Thesis Ref. No. _____

ADDIS ABABA UNIVERSITY COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

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

MSc THESIS

BY

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DEPARTMENT OF CLINICAL STUDIES
MASTERS PROGRAM IN TROPICAL VETERINARY EPIDEMIOLOGY

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BISHOFTU, ETHIOPIA

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A Thesis Submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Tropical Veterinary Epidemiology

By

Frehiwot Mesele

June, 2018

Bishoftu, Ethiopia

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Addis Ababa University

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STATEMENT OF THE AUTHOR

First, I pronounce that this thesis is my truthful work and that all sources of material used for this thesis work have been duly recognized. This thesis has been submitted in partial fulfillment of the requirements for M.Sc. degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is placed at the University/College library to be made accessible to debtors under rules of the Library. I seriously declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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LIST OF ABBREVATIONS

AE	Attaching and effacing lesions (eaeA)
ATJK	Adami Tulu Jido Kombolch a
Aw	Water activity
BPW	Buffered pepton water
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CVMA	Collage of Veterinary Medicine and Agriculture
DNA	Deoxyribonucleic acids
EHEC	Enterohemorrhagic Escherichia coli
EIEC	Enteroinvasive Escherichia coli
ELISA	Enzyme-linked immunosorbent assay
EMB agar	Eosin methylene blue agar
EPEC	Enteropathogenic E. coli
HC	Hemorrhagic colitis
HUS	Hemolytic uremic syndrome
IMS	Immunomagnetic seperation
ISO	International standardization office
MAR index	Multi antimicrobial resistance index
MDR	Multi antimicrobial resistance
NSF	Non-Sorbitol Fermenting
OIE	Office International des Epizooties
RNA	Ribonucleic acid
PCR	Polymerase Chain Reaction
SLT	Shiga like toxin
SMAC	Sorbitol MacConkey agar
STEC	Shiga-toxigenic Escherichia coli
Stx	Shiga toxin gene
TSB	Tryptose Soya Broth
TTP	Thrombotic thrombocytopaenic purpura

ABSTRACT

A cross sectional study was conducted from December 2017 to June 2018 on apparently healthy lactating cows at Adami Tulu Jido Kombolcha (ATJK) district in order to assess the occurrence of *Escherichia coli* O157:H7 in lactating cows and in dairy environment and to detect its antimicrobial resistance pattern. Total of 408 samples were collected and processed according to OIE terrestrial manual 2016. From 408 samples collected and processed, 19 were positive for *E. coli* O157:H7. The overall prevalence of *E. coli* O157:H7 was 4.7% (95% CI: 2.6; 6.7). Of 19 *E.coli* O157:H7 isolates, 4/50 were from water sample, 7/154 were from milk samples, 2/50 were from manure and 6/154 were from feces. The multivariable logistic regression indicate that, the prevalence of *E. coli* O157:H7 was significantly ($p < 0.05$) affected by factors such as area (urban, rural), floor type, cleaning of pens, milking location and hand washing during the time of milking. On the contrary, factors such as breed of the animal, herd size, use of towel and detergent, and history of mastitis did not show significant difference ($p > 0.05$). All 19 *E.coli* O157:H7 isolates were subjected to *in vitro* antimicrobial sensitivity test to ten commonly used antimicrobials. The test indicates varying degree of resistance; 100% resistance was observed for Ampicillin, Cephalothin and Rifampin and 100% susceptibility was observed for chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, kanamycin and tetracycline. With regard to streptomycin, 63.15% of the isolates were susceptible and 36.8% were intermediate. All 19 *E.coli* O157:H7 isolates showed the presence of multidrug resistance. In conclusion, the occurrence of *E. coli* O157:H7 was observed both in lactating cows (milk and feces) and dairy farm environment (manure and water) sustaining a continuous transmission of the bacterial. The development of multidrug resistance could hamper the control and prevention effort. Therefore, strict control measures such as treatment of positive cases using effective drugs and prevention measures such as strict hygiene practices should be established, including cleaning of floor, pens and milking barns as well as proper hand cleaning.

Keywords: *Occurrence, Lactating cows, E. coli O157:H7, Dairy farms, Antimicrobial, ATJK district. Susceptibility test,*

1. INTRODUCTION

Escherichia coli is a Gram-negative, coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestine of most mammalian species (Tenaillon *et al*., 2010). Most *E. coli* are commensal and harmless, but small proportions are an important cause of disease worldwide. The pathogenic *E. coli* are classified into different strains based on the production of virulence factors and on the type of clinical disease they cause (Caprioli *et al*., 2005; Fairbrother and Nadeau, 2006; Pennington, 2010). The category of *E. coli* strains producing Shiga toxins (Stx) is referred to as verotoxinogenic *E. coli* and Stx producing *E. coli* (STEC). Shiga toxin-producing *E. coli* (STEC), also called verotoxinogenic *E. coli*, usually do not cause clinical disease in animals but may cause a life threatening disease in humans. Zoonotic STEC include *E. coli* O157:H7 strains and these strains are increasing being reported globally (Fairbrother and Nadeau, 2006; Preussel *et al*., 2013).

Strains of *E. coli* O157:H7 are commonly found in a wide range of farm animals. The primary reservoir host of *E. coli* O157:H7 is cattle, in the cattle gut, the bacteria lives in symbiosis with its host (Fairbrother and Nadeau, 2006; Money *et al*., 2010). The bacteria can sometimes be found in other mammals including pigs, rabbits, horses, dogs, domestic, and wild birds (Caprioli *et al*., 2005; Faith *et al*., 1996; Rice and Johnson, 2000; Saeedi *et al*., 2017; Wallace *et al*., 1997). The bacteria are transmitted to humans through the ingestion of foods or water contaminated with animal feces, or through direct contact with the infected animals or contaminated farm environment. The main sources of *E. coli* O157:H7 infections for cattle are contaminated feed and water, and the immediate environment of the animal (Caprioli *et al*., 2005; Fairbrother and Nadeau, 2006). Risk factors that have been identified for infection of animals with *E. coli* O157:H7 include age, weaning, movement of the animals, season, feed composition, and the ability of the bacteria to persist in the environment (Fairbrother and Nadeau, 2006; Saeedi *et al*., 2017).

Farm environment is the main factor when it comes to sustaining a population of viable bacteria. The bacteria have been shown to survive in feces, manure, on pen surfaces, bedding and flooring and water (Awadallah *et al*., 2016; Davis *et al*., 2005; LeJeune *et al*., 2001;

LeJeune and Wetzel, 2007; Money *et al*., 2010; Sargeant *et al*., 2004). Combinations of urine and bedding have been found to enhance the growth of *E.coli* O157:H7 (Davis *et al*., 2005; LeJeune and Wetzel, 2007) further emphasizing the importance of clean pens and beds. Cleaning, change of bedding material and frequent removal of feces could ensure that populations do not buildup and colonize the space. Bedding moistened with bovine urine was found to more frequently contain higher levels of *E.coli* O157:H7. Manure could allow prolonged survival of the bacterium outside the host (Kudva *et al*., 1998) and should therefore be removed as frequently as possible. Contaminated animal drinking water may contribute to the dissemination and/or maintenance of *E. coli* O157:H7 on farms (LeJeune *et al*., 2001).

Antibiotics are often used for therapy of infected humans and animals as well as for prophylaxis. Inadequate selection and abuse of antimicrobials may lead to resistance in various bacteria and make the treatment of bacterial infections more difficult (Kolar, 2001). Antimicrobial resistance in *E. coli* has been reported worldwide. Treatment for *E. coli* infection has been increasingly complicated by the emergence of resistance to most first-line antimicrobial agents (Sabate, 2008) including ampicillin, amoxicillin, ceftriaxone, chloramphenicol, ciprofloxacin, cotrimoxazole, methicillin, tetracycline and vancomycin (Constable *et al*., 2017; Vijayarani *et al*., 2010). The development of antimicrobial resistance in *E. coli* O157:H7 is the matter of increase concern and generate new public health challenge (Newell *et al*., 2010).

The safety of food with respect to *E. coli* O157:H7 is one of great concern around the world. This is especially true in developing countries like Ethiopia, where production of food often takes place under unsanitary conditions. Consumptions of different food items particularly animal origin like milk which can be contaminated by feces, manure and contaminated water in dairy farms are the major source of infection for humans. Different research works have been undertaken in Ethiopia on the prevalence of *E.coli* O157:H7 and antimicrobial susceptibility test were also performed on *E.coli* O157:H7 isolated from samples such as carcass, meat and environmental samples but additional studies is needed to verify gap on epidemiological linkages on raw milk, feces, water and manure in dairy farms particularly in the study area.

Thus, this study aimed to investigate the occurrence of *E.coli* O157:H7 from dairy cattle and dairy environment. In addition, risk factors associated with *E.coli* O157:H7 occurrence and antimicrobial profile of the isolates were evaluated.

General objective

The general objective of this study is to assess the occurrence of *E. coli* O157:H7 in lactating cows and dairy farm environment and its antimicrobial susceptibility profile at Adami Tulu Jido Kombolcha district.

Specific objectives

- To assess the occurrence of *E.coli* O157:H7 in milk, feces, water and manure
- To estimate prevalence and associated risk factors of isolates
- To determine the antimicrobial susceptibility profile of the *E.coli* O157:H7 isolates

2. LITERATURE REVIEW

2.1. Etiology

E. coli is the most common species of facultative anaerobe in Family *Enterobacteriaceae* Genus *Escherichia* and Species *coli* (Hogg, 2005). It is gram-negative short rods; 1.1 - 1.5 x 2.0 - 6.0µm (Fernands, 2008) with flagella (peritricha), ferments lactose, with a number of serovars (160 antigenic types O, 56 types H, 80 types K/Vi). The strains pathogenic for human are labeled as *E. coli* enteropathogenic (EPEC), enterohaemorrhagic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC) or enteroaggregative (EAEC); usually formed toxins include verotoxin (VTEC: e.g. in O157) or thermostable "shiga-like toxin" Stx 1 and Stx 2 (STEC); the most frequent antigenic types of these bacteria are O157:H7, further O111 and O26 (Constable *et al*., 2017; Hubalek and Rudolf, 2011).

2.2. Growth characteristics

The growth range for *E. coli* O157:H7 is thought to be between 7 and 45°C, with an optimum of approximately 37 °C. *E.coli* O157:H7 is not notably heat resistant, and is 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)

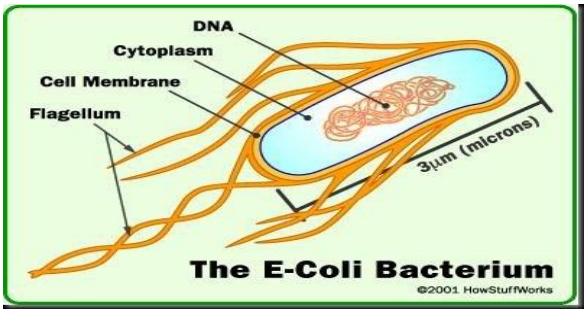

Figure 1: Structure of *E.coli*

Source: http://www.nature-education.org/water-ecoli.html

2.3. Epidemiology

2.3.1. Geographical distribution

E. coli O157:H7 infections occur worldwide and also have been stated on all continent except Antarctica (CFSPH, 2009). *E. coli* O157:H7 related diarrheal cases have been reported from a number of African countries including South Africa, Swaziland, Central African Republic, Kenya, Ethiopia, Uganda Gabon, Nigeria and Ivory Coast (Raji *et al*., 2006).

Table 1: Estimated pooled prevalence of *E. coli* O157:H7 in cattle by region

World region	No. of study	No. cattle sampled	No. of positive cattle	Pooled estimate (%)
Global estimate	140	220,427	12,683	5.68
Africa	4	626	118	31.20
Asia	22	14,916	937	4.69
Europe	53	88,643	5,425	5.15
Latin America and Caribbean	11	4,313	73	1.65
Northern America	46	110,641	6,059	7.35
Oceania	4	1,288	71	6.85

Source:(Islam *et al*., 2014)

2.3.2. Reservoir of E. coli O157:H7

E. coli is a species of bacteria naturally occurring in digestive tracts of warm blooded mammals. The major reservoirs are healthy domesticated ruminants, primarily cattle and, to a lesser extent, sheep and possibly goats (Calderwood *et al*., 1996). *E. coli* O157:H7 strains are occasionally found in other animals including pig, deer, geese, dogs, cats, rodents, birds and fish (Ferens and Hovde, 2011). *E. coli* O157:H7 is one of the dangerous strains and is shed in the manure of many warm-blooded animals. *E. coli* O157:H7 is harmless to most animals but is dangerous to humans, especially to those with an immature or weakened immune system, because it produces a toxin that can cause severe illness (Kang *et al*., 2004).

2.3.3. Source of infection and mode of transmission

Cattle are the most common farm animals to have *E. coli* O157:H7 and cattle and other ruminants naturally have *E. coli* O157:H7 in their intestines. The bacteria can be found in any premises that are contaminated by feces (Sargeant *et al*., 2000) including as animal bedding, food and water containers, and on gates and pens. People can become infected by consuming undercooked foods, raw milk and meat or water that was contaminated by *E. coli* O157:H7 (Kang *et al*., 2004).

2.3.4. Occurrence of E. coli *O157:H7*

E. coli O157:H7 appears to be most common in ruminants. Depending country, type of herd, detection method and methodology and other factors, its occurrence could range between 1 to 67% of cattle. Animals in feedlots appear more likely to shed *E. coli* O157:H7 than animals on pasture or dairy cattle. Young cattle are more likely to be infected than older animals, though it seems to be rare in pre weaning calves. *E. coli* O157:H7 infections look to be influenced by the season and more common from spring to early autumn. Management and climatic factors (e.g., warm climates) were reported to influence its occurrence (Islam *et al*., 2014).

2.4. Pathogenesis

According to Lim *et al*. (2010), *E. coli* O157:H7 is mainly pathogenic to human but in cattle and other animals, it did not induce any clinical disease except diarrhea. So, these animals act as reservoir. The pathogenicity of *E. coli* O157:H7 is associated with a number of virulence factors, including shiga toxins (Stx1 and Stx2; encoded by the stx1 and stx2 genes), intimin (encoded by the eae gene) and the enterohaemolysin (encoded by the hlyA gene). Following the ingestion of the bacteria, organisms adhere to and colonize the bowel mucosa (Nguyen and Sperandio, 2012). This may be mediated in part by the gene Shiga toxins bind to receptors on the bowel mucosa and are elaborated and translocated into the cell interior and inactivate ribosomal RNA leading to the inhibition of protein synthesis in cells expressing glycolipid G3b (globotriaosylceramide) and eventually causes death of host cells (Mohawk and O'Brien, 2011; Kiranmayi *et al*., 2010).

2.5. Clinical signs and symptoms

Cattle with *E. coli* O157:H7 in their digestive tract show no clinical signs. Most infections in cattle are temporary, lasting approximately 4 weeks, and the numbers of *E. coli* O157:H7 shed by infected cattle can vary widely, even within one day. Infections come and go within animals. An infection may not produce a strong enough immune response to prevent subsequent infections. In young domestic mammals (calf, lamb, and piglet) and fowl diarrhea could occurred apparently

2.6. Clinical diagnosis

Clinical cases can be diagnosed by finding the organisms in fecal samples, but food and environmental samples may also be tested to determine the source of the infection. There is no single technique that can be used to isolate all *E.coli* serotypes (CDC, 2016). In humans, infection with *E. coli* O157:H7 is associated with a broad spectrum of illness ranging from mild diarrhea and hemorrhagic colitis to the potentially fatal hemolytic uremic syndrome (HUS). These clinical symptoms could be used as one diagnoses technique (Rahal *et al*., 2012). Common sample are diarrheic feces in animals, predictable food item in both animal and human food (Elhadidy *et al*., 2015). The most sensitive sampling method from animal for *E. coli* O157:H7, is the rectal swab, because *E. coli* O157:H7 specifically colonize the recto-anal junction of the intestinal mucosa that is directly sampled with the swab approach (Constable *et al*., 2017).

Immunoassays and polymerase chain reaction technology have led to more rapid detection of *E. coli* in stools, food, and water. Techniques included in this category are PCR and DNA-based techniques, immunomagnetic separation, and enzyme-linked immunosorbent assays (ELISAs) (Bavaro, 2009; Clifton, 2000). Molecular-based techniques are distinctly advantageous because of their sensitivity, selectivity, and their rapid results (Parsons *et al*., 2016). However, molecular-based techniques are appreciably more expensive than traditional plating techniques and are also more novel and unfamiliar. Therefore, the integration of molecular-based approaches into quality control procedures depends on the overall needs and resources of the food processing plant (Robinson and McKillip, 2010). Latex agglutination test

is often used for the rapid identification of *E. coli* O157:H7. The test is best used in conjunction with Sorbitol MacConkey Agar. A positive result is indicated by agglutination with the test reagent, whilst the control reagent should appear milky and smooth (Al-Dragy and Baqer, 2014).

2.7. Treatment

Treatment of *E. coli O157:H7* infections with antibiotics may worsen the illness. The use of antibiotics could results in breaking up of the bacteria that increases production and secretion of Shiga toxins (Hiko *et al*., 2008) In vitro data have demonstrated that ciprofloxacin or sub inhibitory concentrations of trimethoprim-sulfamethoxazole induce shiga toxin production by *E. coli* O157:H7. Therefore, treatment is mainly supportive to limit the duration of symptoms and prevent systemic complications (Lim *et al*., 2010). Clear liquids are recommended for persons with diarrhea to prevent dehydration and loss of electrolytes (Dulo, 2014).

2.8. Prevention and control

E. coli O157:H7 can be found on nearly all cattle farms. Because *E. coli* O157:H7 is very common in farm animals and they don't show signs of illness, it is not practical to identify and remove infected animals. If an animal does have diarrhea, it is important to isolate the animal so it can't spread the bacteria to other animals or the environment. Good hygiene and sanitation practices can lower the levels of *E. coli* O157:H7 found on the farm; feeders and waterers should be designed in the way to keep manure out). Cleaning of pens and beddings with proper water drainage will minimize the transmission potential of the bacteria (Desta *et al*., 2012).

The transmission of *E.coli* O157:H7 from animal to human could be prevented by washing hands with soap and running water for 20 seconds before eating or drinking, especially after working on the farm or handling materials that might be contaminated with manure (Ziemer *et al*., 2010). Generally, hazard analysis critical control points (HACCP) could be implemented to avoid the transmission of *E.coli* O157:H7 from animal to human. HACCP is the universally accepted food safety management system (Pennington, 2010).

A vaccine for cattle has recently been developed to aid in the reduction of *E. coli* O157:H7 shedding in cattle. The vaccine works by immunizing cattle against the proteins that are expressed on the surface of *E. coli* O157:H7 cells. These proteins act as a receptor in intestinal walls, allowing the bacteria to colonize and the vaccine has been shown to reduce shedding (Smith, 2014).

2.9. Public health and economic importance

E. coli has been implicated in food borne illnesses with increasing frequency over the last 2 decades. Among this, *Escherichia coli* O157:H7 is the most common member of a group of pathogenic *E. coli* strains known variously as enterhemorrhagic, verocytotoxin producing or Shiga-toxin producing organisms (Kiranmayi *et al*., 2010). Shiga toxin-producing *E. coli* O157:H7 (STEC O157:H7) is a significant public health concern, causing severe, sometimes life-threatening, human illness (Niu *et al*., 2009) and major public health concern in North America, Europe, and other areas of the world (Lim *et al*., 2010).

Human infection caused by *E. coli* O157:H7 can present a broad clinical spectrum ranging from asymptomatic cases to death. Most cases initiate with non-bloody diarrhea and self-resolve without further complication. However, some patients progress to bloody diarrhea or HC in 1–3 days. In 5–10% of HC patients, the disease can progress to the life-threatening sequelae, HUS or thrombocytopenic purpura (TTP) (Lim, 2010 10; Ahmadi *et al*., 2007) .

The incidence of *E. coli* O157:H7 in humans is difficult to determine, because cases of uncomplicated diarrhea may not be tested for these organisms. In 2004, the estimated annual incidence of *E. coli* O157:H7 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:H7 causes approximately 73,000 illnesses, 2,000 hospitalizations, and 50-60 deaths each year (Dulo, 2014; Schroeder *et al*., 2002; Tassew, 2015).

The *E. coli* infection is a disease of economic importance because of medical and outbreak control, and productivity loss (Lu and Breidt, 2015; Mohawk and O'Brien, 2011). Due to *E.*

coli infection milk, meat and wool production could decline dramatically (Aklilu *et al*., 2013) The diseases caused by *E. coli* O157:H7 showed much higher hospitalization and fatality rates (Lim *et al*., 2010) resulting in considerable economic losses.

2.10. Occurrence of Escherichia coli O157:H7 in Ethiopia

Considerable number of studies have reported occurrence of *E. coli* and *E. Coli* O157: H7 from food of animal origin (mainly meat and milk), animal surfaces and feces in Ethiopia. Thogh most of the studies were from the central Ethiopia, there are also reports from southern, eastern, western and northern parts of the country. The sample sources includes abattoirs, butcher shops, retail shops, resturants, farms and milk vendors (Abayneh *et al*., 2014; Abdissa *et al*., 2017; Abebe et al., 2014; Atnafie *et al*., 2017; Balcha *et al*., 2014; Bekele *et al*., 2014; Beyi *et al*., 2017; Bedasa et al., 2018; Disassa *et al*., 2017; Dulo, 2014; Haile, 2017; Hiko *et al*., 2008; Mersha *et al*., 2010; Messele, 2016; Mohammed *et al*., 2014; Shecho *et al*., 2017; Shunda *et al*., 2013; Tassew, 2015; Taye *et al*., 2013). The study area, the sample processed and prevalence reports was sumerized in table 2.

Table 2: Prevalence of *E. coli* O157: H7 in different parts of Ethiopia

Study area	Sample	Prevalence (%)	Authors
Afar Arsi-bale Harar Ogaden Yabello Wollo Wolayta	Meat from abattoirs	1.32 9.17 4.62 9.80 0.76 9.57 5.20	Tassew, 2015
Asosa	Traditionally marketed raw cow milk	2.9	Disassa *et al*., 2017
Addis Ababa/ Debre Birhan	Fecal swab Intestinal mucosal swab Skin swab Carcass internal swab Cutting board swab Carcass swab	1.89 0.81 0.54 0.54 0.8 0.8	Abdissa *et al*., 2017

Dire Dawa	Cecal contents Carcass swab Environmental samples	2.15 3.22 2.04	Dulo, 2014
Hawassa	Fecal sample Carcass swabs Meat sample from butcher shop Cutting board swabs	4.7 2.7 2 3.3	Atnafie *et al*., 2017
Central Ethiopian (Addis Ababa, Bishoftu, Batu, and Holetta)	Carcass swabs Cutting board swabs	4.5 3.6	Beyi *et al*., 2017
Mekelle Quiha Wukro	Meat sample	0.04 0.06 0.01	Balcha *et al*., 2014
Debre-Zeit and Modjo towns	Beef Lamb and mutton meat Goat meat	8 2.5 2	Hiko *et al*., 2008
Addis Ababa	Beef Sheep meat Goat meat	13.3 9.4 7.8	Bekele *et al*., 2014
Addis Ababa	Abattoir sample Butcher houses sample	1.03 0.43	Haile, 2017
Haramaya University Slaughter House	Carcass swab	2.65	Taye *et al*., 2013
Mojo	Feces Skin swabs Carcasses before washing Carcasses after washing Water samples	4.7 8.7 8.1 8.7 4.2	Mersha *et al*., 2010
Haramaya	Cloacae samples	13.4	Shecho *et al*., 2017
Bishoftu	Raw milk Cheese Meat	12 5.71 3.07	Bedasa *et al.*, 2018

3. MATERIALS AND METHODS

3.1. Study area

Adami Tulu Jido Kombolcha (ATJK) district is found in the mid-Rift Valley at 7° 9‘N latitude and 38° 7 ‘E longitude. The altitude of the district is ranges 1500-2300 m above sea level. The average annual rainfall of the area is 760.9 mm. The main climate type is semi-arid. Minimum and maximum annual mean temperatures are 14 and 27 ^{0}C respectively. Livestock production is the dominant farming system and crop production is not common. Dairy cattle are mostly reared in small-scale dairy operations, in which animals are managed both intensively and extensively. It has a light texture soil class, which is vulnerable to both wind and soil erosion. Fishing and horticulture are the major basis of economy in the area (Abdissa *et al*., 2011; Jergefa *et al*., 2009).

The district is characterized by bimodal pattern of rainfall; with short rainy season running from February to April and long rainy season from June to September. However, the pattern of rainfall is usually erratic with fluctuations in the start and end of the season, in addition to the total absence of rainfall at times (Shiferaw, 2008).

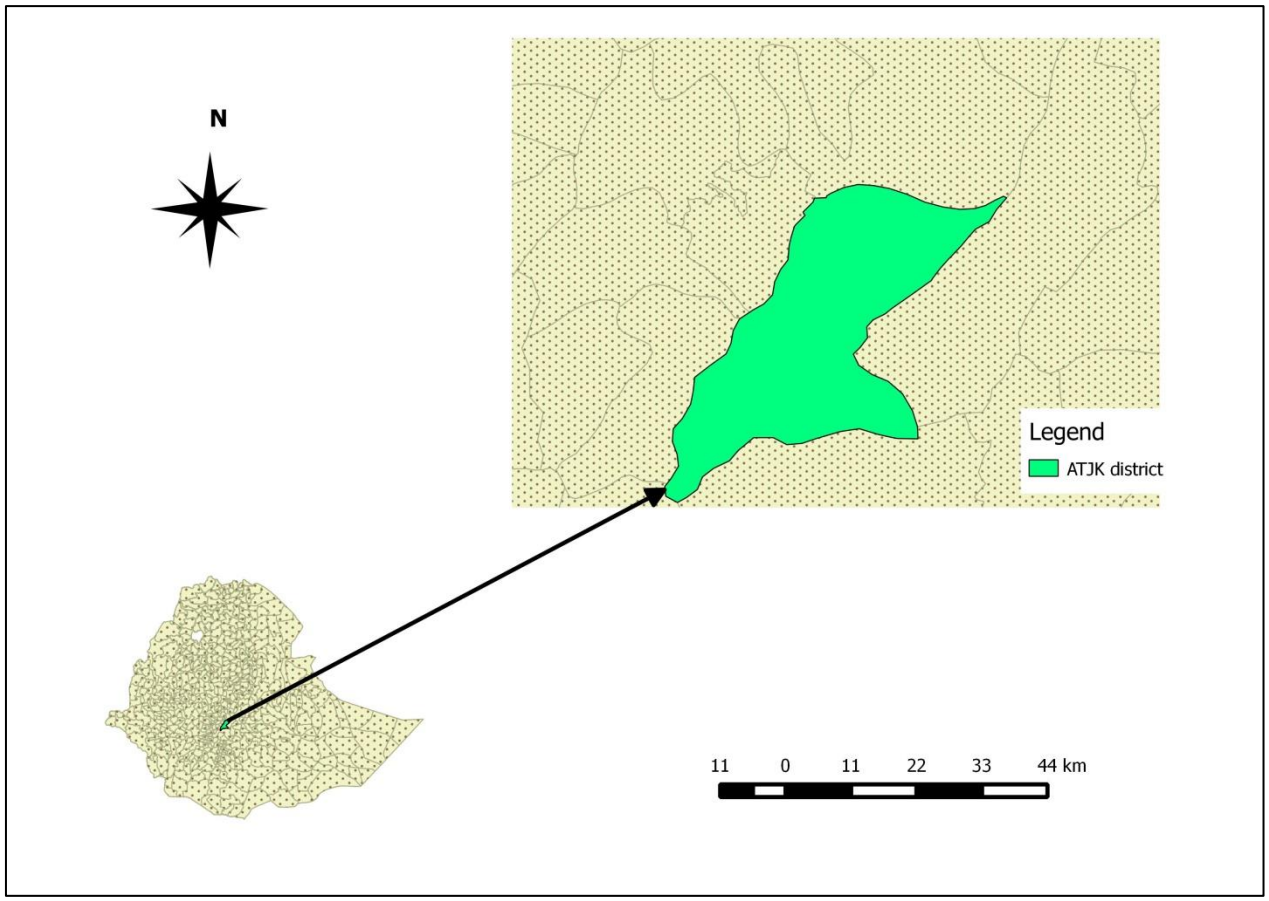

Figure 2: Map of the study area

3.2. Study population

The study population was lactating dairy animals in the district. There is an estimated 68 small and 22 medium scale dairy farms in the district. The study population comprises exotic, cross and local breeds in small and medium scale dairy farms managed under intensive and extensive management conditions. They are often provided with some supplementary diet in addition to the natural pasture and agricultural by products.

3.3. Study design

A cross-sectional study was conducted from December 2017 to June 2018 to estimate the occurrence of *E. coli* O157:H7 and to assess the antimicrobial susceptibility profile of isolates. Raw milk and feces samples were collected from teat and rectum of selected dairy cows, respectively. From dairy farm environment, manure and water samples were also collected. Semi structured questionnaire was used to collect risk factors associated with the occurrence of *E. coli* O157:H7 in lactating cows and dairy farm environment.

3.4. Sample size determination

Sample size were determined by using the formula given by (Thrusfield, 2005) with assumption of 95% confidence interval and 5% precision.

$$n = \frac{z^2 * P_{exp}(1 - P_{exp})}{d^2} = \frac{1.96^2 * 0.104(1 - 0.104)}{0.05^2} = 143$$

Where, n= required sample size

Z= alpha value of 95% confidence interval

d=desired absolute precision

The expected prevalence was set at 10.4% based on previous study conducted by (Abebe et al., 2014). Additional 11 samples were incorporated and the sample size became 154. Thus, 154 dairy cows in 50 dairy farms were sampled. The total sample size then became 408 (154 milk sample, 154 feces sample, 50 water and 50 manure samples).

3.5. Sampling methods

Stratified random sampling method were undertaken to categorize farms based on their herd size in to three stratums (small scale, medium scale and large scale commercial farms) using the classification made by Megersa *et al.* (2011) as a reference, small scale (<10 animals), medium (10 to 50 animals) and large (>50 animals). Based on the data obtained from the district livestock and fishery agency, there are no large scale dairy farms in the district. The district has 22 medium and 68 small scale dairy farms. A simple random sampling method was

used to select farms from each stratum and to select individual animals in the selected farm. The farm owners were then interviewed using a semi structured questionnaire.

3.6. Sample collection

All collected samples were tagged by sample ID, date of sampling and sample type. The samples were transported to the veterinary bacteriology laboratory, College of Veterinary Medicine and Agriculture of the Addis Ababa University using ice box in cold chain for microbiological analysis. Up on arrival, the samples were stored in refrigerator at 4°C for 24 hours until being processed for isolation as described by (Quinn *et al.,* 2004).

3.6.1. Milk sample

Milk samples were collected directly from teats. The udder and teats were thoroughly cleaned and dried before sampling; each teat was rubbed gently with cotton swabs moisturized with 70% ethyl alcohol. The first 3–4 streams of milk were discarded, and approximately 5ml of milk was collected aseptically by sterile screw topped universal bottle and the sample were transported using an ice box (4^{o}C) for further processing and microbiological analysis. Isolation and identification of *E. coli* O157:H7 from milk samples were passed on the basis of colony morphology in different media, staining characteristics and biochemical properties (ISO 18593, 2004).

3.6.2. Fecal sample

The fecal samples were collected using sterile stomacher bag aseptically directly from rectum and stored in ice box and ice pack until analysis (within 24 hours). Milk and feces samples were collected from the same dairy animals.

3.6.3. Water sample

Water samples (10ml) were collected using sterile capped universal bottle from the selected dairy farms pipe, and wide mouthed containers such as barrels, plastic buckets and jugs that was used for animal drinking, hand and udder washing.

3.6.4. Manure sample

Pooled manure samples were also collected from the selected dairy farms using sterile stomacher bag from different points including pen, floor surface and dung storage area.

3.7. Questionnaire survey

A pre-tested semi structured questionnaire was used to collect additional data on demographic characteristics, milking system, milking and hygienic practices (washing of milkers' hand, milk utensils and udder before milking). The interview was made in local language (Afaan Oromo/Amharic). All 50 farm owners were interviewed through face to face conversation.

3.8. Laboratory work

3.8.1**.** *Culture and identification of E. coli* O157:H7

Stage 1: Liquid enrichment media

Nonselective pre-enrichment is necessary for the effective recovery of low levels of stressed *E. coli* O157. Then enrichment broths were pre-warmed to prevent cold-shocking the organisms and slowing their initial growth. Milk and water samples were enriched using 1ml/9ml buffered peptone water and incubated at 37°C and 41.5°C, respectively for 24 hours. Similarly feces and manure samples were enriched with 25g/225ml BPW and incubated at 37°C for 24 hours to increase recovery of the organisms (OIE, 2016.).

Stage 2: Culturing of extracted enrichment

All enriched samples were cultured on sterilized Sorbitol MacConkey agar plate, (CM0813, Oxoid Basingstoke, England). Then the plates were incubated at 37°C for 24 hours. The SMAC agar plates were examined for the presence of non-sorbitol fermenting (colorless) colonies. Then, up to six colorless colonies (non- Sorbitol fermenters) on SMAC agar were taken and sub-cultured separately on MacConkey agar (Oxoid) and incubated for 24 hours at 37°C for clarification. Then the confirmed pure cultures considered as *E. coli O157:H7* positive was transferred to nutrient agar to be used for additional biochemical and serological confirmation (Quinn *et al.*, 2004).

Stage 3: Biochemical tests

The sub-cultured and purified colonies were tested for hydrogen sulphide and indole production using Triple Sugar Iron agar (TSI) slant (Oxoid) and Indole production test. The isolates giving a result of yellow slant and butt with gas but no hydrogen sulfide (Y/Y/ H2S -) production on TSI slant agar after incubation of the media at 37°C for 24 hours were kept with tubes capped loosely to maintain aerobic conditions. Indole test was carried out using one pure colony inoculated into 4 ml of tryptone soya broth (Oxoid) with a straight inoculation wire. Incubation was done for overnight at 37°C. Then one drop of Indole (Kovac's) reagent was added to the tryptone soya broth culture to test for indole production (formation of red ring indicating positive reaction) as stated at annex 2.

Stage 4: Confirmation of E.coli O157:H7

Tryptone soya broth and indole positive colonies were then confirmed using Oxoid Dryspot *E. coli* O157 latex test kit. The Dry spot *E. coli* O157 latex test was confirmed by agglutination of *Escherichia* strains possessing the O157 serogroup antigen. One drop of saline was dispensed to the small ring (at the bottom of each oval) in both the test and control reaction areas ensuring that the liquid did not mixed with the dried latex reagents (Annex 2). Using a sterile single use plastic loop, a portion of the colony to be tested was selected and carefully mixed in the saline drop until the suspension was smooth. Then, using paddle the suspension was mixed into the dry latex spots until completely suspended and spread to cover the reaction area. The test card picked up and stirred for up to 60 seconds and looked for agglutination under normal lighting conditions. A result was considered positive if agglutination of the latex particles occurs within 1 minute. This indicates the presence of *E. coli* serogroup O157. A negative result is obtained if no agglutination occurs and a smooth blue suspension remains after 60 seconds in the test area.

3.8.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed by standard disc diffusion technique using commercially available antimicrobial disks (Annex 5) and recommended from the guideline of antimicrobial susceptibility testing from CLSI (2015). Antimicrobial disks containing

Ampicillin (10µg), Cephalothin (30µg), Ciprofloxacin (5µg), Chloramphenicol (30µg), Gentamicin (10µg), kanamycin (30µg), Nalidixic acid (30µg), Rifampin (5 µg), Streptomycin (10µg) and tetracycline (30µg) (HI media, India) were used.

Serologically confirmed colony from pure fresh culture was transferred in to a test tube of 5 ml tryptone soya broth (TSB) (Oxid, England) and incubated at 37^{o}C for 6 hours. Then turbidity of the culture broth was adjusted using sterile saline solution or by addition of more isolated colonies to get turbidity analogous with that of 0.5 McFarland standards (approximately 3x108 CFU per ml). A Mueller-Hinton agar (Oxid, England) plate was ready according the manufacturer. Then after sterile cotton swab was immersed into the suspension and revolved against the side of the tube to remove the excess fluid and then swabbed in three directions homogeneously on the surface of the plates. After the inoculated plates dried, antibiotic disks were placed by the help of sterile forceps. The disks were slowly pressed onto the agar to ensure stable contact with the agar surface, and incubated at 37^{o}C for 24 hours. Subsequent the diameter of inhibition zone created around each disk was measured using digital caliper by laying it over the plates. The results were classified as sensitive, intermediate and resistant according to the standardized table supplied by the manufacturer CLSI (2015) as described on table 3.

Table 3: Antimicrobial susceptibility test interpretive criteria for *Enterobacteriaceae*

Antimicrobial Agent	Disk concentration	Zone Diameter: Interpretive Criteria (nearest whole millimeter)		
		S	I	R
Ampicillin (AMP)	10 µg	≥17	14–16	≤13
Cephalothin (CEP)	30 µg	≥18	15–17	≤14
Ciprofloxacin (CIP)	5 µg	≥31	21–30	≤20
Chloramphenicol (C)	30 µg	≥18	13–17	≤12
Gentamicin (GEN)	10 µg	≥15	13–14	≤12
Kanamycin	30 µg	≥18	14–17	≤13
Nalidixic acid (NA)	30 µg	≥19	14–18	≤13
Rifampin (R)	5 µg	≥20	17–19	≤16
Streptomycin (S)	10 µg	≥15	12–14	≤11
Tetracycline (TE)	30 µg	≥15	12–14	≤11

Source: Clinical Laboratory Institute Standards (CLIS, 2015)

3.9. Data management and statistical analysis

Data were entered to a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA). Descriptive statistics (determination of proportions) were used to summarize the data. The overall prevalence of *E. coli* O157: H7 in milk, feces and environmental samples were estimated using standard formula. The number of positive samples were divided by the total number of samples examined multiplied by 100. R statistical software Version 3.3.2 (R Core Team, 2016) was used to analyze the data (Annex 4). Pearson chi-square, Pearson's Chi-squared test with Yates' continuity correction and fisher exact tests were used to association of different risk factors with the occurrence of *E. coli* O157:H7. Univariable and multivariable logistic regression analysis were performed to quantify crude and adjusted effect of the risk factors on the occurrence of *E. coli* O157: H7. The stepAIC function in 'MASS' package was used to select the final multivariable logistic regression model. P-value less than 5% ($P<0.05$) was considered statistically significant. Conditional logistic regression was used to assess the presence stratification and matching in the data using 'clogit' function in 'survival' package, but there is no difference between the binary and conditional regression models and thus, the binary logistic regression results were used. In cases of estimating the effect of different risk factors in terms of Odds ratio (OR) with corresponding 95% confidence interval, statistical significance was assumed if the confidence interval did not include one among its value. For antimicrobial susceptibility test the results were interpreted according to Clinical and Laboratory Standards Institute (CLSI, 2015) 'interpretive criteria for *Enterobacteriaceae* (Table 3).

3.10. Ethical consideration

Before collection of samples undertaken the protocol approved by Addis Ababa University, co llage of veterinary medicine animal research ethical committee with reference number VM/ER C/28/05/10/2018.

4. RESULTS

4.1. Occurrence of *E.coli* O157:H7

Out of 408 samples collected and processed, 19 were positive for *E. coli* O157:H7. The overall prevalence of *E. coli* O157:H7 was 4.7% (95% CI: 2.6; 6.7). Of these positive cases, the isolation of *E.coli* O157:H7 was the highest in water sample 4(8%), followed by milk samples 7 (4.54%), in manure 2(4%) and 6 (3.89%) in feces as presented table 4.

Table 4: Occurrence of E.coli O157:H7 in different sample type

Sample type	Total sample examined	*E. coli* isolates (%)	*E. coli* O157:H7 strains (%)
Milk	154	15(9.74)	7(4.54)
Feces	154	16(10.4)	6(3.9)
Water	50	6(12)	4(8)
Manure	50	5(10)	2(4)
Overall	408	42(10.3)	19(4.7)

4.2. Univariable analysis of the association of *E. coli* O157:H7 with different risk factors

The effect of potential risk factors on the occurrence of *E.coli* O157:H7 was assessed and from the risk factors considered, cleaning of pens, milking location, use of towel and hand washing during the time of milking had a statistically significant impact on the occurrence of *E. coli* O157:H7 ($P< 0.05$) using Univariable logistic regression analysis. On the contrary, factors such as breed of the animal, herd size, area, floor type, use of detergent and history of mastitis did not show significant difference ($p > 0.05$) (table 5).

Table 5: Univariable logistic regression analysis of *E.coli* O157:H7 occurrence with various risk factors

Risk factors	*E. coli* O157:H7					
	No. examined	No. of positive	X^2	P-value	Crude OR	95% CI OR
Area						
Rural	120	8	0.61	-	-	-
Urban	269	11		0.306	0.61	0.24 - 1.6
Breed						
Exotic	101	1	*	-	-	-
Cross	183	13		0.059	7.17	0.93 - 55.65
Local	105	5		0.155	4.81	0.55 - 41.89
Cleaning of pens						
No stay overnight	133	13	7.81#	-	-	-
Stay overnight	256	6		0.005**	4.17	1.61 - 12.5
Herd size (Farm scale)						
Medium scale	137	3	2.23	-	-	-
Small scale	252	16		0.095	2.90	0.94 - 12.62
Milking location						
In barn	151	13	5.43#	-	-	-
Anywhere	238	6		0.005**	3.45	1.32 – 10.0
Hand wash						
Before and after milking	377	15	*			
Only before milking	12	4		0.000***	8.37	2.15- 27.49
Floor type						
Earthen	214	8	0.75#	-	-	-
Concrete	175	11		0.275	1.68	0.67 - 4.42
Use of towel						
No use of towel	96	8	*	-	-	-
Before milking	174	9		0.310	0.6	0.22 -1.61
Before and after milking	138	2		0.023	0.16	0.03- 0.78
Use of detergent						
No	52	2	*	-	-	-
Yes	356	17		0.767	1.25	0.35-8.06
History of mastitis						
No	104	8	*	-	-	-
Yes	304	11		0.096	0.45	0.18-0.19

Keys: * ** Significant level (P< 0.001), **Significant level (P< 0.01), *Computed using fisher exact test, - reference, # Pearson's Chi-squared test with Yates' continuity correction

4.3. Multivariable analysis of the association of *E. coli* O157:H7 with different risk factors

From potential risk factors considered in this study (table 6), area, floor type, cleaning of pens, milking location and hand washing during the time of milking were significantly associated ($P< 0.05$) with the occurrence of *E. coli* O157:H7. As shown in table 6, the odds ratio of *E. coli* O157:H7 occurrence of was 9.32 times higher in urban areas than rural areas. This could be contradicted from normal assumptions but during the study observed higher number of animals in urban area than in rural area that leads to snags of easily farm management; and also in rural area all farms managed by farm owners themselves nevertheless in the urban area almost all of them were managed by labor works and the farms were not frequently visited by the owners. In pens where the feces stay overnight, the odds ratio of *E. coli* O157:H7 occurrence is 50 times higher. Animals which were milked anywhere in the farm had 16.67 times at higher risk compared to animals which are milked in a milking barn. Hand washing practice had also a significant impact on the occurrence of *E. coli* O157:H7, the odds ratio of *E. coli* O157:H7 occurrence in farms where hand washing is practiced only before milking were 8.51 times higher when compared with farms where before and after milking hand wash is practiced. Farms with concrete floor were 48.74 times at higher risk when compared with farms with ordinary floor type (earthen floor).

Table 6: Multivariable logistic regression analysis of *E.coli* O157:H7 occurrence with various risk factors

Risk factors	Adjusted OR	95% CI OR	p-value
Area			
Rural	-	-	-
Urban	9.32	1.79 - 48.60	0.008**
Cleaning of pens			
No stay overnight	-	-	-
Stay overnight	50	7.69-500	0.000***
Milking location			
In barn	-	-	-
Anywhere	16.67	3.03-83.33	0.001**
Hand wash			
Before and after milking	-	-	-
Only before milking	8.51	1.88 - 38.49	0.005**
Floor type			
Earthen	-	-	-
Concrete	48.74	3.49 - 680.66	0.004**

Keys: * ** Significant level (P< 0.001), ** ** Significant level (P< 0.01), * * computed using fisher exact test, - reference

4.4. Antimicrobial susceptibility pattern of isolates

All isolates were subjected to *in vitro* antimicrobial sensitivity test to ten commonly used antimicrobials. The test result indicates (Figure 3) varying degree of resistance; 100% resistance was observed for Ampicillin, Cephalothin and Rifampin and 100% susceptibility was observed for chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, kanamycin and tetracycline. With regard to streptomycin, 63.15% of the isolates were susceptible and 36.8% were intermediate. All of them showed the presence of multidrug resistance. Multidrug resistance refers to resistance of single isolate against more than two drugs).

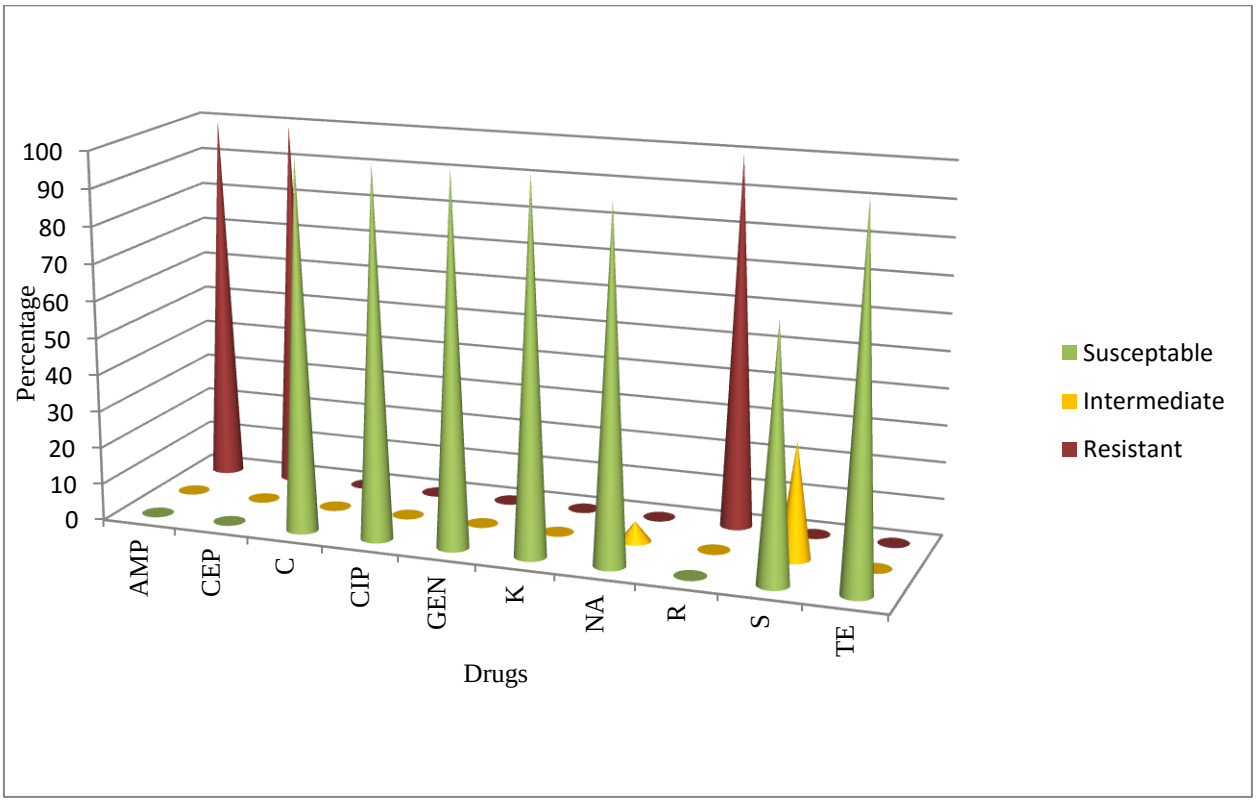

Figure 3: Antimicrobial susceptibility pattern of *E. coli* O157:H7 to ten antimicrobials

Key: AMP: ampicillin, C: chloramphenicol, CIP: ciprofloxacin, K: kanamycin, NA: nalidixic acid, S: streptomycin, TE: tetracycline.

5. DISCUSSION

In Ethiopia, of *E. coli* O157:H7 is considered to be an important challenge for the dairy development and public health. This study also indicates *E. coli* O157:H7 to be the major dairy development challenge in the study area. The prevalence of *E. coli* O157:H7 in lactating cows (milk and feces) and dairy farm environment (water and manure) at ATJK district found to 4.7%. The result is in line with the result reported by Bedasa *et al.* (2018) who reported a prevalence of 3.5% in food of animal origin in Bishoftu town, Central Ethiopia. This result also supports previous evidence (Fairbrother and Nadeau, 2006; Ferens and Hovde, 2011; Hancock *et al.*, 1998; Sami and Firouzi, 2007; Wells *et al*., 1991) that cattle are asymptomatic animal carriers a major reservoir of *E. coli* serotype O157:H7. Carrier animal excretes the organism occasionally and in low numbers in feces (Rahn *et al*., 1997). Cattle and their feces have been considered as the primary source of *E. coli* O157:H7 (Shere *et al*., 2002; Venegas-Vargas *et al*., 2016).

Raw milk can be a vehicle of transmission for *E. coli* O157:H7 (USDA, 1997). Risk of *E. coli* O157:H7 infection related to consumption of raw milk is high, indicating that there is risk of *E.coli* O157:H7 infection even though the prevalence detected is relatively low (Lye *et al*., 2013). Isolation rate of *E. coli* O157:H7 from raw milk samples similar to that recorded in the current study (4.54%) was slightly in agreement with the report of 2.9% by Disassa *et al*. (2017). But, the prevalence is far lower when compared to the reports of Bedasa *et al*., (2018) who reported 12% and Abebe *et al*., (2014) who reported 10.4% from Bishoftu town and Tigray, Ethiopia. In the result of the present study was also comparable with the 8.75% result reported by Lye *et al*. (2013) from Malaysia. The highest occurrence of *E. coli* O157:H7 was reported by Chye *et al*. (2004) who reported 33.5% from Malaysia and Msolo *et al*. (2016) who reported 55% from South Africa. The differences might be attributed to the differences in animal management, milking systems, and milk hygiene and handling practices among different farms in different countries. The detection of *E. coli* O157:H7 in milk is not only a reliable indicator of fecal contamination, but is also an indicator of poor hygiene and sanitary conditions during milking and handling.

In the present study, 3.9% isolation rate of *E. coli* O157:H7 was recorded from feces sample. This is in agreement with prevalence reported by Atnafie *et al.* (2017) and Mersha *et al*., (2010), both reported 4.7% in Hawasa and Modjo, respectively. The result is slightly lower than the 7.26% report by Nazareth (2017) and 9% report by Lupindu *et al.* (2014) from USA and Tanzania, respectively. The result of the present study, is higher than the prevalence reported by (Sami and Firouzi, 2007; Hancock *et al*., 1994; Swirski *et al*., 2014; Faith *et al*., 1996 and Hancock *et al.*, 1998) who reported a prevalence of 0.51, 0.71, 1 and 1.8 and 2.3%, respectively. The difference could be attributed to difference in bacterial isolation and identification method used and differences in farm management and hygienic practices. Isolation of *E. coli* O157:H7 from feces regarded as an important epidemiological information. Infected cattle could shade 10^1 to 10^7 cfu of *E. coli* O157:H7 per gram of feces. Given that typical cattle excrete 20 to 50 kg of feces per day, this provides a large inoculum of *E. coli* O157:H7 for the farm environment and could contaminate dairy products in the presence of poor hygienic practices (Mathhews *et al*., 2014).

From 50 water samples, 4 (8%) were *E. coli* O157:H7 positive. Four of the positive samples were from animal drinking water. The presence of *E. coli* O157:H7 in the drinking water may contribute to the occurrence of infection in cattle, a factor directly related to the contamination of dairy products and the environment. Contaminated water can serve as a vehicle of *E. coli* O157:H7 transmission in cattle, although there was variation among animals in the doses necessary to initiate shedding (Shere *et al*., 2002). Animal drinking water was identified as one source of *E. coli* O157:H7 in the farm (Faith *et al.*, 1996).

In this study, 4% isolation rate of *E. coli* O157:H7 was recorded from manure sample. Farm manure may disseminate, transmit, or propagate *E. coli* O157:H7. Manure is a good vehicle of *E. coli* O157:H7. Manure sewages from cattle houses could result in contamination of the surrounding land, with cattle keepers and their household members being at increased the risk. The survival of *E. coli* O157:H7 in manure depends on many variables, including the level of pathogen shedding by animals, conditions, and duration of manure storage, extraneous microbial interactions within stored manure, and interactions with water (Ziemer *et al*., 2010). A number of researchers have investigated the survival of *E. coli* O157:H7 in manure from various animals, under different conditions such as temperature or aeration, presence of

different manure amendments, and at a range of manure-to-soil ratios (Duffy, 2003). Kudva *et al.* (1998) found that *E. coli* O157:H7 survived for more than 21 months in ovine manure at levels ranging up to 106 cfu/g manure. Experiments with artificially inoculated bovine feces have also confirmed the survival of *E. coli* O157:H7 for greater than 40 days, dependent on initial inoculum and holding temperature (Wang *et al*., 1996).

The perceived dissimilarity in the outcome of the current study from other studies could be the differences in husbandry practices and climate usual climatic conditions which may account for the varied prevalence of *E.coli* O157:H7 for different geographical locations. The method and techniques used for identification of bacteria in this study could accountable; immunomagnetic separation (IMS) technique with supplemented enrichment in broth culture has been reported to improve the identification of *E.coli* O157:H7 from samples with low concentration (Ojo *et al*., 2010). In present study buffered peptone water without supplement and IMS was used for enrichment of the samples as described in OIE (2016) that gives good recovery of stressed organism. Enrichment before plating on selective media agar might increase E.coli O157:H7 isolation compared to direct plating of the test samples on selective agar (Hashemi *et al*., 2010).

Many factors were tested for associations with E. coli O157:H7, yet relatively few were significant in the final model. Factors such as area (urban, rural), floor type, cleaning of pens, milking location and hand washing during the time of milking was found to be significantly associated with the occurrence of E. coli O157:H7. E. coli O157:H7 shedding in cattle and its survival in the environment could be multifactorial. No single factor could stands out as the major risk factor for shedding (USDA, 1997). But, factors related to poor hygienic practices were found to affect the occurrence of the bacteria which is consistent with observations in the literature. The multivariable analysis demonstrated a significant association between the presence of *E. coli* in and cleaning pens (OR, 50; 95% CI, 7.69–500; P = 0.000) in farms where the feces stay overnight, the odds ratio of *E. coli* O157:H7 occurrence is 50 times higher. Milking location and hand wash practice were also significantly associated with the occurrence of *E. coli* O157:H7 and animals which were milked anywhere in the farm and hand washing practice ‘only before milking’ being a risk for high occurrence. Floor type was also significantly associated the occurrence of *E. coli* O157:H7. Farms with concrete floor were

significantly at higher risk when compared with earthen floor (OR, 48.74; 95% CI, 3.49–680; $P = 0.004$). It is important to note that, the use of towel and detergent and history of mastitis were not significantly associated with the occurrence of *E. coli* O157:H7. This suggests that use of towel and detergent alone is unlikely to prevent the presence of E. coli O157: H7 while the other hygienic are poorly practiced. Thus, general hygienic practices might represent a critical control point for reducing transmission of E. coli O157 in dairy farms.

In this study all isolates were resistant to ampicillin, cephalothin and rifampin. Previous study reported high degrees of resistance for *E.coli* O157:H7 originating from cattle (Um *et al.*, 2018). Cabal *et al*., (2013) compared the resistance of *E. coli* O157:H7 strains versus non-O157:H7 *E. coli* isolated from cattle feces. These authors reported a significantly higher proportion of resistant isolates among *E. coli* O157:H7 isolates than in non-O157:H7. The appearance and distribution of multidrug resistance in *E.coli* O157:H7 can serve as a reservoir for different antimicrobial resistance genes (Srinivasan et al., 2007). It is important to note that the isolates are susceptible to most commonly used antibiotics including chloramphenicol, ciprofloxacin, gentamicin (Kibret and Abera, 2011) and tetracycline. Um *et al*. (2018) reported resitant of *E.coli* O157:H7 to tetracycline from France.

6. CONCLUSION AND RECOMMENDATIONS

The current study revealed a substantial occurrence of *E. coli* O157:H7 in lactating cows and dairy farm environment at Adami Tulu Jido Kombolcha district. *E. coli* O157:H7 was isolated from feces, manure, milk and water designating a sustaining transmission of the bacteria. The occurrence of *E. coli* O157:H7 in milk samples suggests a potential zoonotic risk of raw milk consumption in the area. Factors related to poor hygienic practices such as cleaning of pens, milking location and hand washing were the main factors that backed the occurrence of *E. coli* O157:H7 in the dairy farms. *E. coli* O157:H7 isolates manifested a multi drug resistance; 100% resistance to Ampicillin, Cephalothin and Rifampin was observed. Antibiotics such as chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, kanamycin and tetracycline could be considered as first choice drugs as the isolates are susceptible to these drugs.

Based on the above conclusions the following recommendations are forwarded:

- Comprehensive trainings should be given to farm owners, milkers and other personnel involved in dairying activity to improve the hygienic practices.
- Strict animal and environment level hygienic should be practiced to break a sustained transmission of the bacteria in the farms.
- Awareness should be created on the risk of raw milk consumption in the area.
- Physicians in the area should consider ciprofloxacin, gentamicin, nalidixic acid, kanamycin and tetracycline as first choice drugs in the treatment of clinical diseases associated with *E. coli* O157:H7.

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8. ANNEXES

Annex 1: Bacteriological Medias used for isolation, identification and antimicrobial susceptibility test of *E. coli* O157:H7

Nutrient Agar

Nutrient agar (OXOID® Ltd., Basingstoke, U.K.) containing 1 g/l of '*lab-lecmo'* powder, 2 g/l of yeast extract, 5 g/l of peptone, 5 g/l of sodium chloride and 15 g/l of agar will be prepared according to the manufacturer's instructions. Briefly, 28 g of the powder will be dissolved in 1 liter of distilled water. The solution will be boiled to dissolve completely and sterilized by autoclaving at 121 ºC for 15 minutes. Before use, the media will be cooled up to 45 ºC.

Buffered Peptone Water (BPW)

The medium (BPW powder, Oxoid® Ltd., Basingstoke, Hampshire, England, CM0509, Lot 1442805) is composed of 10 g/l Peptone, 5 g/l Sodium chloride, 3.5 g/l Di-sodium phosphate and 1.5 g/l Potassium di-hydrogen phosphate. The medium will be prepared according to manufacturer's instructions whereby 20 g of the powdered medium will be dissolved in 1 liter of distilled water. The culture medium is mixed well and each 10 ml will be dispensed into capped test tubes. Then, the test tubes are sterilized by autoclaving at 121°C for 15 minutes and cooled to 25°C before use. All the unused prepared media will be stored under refrigeration temperature.

Normal saline solution

The solution will be prepared by dissolving 0.85 g of Sodium chloride (Sigma-Aldrich, Co., USA, and Cat. S5886, Lot SLBC3215V) into 100 ml of sterile distilled water, mixed well and sterilizing by autoclaving at 121°C for 15 minutes and cooled to below 45°C, the solution will be ready for use.

Mueller-Hinton (MH) Agar

The medium (Oxoid® Ltd., Basingstoke, Hampshire, England, CM0337 Lot 744451) is composed of 300 g/l Beef, dehydrated infusion, 17.5 g/l Casein hydrolysate, 1.5 g/l Starch, 17

g/l Agar and final pH of 7.3±0.1 at 25°C. The medium will be prepared according to manufacturer's instructions whereby 38 g of the powdered medium is suspended into 1 liter of distilled water, mixed well and brought to boil to dissolve the medium completely. Then, the medium is sterilized by autoclaving at 121°C for 15 minutes, cooled to below 45°C and poured into sterile Petri dishes. The plates are left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility and to dry the condensed vapour on the plate cover.

Sorbitol MacConkey (SMAC) Agar

Sorbitol MacConkey (SMAC) Agar (Oxoid®, Ltd., Basingstoke, Hampshire, England, CM0813 and Lot 1116827) is composed of Peptone (10 g/l), Sorbitol (10 g/l), bile salts No.3 (1.5 g/l), Sodium chloride (5g/l), Neutral red (0.03 g/l), crystal violet (0.001 g/l) Agar (15 g/l) and final pH of 7.1±0.2 at 25°C. It will be prepared according to manufacturer's instruction, whereby 51.5g powder medium will be suspended in one liter of distilled water and brought to the boil to dissolve completely. Then it will be sterilized by autoclaving at 121°C for 15 minutes. Thereafter it will be 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.

Methyl Red and Voges-Proskauer (MR-VP) medium

MR-VP Medium (Himedia Laboratories Pvt, Ltd., Mumbai-400086, India, M070I Lot 0000219697) is composed of Buffered peptone (7g/l), Dextrose (5 g/l), Dipotassium phosphate (5g/l) and final pH 6.9±0.2 at 25°C. The medium will be prepared according to manufacturer's instruction by which 17 grams of powder will be suspended in one liter of distilled water. The medium will be heated (if necessary) to dissolve it completely and then sterilized by autoclaving 121°C for 15 minutes. Then it will be allowed to cool and two sets of test tubes were dispended by MR-VP broth (3 mL, Voges-Proskauer and 5 mL, Methyl Red). The tubes will be placed in refrigerator if not meanwhile the inoculum is prepared.

Simmons citrate agar

The medium (Himedia Laboratories Pvt, Ltd., Mumbai-400086, India, M099, Lot 0000163279) is composed of Magnesium Sulfate (heptahydrate) (0.2 g/l), Ammonium

Dihydrogen Phosphate (1 g/l), Dipotassium Phosphate (1 g/l), Sodium Citrate (dehydrate) (2 g/l), Sodium Chloride (NaCl) (0.08 g/l), Bromothymol blue (0.08 g/l) and Agar (15 g/l) adjusted at final pH of 6.8±0.2 at 25°C. It will be prepared according to manufacturer's instruction by which 24.28 gram of powder will be suspended in one liter of distilled water. The medium will be heated to boil to dissolve the medium completely and then sterilized by autoclaving 121°C for 15 minutes. Then after it will be cooled to 45-50^{0}C and poured in to sterile test tubes, in so doing cooled in slanted position and finally stored in a refrigerator to ensure a shelf life.

Wash buffer: modified phosphate buffer

The medium is composed of sodium chloride (8g), potassium chloride (0.2g), Disodium hydrogen phosphate (1.44g), potassium dihydrogen phosphate (0.24g) and polyoxylethylene sorbitan monnolaurate (0.2g) adjusted at final pH of 7.3±0.1 at 25^{0}C. It will be prepared according to manufacturer's instruction and the medium will be heated to boil to dissolve the medium completely and then sterilized by autoclaving 121°C for 15 minutes.

Annex 2: Biochemical test procedure

Indole Test**:** Peptone water will be prepared and about 3 ml of it will be dispensed in bijou tubes using a sterile pipette. Then, fresh sterile loops will be used to pick a well-isolated colony of bacteria and inoculated into bijou tubes, thereafter, the tubes will be incubated at 37ºC for 48 hours. After incubation period, 0.5 ml of Kovac's Indole Reagent (Loba Chemie Pvt. Ltd, Lot LM01131303) will be added to the inoculated bijou tubes. The tubes will be subjected to gentle shaking and examined for red colour in the surface layer within 10 minutes (Cheesbrough, 2000). A red ring on top of the tube indicated indole positive reaction.

Annex 3: Questionnaire format

Format 1: Questionnaire format for farm owner

Farm ID or No: ____ Date_____

1. Farm name_____ Address_____

2. Scale and type of dairy farm: _____ Government_____

Private_____

3. Herd size_____ Breed: local_____ cross _____ exotic_____

5. Feed and water hygiene and storage: Excellent ☐ very good ☐ Good ☐ Fair ☐ Poor ☐

6. If your animals are enclosed, what type of animal house floor is in? (Only single choice)

Covered with manure ☐ Concrete ☐ Earthed floor ☐ Others (specify)_____

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

Twice a day ☐ Once a day ☐ Once per two days ☐ Others (sepecify)_____

8. Where do cows milked? In barn ☐ In milking room ☐ Any where ☐

9. How do you milk your cows? By hand milking ☐ By milking machine ☐

10. Milking frequency per day: Once ☐ Twice ☐ Three times ☐ If more label_____

11. When do you wash your hands? (Encircle)

a. Before and after milking
b. Between milking
c. Only before milking
d. Only after milking
e. Not at all

12. When do you use teat bathe and towel? (Encircle)

a. Before milking
b. After milking
c. Between milking
d. before and after milking
e. Don't use any dip and towel

13. Have you transportation facility to deliver milk for your customer?

13.1. Where does the milk go? To household consumption____ To restaurants____ To___

14. How do you keep milk containers and milking buckets? Washing with:

a. Warm water
b. Cold water
c. Both warm and cold water
d. Detergents

15. Milker's clothing: Boot_____Clean outer garment_____ Ordinary own cloth_____

Apron_____ others (specify) _____ Interviewer observation_____

16. Do you sell raw milk to customers? Every day ☐ Sometimes ☐ Never ☐

16.1. When you sell? Every morning ☐ Afternoon ☐ Evening ☐

16.2. Have you transportation facility to deliver milk for your customer? Yes ☐ No ☐

16.3. Where does the milk go?

17. Do lactating herd experience mastitis at the farm? Yes_____ No_____

17.1. If your answer is yes, who treated mastitis cow?

_____

18. Is there any practice of record keeping? Yes_____ No_____

If yes: Breeding records_____ Calving records_____ Production records_____ Health records_____ Financial records_____ Feeding records_____ Others_____

Annex 4: R analysis

```
R version 3.3.2 (2016-10-31) -- "Sincere Pumpkin Patch"
Copyright (C) 2016 The R Foundation for Statistical Computing
Platform: x86_64-w64-mingw32/x64 (64-bit)
#################### E. coli analysis ########################
############# Tabulation, chi-square and fisher exact test ####################
>Fre.data.cleared <- read.csv("C:/Users/Frehiwot/Desktop/thesis data/Fre data cleared.csv")
>attach(Fre.data.cleared)
>names(Fre.data.cleared)
>table(Area,Result); chisq.test(Area,Result)
>table(Frm_sc,Result); chisq.test(Frm_sc,Result);
>table(Breed,Result); chisq.test(Breed,Result); fisher.test(Breed,Result, hybrid = T)
>table(Floor_typ,Result); chisq.test(Floor_typ,Result)
>table(Milking_loc,Result); chisq.test(Milking_loc,Result)
>table(Cleaning,Result); chisq.test(Cleaning,Result)
>table(Hand_wash, Result); chisq.test(Hand_wash, Result); fisher.test(Hand_wash,Result)
>table(Towl_use, Result); chisq.test(Towl_use, Result); fisher.test(Towl_use, Result, hybrid = T)
>table(use_detergents, Result); chisq.test(use_detergents, Result); fisher.test(use_detergents,
Result)
>table(Mastitis_affected, Result); chisq.test(Mastitis_affected, Result);
fisher.test(Mastitis_affected, Result)

###################### univariable logistic regression ###################
>Area_fit<-glm(Result~factor(Area),family = binomial(link = "logit"))
>summary(Area_fit)
>exp(coefficients(Area_fit))
>exp(confint(Area_fit))

>Breed_fit<-glm(Result~factor(Breed),family = binomial(link = "logit"))
>summary(Breed_fit)
>exp(coefficients(Breed_fit))
>exp(confint(Breed_fit))
```

```
>?relevel
>Fre.data.cleared$Breed <- relevel(Fre.data.cleared$Breed2, ref = "exotic")
>Breed_fit<-glm(Result~factor(Breed),family = binomial(link = "logit"))

>Cleaning_fit<-glm(Result~factor(Cleaning),family = binomial(link = "logit"))
>summary(Cleaning_fit)
>exp(coefficients(Cleaning_fit))
>exp(confint(Cleaning_fit))

>frmSCALE_fit<-glm(Result~factor(Frm_sc),family = binomial(link = "logit"))
>summary(frmSCALE_fit)
>exp(coefficients(frmSCALE_fit))
>exp(confint(frmSCALE_fit))

>milkingLOCATION_fit<-glm(Result~factor(Milking_loc),family = binomial(link = "logit"))
>summary(milkingLOCATION_fit)
>exp(coefficients(milkingLOCATION_fit))
>exp(confint(milkingLOCATION_fit))

>HandWASH_fit<-glm(Result~factor(Hand_wash),family = binomial(link = "logit"))
>summary(HandWASH_fit)
>exp(coefficients(HandWASH_fit))
>exp(confint(HandWASH_fit))

>floorTYPE_fit<-glm(Result~factor(Floor_typ) , family = binomial(link = "logit"))
>summary(floorTYPE_fit)
>exp(coefficients(floorTYPE_fit))
>exp(confint(floorTYPE_fit))

>TowlUSE_fit<-glm(Result~factor(Towl_use),family = binomial(link = "logit"))
>summary(TowlUSE_fit)
>exp(coefficients(TowlUSE_fit))
>exp(confint(TowlUSE_fit))

>useDETERGENT_fit<-glm(Result~factor(use_detergents),family = binomial(link = "logit"))
```

```
>summary(useDETERGENT_fit)
>exp(coefficients(useDETERGENT_fit))
>exp(confint(useDETERGENT_fit))

>MastitisAFFECTED_fit<-glm(Result~factor(Mastitis_affected),family = binomial(link =
"logit"))
>summary(MastitisAFFECTED_fit)
>exp(coefficients(MastitisAFFECTED_fit))
>exp(confint(MastitisAFFECTED_fit))

################## Conditional logistic regression ######################
>clogit_fit<-clogit(Result~factor(Area)  + factor(Breed) + factor(Floor_typ) + factor(Cleaning) +
factor(Milking_loc)+ factor(Hand_wash)+factor(Towl_use) + factor(use_detergents) +
factor(Mastitis_affected) + strata(Frm_sc))
>summary(clogit_fit)

################## multivariable logistic regression ######################
multi_fit<-glm(Result~factor(Area) + factor(Frm_sc) + factor(Breed) + factor(Floor_typ) +
factor(Cleaning) + factor(Milking_loc)+ factor(Hand_wash)+factor(Towl_use) +
>factor(use_detergents) + factor(Mastitis_affected),family = binomial(link = "logit"))
>summary(multi_fit)
>exp(coefficients(multi_fit))
>exp(cbind(OR=coefficients(multi_fit), confint(multi_fit)))
>anova(multi_fit, test = "Chisq")
>anova(multi_fit, test = "LR")
>library(MASS)
>step <- stepAIC(multi_fit, direction = "both")

################## Final multivariable logistic regression model ######################
>final_fit<-glm(Result ~ factor(Area) + factor(Floor_typ) + factor(Cleaning) +
factor(Milking_loc) + factor(Hand_wash), na.action = na.omit , family = binomial(link = "logit"))
>summary(final_fit)
```

>exp(coefficients(final_fit))

>exp(cbind(OR_F=coefficients(final_fit), confint(final_fit)))

Annex 5: Antimicrobial sensitivity test

Annex 6: Ethical consideration approval letter

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu/Debre Zeit

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/28/05/10/2018

Name of Applicant: Frehiwot Mesele (DVM, MVSc fellow)

Address: College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: ***Occurrence of E.coli O157:H7 in lactating cows and dairy farm environment and its antimicrobial susceptibility pattern at Adami Tulu Jiddo Kombolcha District, mid-rift valley, Ethiopia***

Date of application:	**14/11/2017**
Nature of the project:	**non-invasive**
Target animal species:	**Cattle**
Number of animals involved:	308
Study area:	**Mid Rift Valley, Ethiopia**

Minutes No. and date of review: **VM/ERC/05/10/018, 03/01/2018**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is allowed to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee whenever this is deemed necessary

Dr Getachew Terefe
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University College of Veterinary Medicine and Agriculture

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Please quote Our Ref. No. When replying

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