9).Virtually, the current result agreed with Bruktawit Shimeles (2016)reported an average 83.1% of respondents on the study area clean their milking sheds once a week while the rest 16.9% of the respondents clean their milk shed more than once a week.

This is in agreement with Zelalem Yilma, (2010) reported that about 87% of the respondents cleaned their barn on a daily basis, while few (9%) of them cleaned only three times a week in the Ethiopian highlands.

In the current study, about 17.24%of respondents of dairy farmers in the study area do not washed their hands before milking, while the remaining (82.76%) did wash(Table 4.9).

The current result has also found that 54.17% and 45.83% of the dairy farmers washed their hands both before and after milking and only before milking, respectively (Table 4.9).This result in line with Almaz Kehase (2014)reported 65.52% of the dairy farmers respondents wash their hands both before and after milking and while 34.48% wash their hands before milking in Mekelle city. Contrary to Almaz Kehase (2014) reported all of the respondents washed their hands before milking their cows in Mekelle city.

Cleaning of the udder and teats of cows before milking is one of the essential hygienic practices required to ensure clean milk production. This is important since the udder and teats of the milking cows could have direct contact with the ground, urine, dung and feed refusals (Zelalem Yilma, 2010).

Gran *et al.* (2002) reported that insufficient cleaning of the udder and teats might result in contamination of milk the use of detergent and good-quality water for cleaning could be expected to remove milk remains, including microorganisms that affect the microbial quality of milk.

The current result also indicated that 65.52% of the dairy farm milk producers were practiced udder and teats washing both before and after milking(Table 4.9). This result contrary to Mesfin Zewdu (2015) from Mekelle and Haile Welearegay*et al.* (2012) from Hawassa city reported, about 35%and 82.5% of the respondents practiced udder and teats washing before

milking, respectively. However, 68.42% and 31.58% of the producers both before and after milking, and only before milking, respectively, practiced udder and teats washing(Table 4.9). This result contrast with Haileyesus Dejene(2018reported 12.1% of the producers were practiced udder and teats washing both before and after milking.

This is a potential source of contamination of milk microorganisms during milking. Since one of the objectives in dairy farming in the study area to produce good quality desirable, milk which is saleable to the processors and acceptable by the consumers. The provision of milk and milk products of good hygienic quality is beneficial from consumers health points of view. However, as observed in the current study,all of the respondents useda collective towel (Table 4.9).This result in line with Almaz Kehase (2014)from Mekelle city and Haileyesus Dejene (2018) from central Ethiopia. They reported 68.97% and 98% of dairy farmers respondents utilizedcollective towelsto dry the udder and teats of their dairy cows.

Table 4.8: Occurrence of *E. coli* O157 and *salmonella* associated milking hygienic practice in dairy farms from Bahir Dar city and Bahir Dar *zuriaworeda*byusing Fisher's Exact Test .

Risk factors	**Overall (No=29)**	***E. coli* O157**		***Salmonella***	
		Positive (%)	**Fisher's exact test(p-value)**	**Positive (%)**	**Fisher's exact test (p-value)**
Milking area					
In barn	7(24.14)	0(0)	0.33	0(0)	0.11
In milking room	22(75.86)	1(100)	(0.57)	1(100)	(0.566)
Frequency of cleaning the barn/milking room					
Twice a day	11(37.93)	1(100)		0(0)	
Once a day	12(41.38)	0(0)	1.7	0(0)	14
Once per two day	4(13.79)	0(0)	(0.638)	0(0)	(0.003)*
Once a week	2(6.9)	0(0)		1(100)	
Hand washing before milking					
Yes	24(82.76)	0(0)	4.97	1(100)	0.21
No	5(17.24)	1(100)	(0.026)	0(0)	(0.642)
The practice of handwashing					
Only before milking	11(45.83)	0(0)	4.97	1(100)	1.69
Both before and after milking	13(54.17)	0(0)	(0.083)	0(0)	(0.429)
Udder and teats washing before milking					
Yes	19(65.52)	1(100)	0.55	0(0)	1.97
No	10(34.48)	0(0)	(0.46)	1(100)	(0.161)
The practice of udder and teats washing					
Only before milking	6(31.58)	0(0)	1.275	0(0)	1.97
Both before and after milking	13(68.42)	1(100)	(0.529)	0(0)	(0.374)
Type of towel used					
Collective towel	19(100)	1(100)	0.545(0.46)	0(0)	1.97(0.161)

No= number, * =Statistically significant.

4.5.2. Occurrence related to milk utensils hygienic practicein dairy farms

Proper cleaning of utensils and equipment utilized within the dairy requires scrubbing during warm water with a detergent solution, followed by rinsing with clean warm water and sterilization using wet heat during the steam cabinet. Since dairy workers clean their utensils but do not sterilize them, there is a risk of bacterial build-up and thus the contamination of subsequent consignments of milk. Efficient cleaning by using the right detergent at the recommended strength is essential for keeping microbial contamination of the products to a minimum.

Type of equipment used for milking and storage contributes to the quality and safety of milk and milk products. Therefore, milk producers have to be attention tothe cleanliness of milk equipment. Additionally, it should be better to used aluminum equipment because this equipment is easy to clean. Efficient cleaning of utensils by using the right detergent at the recommended strength is essential for keeping microbial contamination of the products to a minimum. It was evident that 74.41 % of the dairy farmers use Aluminummilk utensils (Table 4.10).This result in line with Haile Welearegay *et al.*, 2012)reported about 74.41 % of the dairy farmers used Aluminumin Hawassa city.

About 89.66%of the respondents used tape water, and the remaining 10.34% used pond water as primary source water for cleaning the udder or teats, washing their hands, and milking equipment (Table 4.10). This result in line with Bruktawit Shimeles (2016) reported about 98.9% of the respondents used tape water and other 1.1% used pond water source for cleaning and washing purpose in Addis Ababa and Abera Jabessa (2018) all respondents of the dairy farmers obtained water from tap in Bishoftu and Asella town. This result higher than with Almaz Kehase (2014)reported about 60% of the respondents of dairy farms using only tap water in Mekelle city.

About 48.3% of the respondents used warm water, followed by 31.03% cold water (Table 4.10). This result, contrary toAlmaz Kehase (2014)reported about 73.3 % of the respondents of dairy farms, usedcold water for washing milking utensils in Mekelle city.

Table 4.9: Occurrence of *E. coli* O157 and *salmonella* associated milk utensils hygienic practice in dairy farms from Bahir Dar city and Bahir Dar *zuriaworeda*byusing Fisher's Exact Test .

Risk factors	Overall (No=29)	*E. coli* O157		*Salmonella*	
		Positive (%)	Fisher's exact test(p-value)	Positive (%)	Fisher's exact test(p-value)
Type of milk utensils					
Clay pot	3(10.34)	0(0)	4.97 (0.083)	0(0)	0.4 (0.821)
Aluminum	21(74.41)	0(0)		1(100)	
Plastic equipments	5(17.24)	1(100)		0(0)	
Total	29(100)	1(100)		1(00)	
Source of water for milk utensils washing					
Pond water	3(10.34)	0(0)	0.12(0.73)	0(0)	0.12 (0.73)
Tap/pipe water	26(89.66)	1(100)		1(100)	
Total	29(100)	1(100)		1(00)	
Types of water used for milk utensils washing					
Cold water	9(31.03)	1(100)	2.3 (0.512)	0(0)	6.47 (0.091)
Warm water	14(48.3)	0(0)		0(0)	
Cold water with soap	4(13.79)	0(0)		1(100)	
Warm water with soap	2(6.9)	0(0)		0(0)	
Total	29(100)	1(100)		1(00)	

No=number.

4.6. The occurrence of *E. coli* O157and*Salmonella*in Milk Collection Center

4.6.1. Occurrence related to milk hygienic practice

Milk storage utensils are properly cleaned and maintained. Therefore, cleaning and disinfection of equipment after each usage is essential for the reduction of milk contamination from the equipment (Murphy, 1996) and the utilization of Aluminumcontainers for milk storage, as they are milk collection center can compromise milk quality to enhance and advised in milk storage as they are easily cleaned. Aluminumcontainers are recommended because they do not have adhesive properties and, therefore, easy to clean when compared with plastic containers (Karuga, 2009).

As indicated in Table 11, about 80% of the interviewed milk collection center was used Aluminumis used during storage. The current finding contradicts the finding of Almaz Kehase (2014)reported about 70% of milk collection centers used plastic containers (Jerry cans). This might be a contributing factor for the rapid spoilage of milk, as plastic jerry cans cannot be cleaned properly due to their narrow mouths and the inaccessible cavities of their handles in which microorganisms may form biofilms, which cannot be removed easily.

About,all most 85.7% of the interviewed milk collection center was tape/pipe water while the remainder 14.3% used Pond water. Thisresult contrary toHaileyesus Dejene (2018)reported all the milk collection centers used pipe water.

In virtually, the currents result contradict with Saba Haile (2015) reported, 67% milk collection center obtained water from Tape waterin Adea Berga and Ejerie districts of west Shoa zoneand Nigatu Disassa *et al.* (2017) reported that 30% of the milk collection centers in and around Asosa town, used pipe water.

About 34.29 % of the respondent washed their milk container with cold water, while 45.7% used both alternatively cold water and warm water (Table 4.11). In contrary to Saba Haile (2015) reported about 77% of the respondent washed their milk container with cold water and soap while 23% used hot water and soap Adea Berga and Ejerie districts of west Shoa zone and Haile Welearegay *et al.* (2012) reported about 85.6% of the producers used warm water

together with detergents to wash milk handling equipment while 12.1% of them cleaned with cold water.

The plants used for smoking milking and milk storage utensils are indicated in Table 4.11. To extend the shelf life of milk and milk products, milk producers of the study area exercise smoking for milk utensils, *Abalo (Terminalia brownii*) and *Woyira* (*Olea Africana)* are most commonly used and the other smoking plant species were occasionally used. It is essential to notice that the utilization of regularly use smoke to utensil and types of plants used for smoking of the milk utensil practices might represent a critical control point for reducing the occurrence rate of *E. coli* O157 and *salmonella* in the milk collection center.

About Majority of the respondents reported that the most common plant species used for smoking milk vessels in the study area was *Abalo (Terminalia brownii*) (37.14%) and *Woyira* (*Olea Africana*)(37.14%) for increasing the shelf life of milk)(71.43%) and to give good flavor and aroma of milk utensils)(28.57%) (Table 4.11). The current result in line with Birhanu Yeserah (2018) reported *Abalo (Terminalia brownii*) (63.5%) followed by *Woyira* (*Olea Africana*)(10.2%) were occasionally used in and around Bahir Dar city and Kassu Tsegaye (2016) reported majority of the respondents, *Woyira* (*Olea Africana*)are used for smoking of milk and milk product containers in Bona Zuria district of Sidama Zone.

After milking proper milk cooling method is essential to maintain the quality of milk. However, Out of the total (37.14%) milk collection centerrespondentsused a cooling system in the study area were all milk collection centers (100%) used Refrigerator to put raw milk (Table 4.11). This result agreed with Saba Haile (2015) from Adea Berga and Ejerie districts, West Shoa Zone andKassu Tsegaye (2016)from Sidama Zone reported allcollection center respondents used With container at room temperature.

Table 4.10: Occurrence of *E. coli* O157 and *Salmonella* associated with milk hygienic practice in milk collection center from Bahir Dar city and Bahir Dar *zuriaworeda*byusing Fisher's Exact Test .

Risk factors	Overall (N=35)	*E. coli* O157		*Salmonella*	
		Positive (%)	Fisher's exact test (p-value)	Positive	Fisher's exact test (p-value)
Type of utensils used					
Aluminum	28(80)	1(50)	1.2	0(0)	8.5
Plastic equipment's	7(20)	1(50)	(0.275)	2(100)	(0.004)*
Source of water for washing utensils					
Pond water	5(14.29)	1(50)	2.2	1(50)	2.2
Tape/pipe water	30(85.71)	1(50)	(0.137)	1(50)	(0.137)
Type of water used					
Cold water	12(34.29)	1(50)	0.59 (0.746)	1(50)	2.08 (0.354)
Warm water	7(20)	0(0)		1(50)	
Both alternatively	16(45.7)	1(50)		0(0)	
Types of plants used to smoke utensils					
Abalo*(Terminalia brownii*)	13(37.14)	0(0)	6.13 (0.043)*	0(0)	6.13 (0.043)*
Weyira(*Olea Africana*)	13(37.14)	0(0)		0(0)	
Other plants	9(25.714)	2(100)		2(100)	
Purpose of these plants used					
Good flavor and aroma	10(28.57)	1(50)	0.477	2(100)	5.3
Increase the shelf life	25(71.43)	1(50)	(0.49)	0(0)	(0.076)
Used cooling system					
Yes	13(37.14)	0(0)	1.25	0(0)	1.25
No	22(62.86)	2(100)	(0.263)	2(100)	(0.263)
Milk cooling methods					
In refrigerator	13(100)	0(0)	1.25 (0.263)	0(0)	1.25 (0.263)

No= number, * =Statistically significant.

4.7. Occurrence of *E. coli* O157 and *Salmonella* in Cafeteria

4.7.1. Occurrence related to milk hygienic practice

The assessment on milkhygienic practicein cafeteriaassociated with*E. coli* O157 and *Salmonella*. The 5 checklist parameters were listed as follows with their percentage contribution for the occurrence *E. coli* O157 and *Salmonella* in raw cow milk samples.

As shown in Table 4.12, about 43.75%, 31.25% and 25% of the cafeterias were sources of raw milk used from milk collection centers, dairy farm and smallholder milk producers (Table 4.12). About 100% of the respondents from cafeterias in the study area used plastic milk storage containers. Contrary to the reports of Haileyesus Dejene (2018) reported 84.6% of the cafeterias used plastic milk storage containers in central Ethiopia and Nigatu Disassa*et al.*(2017) in and around Asosa town have reported that 66.6% of the cafeterias used plastic storage containers.Additionally, in the current study, 100% of the cafeterias used pipe water for sanitation purposes. However, the current finding was higher in comparison with the reports of Haileyesus Dejene (2018 from central Ethiopia,Tsedey Azeze and Asrat Tera *et al.* (2016) in and around Sodo town, Wolaita Zone, Nigatu Disassa *et al.* (2017) in and around Asosa town and Amistu Kuma *et al.* (2015) in Sebeta town have reported 42.3%, 56.6%, 45% and 76.7% of the cafeterias,respectively, used pipe water.

As observed in the current result, about 62.5% and 37.5% of the cafeterias in the study area have stored milk in the refrigerator and within the container at room temperature before it was delivered to the consumers, respectively (Table 4.12).This result agreed with Haileyesus Dejene (2018) from Central Ethiopiareported all cafeterias respondents used 23.1% and 76.9% store milk in the refrigerator andwitha container at room temperature until used by consumers. In contrast with Kassu Tsegaye (2016) reported all of the respondents to store milk at room temperature in Bona Zuria district of Southern Ethiopia.

Table 4.11: Occurrence of *E. coli* O157 and *Salmonella* associated with milk hygienic practice in the cafeteria from in Bahir Dar city and Bahir Dar *zuriaworeda*byusing Fisher's Exact Test .

Risk factors	**Overall**	***E. coli* O157**		***Salmonella***	
	(No=16)	**Positive (%)**	**Fisher's exact test (p-value)**	**Positive (%)**	**Fisher's exact test (p-value)**
Source of milk					
Smallholder	4(25)	1(100)		1(100)	
Dairy farm	5(31.25)	0(0)	3.2(0.202)	0(0)	3.2(0.202)
Milk Collection center	7(43.75)	0(0)		0(0)	
Total	16(100)	1(100)		1(100)	
Form of milk sell for consumers					
Boiled	3(18.75)	0(0)		1(100)	
Yoghurt	1(6.25)	0(0)	0.36(0.837)	0(0)	4.6(0.099)
Both alternatively	12(75)	1(100)		0(0)	
Total	16(100)	1(100)		1(100)	
Store milk until used					
In refrigerator	10(62.5)	1(100)	1.1(0.587)	1(100)	1.1(0.587)
Within container at room temperature	6(37.5)	0(0)		0(0)	
Total	16(100)	1(100)		1(100)	

No= number.

4.8. Antimicrobial Susceptibility

The antimicrobial sensitivity tests results showed a high level of resistance to most of the antibiotics used. Thus, the development of antimicrobial resistance by the bacteria to these drugs poses a significant challenge in both human and animal medicine since they are commonly used in the treatment of human patients and veterinary practice. Moreover, uncontrolled and indiscriminate usage of antibiotics in the treatment of animals and their incorporation in animal feeds has been alleged to account significantly to the increase in antibiotic resistance bacterial isolates (Reuben and Owuna, 2013).

All(9)of *E. coli* O157and seven of *Salmonella*isolatedwere subjected to sevencommercially available antimicrobial agents, as summarized in (Table 4.13) using diskdiffusion methods. The results were classified into resistant, intermediate, orsusceptiblebased on CLS I, (2018).

4.8.1. Antimicrobial susceptibility result of *E. coli* O157

In the current study, 100% isolates of *E. coli* O157 from the total 9 isolated tested were susceptible to Gentamicin and Ciprofloxacin, However, all (100%) E. *coli* O157 isolated were resistant to Ampicillin and Chloramphenicol. Additionally, all isolated of *E. coli* O157 were67%, 56% and 45% susceptible to Tetracycline, Kanamycin and Streptomycin, respectively (Table 4.13). This result in line with the reports ofSegn Bedasa *et al.* (2018) 100% and 85.7% from Bishoftu town, Diriba Hunduma (2018) 100% and 100% from Borena pastorial area and Seyum Firew (2019) 100% and 100% from Bishoftu town were susceptibility of *E. coli O157* isolated to Gentamycin and Ciprofloxacin, from raw cow milk samples, respectively, and also this result inline from abroad with Reuben and Owuna (2013) 89.5% and 78.9%in Nigeria and Alam *et al.* (2017) 50% and 66.67%; in Bangladesh were susceptibility of *E. coli O157* isolated to Gentamycin and Ciprofloxacin, respectively.

The current finding in line with the report of Zelalem Addis *et al*., (2011) also indicated resistance of*Salmonella* isolates to commonly used antimicrobials including Ampicillin, Streptomycin, Kanamycine and Tetracycline, with a resistance rate of 100%, 66.7%, 58.3% and33.3%, respectively.

In Ethiopia, different studies showed that drug resistance of *E .coli* O157 from animal origin foods (Adem Hiko *et al.,* 2008; Tizeta Bekele*et al.,* 2014; Mengistie Taye*et al.,* 2013; Seyum Firew, 2019).The current study showed that *E. coli* O157 isolates were highly sensitive to ciprofloxacin and Gentamicin and help for suggesting the use of these antibiotics.

4.8.2. Antimicrobial susceptibility result of *Salmonella*

In the current study, 100% isolates of *Salmonella* from a total of 7 isolated tested were susceptible to Gentamicin and Ciprofloxacin. However, all (100%) isolates of *Salmonella* were resistant to Ampicillin. Additionally, all isolated of *Salmonella*were 29%, 57% and 86% of isolatedsusceptible to Streptomycin, Kanamycin and Chlorophenicol, respectively (Table 4.13).The current finding of *Salmonella*was in line with the reports ofSeyum Firew (2019) in Bishoftu town,Diriba Hunduma(2018) in Borena pastoral areaand Olana Marera in Bishoftu townfrom raw cow milk samples reported were63% and 93%; 100% and 100% and 100% and 100% of *Salmonella*isolated susceptibility to Gentamycin and Ciprofloxacin, respectively.

Table 4.12: Antimicrobial susceptibility test result of *E. coli* O157 and *Salmonella* isolates

Antimicrobial agent	**Disk Content**	**Susceptibility and resistance patterns**					
		Escherichia coli O157(No=9)			*Salmonella* (No=7)		
		S (%)	**R (%)**	**I (%)**	**S (%)**	**R (%)**	**I (%)**
Ampicillin (AMP)	10 µg	0(0)	9(100)	0(0)	0(0)	7(100)	0(0)
Chloramphenicol (C)	30 µg	9(100)	0(0)	0(0)	6(86)	0(0)	1(14)
Ciprofloxacin (CIP)	5 µg	9(100)	0(0)	0(0)	7(100)	0(0)	0(0)
Gentamicin (GEN)	10 µg	9(100)	0(0)	0(0)	7(100)	0(0)	0(0)
Kanamycin (K)	30 µg	5(56)	3(33)	1(11)	4(57)	3(43)	0(0)
Streptomycin (S)	10 µg	4(45)	3(33)	2(22)	2(29)	1(14)	4(57)
Tetracycline (TE)	30 µg	6(67)	0(0)	3(33)	1(1)	0(0)	6(86)

Key: S = Susceptible R = Resistant I = Intermediate
AMP= Ampicillin, C=Chloramphenicol, CIP= Ciprofloxacin, GEN= Gentamicin, K= Kanamycin,S= Streptomycin, TE= Tetracycline.

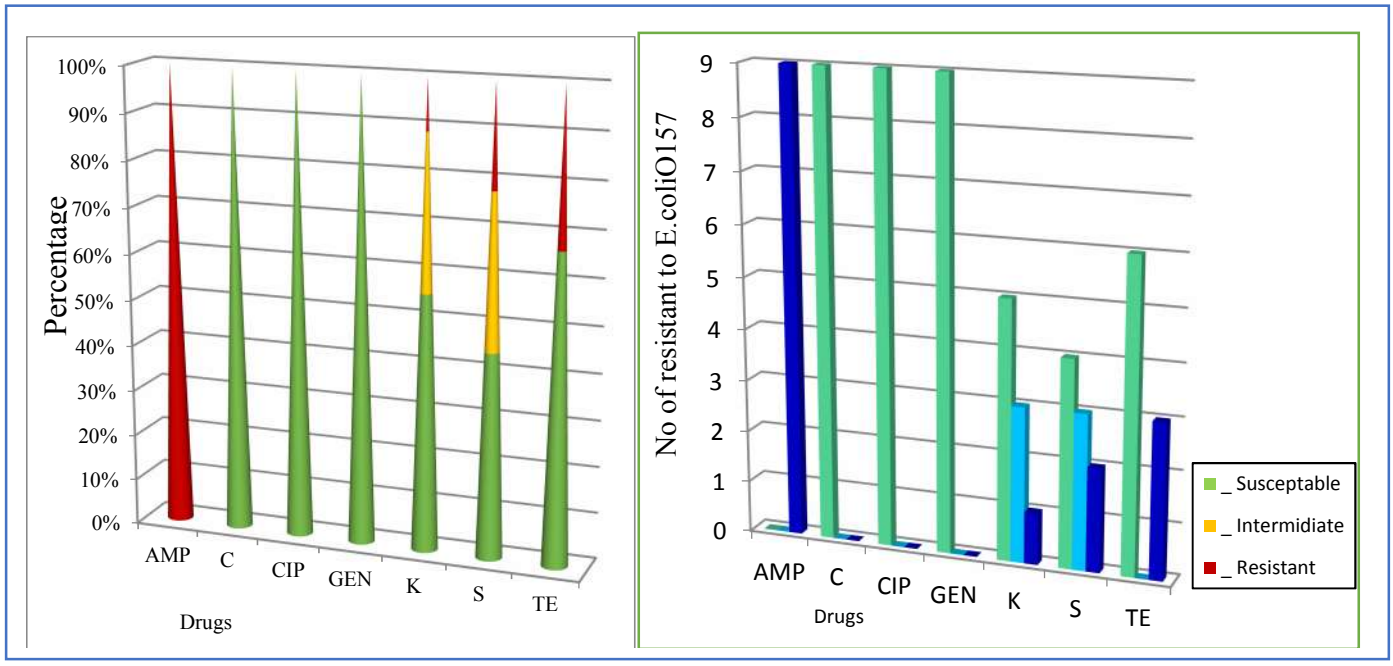

Figure 4.1: Antimicrobial susceptibility test result of *E. coli* O157

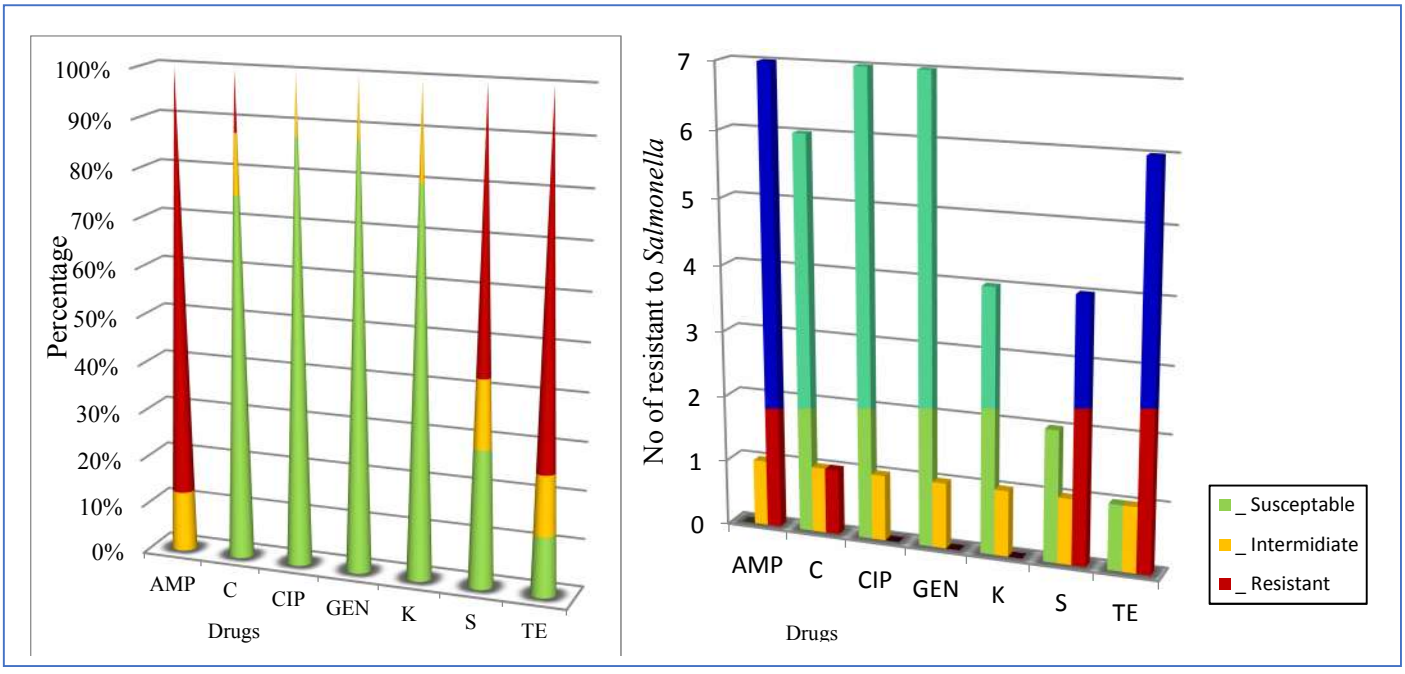

Figure 4.2: Antimicrobial susceptibility test result of *Salmonella*

Multi-drug resistance result of *E. coli* O157 and *Salmonella*

According to the definition of Magiorakos *et al*. (2012) Multi-drug resistance (MDR) was defined as acquired non-susceptibility to a minimum of one agent in three or more antimicrobial categories. The increasing developing multi-drug resistant bacteria is a significant alarm from treatment point of view or the possible transforming of resistance genes to other related pathogens (Osaili *et al*., 2013).

Regarding multi drug resistance of the current study, 11% (1/9) of *E. coli* O157isolated were found to be MDR. Only one isolated (11 %) of*E. coli* O157 showed resistance for three antimicrobials drug.

Virtually, the current study, 42.9 % (3/7) of *Salmonella* isolated were found to be MDR.Most of the isolated showed resistance against one or two antimicrobials, while three isolated (42.9%) showed resistance against three antimicrobials.

Virtually, both *E. coli* O157and *salmonella* isolates were not found to be resistant against four or more antimicrobials though most of them showed similar resistance patterns.

Chapter 5. CONCLUSION AND RECOMMENDATIONS

The current study showed considerable occurrence of*Escherichia coli* O157 and *Salmonella*in raw cow milk from smallholder milk producers, dairy farms, milk collection centers and cafeterias in and round Bahir Dar city. Out of150 raw milk sample examined, nine (6%) and seven (4.7%) were found to be contaminated with *E. coli*O157 and *Salmonella*, respectively. Also showed, Hygienic practice of smallholder milk producers, dairy farms, milk collection centers and cafeteriasdo not follow washing milker hand, udder and teats before and after milking, and alsodo not use clean tap or pipe waterfor thesanitation practices,Aluminumcontainers for thestorage of milkand store in a refrigerator until delivering to consumers might be the cause of contamination of *E. coli*O157 and *Salmonella*. The antimicrobial susceptibility profile showed that all isolates were 100% susceptible to Gentamicin and Ciprofloxacin, and resistant to Ampicillin. While, 11% (1/9) of *E. coli* O157 and42.9% (3/7) of *Salmonella*isolates were found to be multidrug resistance. Generally, the current study provided an initial baseline data regarding to the occurrence of *E. coli* O157 and *Salmonella* in the milk value chains.

Based on the above conclusions the followingrecommendations are forwarded:

- ✓ Smallholder milk producers, dairy farms, milk collection centers and cafeterias should use clean tap or pipe water, and aluminum containers for thesanitation practices and storage of milk, respectively and therefore the milk should be stored during a refrigerator until delivering to consumers.
- ✓ Preventive measures such as regular washing of milker's hand, udder and teats of the cows, dairy equipment and utensils highly recommended.
- ✓ Physicians within the area should consider Ciprofloxacin and Gentamicin as first choice drugs within the treatment of clinicaldiseases associated with*E. coli* O157 and *Salmonella.*
- ✓ Therefore further detailed studies should be conducted to describe the common *Salmonella* serovars isolated from animals and humans in the study area and molecular characterization of spp and serotypes of *Salmonella.*

6. REFERENCES

Abebe Bereda, Mohammed Yesuf and Zelalem Yilma, 2014. Handling, processing and utilization of milk and milk products in Ethiopia. A Review. *World Journal of Dairy and Food Sciences*. **9:** 105-112.

Abebe Bereda, Zelalem Yilma and Ajebu Nurfeta, 2012. Hygienic and microbial quality of raw whole cow's milk produced in *Ezha* district of the Gurage zone, Southern Ethiopia. *Journal of Agricultural Research*. **1:** 459-465.

Abera Jabessa Fufa, 2018. *Assessment of resilience of dairy farming in Bishoftu and Asella areas, Oromia regional state, Ethiopia*. MVSc Thesis, Addis Ababa University, Ethiopia.

Abeygunawardana D., Ranasinghe R. and Deshapriya R., 2017. Hygienic practices and quality of raw milk produced in a small scale dairy farming area in Sri Lanka. *International Journal of Scientific and Research Publications.* **7:** *2250-3153.*

Adam M.R and Moss M.O., 2008. Food Microbiology. 3rd Edition. Royal Society of Chemistry, Cambridge. Pp. 216-224.

Addo K., Mensah G., Aning, K., Nartey, N., Nipah, G., Bonsu, C., Akyeh M. and Smits H., 2011. Microbiological quality and antibiotic residues in informally marketed raw cow milk within the coastal savannah zone of Ghana. *Journal of Tropical Medicine and International Health.***16:** 227-232.

Adebabay Kebede, 2009. *Characterization of milk production systems, marketing and on farm evaluation of the effect of feed supplementation on milk yield and milk composition of cows at Bure district, Ethiopia*. M.Sc. Thesis, Bahir Dar University, Ethiopia.

Adem Hiko, Daniel Asrat and Girma Zewde, 2008. Occurrence of *E. coli* O157:H7 in retail raw meat products in Ethiopia. *Journal of Infectious Disease.* **2(5):** 389-93.

Ahmed A and Shimamoto T., 2014. Isolation and molecular characterization of *Salmonella enterica,E. coli* O157 and Shigella spp. from meat and dairy products in Egypt. *International Journal of Food Microbiology.* **168:** 57-62.

Alehegn Wubet, 2004. *Bacteriological quality of Bovine milk in small holder dairy farms in Debre Zeit, Ethiopia.* Msc Thesis, Addis Ababa University, Ethiopia.

Alemu Ayano, Fikiru Hiriko, Alemant Simyalew and Aster Yohannes, 2013. Prevalence of subclinical mastitis in lactating cows in selected commercial dairy farms of Holeta district. *Journal of Veterinary Medicine and Animal Health.***5:** 67-72.

Alganesh Tola and Fekadu Beyene, 2012. Traditional milk and milk products handling practices and raw milk quality in Eastern Wollega, Ethiopia. In: Laura Dean (ed.) LAP LAMBERT Academic Publishing. pp85.

Ali A.A, Irshad N, Razaz S. and Manahil A., 2010. Microbiological safety of raw milk in Khartoum state, Sudan: 1-Khartoum and Omdurman cities. *Pakistan Journal of Nutrition.* **9:** 426-429.

Allerberger F., Wagner M., Schweiger P., Rammer H., Resch A., Dierich M.P., Friedrich A.W. and Karch H., 2001. *E. coli* O157 infections and unpasteurised milk. *Eurosurveillance***6:**147-151.

Allocati N., Masulli M., Alexeyev M. and Di Ilio C., 2013 *E. coli* in Europe: An overview. *International Journal Environment Research Public Health.***10(12):** 6235-6254.

Almaz Kehase, 2014. *The quality and safety of raw cow milk produced and marketed by three dairy farms in Mekelle, Northern Ethiopia.*M.Sc Thesis, Haramaya University, Ethiopia.

Amistu Kuma, Melese Abdisa and Degefa Tolossa, 2015. Evaluation of hygienic status and marketing system of raw cow milk in different critical points of oromia special zone. *Global Journal of Science Frontier Research*: *C Biological Science*. **15:** 21-30.

Arafa M and Soliman M., 2013. Bacteriological quality and safety of raw cow's milk and fresh cream. *Slovenian Vetetrinar Research*. **50 (1):**21-30.

Asaminew Tasew, 2007. *Production, handling, traditional processing practices and quality of milk in Bahir Dar milk shed area, Ethiopia.* M.Sc, Thesis, Haramaya University, Ethiopia.

Asaminew Tassew and Eyassu Seifu, 2009. Smallholder dairy production system and emergence of dairy cooperatives in Bahir Dar zuria and Mecha woredas, Northwestern Ethiopia. *World Journal of Dairy and Food Sciences*.**4:** 185-192.

Asaminew Tassew and Eyassu Seifu, 2011. Microbial quality of raw cow's milk collected from farmers and dairy cooperatives in Bahir Dar Zuria and Mecha district, Ethiopia. *Journal of Nutrition Agriculture and Biology*.**2:** 29-33.

Asfawwossen Asrat, Adey Feleke and Ermias B., 2016. Characterization of dairy cattle production systems in and around Wolaita Sodo town, southern Ethiopia. *Scholarly Journal of Agricultural Science.***6:** 62-70.

Assaminew Shewangizaw, 2014 *Assessment of Feed Formulation and Feeding Practices For Urban and Peri-urban Dairy Cows Around Holetta, Ethiopia.* MVSc Thesis, Addis Ababa University, Ethiopia.

Ayalew Assefa and Amare Bihon, 2018. A systematic review and meta-analysis of prevalence of *Escherichia coli* in foods of animal origin in Ethiopia. Heliyon. **4:** 716.

Azage Tegegne, Berhanu Gebremedhin, Dirk Hoekstra, Berhanu Belay and Yoseph Mekasha, 2013. Smallholder dairy production and marketing systems in Ethiopia: IPMS experiences and opportunities for market-oriented development. IPMS (Improving Productivity and Market Success) of Ethiopian Farmers Project Working Paper 31. Nairobi.

Baker C. A., Rubinelli P. M., Park H., Carbonero F and Ricke C., 2016. Shiga toxinproducing *E. coli* in food: Incidence, ecology, and detection strategies, *Food Control*. **59:** 407-419.

Basil A., Khudor M and Abid-Smeasem O., 2012. Detection of verotoxigenic *E. coli* O157:H7 in raw milk using duplex PCR in Basra city, Iraq. *Mirror of Research in Veterinary Sciences and Animals*.**1:** 25-33.

Bayeh Abera, Fantahun Biadegelgen and Balay Bezabih, 2010. Prevalence of *Salmonella typhi* and intestinal parasites among food handlers in Bahir Dar Town, Northwest Ethiopia.*Ethiopian Journal of Health Development*. **24:** 1.

BCOARD, 2017. Annual Budget report of Bahir Dar City Office of Agriculture and Rural Development (BCOARD), Bahir Dar, Ethiopia.

Belay Duguma, 2016. Assessment of Dairy Farmers' Hygienic Milking Practices and Awareness of Cattle and Milk-Borne Zoonoses in Jimma, Ethiopia. *Food Science Quality Manag.* 45.

Birhanu Yeserah, 2018. *Handling Practices and Microbiological Quality of Raw Cow's Milk in and around, Bahir Dar city, Ethiopia.* M.Sc. Thesis, Bahir Dar University, Ethiopia.

Biswas, P., Uddin, G., Barua, H., Roy, K., Biswas, D., Ahad, A. and Debnath, N., 2006. Causes of loss of Sonali chickens on smallholder households in Bangladesh. *Journal of Preventive Veterinary Medicine*. **76:** 185-195.

BoARD., 2016. Bureau of Agriculture and Rural Development (BoARD) Amhara Region, Statistics section. Bahir Dar, Ethiopia.

Bramley A.J and Mckinnon C.H., 2004. The microbiology of raw milk, in R. K. Robinson (ed.), Dairy Microbiology, 2nd edn. Elsevier Applied Science, London. **1:** 63-208.

Brenner FW, Villar RG, Angulo FJ, Tauxe R, Swaminathan B. 2000. *Salmonella* nomenclature. *Journal of Clinical Microbiology*.**38(7):** 2465-2467. 13.

Brown MH, Gill CO, Hollingsworth J, Nickelson R II, Seward S, *et al.* (2000) The role of microbiological testing in systems for assuring the safety of beef. *International Journal of Food Microbiology*.**62:** 7-16.

Bruktawit Shimeles, 2016. *Physicochemical Properties And Microbial Quality Of Cow Milk Collected From Selected Subcity Of Addis Ababa, Ethiopia.* MVSc Thesis, Addis Ababa University, Ethiopia.

Bryan A., Youngster I and McAdam J., 2015. Shiga Toxin Producing *Escherichia coli*, Clinics in Laboratory Medicine. **35:** 247-272.

BZOARD, 2016. Annual Budget Report of Bahir Dar zuria Office of Agriculture and Rural Development (BZOARD), Bahir Dar, Ethiopia.

Canizalez-Roman A., Sui B.Q., Nataro J.P and Azamar Y., 2013. The serine protease motif of EspC from enteropathogenic *E. coli* produces epithelial damage by a mechanism different from that of Pet toxin from enteroaggregative *E. coli*. *Infectious Immunology.***72:** 3609-3621.

Carr J.H., 2017. *Escherichia coli.* https://pixnio.com/science/microscopy images/escherichiac oli/gram-negative-escherichia-coli-bacteria-o157-h7-e-coli-o157h7-bacterium (accessed 2018 January 22).

CDC (Center of Disease Control and Prevention), 2015. Enterohemorrhagic *E. coli* and Other *E. coli* Causing Hemolytic Uremic Syndrome. Iowa state university, *Institute for international Cooperation in Animal Biolocs.*

CDC(Center of Disease Control and Prevention), 2016. Enterohemorrhagic *E. coli* and Other *E. coli* Causing Hemolytic Uremic Syndrome. Iowa state university, *Institute for international Cooperation in Animal Biolocs.*

Chagunda M G. G., Mwangwela A., Mumba C., Anjos FD. and Kawonga BS. *et al.,* 2015. Assessing and managing intensification in smallholder dairy systems for food and nutrition security in Sub-Saharan Africa. Reg Environ Change.

Chapman P., Malo AT., Ellin M., Ashton R. and Harkin M., 2001. *E. coli* O157 in cattle and sheep at slaughter, on beef and lamb carcasses and in raw beef and lamb products in South Yorkshire, UK. *International Journal of Food Microbiology*. **64:** 1, 139-150.

Chye F.Y, Abdullah A, Ayob M.K., 2004. Bacteriological quality and safety of raw milk in Malaysia. *Food microbiology.* **21:** 535-541.

Clermont M.J., Lapeyraque A.L., Malina M, Fremeaux-Bacchi V, Boppel T, Kirschfink M, Oualha M, Proulx F, Le Deist F, Niaudet P, Schaefer F., 2011. Eculizumab in severe Shiga-toxin-associated HUS. N. *England Journal of Medicine.***364:** 2561-2563.

CLSI (Clinical and Laboratory Standards Institute). 2018. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement. document Wayne, *PA, Clinical and Laboratory Standards Institute*. *M100-S25*. Vol. 35 No. 3.

CSA. 2017. Agricultural sample survey. Report on livestock and livestock characteristics. Addis Ababa, Ethiopia. 194pp.

De Boer E and Heuvelink A.E., 2000. Methods for the detection and isolation of Shiga toxinproducing *E. coli. Journal of Applied and Microbiol Symptom.***88:** 133S-43S.

Dessalegn Alehegn, 2018. *Comparative analysis of microbial quality and safety of raw cow milk in andassa livestock research center and dairy cooperatives in Bahir Dar city,*M.Sc. Thesis, Bahir Dar University, Ethiopia.

Dessalegn Genzebu., Berhan Tamir. and Gebreyohanes Berhane., 2016. Dairy Cattle Husbandry Practices and Constraints Associated with Milk Production in Bishoftu and Akaki towns of Oromia Region, Ethiopia. *World Journal of Dairy and Food Sciences*, **11(2):** 141-149.

Diriba Hunduma, 2018. *Isolation, identification and antimicrobial susceptibility test of Escherichia coli o157 and non-typhoid salmonella species in milk and feces of lactating cows and camels in borana pastoral area, southern Ethiopia* MVSc Thesis, Addis Ababa University, Ethiopia.

EFSA, 2013. The European Union summary report on trends and sources of zoonoses, zoonotic agents and foodborne outbreaks in 2011. *EFSA Journal*. **11(4):** 3129

Escherich T, 1885. Die Darmbacterien des neugeborenen und des Säuglings. *Fortschritte in der Medizin.***3:** 515-522.

FAO, 2020: Cattle dairy production and products. http://www.fao.org/dairy-production-products/production/dairy-animals/en/.

Farrokh E., Rostami J. and Laughton C., 2012. Study of various models for estimation of penetration rate of hard rock TBMs. *Tunnelling and Underground Space Technology.***30:** 110-123.

Frehiwot Mesele, 2018. *Occurrence of Escherichia coli O157:H7 in lactating cows and dairy farm environment and its antimicrobial susceptibility pattern at adami tulu jido kombolcha district, mid rift valley, Ethiopia*. MVSc Thesis, Addis Ababa University, Ethiopia.

Frenzen, P., Drake, A. and Angulo, F. 2005. Economic cost of illness due to *E. coli* O157 infections in the United States. *Journal of Food Protection.***68:** 2623-2630.

Fufa Abunna, 2017. Isolation, Identification and Antimicrobial Susceptibility Profile of *Salmonella* Isolates from Abattoir and Dairy Farms in and Around Holeta Town, Oromia, Ethiopia. *Journal of Veterinary Medicine and Research.*

Fufa Abunna, Debebe Ashenafi, Takele Beyene, Dinka Ayana, Bedaso Mamo and Reta Duguma. 2017. *Isolation, identification and antimicrobial susceptibility profiles of Salmonella isolates from dairy farms in and around Modjo town, Ethiopia.* MVSc Thesis, Addis Ababa University, Ethiopia.

Garbaj, A., Awad, E., Azwai, S., Abolghait, S., Naas, H., Moawad, A., Gammoudi, F., Barbieri, I. and Eldaghayes, I., 2016. Enterohemorrhagic *E. coli* O157 in milk and dairy products from Libya: Isolation and molecular identification by partial sequencing of 16S rDNA. *Veterinary World*. **9:** 1184-1189.

Gashaw Andargie, Afework Kassu, Feleke Moges, Moges Tiruneh and Kahsay Huruy, 2008. Prevalence of Bacteria and Intestinal Parasites among Food handlers in Gondar Town, Northwest Ethiopia. *Journal of health population and nutrition*. **26(4):** 451-455.

Geremew Zemenu, 2017. *Bacteriological quality of raw cows' milk: Prevalence and antimicrobial susceptibility profile of Escherichia coli isolated from milk and milking equipment in Bishoftu, Ethiopia*. DVM Thesis, Addis Ababa University, Ethiopia.

Getachew Felleke, 2003b. A Review of the Small Scale Dairy Sector in Ethiopia. FAO prevention of food losses programme. Milk and milk products, post-harvest losses and food safety in sub-SaharanAfrica and the NearEast.

Getachew Tadesseand Tesfaye Tessema, 2014.Ameta-analysisoftheprevalenceof*Salmonella*infoodanimals in Ethiopia. *BMC. Microbiology.***14(1):** 270.

Gizachew Yismaw, Negeri C, Afework Kassu and Moges Tiruneh., 2007. Antimicrobial Resistance Pattern of Salmonella Isolates from Gondar University Hospital, Northwest Ethiopia. *Ethiopian Pharmaceutical Journal*. **25:** 2.

Gran H.M., Mutukumira A.N., Wetlesen A. and Narvhus J.A., 2002. Smallholder dairy processing in Zimbabwe: hygienic practices during milking and the microbiological quality of the milk at the farm and on delivery. *Journal of Food Control*. **13:** 41-47.

Gray J.T and Fedorka-Cray P.J., 2002. *Salmonella*. In: Foodborne diseases. San Diego: Academic Press. 55-68.

Gyles C.L., 2007. Shiga toxin-producing: An overview. *Journal of Animal Science.***85:** E45-E62.

Haile Welearegay, Zelalem Yilma and Yosef Tekle-Giorgis., 2012. Hygienic practices and microbiological quality of raw milk produced under different farm size in Hawassa, southern Ethiopia. *Agricultural Research and Reviews*. **1:** 132-142.

Haileyesus Dejene., 2018, *Epidemiology and assessment of critical control points of E. coli O157:H7 along dairy value chainin central Ethiopia*. MSc Thesis, Addis Ababa University, Ethiopia.

Havelaar A.H., Cawthorne A., Angulo F., Bellinger D., Corrigan T., Cravioto A. and Lake, R., 2013. WHO initiative to estimate the global burden of foodborne diseases. The Lancet, 381, S59. Heiman K. E., Mody R. K., Johnson

Hoffmann, S.M. Batz, and J.G. Morris J.R., 2012. "Annual cost of illness and quality-adjusted life year losses in the united states due to 14 foodborne pathogens," *Journal of Food Protection*. **7:** 1292-1302.

Hunt M., 2010. Shiga toxin-producing *E. coli* (STEC), Clinics in Laboratory Medicine. 30: 21–45.

Hussein H. S. and Sakuma T., 2005. Invited Review: Prevalence of Shiga Toxin-Producing *E. coli* in Dairy Cattle and Their Products, *Journal of Dairy Science. Elsevier***88:** 450-465.

Islam M., Musekiwa A., Islam K., Ahmed S., Chowdhury S., Ahad A. and Biswas, P., 2014. Regional variation in the prevalence of *E. coli* O157 in cattle: *A meta-analysis and meta regression*. PloS one. **9:** 93299.

ISO (International Organization for Standardization) 16654, 2001. 1st ed. Microbiology – Horizontal method for the detection of *E. coli* O157, Geneve, Switzerland.

ISO (International Organization for Standardization) 6579, 2002. Microbiology. 4th ed. General guidance on methods for the detection of *E. coli O157.,* Geneve and Switzerland. Pp. 5-10.

ISO (International Organization for Standardization) 6887, 2017. Microbiology of the food chain -Preparation of test samples, initial suspension and decimal dilutions for microbiological examination, Geneve, Switzerland.

Jordan D., 2007. Antimicrobial resistance in animals and impacts on food safety and public health. *Microbiol Australia*. **28:** 163-164.

Karmali A., Gannon V and Sargeant J. M., 2010. Verocytotoxin-producing *E. coli* (VTEC). *Veterinary Microbiology.***140:** 360-370.

Kassu Tsegaye., 2016. *Assessment of milk production and marketing systems, and evaluation of the productive performances of crossbred dairy cows in Bona Zuria district of Sidama Zone, Southern Ethiopia*. Hawassa University, Ethiopia.

Kauffmann F., 1966. The Bacteriology of Enterobacteriaeceae. Munksgaard, Copenhagen, Denmark.

Ketema Worku, 2014. *Assessment of dairy cattle feed resources and milk yields under smallholder farmers in Kersa Malima Woreda, Ethiopia.* M.Sc. Thesis, Addis Ababa University, Ethiopia.

Khan, M.T.G., Zinnah, M.A., Siddique, M.P., Rashid, M.H.A., Islam, M. A. and Choudhury, K.A. 2008. Physical and microbial qualities of raw milk collected from Bangladesh Agricultural University Dairy Farm and the Surrounding Villages, Bangladesh Agricultural University, Bangladesh. *Journal of Veterinary Medicine.* **6:** 217-221.

Kiros Abebe Embaye, 2019. *dairy production systems characterization in urban and peri-urban areas of central oromia, ethiopia, and effects of concentrate supplementation on pro ductive and reproductive performances of crossbred dairy cows*. PhD Dissertation. Addis Ababa University, Ethiopia.

Lee J.H. and Choi S.J., 2015. Isolation and characteristics of sorbitol-fermenting *E. coli* O157 strains from cattle. *Microbes and Infection*. **8(8):** 2021-026.

Lemma Fita, 2004. *Assessment of butter quality and butter making efficiency of new churns compared to smallholders' butter making techniques in east Shewa zone of Oromia*, Ethiopia. M.Sc Thesis, Alemaya University, Ethiopia. 129pp.

Lencho Getechew and Seblewongel Ayichew, 2018. Assessment of dairy farmer's hygienic milking practices and awareness on cattle milk-borne zoonoses in Bishoftu, Ethiopia. *Journal of Veterinary Medicine and Animal Health.***10:** 45-54.

Liyuwork Tesfaw, Biruhalem Taye, Sefinew Alemu, Haile Alemayehu, Zufan Sisay and Haileleul Negussie, 2013. Prevalence and antimicrobial resistance profile of

Salmonella isolates from dairy products in Addis Ababa, Ethiopia. *Africa Journal Of Microbiology Research*. **7(43):**5046-5050.

Magiorakos A.P., Srinivasan A., Carey R.B., Carmeli Y., Falagas M.E., Giske C.G. *et al.* 2012. Multidrug-resistant, extensively drug-resistant and pan drugresistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infectious*. **18:** 268-81.

Majowicz S., Scallan E., Jones-Bitton A., Sargeant J., Stapleton J., Angulo F., Yeung D. and Kirk M. 2014. Global incidence of human Shiga toxin-producing *E. coli* infections and deaths: A systematic review and knowledge synthesis. *Journal of Foodborne Pathogens and Disease.***11:** 447-455.

Majowicz S.E., Musto J., Scallan E., Angulo F.J., Kirk M., O'Brien S.J., Jones T.F., Fazil A and Hoekstra R.M., 2010. International Collaboration on Enteric Disease 'Burden of Illness' Studies: The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clinical Infectious Disease.***50(6):** 882-889.

Mekonnen Tola, Bekana Merga, Abayneh Tesfaye., 2010. Reproductive performance and efficiency of artificial insemination smallholder dairy cows/heifers in and around ArsiNegelle, Ethiopia. *Livestock Research for Rural Development*. **22(3):** 153-162.

Mengistie Taye, 2013. Study on carcass contaminating *Escherichia coli* in apparently healthy slaughtered cattle in Haramaya University slaughter house with special emphasis on *Escherichia coli* O157: H7, Ethiopia. *Journal of Veterinary Science and Technology.***4:** 4-6.

Mesfin Zewdu, 2015. *Hygienic Practices, Bacteriological Quality Of Cow Milk And It' S Public Health Importance Along The Dairy Value Chain In Sidama High Lands Of Southern Ethiopia*. MVSc Thesis, Addis Ababa University, Ethiopia.

Misganaw Birhaneselassie and Williams D., 2013. A study of *Salmonella* carriage among asymptomatic food-handlers in southern Ethiopia. *International Journal of science.***2(5):** 243-5.

Mogessie Ashenafi, 2006. A Review on microbiology of indigenous fermented foods and beverages of Ethiopia. *Ethiopian Biology Sciencie.***5:** 189-245.

Montville T.J and Matthews K.R., 2008. Food microbiology: An introduction. 2nd edition. Washington D. C.: ASM Press.

Moxley R., 2017. Detection and Diagnosis of *E. coli* O157:H7 in food-producing Animals. In: Torrence, M. E. and Isaacson, R.E. (eds). Microbial Food Safety in Animal.

Msolo L., Igbinosa E.O. and Okoh A.I., 2016. Prevalence and antibiogram profiles of *E. coli* O157: H7 isolates recovered from three selected dairy farms in the Eastern Cape Province, South Africa. *Asian Pacific Journal Tropical. Disease.* **6(12):** 990-995.

Murphy S.C., 1996. Sources and causes of high bacteria count in raw milk: Abbreviated Review. Cornell University, Ithaca, N.Y. PP 1-4.

Nataro J.P., Bopp C.A., Fields P.I., Kaper J.B and Strockbine A., 2007. *Escherichia, Shigella* and *Salmonella*. In: Manual of clinical microbiology. 9th edition, Vol. 1. Washington D.C.; AMS Press. 679-683.

Newell DG., Koopmans M., Verhoef L., Duizer E., Aidara-Kane A, *et al.* (2010) Food-borne diseases - the challenges of 20 years ago still persist while new ones continue to emerge. *International Journal of Food Microbiology.* **1:** 3-15.

Nguyen Y. and Sperandio V., 2012. Enterohemorrhagic E. coli (EHEC) pathogenesis. Frontiers in Cellular and Infection Microbiology. **2:** 1-7.

Nigatu Disassa, Berhanu Sibhat, Shimelis Mengistu, Yimer Muktar and Dinaol Belina, 2017. Prevalence and antimicrobial susceptibility pattern of *E. coli* O157: H7 isolated from traditionally marketed raw cow milk in and around Asosa town, western Ethiopia. *InternationalJournal of Veterinary Medicine.***4:** 7.

Olana Merera, 2018. *Detection and antimicrobial susceptibility test of salmonella species along beef value chainin Bishofitu Town.* MVSc Thesis, Addis Ababa University, Ethiopia.

Oliver S.P., Boor K.J., Murphy S.C and Murinda S.E., 2009. Food Safety Hazards Associatedwith Consumption of Raw Milk. *Foodborne Pathogen. Disease*. **6:** 793.

Oporto B., Esteban J.I., Aduriz G., Juste R.A and Hurtado A., 2008. *E. coli* O157:H7 and non-O157 Shiga toxin-producing *E. coli* in healthy cattle, sheep and swine herds in Northern Spain. Zoonoses Public Health **55:** 2, 73-81.

Pandy, G.S and Voskuil G.C., 2011. Manual on milk safety, quality and hygiene. Golden Valley Agricultural Trust. Pp. 1-50.

Pennington H., 2010. Review: *E. coli* O157. The Lancet, **376:** 1428-1435.

Persson Y., Nyman A and Grönlund-andersson U., 2011. Etiology and antimicrobial susceptibility of udder pathogens from cases of subclinical mastitis in dairy cows in Sweden. *Acta Vet. Scand.***53(1):** 36.

Popoff M.Y and Le Minor L., 2001.Antigenic formulas of the *Salmonella* serovars. World Health Organization Collaborating Centre for Reference and Research on *Salmonella*, Pasteur Institute, Paris. 8th edition.

Quinn P.J., Markey B.K., Leonard F.C., Hartigan P., Fanning S and Fitzpatrick E.S., 2011. *Inernational Journal of Veterinary Microbiology and microbial Disease.* 400.

Quinn, G.P and Keough M.J., 2002. Experimental design and data analysis for biologists. Cambridge University Press.

Radiostitis O.M., Blood D.C and Gay C.C., 1994. Veterinary Medicine Text Book of the Disease of Cattle, Sheep, Pigs, Goats and Horses. 8thed. Baillière Tindall. 563- 574p.

Rahimi E., Khamesipour F., Yazdi F. and Momtaz H., 2012. Isolation and Characterization of Enterohaemorragic *E. coli* O157:H7 and EHEC O157: NM from Raw Bovine, Camel, Water Buffalo, Caprine and Ovine Milk in Iran. *Kafkas Univ Vet Fak Derg.* **18 (4):** 559-564.

Rangel J., Sparling P., Crowe C., Griffin P. and Swerdlow D., 2005. Epidemiology of *E. coli* O157:H7 outbreaks in United States, 1982-2002. *Emerging InfectiousDisease Journal.***11:** 603-609.

Reuben, R and Owuna G., 2013. Antimicrobial resistance patterns of *Escherichia coli* O157:H7 from Nigerian fermented milk samples in Nasarawa state, Nigeria. *International Journal of Pharmaceutical Science Invention.* **2:**38-44.

Rosa Abdissa., Woynshet *Haile.*, Akafete Teklu *Fiteet al.,*2017. Prevalence of*Escherichia coli* O157:H7 in beef cattle at slaughter and beef carcasses at retail shops inEthiopia. *BMC Infectious Disease.* **17:** 277.

Rosser T., Dransfield T., Allison L., Hanson M., Holden N., Evans, J., Naylor S., La Ragione R., Low J and Gally D., 2008. Pathogenic potential of emergent sorbitol-fermenting *E. coli* O157:NM. *Journal of Infection and Immunity.***76:** 5598-5607.

Rundasa Megersa, Mesfin Mathewos and Haben Fesseha, 2019. Isolation and Identification of *Escherichia coli* from Dairy Cow Raw Milk in Bishoftu Town, Central Ethiopia. *Arch Veterinaryand Animal Science,* 1.

Ryan K.J and Ray C.G., 2004. An Introduction to Infectious Disease. New York: McGraw-Hill. *Sherris Medical Microbiology.*

Saba Haile, 2015. *Quality assessment of cattle milk in Adea berga and Ejerie districts of west Shoa zone, Ethiopia*. M.Sc. Thesis, Haramaya University, Ethiopia. 32pp.

Sanath Kumar H., Otta K and Karunasagar I., 2001. Detection of Shiga-toxigenic *E. coli* (STEC) in fresh seafood and meat marketed in Mangalore, India by PCR, *Letters in Applied Microbiology.* **33:** 334-338.

Sancak Y., Sancak H and Isleyici O. 2015. Presence of *E. coli* O157 and O157:H7 in raw milk and van herby cheese. Bulletin of the Veterinary Institute in Pulawy. **59:** 511-514.

Schroeder C., Zhao C., DebRoy C., Torcolini J., Zhao S., White G., Wagner D., McDermott F., Walker D. and Meng J., 2002. Antimicrobial Resistance of *E. coli* O157:H7

Isolated from Humans, Cattle, Swine, and *Food. Journal of Applied and Enviromental Microbioogy.***68:** 576-581.

Segni Bedasa, Daniel Shiferaw, Ashebr Abraha and Tesfanesh Moges, 2018. Occurrence and antimicrobial susceptibility profile of *E. coli O157:* H7from food of animal origin in Bishoftu town, Central Ethiopia. *International Journal of Food Contamination.* **5:** 2.

Seyum Firew, 2019. *Investigation of the Occurrence of Selected Food-Borne Pathogen in the Slaughter Houses and their drug susceptibility Profiles at Bishoftu Town, Central Ethiopia.* MVSc Thesis, Addis Ababa University, Ethiopia.

Shewangzaw Addisu, Ahmed Muhammed and Nunu Haile, 2016. Handling, processing and utilization of milk and its products in Gondar town, Ethiopia. *Journal of Life Science and Biomedicine.***6:** 120-126.

Shunda D., Habtamu Tasew and Endale Balicha, 2013. Assessment of bacteriological quality of raw cow milk at different critical points in Mekelle, Ethiopia. *International Journal of Livestock Research*. **3(4):** 42-48.

Smith L., Fratamico M and Gunther W., 2014. Shiga toxin-producing *E. coli*. *Advances in Applied Microbiology*. **86:** 145–197.

Solomakos N., Govaris, A., Angelidis A., Pournaras S., Burriel A., Kritas S and Papageorgiou D., 2009. Occurrence, virulence genes and antibiotic resistance of *E. coli* O157 isolated from raw bovine, caprine and ovine milk in Greece. *International Journal of Food Microbiology.* **26:** 865-871.

Solomon Dadi, Matios Lakew, Mohamed Seid, Tafesse Koran, Abebe Olani, Letebrihan Yimesgen, Mekdes Tamiru, Eyob Eshetu, 2020. Isolation Of *Salmonella* and *E. coli* (*E. coli* O157:H7) And Its Antimicrobial Resistance Pattern From Bulk Tank Raw Milk In Sebeta Town, Ethiopia.*Journal of Animal Research and Veterinary Science.***4:** 021.

Solomon Mebrahtu, 2014. *Exploration of Challenges and Prospects of Milk Production, a Survey Study on Mekelle City*. M.Sc. Thesis, Mekelle University, Ethiopia. 31 pp.

SPSS (Statistical Package for Social Sciences). SPSS version 20, 2011. Application guide, SPSS Inc.

Swai E and Schoonman L., 2011. Microbial quality and associated health risks of raw milk marketed in the Tanga region of Tanzania. *Asian Pacific Journal of Tropical Biomedicine.***1:** 217-222.

Takele Beyene, Habtie Yibeltie, Bulako Chebo, Fufa Abunna, Ashenafi Feyisa Beyi, Bedaso Mammo, Dinka Ayana and Reta Duguma., 2016. Identification and Antimicrobial Susceptibility Profile of *Salmonella* Isolated from Selected Dairy Farms, Abattoir and Humans at Asella Town, Ethiopia. *Journal of Veterinary Science and Technology.***7:** 320.

Tarr P.I, Bilge S.S, Vary J.C, Jr, Jelacic S, Habeeb R.L, Ward T.R, Baylor M.R, Besser T.E., 2005. Iha: a novel *E. coli* O157:H7 adherenceconferring molecule encoded on a recently acquired chromosomal island of conserved structure. *Infectous Immunology*. **68:** 1400 -1407.

Teklemichael Tesfaye, Ameha Kebede and Eyassu Seifu, 2013. Quality and safety of cow milk produced and marketed in Dire Dawa town, Eastern Ethiopia. *International Journal of Science.***2:** 01-05.

Tesfa Mossie and Assefa Dires, 2016. Prevalence of Antimicrobial Resistant *Salmonella* Isolated from Bulk Milk of Dairy Cows in and around Debre Zeit, Ethiopia. *World's Veterinary Journal.***6(3):** 110-116.

Teshome Gemechu, Fekadu Beyene and Mitiku Eshetu, 2014. Handling practices and microbial quality of raw cow's milk produced and marketed in shashemene town, southern Ethiopia. *International Journal of Agriculture Research and Soil Science.* **2:** 153-162.

Thaker C.H., Brahmbhatt N.M and Nayak B.J. 2012. Study on occurrence and antibiogram pattern of *E. coli* from raw milk samples in Anand, Gujarat, India. *Veterinary World*. **5(9):** 556-559.

Thrusfield M., 2005. Veterinary epidemiology. 3rd Edition. London, UK: Blackwell Science Ltd. Pp. 229-254.

Tizeta Bekele, Girma Zewde, Tefera Genene, Aklilu Feleke and Zerom K., 2014. *E. coli* O157: H7 in raw meat in Addis Ababa, Ethiopia: prevalence at an abattoir and retailers and antimicrobial susceptibility. *International Journal of Food Contamination.***1:** 1-4.

Tsadkan Zegeye, 2012. *Study on cattle milk production, processing and marketing system in Enderta district, Tigray regional state of Ethiopia.* M.Sc. Thesis, Addis Ababa University, Ethiopia, 137pp.

Walker R.D., 2000. Antimicrobial susceptibility testing and interpretation of results. Antimicrobial Therapy in Veterinary Medicine, RD Walker Iowa State Press. **2:** 12-26.

Wangalwa R., Tolo C., Kagoro G and Wafula J., 2016. Assessment of on-farm milk handling practices in Mbarara district southwestern Uganda. *African Journal of Dairy Farming and Milk Production.***3:** 141-148.

Wilson D., Dolan G., Aird H., Sorrell S., Dallman J., Jenkins C., Robertson L and Gorton R., 2018. Farm-to-fork investigation of an outbreak of Shiga toxin-producing *E. coli* O157, Microbial Genomics. **4:** 1-7.

Xia X., Meng J., McDermott P., Ayers S., Blickenstaff K., Tran T., Abbott J., Zheng J and Zhao S., 2010. Presence and characterization of Shiga toxin-producing *E. coli* and other potentially diarrheagenic *E. coli* strains in retail meats. *Journal of Applied and Environmental Microbiology.***76:** 1709-1717.

Yien Deng, 2014. *Assessment of Hygienic Milk Production and Prevalence of Mastitis in Dairy Cow in Jikawo Woreda of Nuer Zone, Gambella Region, Ethiopia*. MVSc Thesis, Addis Ababa University, Ethiopia.

Yuen S.K, Yee C.F and Yin F.H. 2012. Microbiological Quality and the Impact of Hygienic Practices on the Raw Milk Obtained from the Small-scale Dairy Farmers in Sabah, Malaysia. *International Journal of Agricultural and Food Sciece.* **2:** 55-59.

Zelalem Addis, Nigatu Kebede, Zufan Sisay, Haile Alemayehu, Alehegne Yirsaw and Tesfu Kassa, 2011. Prevalence andantimicrobial resistance of *Salmonella* isolated from lactating cows and in contact humans indairy farms of Addis Ababa: a cross sectional study. *BMC infectious Disease.* **11**(1)**:** 222.

Zelalem Yilma and Bernared Faye, 2006. Handling and microbial load of cow‘s milk and Irgofermented milk collected from different shops and producers in central highlands ofEthiopia. *Ethiopian Journal of Animal Production*. **6:** 7-82.

Zelalem Yilma, 2003. "Sanitary conditions and microbial qualities of dairy products in urban and peri-urban dairy shed of the central Ethiopia." DEA. Lyon, France.

Zelalem Yilma, 2010. Quality factors that affect Ethiopian formal milk business; experience from selected dairy potential areas. Addis Ababa, Ethiopia. Netherlands Development Organization (SNV).

Zelalem Yilma, 2012. Microbial properties of Ethiopian marketed milk and milk products and associated critical points of contamination: An epidemiological perspective, epidemiology insights, Dr. Maria De Lourdes Ribeiro De Souza Da Cunha (Ed.), ISBN: 978-953-51-0565-7.

Zelyas N., Poon A., Patterson-Fortin L., Johnson P., Lee W and Chui L., 2016. Assessment of commercial chromogenic solid media for the detection of non-O157 Shiga toxin- 66 producing *E. coli* (STEC), *Diagnostic Microbiology and Infectious Disease.***85:** 302-308.

Zhang W., Qi, W., Albert T., Motiwala A., Alland D., Hyytia-Trees E., Ribot E., Fields P., Whittam T. and Swaminathan B., 2006. Probing genomic diversity and evolution of *E. coli* O157 by single nucleotide polymorphisms. Genome Research. **16:** 757-767.

APPENDICES

1:Questionnaire survey

Title: **"OCCURRENCE AND ANTIMICROBIAL RESISTANCE PATTERNS OF *ESCHERICHIA COLI* O157 AND *SALMONELLA* ISOLATES FROM RAW COW MILK IN AND AROUND BAHIR DAR CITY, AMHARA REGION, ETHIOPIA,"**

Dear participant,

To ensure the hygienic handling practice of milk is essential in this area, examining the problem in detail is important. Thus, this survey is an investigation of sound hygienic practices of dairy farm owners and employees in contact with animals and their products and also milk collection centers and cafeterias owners and employees in contact with milk in order to collect the base line data which is necessary to identify the point of intervention and to take the important measurement to prevent food-borne infections. The results of this study will help public institutions in designing control strategies.

All responses to this survey are completely confidential.

Thank you for your participation in this study!

Questionnaire format for smallholder milk producers

I. General Information

- Questionnaire No.
- Sampling date
- Name of *woreda*
- Name of *kebele*
- Name of owner

III. Feeding Practice

1. What type of feeding system practiced?
    a. Grazing B. Stall feeding C. Both alternatively
2. What types of feed you provide?
    a. Roughages B. Concentrates C. Both alternatively
3. Sources of water using for dairy cattle?
    a. Pond water B. River/stream water C. Tap/pipe water
4. II. Milking Procedures
5. Do you use any special order between dairy cows for milking?

a. Yes B. No

6. If yes, which one is the first one for milking?
    a. Healthy B. Mastitis
7. What types of cleaning agent used for hand washing?
    a. Cold water B. Warm water C. Cold water with soap D. Warm water with soap
8. Did you wash your hands between milking?
    a. Yes B. No
9. Do you wash the udder and teats during milking?
    a. Yes B. no
10. If yes, when do you wash the udder and teats?
    a. Only Before milk B. only After milking C. before and after milking
11. Do you use towel or other material for drying udder and teats?
    a. Yes B. no
12. If yes, what type of towel you used for drying udder and teats?
    a. Individual towel B. Common towel C. Disposable towel D. Massage with bare
13. What is average daily milk yield per cow? _____.

III. Milk Hygienic Practices

1. What type of equipment/material do you use for milking?
    A. Clay pot B. AluminumC. Plastic equipment's D. Calabash
2. What do you do for cleaning the milking container?
    A. Washing B. smoking C. Both alternatively
3. If wash, what types of material or detergent do you use?
    A. Soap with Sponge B. Ash with sponges C. Sonly soap D. do not use any detergent/material
4. What is the source of the water used for washing milking equipment's?
    A. Pond water B. River/stream water C. Tap/pipe water
5. If you smoke, what types of plant do you use for smoking?
6. Which plant parts are used for smoking?
    A. leaves B. steam C. both

7. Why do you use these plants?

   A. Give good flavor and aroma B. Increase the shelf life C. It just a tradition

8. Is milking containers cleaned regularly before and after milking?

   A. Yes B. No

9. If yes, how is the frequency of cleaning milking container?

   A. After each usage using cold water B. After each usage using warm water C. Both alternatively

10. Do you use cooling system for raw cow's milk?

    A. Yes B. No

11. If yes how?

    A. Refrigerator B. Traditional cooling C. Both alternatively

IV. Public Health Aspects

1. Do animals tested for tuberculosis? If yes, what is the frequency of the tests?
2. Do animals tested for brucellosis? If yes, what is the frequency of the tests?
3. Do animals screening tested for mastitis? If yes, frequency of the tests?
4. Do humans habit of raw milk consumption: yes/no

This completes the questionnaire. Do you have any question?

Thank you, I really appreciate your participation in this important study.

Questionnaire format for dairy farm owners

I. General Information

Questionnaire No.

- Sampling date
- Name of *kebele*
- Name of *woreda*
- Name of dairy farm

1. Where do the cows milked?

a. In barn b. In milking room c, every were

2. Type of milk bucket used?

   a. Clay pot b. Aluminumc. Plastic equipment's d. Calabash

3. How often the barn and/or the milking room are/is cleaned?

a. Twice a day b. Once a day c. Once per two day d. Once a week

4. Do you wash your hands?

   a. Yes b. No

4.1. If yes, when you wash?

a. Before milking b. after milking c. Before and after milking d. some times

5. Source of water for sanitation?

   a. Pond b. River/stream water c. Tap/pipe water

6. Do you wash the udder and teats, and use towel?

   a. Yes B. No

      6.1. If yes, when do you wash the udder and teats?

         a. Before milking b. only After milking c. Before and after milking

      6.2. If yes, use of towel practice?

         a. Collective towel b. Individual towel c. With bare hand

7. How do you keep the hygiene of milking buckets?

a. Cold water b. Warm water c. Cold water with soap d. Warm water with soap

8. Use of detergents:

   a. Yes B. No

9. Milkers' clothing:

   a. Boots b. Clean outer garment c. Own cloth d. Apron

10. Where does the milk go?

a. To household consumption b. To collection centers c. To cafeteria d. all

11. When does the milk collection center take the milk?

   a. Every day b. Every other day

This completes the questionnaire. Do you have any question?

Thank you, I really appreciate your participation in this important study

Questionnaire format for milk collection center owner/workers

I. General Information

- Questionnaire No.
- Sampling date
- Name of *woreda*
- Name of *kebele*
- Name of milk collection center

II. Milk Hygienic Practice

1. How much litter of milk produced/collected per day? _____.
2. What type of storage container used for milk storage?

   A. Clay pot B. AluminumC. Plastic equipment's D. Calabash
3. What is the source of the water used for washing storage container?

   A. Pond water B. River/stream water C. Tap/pipe water
4. What type of water used for cleaning collection and storage equipment's?

   A. Coldwater B. Warm water C. Both alternatively
5. Do you smoke the milk storage container?

   A. Yes B. No
6. If yes, what types of plant do you use for smoking?
7. Why do you use these plants?

   A. Give good flavor and aroma B. Increase the shelf life C. It just a tradition
8. Do you use cooling system for milk?

   A. Yes B. No
9. If yes how?

   A. Refrigerator B. With container at room temperature C. Both alternatively

This completes the questionnaire. Do you have any question?

Thank you, I really appreciate your participation in this important study.

Questionnaire format for cafeteria owner/workers

I. General Information

- Questionnaire No.
- Sampling date
- Name of *kebele*
- Name of *woreda*
- Name of cafeteria

2. Source of milk for the cafeteria?

a. Smallholder b. dairy Farms c. milk Collectioncenter d, both alternatively

3. Type of milk storage container used?

   a. Clay pot b. Aluminumc. Plastic equipment's d. Calabash

4. Source of water for sanitation?

a. Pond water b. River/stream water c. Tap/pipe water

5. Which form of milk do you sell for the consumers?

a. Boiled b. Raw c. Yoghurt d, both alternatively

6. Where do you store milk until used by the consumers?

a. In the refrigerator b. With container at room temperature c, both alternatively

This completes the questionnaire. Do you have any question?

Thank you, I really appreciate your participation in this important study.

2: Data record sheet

Sample collection and laboratory analysis work sheet for *Escherichia Coli* O157

Sample collection		Laboratory Analysis						
No	Code	Enrichment	Selective Media	Purification	Biochemical Test	Latex Kit	Antimicrobial susceptibility test	Result
		TSB+N	CT-SMAC	NA				
1.								
2.								

Key:TSB+N: Tryptic Soy Broth+Novobiocin; CT-SMAC: Cefixime Tellurite – Sorbitol MacConkey agar;NA: Nutrient Agar.

Sample collection and Laboratory Analysis work sheet for *Salmonella*

Sample collection			Laboratory Analysis								
No	Date/2012	Code	Pre enrichment	Selective enrichment		Selective media		Purification	Biochemical test	Antimicrobial Susceptibility Test	Result
			BPW	SC	RVS	XLD	BGS	NA			
1.											
2.											

Key: BPW: Buffered Peptone Water; RVS: Rappaport-Vassiliadis soya broth; SC:Selenite Cysteine broth;XLD: Xylose Lysine Deoxycholate Agar; BGS: Brilliant Green Sulfa Agar; NA: Nutrient Agar; TSI: Triple sugar iron.

3: Type, composition and preparation of microbiological media used for isolation and detection of *Escherichia coli* O157 and *Salmonella*.

1. Sorbitol MacConkey (SMAC) Agar(CM0813, Oxoid Ltd., Basingstoke, Hampshire, England)

Composition (g/l): peptone 20 , sorbitol 10, bile salts No.3 31.5, sodium chloride 5, neutral red 0.03, crystal violet 0.001 and agar 15

Preparation:51.5g of the powder medium was suspended in one liter of distilled water and brought to the boil to dissolve completely. Then it was sterilized by autoclaving at 121°C for 15 minutes. Thereafter, it was allowed to cool to 50 °C and poured into sterile Petri dishes, and lastly allowed to solidify at room temperature, and stored upside down at 4 to 8°C, refrigerator, for subsequent use and finally adjusted at pH of 7.1±0.2 at 25°C.

2. Buffered Peptone Water (MH 14941-500G, HIMEDIA, Mumbai, India)

Composition (g/l): tryptone10.0gm; Sodium chloride 5.0gm; disodium hydrogen phosphate 9gm ; potassium hydrogen phosphate 1.5gm.

Preparation: suspend 20.07 grams (the equivalent weight of dehydrated medium per litre) in 1000ml of distilled water. Heat if necessary to dissolve the medium completely. Distribute in tubes or flasks as desired. Sterilize by autoclaving at 121°C for (15lbs pressure) 15 minutes. Final PH is 7.0 ± 0.2 at 25°C.

3. Rappaport Vassiliadis soya broth (M1491-500G, HIMEDIA, Mumbai, India)

Composition (g/l): papaic digest of soya bean 4.5gm; sodium chloride 7.2gm; potassium dihydrogen phosphate 1.44gm; dipotasium phosphate 0.4, magnesium chloride hexahydrate 29.00; malachite green 0.036gm.

Preparation: suspend 27.11 gm in 1000ml distilled water. Heat if necessary to dissolve the medium completely. Dispense as desired in to tubes and sterilize by autoclaving at 121°C (10lbs pressure) for 15 minutes. Final PH is 5.2 ± 0.2 at 25°C.

4. KIA media (Oxoid Ltd., England)

Composition (g/l): Peptone 15.0gm, lactose 10.0gm, Proteose peptone 5.0gm, sodium chloride 5.0gm, beef extract 3.0gm, yeast extract 3.0gm, dextrose 1.0gm, sodium thiosulfate 0.3gm, ferrous sulfate 0.2gm, phenol red 0.024gm, agar 12.0gm, final pH 7.4 +/- 0.2 at 25ºC.

Preparation: Suspend 55g in 1 liter of distilled water. Bring to the boil to dissolve completely.

Mix well and distribute into containers. Sterilize by autoclaving at 121°C for 15 minutes. Allow to set as slopes with 1-inch butts.

5. Xylose Lysine Desoxycholate Agar (XLD) (CM 0469, OXOID, Basingstoke, England)

Composition (g/l): yeast extracts 3.0; l-lysine hydrochloric acid 5.0; xylose 3.75; lactose 7.5; sucrose 7.5; sodium desoxycholate 1.0; sodium chloride 5.0; sodium thiosulphate 6.8; ferric ammonium citrate 0.8; phenol red 0.08; agar 15.0.

Preparation: Suspend 53grams in one liter of distilled water. Heat with frequent agitation until the medium boils. Do not over heat. Transfer immediately to a water bath at 50°C. Pour in to plates as soon as the medium has cooled. It is important to preparing large volumes which will cause prolonged heating. PH: 7.4±0.2 at 25°C.

6. Nutrient Agar (AM5074, Accumix, Malaga, Spain)

Composition (g/l): peptone 5gm; sodium chloride 5gm; beef extract 1.5gm; yeast extract 1.5gm; agar 15gm.

Preparation: suspend 28 grams in 100ml distilled water. Mix thoroughly. Boil with frequent agitation to dissolve the powder completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour in to sterile Petri dishes. Final PH (at 25°C): 7.4 ± 0.2.

7. MR-VP Medium (M 070-500g, HIMEDIA, Mumbai, India)

Composition (g/l): buffered peptone 7.00; dextrose 5.00; dipotassium phosphate 5.00

Preparation: suspend 17.0 gram in 1000ml distilled water. Heat if necessary to dissolve the medium completely. Distribute in to test tubes 10ml amounts and sterilize by autoclaving at 15 Ibs pressure (121°C) for 15 minutes.

8. Urea agar base (M112S-500g, HIMEDIA, Mumbai, India)

Composition (g/l): Dextrose 1.000, Peptic digest of animal tissue 1.500, Sodium chloride 5.000, Monopotassium, phosphate 2.000, Phenol red 0.012, Agar 15.000.

Preparation: Suspend 24.51 grams in 950 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 50°C and aseptically add 50 ml of sterile 40% Urea Solution (FD048) and mix well. Dispense into sterile tubes and allow setting in the slanting position. Do not overheat or reheat the medium as urea decomposes very easily. Final PH: 6.5-7 ± 0.2 at 25°C.

9. Lysine Iron Agar (M377-500g, HIMEDIA, Mumbai, India)

Composition (g/l): Peptone 5.000, Yeast extract 3.000, Dextrose (Glucose) 1.000, L-Lysine 10.000, Ferric ammonium citrate 0.500, Sodium thiosulphate 0.040, Bromocresol purple 0.020, Agar 15.000. Final pH 6.7±0.2 at 25°C.

Preparation: Suspend 34.56 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense into tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool the tubes in slanted position to form slants with deep butts.

10. Triptone Soya Broth (TSB) (Oxide, England)

Composition: Pancreatic digest of casein (17.0 g), peptic digest of soyabean meal (3.0 g), sodium chloride (5.0 g), Di-Base potassium phosphate (2.5 g), Glucose (2.5 g)

Preparation: Suspend 30 g of power in 1 litter of purified water. Mixed thoroughly. Heated with frequent agitation and boiled for 1 minute. Autoclaved at 121°C for 15 minutes.

11. Triple sugar agar (CM 0277, OXOID, England)

Composition (g/l): 'meat extract 3.0; yeast extract 3.0g: peptone 20.0; sodium chloride 5.0; lactose 10.0; sucrose 10.0; glucose 1.0; ferric citrate 0.3; sodium thiosulfate 0.3; phenol red 0.024; agar 12.0

Preparation: suspend 65 grams in 1000ml of distilled water. Bring to boil to dissolve completely. Mix well and distribute in to containers. Sterilize by autoclaving at 121°C for 25 minutes. Allow the set as slope with 2.5 cm butts. PH: 7.4 + 0.2 at 25 .

12. Muller-Hintonagarpreparation(Oxoid,England)

Composition:Formula(g/l):BeefExtract2,AcidHydrolysateofCasein17.5,Starch1.5,andAgar17. Final.PH 7.3 ± 0.1 at25°C.

Preparation:Suspend38gofthemediuminoneliterofpurifiedwater.Heatwithfrequentagit ation and boil for one minute to completely dissolve the medium. Autoclave at 121°C for 15minutes.

13. L-LysineDecarboxylationMedium(DIFCO,Becton,Dicknson,USA)

Preparation: 5.25g/500ml and 5g/500ml decarboxylase base moller and L-Lysine mono hydrochloride respectively were dissolved together by heating if necessary and dispense 5ml in to test tubes and sterilize at 1210C for 10 minutes. The broth was clear and yellow tube to amber.

Composition (g/l): L-Lysine mono hydrochloride 5.0; Yeast extracts 3.0; Glucose 1.0 and Bromocresol purple 0.015

14. Tryptone broth for Indoletest

Composition (g/L): tryptone 10.0, Sodium Chloride 5.0, DL-Tryptophan1.0.

Preparation:10g/1000ml,5g/1000and3g/1000mloftryptone,sodiumchlorideand DL_Tryptophanrespectivelyweredissolvedtogetherbyheatingifnecessaryanddispense5 mlinto testtubesandsterilizeat121for15minutes.Thebrothwasclearandyellow.

4: Procedures and interpretation of biochemical tests

Biochemical tests for *E. coli* O157

✓ Indole test procedures

Inoculate the tryptophan broth with bacterial culture or emulsify isolated colony of the test organism in tryptophan broth. Incubate at 37°C for 24-28hrs in ambient air. Add 0.5 ml of Kovac's reagent to the broth culture. results: Positive: Pink colored rink after addition of appropriate reagent. Negative: No color change even after the addition of appropriate reagent.

✓ Sugar fermentation test procedures (lactose and dextrose fermentation)

Procedure: stab the center of the KIA medium into tube butt. Withdraw the needle, and streak surface of the slant. Loosen caps to allow a free exchange of air before incubating at 35°C for 18 – 38hrs. Read tubes for acid production on slant/butt, gas production, and hydrogen sulfide production. Results: An alkaline slant-acid butt (red/yellow) indicates fermentation of dextrose only. An acid slant-acid butt (yellow/yellow) indicates fermentation of dextrose and lactose. An alkaline slant-alkaline butt (red/red) indicates dextrose and lactose did not ferment (non-fermenter). Cracks, splits, or bubbles in the medium indicate gas production. A black precipitate in butt indicates hydrogen sulfide production

Biochemical tests for *Salmonella*

1. TSI Agar test: Streak the agar slant surface and stab the butt. Then incubate at 37**°C**for 24hrs.

Interpretation:

A) Butt: yellow if glucose used, red/unchanged if glucose not used, black if hydrogen sulphide is formed and bubbles if gas is formed.

B) Slant surface: Yellow if lactose and/or sucrose used and red/unchanged if lactose and/or sucrose not used.

2. L-Lysine decarboxylase test: Inoculate the medium just below the surface of the liquid medium. Incubate at 37**°C**for 24hrs.

Interpretation:

Turbidity and purple colour after incubation indicate a positive reaction.

A yellow color indicates a negative reaction.

3. Indole test: inoculate a tube containing 5ml of the tryptone/tryptophan medium with the suspected colony. Then incubate at 37℃ for 24hrs. After incubation, add 1ml of the kovacs reagent.

Interpretation: the formation of red ring indicates a positive reaction. A yellow-brown ring indicates a negative reaction.

5: Antimicrobial susceptibility test, the disc diffusion method

1. Three to five well-isolated colonies of the same morphological type were selected from thenutrient agar medium (Oxoid, England) (non-selective medium), from 18 to 24hrs agarplate, was touched with the loop, and transferred into a tube containing 4 to 5 ml of sterilesaline solution.
2. The inoculum was prepared by making direct colony suspension and was adjusted to match the 0.5 McFarland turbidity standard.
3. Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab.
4. The dried surface of a Mueller-Hinton agar plate (Oxoid, England), already prepared media,was inoculated by streaking the swab over the entire sterile agar surface. The procedure was repeated by streaking two more times, rotating the plate approximately 60º each time to ensure an even distribution of the inoculum. Finally, the rim of the agar was swabbed.
5. The lid was left ajar for 3 to 5 minutes to allow for any excess surface moisture to be absorbed before applying antimicrobial discs.
6. Then after, antimicrobial discs were placed onto the surface of the inoculated agar plate by using sterile forceps, no closer than 24 mm from center to center. The discs were pressed gently down to ensure complete contact with the agar surface.
7. The plates were inverted and incubated at 35 ºC for 18hrs.
8. After incubation, each plate was examined and the diameters of the zones of complete inhibition were measured, using sliding calipers (vernier calliper) on the back of the inverted petridish.
9. The sizes of the zones of inhibition, to the nearest whole millimeter, were interpreted according to CLSI (2018) criteria as described Table 3.2.

6: *E coli* O157 latex kit agglutination test

The *E. coli* O157 latex kit agglutination assay(DR0621M, Oxoid Ltd., Basingstoke, Hampshire, England) containing latex particles coated with antibodies specific for the *E. coli* O157, and*E. coli* O157 antigens. Identification of *E. coli*O157 was carried out following the manufacturer's instructions; hence colonies thatagglutinated were considered to be *E. coli*O157. The control latex reagent identifies the nonspecific agglutination.

The test was done with the following procedures:

1. Suspected colonies (non-sorbitol fermenting colonies) isolated from Sorbitol MacConkey(SMAC) agar (Oxoid, England) which were sub cultured on the nutrient agar (Oxoid,England) was used from 18-24 old culture.
2. For each isolate to be tested dispense one drop of the *E. coli* O157 test latex was dispendedinto the well of the test slide (reaction card).
3. In like manner, one drop of *E. coli* control latex was dispensed into a separate well of thetest slide.Then a drop of sterile saline solution was dispended into each of the test slide.
4. Using a plastic stick (provided), a portion of the colonies was removed from the nutrientagar plate and emulsified in *E. coli* O157 test latex and sterile saline water on the slide aswell it was spread over two-thirds of the reaction area. Lastly, the plastic stick was discardedproperly.
5. Once more using a fresh plastic stick, the process was repeated with the remaining coloniesand emulsified in *E. coli* control latex on the slide.
6. Thereafter, the slide was rotated using circular motions for up to 1 minute and observe forthe presence of precipitation on the *E. coli* O157 test latex and control latex. If agglutinationoccurred with the *E. coli* O157 test latex and the control latex was negative. Then, itindicates positive result for the *E. coli* O157 serogroup.

7:Some of the pictures taken during the processing (analysis)