



ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

**ISOLATION, IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TEST OF
ESCHERICHIA COLI O157 AND NON-TYPHOID *SALMONELLA* SPECIES IN MILK AND
FAECES OF LACTATING COWS AND CAMELS IN BORANA PASTORAL AREA,
SOUTHERN ETHIOPIA**

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Thesis Title

“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”

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STATEMENT OF THE AUTHOR

First, I declare that this thesis is my genuine 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 MSc. degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under 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. Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the department or the Dean of the College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however permission must be obtained from the author.

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ABBREVIATIONS

BPW	Buffered Peptone Water
CDC	Center for disease control and prevention
CFU	Colony Forming Units
CLSI	Clinical and Laboratory Standards Institute
CR-SMAC	Cefixime Rhamnose Sorbitol MacConkey agar
CVMA	College of Veterinary Medicine and Agriculture
DNA	Deoxyribonucleic Acid
EHEC	Enterohemorrhagic <i>Escherichia coli</i>
EIEC	Enteroinvasive <i>Escherichia coli</i>
ELISA	Enzyme-Linked Immunosorbent Assay
EPEC	Enteropathogenic <i>E. coli</i>
ETEC	Enterotoxigenic <i>E. coli</i>
FDA	Food and Drug Administration
HC	Hemorrhagic Colitis
HUS	Hemolytic Uremic Syndrome
ILRI	International Livestock Research Institute
IMS	Immuno-magnet Separation
ISO	International Organization for Standardization
LPS	Lipopolysaccharide
MDR	Multi-Drug Resistant
MPC	Magnetic Particle Concentrator
OIE	Office for International des Epizootics
RVS	Rappaport Vassiliadis Soya Peptone Broth
SMAC	Sorbitol MacConkey agar
STEC	Shiga Toxigenic producing <i>Escherichia coli</i>
TSI	Triple Sugar Iron agar
VTEC	Vero-toxigenic <i>Escherichia coli</i>
XLD	Xylose Lysine Deoxycholate

ABSTRACT

A cross-sectional study was conducted to estimate the prevalence and determine antimicrobial susceptibility profile of *E. coli* O157 and non-typhoid *Salmonella* species in milk and feces of apparently healthy lactating cows and camels managed under extensive husbandry systems in Borana pastoral areas of southern Ethiopia. A total of 300 samples from cattle and 188 samples from camel were systematically collected and examined to isolate and identify both pathogens following the standard techniques and procedures. The prevalence of *E. coli* O157 was 4.67% in both milk and feces samples in cows and the prevalence of *Salmonella* was 4% and 8.6% in cattle milk and feces respectively. The prevalence of *E. coli* O157 in camels' feces was 3.29%. But no isolate was observed in camel milk samples. On the other hand, 1.06% and 2.13% of milk and feces from camels were positive for *Salmonella*, respectively. All of the *E. coli* O157 and *Salmonella* isolates from both animal species showed high susceptibility to Nalidixic acid (100%), Gentamicin (100%) Ciprofloxacin (100%) and Chloramphenicol (90.9%), whereas, 92.86% (13/14) of *E. coli* O157 and 15.79% (3/19) of *Salmonella* isolates from cattle showed resistance to two or more antimicrobial agents. However, none of *E. coli* O157 isolates from camels found resistant to two or more antimicrobial agents while 66.66% (2/3) of *Salmonella* isolates were multiple antimicrobial resistant. The highest level of resistance was observed for Ampicillin (100%) in both animal species for all isolated pathogens followed by Tetracycline (58.8%) for *E. coli* O157 in cattle. In the current study, considerable proportions of milk and fecal samples harbored drug resistant *E. coli* O157 and *Salmonella* species which have a significant public health risk. Therefore, relevant intervention programs and awareness creation on best practice of milk handling are essential to minimize the risk associated with consumption of contaminated milk. Education on the control and surveillance program of antimicrobial usage in animals and animal products are recommended to ensure consumer safety.

Keywords: *Camel, Cattle, Escherichia Coli O157, Prevalence, Salmonella*

1. INTRODUCTION

Nutritionally, milk has been defined as the most nearly perfect food, and it is considered as one of the most important diet items of society (Javaid *et al.*, 2009; Olatunji *et al.*, 2012). However, the fluid or semi-fluid nature of milk and its chemical composition renders it one of the ideal culture media for microbial growth and multiplication (Zelalem and Faye, 2006). As a result, milk produced, handled and processed under unhygienic conditions harbors variety of foodborne pathogens and consequently can cause diseases in humans, if consumed without further treatment such as boiling or pasteurization (Oliver *et al.*, 2009).

Milk-borne pathogens cause human diseases ranging from gastrointestinal disturbances characterized by diarrhea and vomiting to other, generalized, and even life threatening foodborne illnesses (Oliver *et al.*, 2009). They have not only of public health importance but also economic importance. In addition to causing serious economic losses in dairy cattle production, they pose a major barrier for trade of animals and animal products, and this could seriously impair socio-economic progress especially in developing countries like Africa. Furthermore, the level of awareness among farmers of the economic and public health importance of zoonotic diseases in most of these countries is low, and this further stifles efforts to control these diseases (Munyeme *et al.*, 2010; Mosalagae *et al.*, 2010).

The safety of dairy products with respect to food borne disease is of great concern around the world and it is especially true in developing countries where production of milk and various milk products takes place under unsanitary conditions and poor production practices (Mogessie, 1990). Milk from a healthy udder contains few bacteria but it picks up many bacteria from the time it leaves the teat of the animal depending on the hygienic level exercised during milking, cleanliness of the milk utensils, condition of storage, manner of transport for further processing until it is used for human consumption. These microorganisms are indicators of both manner of handling milk from milking till consumption and the quality of the milk (Ahmed, 2009; Fatine *et al.*, 2012; Lunder and Brenne, 1996; Shunda *et al.*, 2013).

The ability of raw or processed milk to support the growth of several spoilage or pathogenic microorganisms can lead to spoilage of the product or infections and intoxications in consumers (Abdissa *et al.*, 2017). Such pathogens include *Salmonella* spp., *Klebsiella* spp., *Enterobacter* spp. and *Escherichia coli*, which have been identified in milk products in Ethiopia (Yilma *et al.*, 2007). Gram positive pathogens such as *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes* and *Enterococcus* spp. have also been frequently isolated from milk (Adugna and Asresie, 2015).

Many countries have milk quality regulations, including limits on the total number of bacteria in raw milk, to ensure the quality and safety of the final product. However, hygienic quality control of milk and milk products in Ethiopia is not usually conducted on routine basis. There is little information on the microbial quality of raw milk (Zelalem and Faye, 2006) especially in the pastoral and agro-pastoral area of southern Ethiopia, where milk consumption plays a significant role in the diet of the community (Worku *et al.*, 2015).

Borana zone, the pastoral area in southern part of Ethiopia, is characterized by arid and semi-arid climate with livestock production which is the mainstay of the people (Coppock, 1994). Milk is a common diet of the pastoral households and provides a quick way of supplying nutrients. So far, there was no documented information regarding the prevalence of some foodborne pathogens like *E. coli* O157:H7 and *Salmonella* species from milk in the study area. In this respect, there is a need for documented information on prevalence of some food borne pathogens circulating in the area to undertake relevant interventions to minimize contamination of milk and milk products. The present study is a component of a project which aimed at improving hygiene and handling practices of milk and milk products in Borana (<http://livestocklab.ifas.ufl.edu/projects/dr-kebede-amenu>).

Therefore, the objectives of the present study were:

- To isolate and identify *E. coli* O157 and non-typhoid *Salmonella* species in milk and feces of lactating cows and camels in Borana pastoral area of Ethiopia.
- To investigate the risk factors associated with the prevalence of *E. coli* O157 and non-typhoid *Salmonella* species in milk and feces of lactating cows and camels in the study area.
- To determine the antimicrobial susceptibility pattern of the isolated pathogens.

2. LITERATURE REVIEW

2.1. Occurrence and Prevalence of *Escherichia coli* O157:H7 and *Salmonella*

2.1.1. Occurrence and Prevalence of *Escherichia coli* O157:H7

Escherichia coli is a normal inhabitant of the intestines of animals and humans but its recovery from food may be of public health concern due to the possible presence of enteropathogenic and/or toxigenic strains which lead to severe gastrointestinal disturbance (Soomro *et al.*, 2002). It is considered as the major indicator of fecal pollution in food production. Its presence in processed foods results from recontamination, because this bacterium usually does not survive food preservation processes (Law, 2000).

The majority of *E. coli* rods do not constitute a serious health hazard, but some serotypes can cause food poisoning and alimentary intoxications. The most dangerous among them are enterohemorrhagic *E. coli* strains, especially serotype O157:H7. *E. coli* O157:H7 has become a pathogen of major concern in both food and dairy industries, and to the public, because of its ability to cause severe illness, in particular, haemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (Picozzi *et al.*, 2005; Reuben *et al.*, 2013).

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 serious disease (Farrokh *et al.*, 2012). *E. coli* O157:H7 is characterized by antigenic structure on its surface such as O, H and LPS. These structure is defined as the bacterium's O-antigen (Ohne), a polysaccharide domain in the bacterium's lipopolysaccharide (LPS) in the outer membrane, and the H-antigen (Hauch) consisting of flagella protein (Ratnam *et al.*, 1988).

Escherichia coli O157:H7 which is also known as verocytotoxin producing or shiga toxin producing *E. coli* O157 is the most important food borne pathogens reported world-wide. The public health concern of *E. coli* O157 came to light at first after its first outbreak reported in the USA in 1982 (Gregory *et al.*, 1996). Globally, STECs are estimated to cause 2.8 million acute illnesses each year. Foodborne *E. coli* O157:H7 is estimated to cause over 60,000 illnesses in the United States each year, resulting in about 2,000 hospitalizations and 20 deaths (Majowicz *et al.*, 2014).

Cattle are the natural reservoir of it contributing as a major source of *E. coli* O157 for human infections (Toth *et al.*, 2009). Although the organism does not produce any clinical illness in their natural reservoir, it can produce a broad spectrum of clinical abnormalities in humans including mild diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), bloody diarrhea and thrombotic thrombocytopenic purpura (TTP). The sources of infections with enterohemorrhagic *E. coli* strains are mostly meat products, especially underdone steaks and hamburgers (Chinen *et al.*, 2001), but also other foodstuffs as unpasteurized milk and dairy products manufactured from raw milk, have been implicated in many outbreaks, (Maher *et al.*, 2001). *E. coli* O157:H7 has been isolated from the feces or gastrointestinal tract of cattle, sheep, horses, pigs, turkeys, dogs, and a variety of wild animal species (Heuvelink *et al.* 1999). It has also been isolated from bodies of water (e.g., ponds, streams), wells and water troughs and has been found to survive for months in manure and water trough sediments (Sargeant *et al.*, 2000).

E. coli O157:H7 is among many pathogenic microorganisms which can get access to milk and dairy products and is considered as a reliable indicator of contamination by manure, soil, and contaminated water (Oliver *et al.*, 2009; Disassa *et al.*, 2017). Although detection of *E. coli* in milk reflects fecal contamination, environmental coli forms have also been detected in milk. The existence of fecal coli form bacteria may not necessarily indicate a direct fecal contamination of milk but it is a precise indicator of poor sanitary practices during milking and further handling processes (Hayes *et al.*, 2001).

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 Bollinger, 2005). The random effects pooled

prevalence estimates of it in Africa, Northern America, Oceania, Europe, Asia and Latin America-Caribbean are likely to be 31.20%, 7.35%, 6.85%, 5.15%, 4.69% and 1.65%, respectively, although between studies heterogeneity was evidenced in most of these world regions. The highest prevalence estimate (31.20%) was in African cattle and the estimates 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:H7, respectively. Allerberger *et al.* (2001) reported 3% of the milk samples tested in Austria to be positive for *E. coli* O157:H7 and Klie *et al.* (1997) found that 3.9% of the raw milk analyzed in Germany was contaminated with *E. coli* O157:H7. Despite greater burden caused by foodborne infections in developing countries than developed countries, there is a great scarcity of information on their occurrences (Havelaar, 2010).

In Ethiopia, few studies have been done on prevalence, distribution and associated virulent genes of *E. coli* O157:H7 in humans, animals or in foods of animal origin (Hiko *et al.*, 2008). The study conducted on the prevalence and antimicrobial susceptibility pattern of *E. coli* O157:H7 isolated from traditionally marketed raw cow milk in and around Asosa town shows 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 (Disassa *et al.*, 2017). The prevalence from raw milk is also reported as 44.4% from Mekelle town (Ethiopia) (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 (Abdissa *et al.*, 2017).

2.1.2. Occurrence and Prevalence of Non typhoid *Salmonella*

Salmonella is the second leading cause of food borne illness in most developed countries causing diarrhea, cramps, vomiting, and often fever. Globally, more than 93.8 million cases of gastroenteritis are caused by non typhoidal *Salmonella* with 155,000 deaths each year. Of these cases, 85.6 million cases were estimated to be food borne (Majowicz *et al.*, 2010). Salmonellosis, the diseases caused by bacteria of the genus *Salmonella*, is a common intestinal illness caused by numerous *Salmonella* serovars with clinical manifestations that vary from severe enteric fever to mild food poisoning (Jones *et al.*, 2004) both in animals (Radostits *et al.*, 2007) and humans (Hohmann, 2001).

The *salmonella* are small, gram negative, non-spore forming rods that are indistinguishable from the *Escherichia coli* under the microscope or ordinary nutrient media. They are widely distributed in the nature with humans and animals being their primary reservoirs. Some significant changes have occurred in the taxonomy of *salmonella*. *Salmonella* is a diverse bacterial species comprising over 2600 serotypes (Guibourdenche *et al.*, 2010). There are two species of *Salmonella*: *Salmonella enterica* and *Salmonella bongori*. Serotyping differentiates the strains and *Salmonella enterica* is further classified into 6 subspecies (*Salmonella enterica* subspecies *enterica*, *S. enterica* Subspecies *salmae*, *S. enterica* Subspecies *arizonae*, *S. enterica* Subspecies *diarizonae*, *S. enterica* Subspecies *hautena* and *S. enterica* Subspecies *indica*). Most of the *Salmonella* serotypes are part of *S. enterica* subspecies *enterica*, and over 99 % of human and animal infections are caused by serotypes under this subspecies (Uzzau *et al.*, 200).

The principal sources of infection are carrier animals and contaminated feeds containing food stuff of animal origin. The primary habitat of *salmonella* species is the intestinal tract of the animals such as farm animals, humans, birds, reptiles and insects. The organisms are excreted in feces from which they may be transmitted by insects and other living creatures to large number of places (Radostits *et al.*, 2007). Foods of animal origin particularly meat, poultry, egg, milk and milk products are considered to be the primary source of human salmonellosis (Acha and Szyfers, 2001).

For epidemiological purposes, the *salmonella* can be placed into three groups; the first are those that infect humans only. This includes, *S. Typhi*, *S. Paratyphi A*, *S. Paratyphi C*. this group includes the

agents of typhoid and paratyphoid fevers, which are the most severe of disease caused by *salmonella*. The second was the host-adapted serovars (some of which are human pathogens and may be contracted from food), included are *S. gallinarum* (poultry), *S. dublin* (cattle), *S. abortu-equi* (equine), *S. abortus-ovis* (sheep) and *S. cholera-suis* (swine). The third is unadapted serovars (no host preference). They are pathogenic for humans and other animals. The epidemiology of the *salmonella* is complex, which often make animals control of the disease is difficult (Quinn *et al.*, 2001).

There is a critical need to develop method to control the spoilage or poisoning of food by *salmonella* ordinary farms by instituting biosecurity and bio-containment practices in addition to enhanced food processing method, preparation and storage practices. Effective heat processing of food of animal origin, which includes pasteurization of milk and eggs, irradiation of meat and poultry thermal processing; good hygiene practices during production of food; vaccination of egg-producing flocks and food producing animals, are crucial measures (Jay, 2000).

Occurrence of non-typhoidal *Salmonella* serotypes commonly infecting humans in dairy cattle, particularly, those stains resistant to antimicrobial agents commonly used in human medicine, are a serious threat to human health. Some multi-drug resistant (MDR) *Salmonella* outbreaks in humans have been linked to exposure to dairy farms or contaminated dairy products (Gupta, 2003).

According to Jayaroo and Henning, *Salmonella* was isolated from 6.1% of bulk tank milk sample from dairy herds in South Eastern Dakata and Western Minnesota (Jayaroo and Henning, 2001). Cheeses made from raw milk have been implicated as sources of several outbreaks (D'Aoust, 1994). The worst food poisoning incident due to *Salmonella* occurred in USA in 1985 and there was a cause of 16,289 human cases and 7 deaths as the result of recontamination of pasteurized milk with a potent strain of *Salmonella typhimurium*. Studies show that antimicrobial resistance *Salmonella* are increasing due to the use of antimicrobial agents in food animals, which are subsequently transmitted to humans usually through the food supply (White *et al.*, 2001).

In Ethiopia, despite attempts to study prevalence of *Salmonella* mainly in poultry and beef, the status in milk and milk products is still unknown. However, studies made elsewhere indicated that milk and milk products are important source of *Salmonella* particularly among those raw consumers.

Ubiquitous nature of *Salmonella*, unhygienic condition prevailing at the farm levels and food handlers, and habit of consuming milk and milk products in raw suggest that milk and milk products can act as source of *Salmonella* organisms in Ethiopia (Tesfaw *et al.*, 2013)..

For example, a study conducted on prevalence and antimicrobial resistance profile 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 (Tesfaw *et al.*, 2013). There is also a report of 7.1% prevalence of *Salmonella* from apparently healthy slaughtered cattle (Alemayehu *et al.*, 2003).

According to Tadesse and Tessema, the prevalence of *Salmonella* in food animals slaughtered including cattle, sheep, goats and pigs were 7.07%, 8.41%, 9.01% and 43.81% respectively. The occurrence of *Salmonella* was significantly higher in pigs than in slaughtered true ruminants, but not significantly different between cattle, sheep and goats (Tadesse and Tessema, 2014). A previous study conducted in Addis Ababa has shown farm level prevalence of 47.8 % and animal level prevalence of 7.7 % (Addis *et al.*, 2011).

2.2. Isolation and Identification of *E. coli* O157 and *Salmonella*

2.2.1. Isolation and Identification of *E. coli* O157

Conventional or traditional methods for detecting microorganisms in foods often involve culturing the organism in selective media and identifying isolates according to their morphological, biochemical, and/or immunological characteristics. This involves enriching in one or more liquid enrichment media that allow for the resuscitation and multiplication of a particular microorganism. Non selective pre enrichment is necessary for the effective recovery of low levels of stressed *E. coli* O157. The commonly used enrichment media is buffered peptone water either without supplements or supplemented with 8 mg/litre vancomycin, 10 mg/litre cefsulodin and 0.05 mg/litre cefixime (BPW-VCC) to suppress the growth of Gram-positive organisms such as *Aeromonas* spp. and *Proteus* spp. Modified trypticase soy broth (mTSB) supplemented with 20 mg/litre novobiocin or 10

mg/litre acriflavin and modified *E. coli* broth supplemented with 20 mg/litre novobiocin (mEC+n) to reduce the growth of Gram positive organisms are also common enrichment media (Radostits *et al.*, 2000).

The most widely used solid plating medium for the detection of non-sorbitol fermenting *E. coli* O157:H7 is sorbitol MacConkey (SMAC) agar and selectivity is also improved by the addition of selective supplements cefixime and potassium tellurite (CT-SMAC). In recent years a variety of chromogenic media have also become available commercially for the detection of *E. coli* O157:H7 in humans, food and animal feed stuffs. These media contain a particular mixture of artificial chromogenic conjugates composed of a substrate for an *E. coli*-specific enzyme coupled to a chromophore that produce a visible and qualitative color change when degraded by specific microbial enzymes. When the *E. coli* enzyme cleaves the colorless conjugate, one or more insoluble chromophores are released, resulting in a distinctive color for the *E. coli* colonies (Bettelheim, 1998b).

Chromogenic agars that are specific for the isolation of *E. coli* O157:H7 from foods include CHROMagar™ O157 (CHROMagar Microbiology, Paris, France) (Bettelheim, 1998b), RAPID'E. coli™ O157:H7 (Biorad, Hercules, CA, USA) and Rainbow® O157 agar (Biolog, Hayward, CA, USA) (Bettelheim, 1998a). CHROM agar O157 is non-inhibitory *E. coli* O157 selective medium specially designed to differentiate *E. coli* O157 from other *E. coli* organisms because of its specific chromogenic properties. This medium can differentiate *E. coli* O157 by its pink-mauve colony colour, from sorbitol negative background micro-organisms such as *Proteus* and *Pseudomonas* found on SMAC. CHROM agar O157 was designed to be used as plating medium after Immunomagnetic separation (IMS) with Dynabeads anti-*E. coli* O157. The vast majorities of other bacterial species are inhibited or give blue or colourless colonies (Bettelheim, 1998b).

Apart from the traditional culture methods relying on biochemical characteristics, various genotypic methods have been proven useful for species identification, epidemiological typing and determining genetic relatedness among pathogenic and nonpathogenic bacteria (Tenover *et al.*, 1995). Besides, the low infectious dose of *E. coli* O157:H7 (from 50 to 100 organisms) necessities the development of sensitive detection techniques. For example, immunomagnetic separation (IMS) with or without

prior broth enrichment has provided an enhanced isolation capacity by aiding in the capture, separation and concentration of a target pathogen from the sample matrix (Chapman *et al.*, 1997; Hepburn *et al.*, 2002).

IMS allows the rapid capture and concentration of bacteria from a range matrix. The magnetic beads used for IMS are commercially available, either pre-coated with antibodies or ready for antibody conjugation. The beads are typically 2-3 μm spheres containing Fe_2O_4 and Fe_3O_4 to make them super paramagnetic (Reinders *et al.*, 2002). They are only magnetic in the presence of a magnetic field and readily separate from each other when the magnetic field is removed. Therefore, by applying a strong magnetic field to the outside of the reaction vessel, the beads and captured bacteria can be immobilized against the vessel wall which allows selective removal of the remainder of the samples including non-target bacteria and other organic particles. The beads are then released by withdrawing the magnet. Recently, immune-magnetic particles for the separation of *E. coli* O157, *E. coli* O26 and O111 have become commercially available which have remarkably improved their isolation from food matrices (Wright *et al.*, 1994).

According to Wright *et al.* (1994), a 100-fold increase in sensitivity of detection by IMS was achieved when compared to direct subculture from enrichment broth. However, manual IMS (MIMS) is labor intensive when large numbers of samples have to be analyzed. So, an automated IMS in combination with an integrated ELISA (EiaFoss; Foss, Denmark) would increase efficiency and lighten the workload. This method can test about 81-108 samples per day, after overnight enrichment (Reinders *et al.*, 2002).

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:H7 positive (De Boer and Heuvelink, 2000).

Immunoassays, depending on the specific interactions between antigen and antibody, also have been developed for the determination of *E. coli* O157:H7, such as enzyme-linked immunosorbent assays (ELISA) (Johnson *et al.*, 1995), immunofluorescent antibody (IFA) techniques, radioimmunoassay, electrochemical biosensors (Akanda *et al.*, 2013; dos Santos *et al.*, 2013) and fluorescent biosensors (Chen *et al.*, 2015). These methods exhibit simple processing, short analysis time, high sensitivity or good potential applications in practice. However, potential interference from bacteria, which can produce protein A or protein G, may cause false positive results in the immunoassays since *E. coli* O157:H7 always presents in a complex biological environment along with many other organisms (Maalouf *et al.*, 2007).

The low infectious dosage of *E. coli* O157:H7 requires a sensitive detection method to detect verotoxin genes (VT) like Stx 1 and Stx 2). PCR technique allows not only detection of VTECs irrespective of their serotypes, but also detects all different variants of VT (Gilgen *et al.*, 1998). A number of specific PCR procedures are described for detection of genes encoding the virulence factors. Because the genes are well known, the direct detection of the genes encoding the virulence factors by PCR in most laboratories is most convenient (Global Salm-Surv, 2003). PCR can be used on pure or mixed plate or broth cultures, and extracts from food or feces (OIE, 2004). It can also be used to detect genes in non-viable organisms so that having potential to be used to screen samples for EHEC in epidemiological studies (Moxley, 2003).

2.2.2. Isolation and Identification of *Salmonella*

The isolation and identification of *Salmonella* spp in food and animal feed can be performed using current ISO horizontal method recommended by International Organizations for Standardization (ISO 6579:2002/FDAM 1, 2007) which was amended in 2007 to include testing of animal feces and environmental samples from primary production and those recommended by the Global *Salmonella* Surveillance (GSS) and National Health Services for Wales (NHS) (Addis *et al.*, 2011). Conventional cultural methods for the detection of foodborne *Salmonella* species generally consist of five distinct and successive steps. These are pre-enrichment in nonselective media and selective enrichment in broth media, plating on differential agar, biochemical screening and serological conformation (D'Aoust, 2001).

The *Salmonella* may be present in small numbers in environmental samples, feces from apparently healthy animals and in animal feed which are often accompanied by considerably larger numbers of other *Enterobacteriaceae* or other families (D'Aoust, 2001). Therefore, the first step in traditional detection methods for most food samples is usually a pre-enrichment culture in a non-selective liquid medium such as buffered peptone water incubated at 37°C for 18 hours (Amagliani *et al.*, 2012)

Success in isolating *Salmonella* is usually enhanced by the inoculation of incubated pre-enrichment broth into selective enrichment media (Read *et al.*, 1994 and Davies, 1995). Three main families of selective enrichment media for *salmonella* are recognized including selenite, tetrathionate (TT) and Rappaport-Vassiliadis (RV). Rappaport Vassiliadis is currently recommended for *Salmonella* recovery from low and highly contaminated foods (Bakr *et al.*, 2008).

The ISO method specifies the XLD agar and one optional selective medium. A variety of alternatives are available, including Bismuth Sulphite agar, Brilliant Green agar and Hektoen Enteric agar. Typical *Salmonella* colonies on selective agar are sub-cultured onto non-selective media prior to confirmatory testing. From each enrichment medium, plating onto agar media plates (one of which is Xylose-Lysine Desoxycholate (XLD) agar) is carried out after 24 h and 48 h of incubation. Up to five colonies per plate have to be confirmed, which may potentially involve the confirmation of up to 40 presumptive colonies (ISO-6579, 2002).

It can take at least three to five days to obtain a result using traditional methods of detection for *Salmonella* species. For this reason a substantial number of alternative rapid screening methods have been developed to produce results more quickly for food and environmental samples. *Salmonella* rapid test and screening kits utilise several different technologies, including novel culture techniques, immunomagnetic separation, EIA and ELISA based assays incorporating fluorescent or colorimetric detection and simple lateral flow assays incorporating immunochromatographic technology. Some methods can be automated to screen large numbers of samples (Oliveira *et al.*, 2002; Gebreyes, 2003).

Preliminary identification based on colony appearance on chromogenic and other selective agar media is traditionally confirmed using classical biochemical and serological testing. Key biochemical tests are fermentation of glucose, negative urease reaction, lysine decarboxylase, negative indole test, H₂S production and fermentation of dulcitol. Serological confirmation tests typically use polyvalent antisera for flagellar (H) and somatic (O) antigens (OIE, 2000; Jay, 2000; Molbak *et al.*, 2006).

Rapid immunological identification and confirmation tests based on latex agglutination, enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) have been developed for *Salmonella*, and simple-to-use lateral flow test strips using immune chromatographic technology have also been developed into commercial products by a number of manufacturers. Molecular methods are also available, notably DNA hybridization and PCR assays for the identification of *Salmonella enterica*. However, these are generally designed for use as part of a method for rapid detection and screening rather than for confirmation. A range of DNA based typing techniques have also been developed for use by specialist laboratories involved in the investigation of food-borne disease outbreaks (Hensel, 2006).

3. MATERIALS AND METHODS

3.1. Study area

The study was carried out in Borana Zone, located in the south-most part of the Ethiopian lowlands which is inhabited by agro-pastoral and pastoral communities and livestock production is the main livelihood of the people. It is located between 3°36'N-6°38'N and 33°43'E-39°30'E, sloping gently from 2702 meters in the northeast to about 496 meters in the extreme south that borders northern Kenya. Yabello town is about 567 km far from Addis Ababa. The area has semi-arid to arid climate with high variability of rainfall and consequent seasonality in the livestock and livestock products off-take. The rainfall distribution of the area is bimodal with the long rainy season extending from March to May (*Ganna*) and the short rainy season from October to November (*Hagayya*). In Borana, June to September is cool dry season while December to February is warm dry season (Coppock, 1994). The mean average of annual rainfall is between 238-896mm with minimum and maximum daily temperatures ranging between 19°C and 26°C. Borana pastoralists were historically cattle-keepers while recently they are diversifying their herds by keeping camels, goats and sheep. Specifically, pastoral households based on market-shed around Yabello town and other selected towns in the area, Surupha, Dubuluk, and Elweya were considered in the present study.

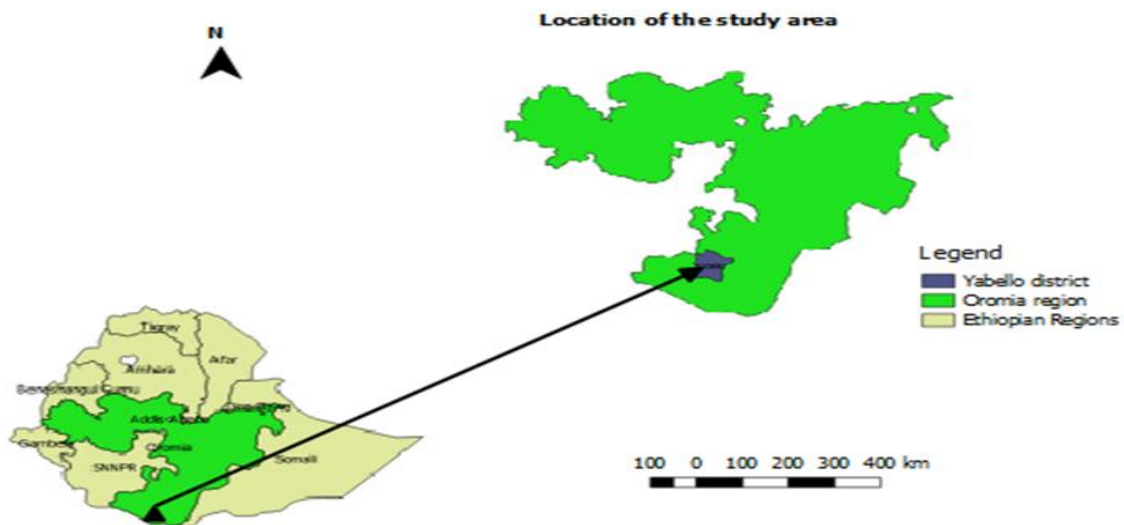


Figure 1: Geographical location of the study area

3.2. Study design and population

A cross-sectional study was conducted to isolate and identify *E. coli* O157 and *Salmonella* species from milk and feces of both lactating cattle and camels in Borana pastoral area of southern Ethiopia and to determine the antimicrobial resistance profile of the isolates. The study population comprised apparently healthy lactating cows and camels which are managed under traditional/extensive husbandry systems in the study area.

3.3. Sample Size and Sampling Strategy

In the present study, both milk and fecal samples from each animal were collected to assess the prevalence of both *E. coli* O157 and *Salmonella*. The number of animals sampled in the study was estimated by the formula described by Thrusfield (1995).

$$N = \frac{(Z_{\alpha/2})^2 \times P(1 - P)}{d^2}$$

Where N is sample size required, d is absolute precession (d=0.05), p is expected prevalence (this is taken based on prevalence of *Salmonella* in different livestock species). For sample size calculation, the prevalence taken was based on previous studies on *Salmonella*: a pooled prevalence of 7.47% *Salmonella* in ruminants from a systematic review (Tadesse and Tessema, 2014), which was taken for both cattle and camels. It was assumed that the prevalence of *E. coli* O157 in both species is less than that of *Salmonella* and the sample size calculated for *Salmonella* can accommodate both pathogens. Similar assumption was made for both species of livestock. Accordingly, 106 animals (each for camel and cattle) were targeted to be sampled by simple random sampling technique and equal sample size was used for both *E. coli* O157 and *Salmonella* species.

The lactating animals were selected after herd (household) selection and to account for this potential herd level clustering of the bacterial infection and contamination, the target sample size was adjusted for intra-cluster correlation coefficient (ρ) of 0.2 as suggested by Otte and Gumm (1997) and the average number of each of the lactating animals was taken as 3 per herd (household). Accordingly, the design effect (deff) of the study was: $deff=1+(m-1)\rho$, where m is cluster size (i.e. 3 in this case) and ρ is 0.2 (as stated above) and this gives deff of 1.4 and the calculated sample size

was adjusted by multiplying by deff (which gave 149 for each species of sampled animals). Finally, 298 animals (paired milk and feces) samples (in total 596) were collected and analyzed. In this study maximum of 3 animals per household for each animal species were sampled. To account for potential specimen loss, the sample size increased to 155 animals from each of the livestock species. At the end paired milk and feces samples were collected from 154 lactating cows and 158 camels. Out of the total collected, it was possible to undertake microbiological analysis for paired samples from 150 cows and 94 camels.

3.4. Sample collection and transportation

Raw milk and fecal samples were collected in April 2018 from lactating animals (Cows and Camels). About $\approx 15\text{g}$ fecal samples were collected through rectal palpation from lactating animals and 30 ml milk samples were collected directly from the animal by the farmer and/or pastoralist (pooled from all quarters). In Borana pastoral area, the animal owners take away their animal to grazing land in the early morning (5 AM) and return to home in the early evening which is late afternoon (5 PM). Therefore sample collection was done at 5 AM in the early morning and at 5 PM in the late afternoon (twice per a day). The overall work sample collection, transportation and processing was highly intensive activities involving three teams: two teams at field for sample collection and transporting the samples as well as another team processing samples in laboratory.

The samples were collected carefully and placed in sterile bottle. After labeling the bottle to identify species, household and date of collection, it was kept in cooler with ice. Finally, the collected samples were transported to Yabello Pastoral and Dryland Agriculture Research Centre and stored in refrigerator at $+4\text{ }^{\circ}\text{C}$ until transported to Addis Ababa on the next day for laboratory analysis at the laboratory of International Livestock Research Institute in Addis Ababa where microbiological analysis was carried out. The specimens were transported from Yabello to Arbaminch (about 4 hrs drive) by car using portable freezer and then transported to Addis Ababa by airplane within the same day and stored at $-20\text{ }^{\circ}\text{C}$ until processed in the laboratory.

3.5. Sample processing and bacteriological examination

3.5.1. Pre-enrichment of the samples

Before processing, the samples were taken from deep freezer a day before and kept in +4⁰C refrigerator and thawed. The next day the specimens were kept at room temperature to initiate the normal physiological activities of bacteria. After this, pre-enrichment of sample was carried out by putting 10 g of feces or 10 ml of milk in a Whirl-Pak filter bag and 90 ml of buffered peptone water (BPW, Oxoid) was added. After that the mixture in the bag was homogenized by using lab blender. This pre-enrichment broth was incubated at 25 ⁰C for 2 hr then at 42 ⁰C for 6 hr and then held at +4 ⁰C until processed the next day for isolation of both *E. coli* O157 and *Salmonella* species.

3.5.2. Isolation of *E. coli* O157 and non-typhoid *Salmonella*

Procedure for immuno-magnetic separation

Isolation and identification of bacteria was done using techniques recommended by International Organizations for Standardization (ISO-6579, 2000) with some modification (Agga *et al.*, 2017). After enrichment, 1 ml aliquot of each pre-enrichment culture (broth) was subjected to 20 µl anti-O157 IMS bead for *E. coli* O157 isolation (Dynabeads anti *E. coli* O157; Applied Biosystems®) or to 20 µl of *Salmonella* specific immunomagnetic separation beads for *Salmonella* isolation (DynaL, Lake Success, NY) as described previously (Durso *et al.* 2005; Keen *et al.* 2006). Briefly, *E. coli* O157 and *Salmonella* specific immunomagnetic separation beads were re-suspended by gentle vortex mixing to ensure that the pellet at the bottom of the vial was completely suspended. Twenty microliter of re-suspended paramagnetic beads was transferred to a screw top Eppendorf tubes (Figure 1) and 1 ml of the enriched culture was added in to the Eppendorf tubes. Then, each tube was briefly vortexed for 10-30 minutes at room temperature. The tubes were then transferred to the manual magnetic particle concentrator (MPC-S) with the magnetic strip in place, inverted several times and left to separate for 3 minutes. Then after carefully opening the cap of the tube the supernatant was aspirated and discarded. Then magnetic strip was removed and 1ml of phosphate buffered saline-Tween 20 (PBST, Sigma chemicals Co. Saint Louis, USA) was added to each tube. The tubes were closed again and the MPC inverted several times with the tubes still in place in order

to re-suspend the beads. The magnetic strip was replaced and the above steps were again performed three times. To prevent cross contamination, separate sterile micropipette tips were used for each sample.

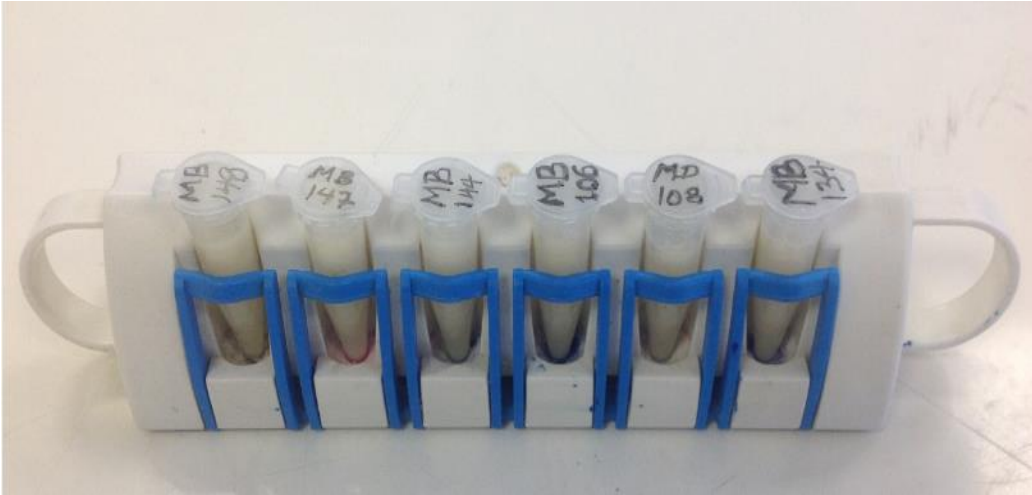


Figure 2: Concentration of *E. coli* O157 and *Salmonella* species by using MPC

Plating of samples for isolation

Following extraction, the final bead-bacteria complexes ($\approx 50 \mu\text{l}$) were plated by spread-planting onto Chromagar plates (CHROMAgar-O157; DRG International, Mountainside, NJ) supplemented with novobiocin (5 mg/liter; Sigma, St. Louis, MO) and potassium tellurite (2.5 mg/liter; Sigma) and incubated at 37°C overnight for the isolation of *E. coli* O157. Following incubation, colony suggestive of *E. coli* O157 on Chromagar plates (a mauve-pink colour) was picked up and inoculated onto nutrient agar slants which were further incubated at 37°C for 18 h (Figure 2). Then the slants were stored at $+4^{\circ}\text{C}$ until further biochemical tests of the isolates were processed.

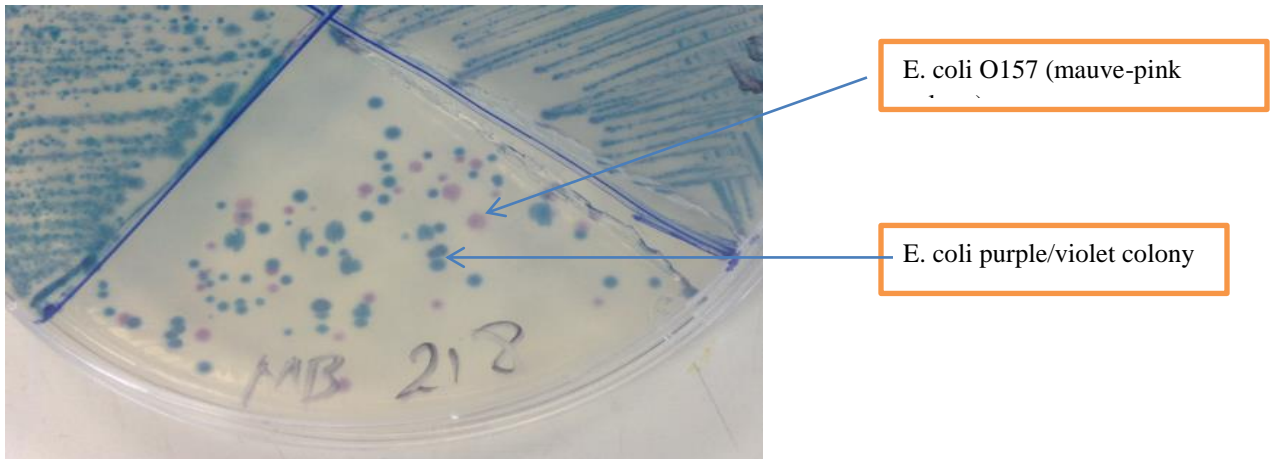


Figure 3: Colony characteristics of *E. coli* and *E. coli* O157 on chromagar plate

For the isolation of *Salmonella*, the recovered bacteria-beads complex were transferred (eluted) into 3 ml of Rappaport Vassiliadis soya peptone broth (RVS: Oxoid) and incubate at 42 °C for 18 hr. After incubation, loop full of RVS broth enrichment culture was plated onto xylose lysine deoxycholate (XLD) agar (Oxoid) supplemented with 4.6 ml/liter tergitol, 15 mg/liter novobiocin and 5 mg/liter cefsulodin (XLDtnc) plate and incubated at 37 °C for 18 hr. Suspected *Salmonella* colony that was presumptively isolated on the basis of characteristic colony appearance on the XLD media plate was inoculated onto nutrient agar slants and incubated at 37 °C for 18 h. Then the slants were stored at +4°C until further biochemical tests of the isolates was carried out.

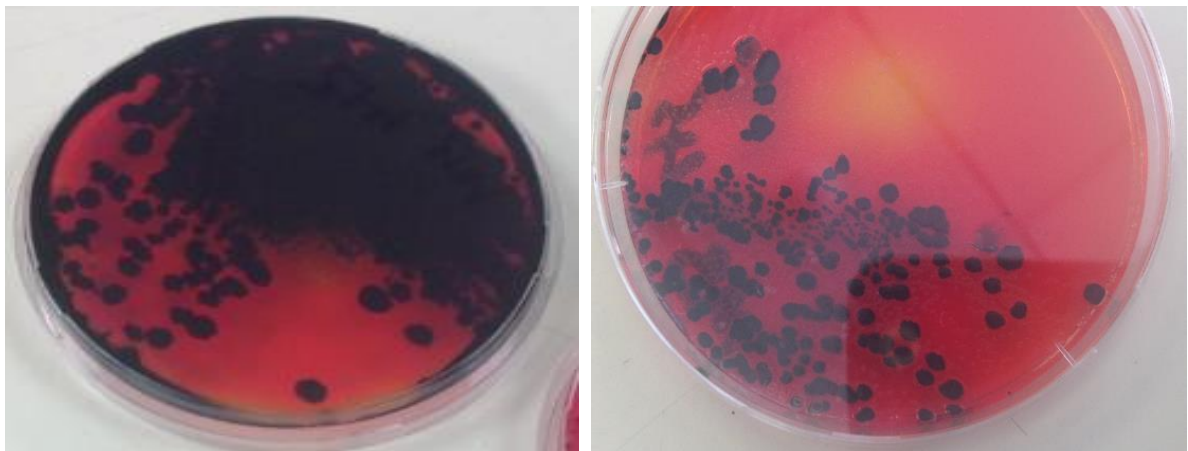


Figure 4: Colony characteristics of *Salmonella* species on XLD plate agar

For both *E. coli* O157 and *Salmonella* species, positive controls were used with known isolates obtained from University of Ghent, Belgium.

3.5.3. Biochemical tests

The selected colonies were streaked onto the surface of pre-dried Nutrient agar (OXOID CM0003, England) plates in a manner that allow isolated colonies to develop and incubated at $37\pm 1^{\circ}\text{C}$ for 24 hr. for further confirmation with biochemical tests. Biochemical tests of both *E. coli* O157 and *Salmonella* isolates were done according to (ISO-6579, 2002) by using different biochemical tests such as triple sugar iron (TSI) agar (oxiod), L-lysine decarboxylation test, indole production, citrate utilization test, methyl red and voges-proskauer tests. Pure colonies from nutrient agar culture were picked and inoculated into biochemical test tubes containing TSI agar, lysine decarboxylase broth, Simon's citrate agar and Tryptone broth and then incubated for 24 hr. at 37°C (for citrate utilization test more than 24 hr. incubation was needed). Colonies producing an alkaline slant with acid (yellow color) butt on TSI with hydrogen sulphide production and gas formation, formation of purple/pink color of L-lysine decarboxylation broth, color change of Simon's citrate agar from green to blue, positive for MR test, negative for VP test and negative for tryptophan utilization (yellow-brown ring) indicating absence of indole production were considered to be *Salmonella* positive (ISO 6579, 1998). For *E. coli* O157, isolates that were positive for indole test, negative for citrate utilization, negative for VP test and positive for L-lysine decarboxylation test were considered as positive isolate for *E. coli* O157.

3.5.4. Antimicrobial susceptibility tests

The antimicrobial susceptibility test was performed according to the National committee for clinical laboratory standards (NCCLS, 2007) by using the Kirby-Bauer disk diffusion method. The antimicrobials used in this study were Ampicillin (AMP, 10 μg), Chloramphenicol (C, 30 μg), Gentamicin (GEN, 10 μg), Ciprofloxacin (CIP, 5 μg), Nalidixic Acid (NA, 30 μg), Streptomycin (S, 10 μg), Tetracycline (TE, 30 μg), Kanamycin (K, 30 μg) and Trimethoprim (TR, 5 μg) (HIMEDIA, India). From each isolate, four to five biochemically confirmed well isolated colonies grown on nutrient agar were transferred to a test tube of 5 ml Tryptone Soya Broth (TSB) (Oxoid, England). The broth culture was incubated at 37°C for 18 hrs until it achieved the 0.5 McFarland turbidity standards.

A sterile cotton swab was dipped into the suspension and then swabbed uniformly three directions over the surface of Muller Hinton agar plate (Oxiod, England). Mueller-Hinton agar plates were prepared according to the manufacturer guidelines and held at room temperature for 30 minutes to allow drying. After the plates dried, antibiotic disks were placed on the inoculated plates using sterile forceps. The antibiotic disks were gently pressed onto the agar to ensure firm contact with the agar surface and incubated at 37°C for 24 hrs. After incubation for 24 hr., the diameter of the zones of inhibition was measured and compared with zone size interpretative guideline described by clinical laboratory and standard institute (CLSI, 2018) for the family *Enterobacteriaceae* (Table 1) and determined to be sensitive, intermediate and resistant.

Table 1: Zone Diameter (mm) and Microbial inhibition concentration for *Enterobacteriaceae*

Antimicrobial	Concentration (µg/disc)	Susceptible (mm)	Resistant (mm)	Intermediate (mm)
Ampicillin (AMP)	10	≥17	≤13	14-16
Chloramphenicol (C)	30	≥18	≤12	13-17
Ciprofloxacin (CIP)	5	≥21	≤15	16-20
Gentamicin (GEN)	10	≥15	≤12	13-14
Nalidixic Acid (NA)	30	≥18	≤13	14-18
Streptomycin (S)	10	≥15	≤11	12-14
Tetracycline (TE)	30	≥15	≤11	12-14
Kanamycin (K)	30	≥18	≤13	14-17
Trimethoprim (TR)	5	≥16	≤10	11–15

3.6. Data management and analysis

All the data were entered in Microsoft Excel and screened for any entry errors. Then it was analyzed by using STATA version 12. Descriptive statistics such as frequencies and chi-square test were used to estimate the prevalence of the pathogens in both milk and feces samples and their association with different risk factors. A p-value < 0.05 was considered as indicative of a statistical significance.

3.7. Ethical considerations

Ethical clearance for the study was obtained from the Academic Commission for Research Ethics Review Committee of Addis Ababa University College Veterinary Medicine (Bishoftu) with Ref. No of VM/ERC/27/05/10/2018 (Annex 5). Informed oral consent was obtained from the animal owners (farmers) at the time of sample collection.

4. RESULTS

4.1. Overall prevalence of *E. coli* O157 and *Salmonella* isolates

The prevalence of *E. coli* O157 and *Salmonella* in milk and feces of both cattle and camel indicated in the Table 2. The prevalence of *E. coli* O157 in cattle was 4.67% (7/150) based on isolation from feces and the same prevalence (4.67%) was also isolated from milk samples of cows. *Salmonella* was isolated from 8.67% and 4.0% of cattle feces and milk respectively. The prevalence of *E. coli* O157 in camel feces were 3.29% but no *E. coli* O157 was isolated from camel milk samples.

Table 2: Prevalence of *E. coli* O157 and *Salmonella* species in milk and feces of both cattle and camel

Species	Type of samples	Number tested	<i>E. coli</i> O157		<i>Salmonella</i>	
			Positive (n)	percent	positive (n)	percent
Cows	Milk	150	7	4.67	6	4.00
	Feces	150	7	4.67	13	8.67
Camels	Milk	94	0	0	1	1.06
	Feces	94	3	3.29	2	2.13

n = number of samples

4.2. Prevalence of *E. coli* O157 and *Salmonella* associated with the risk factors

By taking prevalence of *E. coli* O157 and *Salmonella* in feces of the animals, association with different risk factors were assessed and presented in Table 3 and 4 for cattle and camel respectively. In both animal species, all the risk factors examined did not show any significant association ($p > 0.05$) for the presence of both *E. coli* O157 and *Salmonella* in feces of the two animal species.

Table 3: Prevalence of *E. coli* O157 and *salmonella* in cattle feces in relation to different risk factors

Risk factors		n tested	<i>E. coli</i> O157		<i>Salmonella</i>	
			n (%) positive	Chi-square (p-value)	n (%) positive	Chi-square (p-value)
Age (years)	<=6	57	4(7.01)	4.00 (0.14)	4(7.01)	0.90 (0.64)
	(6, 8]	74	1(1.35)		8(10.81)	
	>8	19	2(10.53)		1(5.26)	
Stage of lactation (months)	<=6	27	1(3.7)	2.48 (0.29)	3(11.11)	0.30 (0.86)
	(6, 12]	90	6(6.67)		7(7.78)	
	>12	33	0 (0)		3(9.09)	
Parity	<=3	118	5(4.24)	0.23 (0.63)	9(7.63)	0.76 (0.39)
	>3	32	2(9.38)		4(12.5)	
Body condition score	good	85	6(7.06)	2.60 (0.27)	7(8.24)	1.63 (0.44)
	medium	53	1(1.92)		6(11.32)	
	poor	12	0(0)		0(0)	
Feces consistency	Normal	76	5(6.58)	1.27 (0.26)	10(13.16)	3.93 (0.05)
	Soft	74	2(2.7)		3(4.05)	

n = number of animals

Table 4: Prevalence of *E. coli* O157 and *salmonella* in camel feces in relation to different risk factors

Risk factors	n tested	<i>E. coli</i> O157		<i>Salmonella</i>		
		N (%) positive	Chi-square (p-value)	n (%) positive	Chi-square (p-value)	
Age (years)	<=8	16	0(0)	0.64 (0.73)	1(6.25)	2.81 (0.25)
	(8, 11]	26	1(3.85)		1(3.85)	
	>11	52	2(3.85)		0(0)	
Stage of lactation (months)	<=6	35	1(2.86)	1.23 (0.54)	1(2.86)	1.62 (0.45)
	(6, 12]	38	2(5.26)		0(0)	
	>12	21	0(0)		1(4.76)	
Parity	<=3	63	2(3.18)	0.00 (0.99)	2(3.18)	0.00 (0.32)
	>3	31	1(3.23)		0(0)	
Body condition score	good	35	2(5.71)	1.29(0.53)	1(2.86)	0.33 (0.19)
	medium	47	1(2.13)		0(0)	
	poor	12	0(0)		1(8.33)	
Feces consistency	Normal	92	3(3.26)	0.07 (0.80)	2(2.17)	0.04 (0.83)
	Soft	2	0(0)		0(0)	

n = number of animals

4.3. Antimicrobial susceptibility test of the isolates

The result of antimicrobial susceptibility test of *E. coli* O157 and *Salmonella* isolates from both animal species and samples to the selected nine different antimicrobial disks is indicated in Table 5.

Table 5: Percent of level of susceptibility of bacterial isolates to different antimicrobial disks

Antimicrobial agent (Code)	Concentration of disk	Susceptibility and resistance pattern for <i>E. coli</i> O157 and <i>Salmonella</i>					
		<i>E. coli</i> O157 (n=17)			<i>Salmonella</i> (n=22)		
		S (%)	R (%)	I (%)	S (%)	R (%)	I (%)
Streptomycin (S)	10 µg	29.4	64.7	5.9	59.1	18.2	22.7
Nalidixic acid (NA)	30 µg	100	0	0	100	0	0
Kanamycin (K)	30 µg	64.7	5.9	29.4	90.9	4.5	4.5
Gentamicin (GEN)	10 µg	100	0	0	100	0	0
Ciprofloxacin (CIP)	5 µg	100	0	0	100	0	0
Chloramphenicol(C)	30 µg	100	0	0	90.9	0	9.1
Ampicillin (AMP)	10 µg	0	100	0	0	100	0
Tetracycline (TE)	30 µg	29.4	58.8	11.8	95.5	4.5	0
Trimethoprim (TR)	5 µg	76.5	4(23.5	-	95.5	4.5	0

Key: S = Susceptible R = Resistant I = Intermediate

In the current study, the bacterial isolates showed varying degree of susceptibility to the antimicrobial agents used. Accordingly, all of both *E. coli* O157 and *Salmonella* isolates were susceptible to Nalidixic acid (100%), Gentamicin (100%) and Ciprofloxacin (100%). For Chloramphenicol, 100% and 90.9% of *E. coli* O157 and *Salmonella* isolates, respectively, were susceptible. In contrast to this, the present study revealed that both *E. coli* O157 and *Salmonella* isolates were developed 100% resistance to Ampicilline (Table 5).

Multidrug resistance defined as resistance for two or more antimicrobials (Dominic *et al.*, 2005) was found in different isolates. Accordingly, 92.86% (13/14) of *E. coli* O157 isolates from cattle showed resistance to two or more antimicrobial agents while only 15.79% (3/19) of *Salmonella* isolates from

cattle showed multidrug resistance. However, none of *E. coli* O157 and 66.66% (2/3) of *Salmonella* isolates from camel developed resistance to two or more antimicrobial agents (Table 6).

Table 6: Multidrug resistance patterns of *E. coli* O157 and *Salmonella*

Sample source	<i>E. coli</i> O157 (n=14)			<i>Salmonella</i> (n=19)			
	Resistance for:	Drugs (number of isolates)	Percent	Resistance for:	Drugs (number of isolates)	Percent	
Cattle	One drug	AMP (1)	7.14	One drug	AMP (16)	84.21	
	Two drugs	AMP, TE (2)				S, AMP (2)	10.53
		S, AMP (2)	35.71	Two drugs	AMP, TE (1)	5.26	
		K, AMP (1)					
	Three drugs	S, AMP, TE (4)	28.57				
Four drugs	S, AMP, TE, TR (4)	28.57					
Camels	<i>E. coli</i> O157 (n=3)			<i>Salmonella</i> (n=3)			
	One drug	AMP (3)	100	One drug	AMP (1)	33.33	
				Two drugs	S, AMP (2)	66.66	

5. DISCUSSION

The present study as part of a milk hygiene improvement project in the study area estimated prevalence of two milk borne enteric pathogens (*E. coli* O157 and non-typhoid *Salmonella*) of public health importance. The prevalence of the two pathogens in lactating cows and camels was estimated by fecal examination and further the contamination level of raw milk at primary production level was determined. Studies which focused on the prevalence of the two foodborne pathogens in lactating animals are very limited. Most studies were carried out on animals destined for slaughter and meat production.

The prevalence of 4.67% in feces of cattle in the present study is comparable with abattoir based study in Hawassa, southern Ethiopia in male cattle feces which reported 4.7% of *E. coli* O157:H7. On the other hand, 1.89% prevalence of *E. coli* O157 was reported by Abdissa *et al.* (2017) in central Ethiopia. Compared to the present study a higher prevalence of *E. coli* O157 (10.7%) was reported by Bosilevac *et al.* (2015) in cattle feces in Riyadh, Saudi Arabia. Bosilevac *et al.* (2015) also reported 2.4% prevalence of *E. coli* O157 in camel feces which is less than the present finding of 3.29%. However, it can be difficult to make valid comparison given that most of the previous studies were carried out involving cattle for slaughter after transportation from the initial production site. The present study was carried out on animals kept under natural conditions in extensive livestock production system of Ethiopia.

In the present study the prevalence of 8.6% of *Salmonella* in feces of cows is higher than the prevalence of 7.69% reported by Abunna *et al.* (2017) in cattle feces from dairy cattle in and around Modjo town of Ethiopia. Compared to the present findings, the lower prevalence of 2.3% and 3.08% were also reported by Egualé *et al.* (2016) and Addis *et al.* (2011) in central Ethiopia respectively. The variation may be due to the difference in geographical location as well as husbandry practices of the studying animal population. The present 2.13% prevalence of *Salmonella* in camel feces is lower when compared to the previous reports of 23.2% by Bosilevac *et al.* (2015) from Riyadh and 13.9% prevalence by Mohamed and Suelam (2010) from camel feces of abattoir based study in Egypt.

Regarding the *E. coli* O157 in cattle milk, the prevalence of 4.67% was observed which is not comparable with that of 8.9% prevalence reported by Abunna *et al.* (2018) in milk of cattle in around Asella town of Ethiopia. However, it is higher than the prevalence of 2.9% and 3.5% reported by Disassa *et al.* (2017) and Bedasa *et al.* (2018) from Asosa and Bishoftu towns of Ethiopia respectively. The prevalence of 3.6% was also reported by Rahimi *et al.* (2012) in cattle milk from Iran. In the present study, *E. coli* O157 not isolated from camel milk sample which is showing agreement with the study conducted by Rahimi *et al.* (2012) from Iran.

The prevalence of 4% *Salmonella* in cattle milk of the present study not in agreement with higher prevalence of 12.1% and 20% reported by Abunna *et al.* (2017) in udder milk, and by Tadesse and Dabassa (2012) in raw milk from Kersa district of Ethiopia respectively. There is also a report of lower prevalence of 3.08% by Addis *et al.* (2011) from central Ethiopia when compared to the present study. On the other hand, the prevalence of 1.06% *Salmonella* was estimated in camel feces samples from the study area. Few studies that reported occurrence of *Salmonella* in camel from abroad were only from camel milk samples but not from camel feces. Furthermore, there is a lack of available literature about the role of camel as a potential reservoir for non-typhoid *Salmonella* in Ethiopia.

Recently, milk and feces of cattle tested for the pathogen in the Nairobi, Kenya country (Kang'ethe *et al.*, 2007) showed positive result indicating that feces may be potential source of milk contamination. The present study may support this report since considerable proportion of cattle and camels milk and feces samples were positive for the pathogens concerned. The risk is potentiated by the ability of the pathogen to survive harsh conditions, such as the low pH of dairy products (Dlamini and Buys, 2009; Tsegaye and Ashenafi, 2005) or in manure for more than four months (Kudva *et al.*, 1998).

Antimicrobial resistance emerges from the use of antimicrobials in animals and human, and the subsequent transfer of resistance genes and bacteria among animals, humans, animal products and the environment (Schroeder *et al.*, 2002). Multi Drug Resistance is defined as resistance of an isolate to two or more than two antimicrobial agents used for test (Dominic *et al.*, 2005). The current study on antimicrobial sensitivity test of *E. coli* O157 and *Salmonella* revealed a varying degree of

susceptibility to antimicrobial agents tested. The degree of susceptibility for *E. coli* O157 isolates ranges from 29.4% up to 100% and that of *Salmonella* ranges from 59.1% up to 100% respectively. All of the isolates from both cattle and camel were 100% susceptible to Nalidixic acid and Gentamicin and Ciprofloxacin which is in agreement with previous studies in Ethiopia (Abunna *et al.*, 2017; Atnafie *et al.*, 2017) and in Sudan (Fadlalla *et al.*, 2012). However, it disagrees with the study conducted in Saudi Arabia that revealed there was resistant strain to nalidixic acid, gentamycin and chloramphenicol (Naser and Wabel, 2007).

Improper use of antimicrobials in both human and veterinary medicine has contributed to development and dissemination of antimicrobial resistant pathogens (Addis *et al.*, 2011). Regarding multi drug resistance (MRD) evidence of the present study, 92.86% (13/14) of *E. coli* O157 isolates from cattle showed resistance to two or more antimicrobial agents while only 15.79% (3/19) of *Salmonella* isolates from cattle showed multidrug resistance. On the other hand, none of *E. coli* O157 and 66.66% (2/3) of *Salmonella* isolate from camel developed resistance to two or more antimicrobial agents used (Table 6). All isolates were 100% resistant to Ampicillin. This result is in line with study conducted by Atnafie *et al.* (2017) that indicated more than half of the isolates were to be resistant to ampicillin. Furthermore, Addis *et al.*, (2011) also indicated resistance of *Salmonella* isolates to commonly used antimicrobials including ampicillin, streptomycin, nitrofurantoin, kanamycin and tetracycline, with resistance rate of 100%, 66.7%, 58.3% and 33.3%, respectively.

Limitations of this study: The two pathogens were identified based on colony characteristics on selective media and further biochemical tests which may not be specific enough to exactly identify the pathogens. In additional samples for the bacteriological analysis were not conducted immediately after collection because of long distance from study site and the place where laboratory is found (Addis Ababa) and this could affect the recovery rate of the pathogens. Moreover, all the collected samples were not analyzed due to shortage of laboratory supplies and long time it took to import. Fortunately, all the bacterial isolates and the samples have been safely stored and analysis will be resumed given the overall project will run till the end of this year. There is a plan that the bacterial isolates will be confirmed through agglutination and further molecular techniques.

6. CONCLUSION AND RECOMMENDATIONS

In the current study, considerable proportions of milk and fecal samples were found contaminated with *E. coli* O157 and *Salmonella* species. The presence of these pathogens in the milk of animals indicated that it might be contaminated from either infected animal or unhygienic conditions during milking and handling at primary production level. This is particularly important in causing several health impacts in consumers who have habit of eating raw or undercooked milk and milk products. Moreover, the higher prevalence of multidrug resistant *E. coli* O157 and *Salmonella* in feces and milk of dairy animals is especially alarming which can have a significant public health risk.

Therefore, based on the present study the following recommendations are forwarded:

- Relevant intervention program and awareness creation on best practice of milk handling should be conducted to minimize contamination of milk and milk products with pathogens of public health importance
- Education on the control and surveillance program of antimicrobial usage in animals is hereby recommended to ensure consumers' safety.
- Further investigation should be conducted in order to further characterize the pathogens and identify their serotypes.

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APPENDICES

Annex 1: Sample collection and laboratory activities work sheet for laboratory analysis

Data collection					Laboratory analysis				
No	Date	Species	Sample type	Sample ID	Pre-enrichment	Enrichment and IMS	Colony Char.	Biochemical test	Antimicrobial Susceptibility Test

Annex 2: Media used for isolation and identification of *E. coli* O157 and *Salmonella*

1. Buffered peptone water (CM0509, Oxoid)

Composition	g/l
Peotone	10.0
Sodium chloride	5
Disodium phosphate	3.5
Potassium dihydrogen phosphate	1.5
Final PH	7.2 + 0.2 @ 25°C

Preparation: Add 20g to 1 litre of distilled water. Mix well and distribute into final containers. Sterilise by autoclaving at 121°C for 15 minutes. It is extremely important that the distilled water used is of a high quality with a low mineral content/conductivity.

2. 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 121oC for 15 minutes.

3. Muller-Hinton agar preparation (Oxoid, England)

Composition: Formula (g/l): Beef Extract 2, Acid Hydrolysate of Casein 17.5, Starch 1.5, and Agar 17. Final.PH 7.3 ± 0.1 at 25°C

Preparation: Suspend 38 g of the medium in one liter of purified water. Heat with frequent agitation and boil for one minute to completely dissolve the medium. Autoclave at 121°C for 15 minutes.

4. Nutrient Agar (Oxoid, ® Hampshire, England)

Ingredients	gm/liter
Peptone digests of animal tissue	5.00
Yeast extracts	1.50
Beef extract	1.50
Sodium chloride	5.00
Agar	15.00

Directions: Suspended 28g Powder in 1 liter of distilled water. Boil to dissolve completely. Sterilize by autoclaving at 121 OC for 15 minutes.

5. CHROMagar™ O157 (DRG International, Mountainside, NJ)

Composition: The product is composed of: 15.0 g/L Agar, 13.0 g/L Peptone and yeast Extract, 1.2 g/L Chromogenic mix. Storage at 15/30°C and Final media PH 6.9 ± 0.2

Preparation:

Disperse slowly 29.2g of powder base in 1L of purified water.

Stir until agar is well thickened.

Heat and bring to boil (100°C) while swirling or stirring regularly.

Do not heat to more than 100°C. Do not autoclave at 121°C.

Cool in a water bath at 45-50°C, swirling or stirring gently.

Pour into sterile Petri dishes and Let it solidify and dry

Store in the dark before use

Prepared media plates can be kept for one day at room temperature.

Plates can be stored for up to 2 weeks under refrigeration (2/8°C) if properly prepared and protected from light and dehydration.

6. Xylose lysine deoxycholate agar (XLD agar) 500 g (CM0469, Germany)

Preparation: Suspend 53 gm. in 1 liter of distilled water Heat with frequent agitation until the medium boils and the agar dissolves. Do not overheat. Adjust the pH, if necessary, so that after sterilization it is 7.4 at 25 °C. Transfer immediately to a water bath at 50 °C, agitate and pour into plates. Allow to solidify.

Composition of XLD agar	(g/Litre)
Yeast extract	3.0
L-Lysine hydrochloride	5.0
Xylose	3.75
Lactose	7.5
Sucrose	7.5
Sodium deoxycholate	1.0
Sodium chloride	5.0
Sodium thiosulphate	6.8
Ferric ammonium citrate	0.8
Phenol red	0.08
Agar	12.5
pH	7.4 ± 0.2 @ 25°C

7. Rappaport-Vassiliadis Soya Peptone Broth (RVS Broth (CM0866, Oxoid)

Composition	(g/litre)
Soya peptone	4.5
Sodium chloride	7.2
Potassium dihydrogen phosphate	1.26
Di-potassium hydrogen phosphate	0.18
Magnesium chloride (anhydrous)	13.58
Malachite green	0.036
pH	5.2 ± 0.2 @ 25°C

Preparation: Suspend 41.8 g of RPVS powder in to 1 liter of distilled water. Heat gently until completely dissolved. Dispense 10 ml volumes into screw capped bottles or tubes and sterilize by autoclaving at 115 °C for 15 minutes.

Annex 3: Media used for Biochemical test of *E. coli O157* and *Salmonella*

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

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°C.

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

2. L-Lysine Decarboxylation Medium (DIFCO, Becton, Dickson, USA)

Preparation: 5.25 g/500ml and 5g/500ml decarboxylase base moller and L-Lysine monohydrochloride respectively were dissolved together by heating if necessary and dispense 5ml in to test tubes and sterilize at 121°Cfor 10 minutes. The broth was clear and yellow to amber.

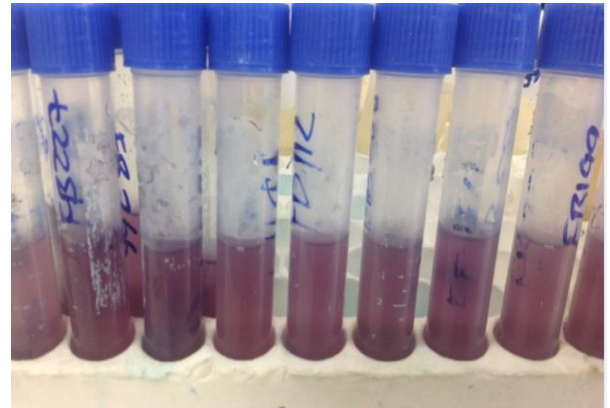
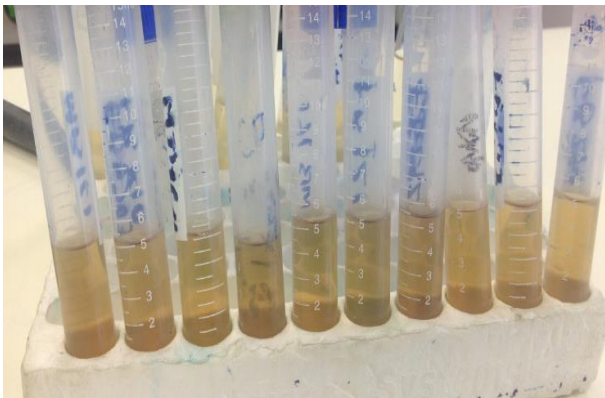
Composition (g/l): L-Lysine monohydrochloride 5.0, yeast extract 3.0, glucose 1.0, bromocresol purple 0.015.

3. Tryptone broth for Indole test

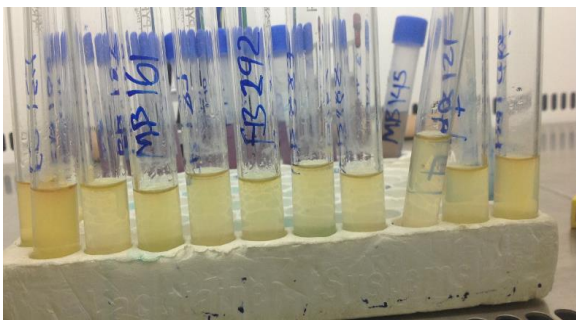
Preparation: 10g/1000ml, 5g/1000 and 3g/1000ml of tryptone, sodium chloride and DL_Tryptophan respectively were dissolved together by heating if necessary and dispense 5ml in to test tubes and sterilize at 121°C for 15 minutes. The broth was clear and yellow.

Composition (g/L): tryptone 10.0, Sodium Chloride 5.0, DL-Tryptophan 1.0.

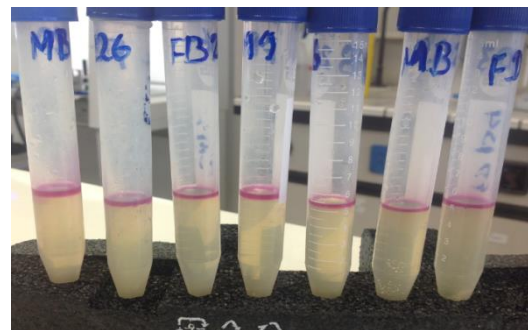
Annex 4: Some of the pictures taken during the processing (analysis)



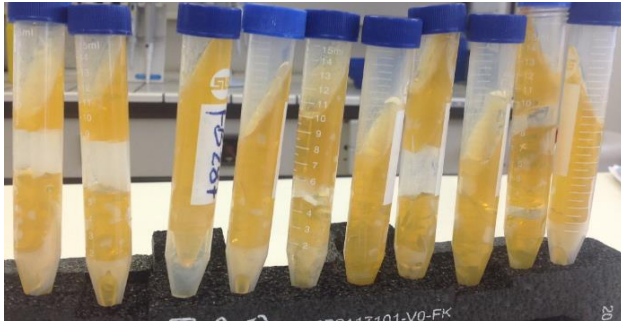
Picture 1: L-lysine decarboxylation test (uninoculated-left side, positive results for both *E. coli* and *salmonella*- on the right side)



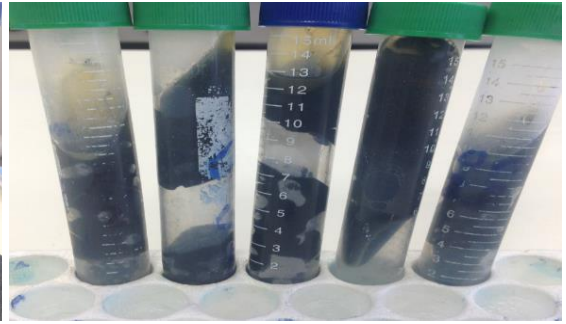
Picture 2: Negative for indole production (left)
Salmonella species



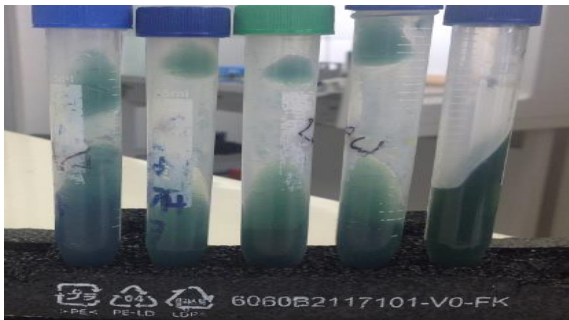
positive for indole production (right)
E. coli O 157



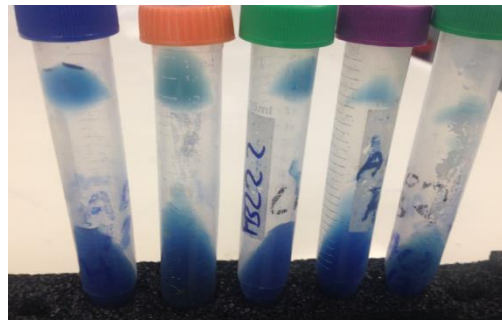
Picture 3: TSI agar test for *E. coli* O157
Gas production but not H2S



TSI agar test for *Salmonella* (Gas and H2S production)

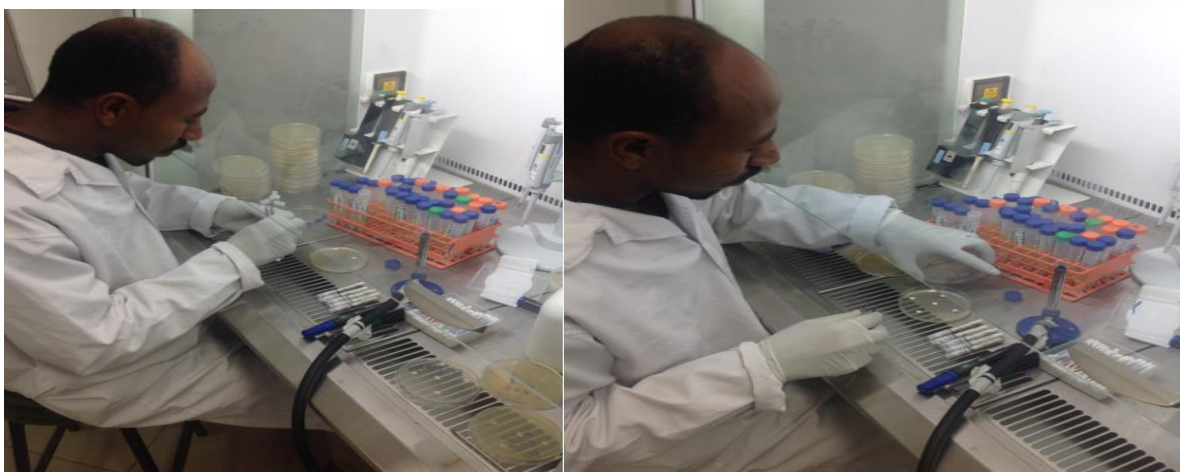


Picture 4: Simmon's Citrate test for *E. coli* O157
(Negative for citrate utilization)

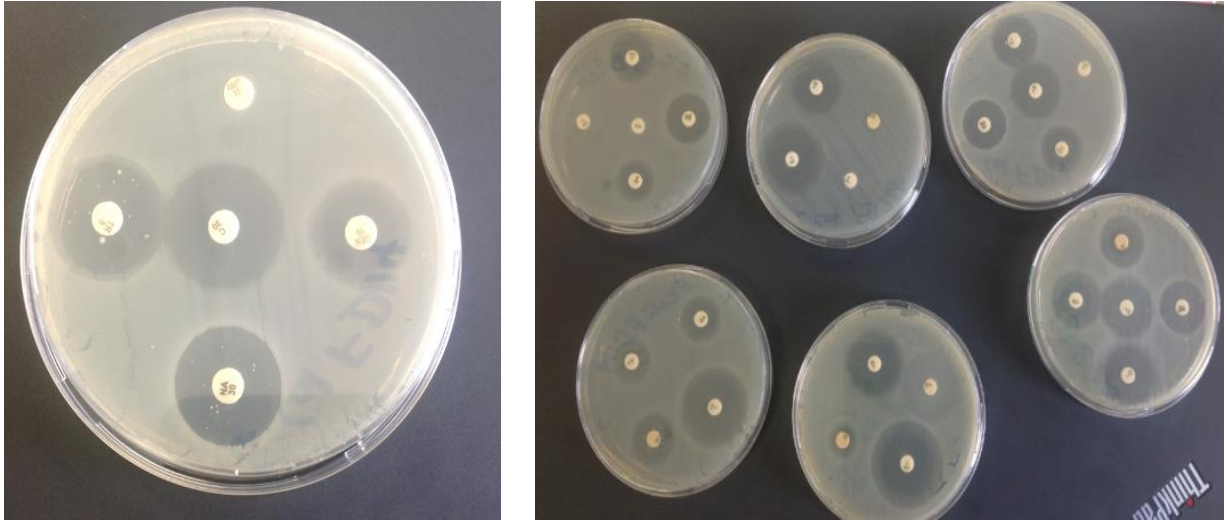


Simmon's citrate test for *Salmonella*
positive for citrate utilization

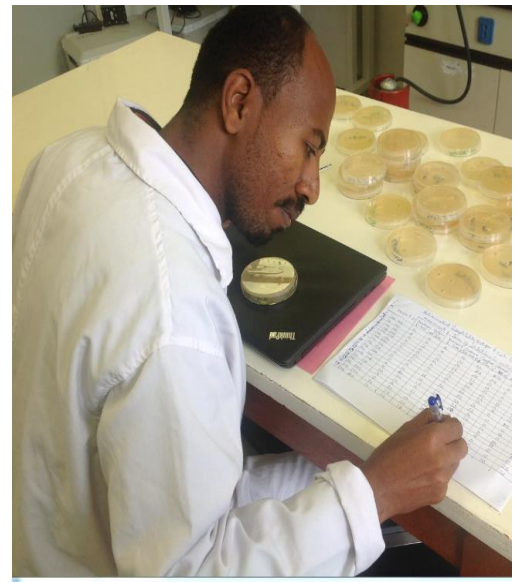
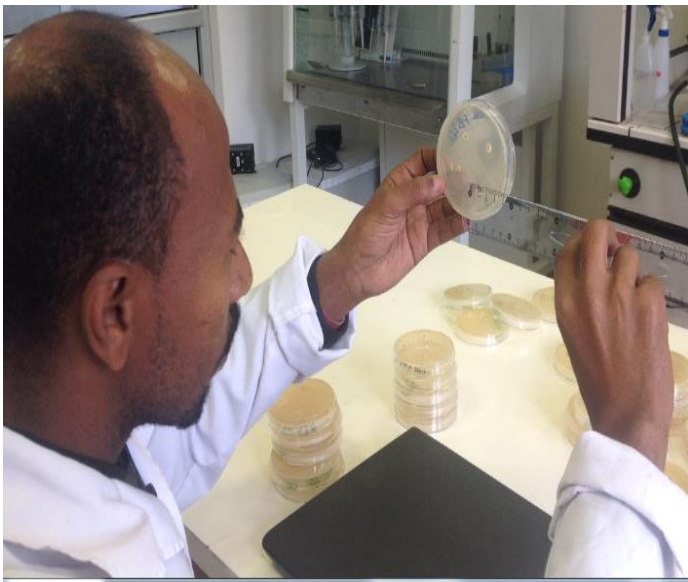
Annex 4: Some pictures showing antimicrobial susceptibility test result



Picture 1: Putting/Dispensing antimicrobial disks on the isolates cultured on Muller-Hinton agar



Picture 2: Different antimicrobial susceptibility patterns of *E. coli* 157 and *Salmonella* isolates



Picture 3: Measuring and recording the diameter of zone inhibition of the isolates

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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/27/05/10/2018

Name of Applicant: Diriba Hunduma (DVM, MSc fellow)

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

Title of the project: Prevalence of *Escherchia coli* 0157 and *Non-typhoid Salmonella species* in lactating cows and camels in Borana pastrol area, Southern Ethiopia

Date of application: 14/11/2017

Nature of the project: non-invasive

Target animal species: Cattle and Camel

Number of animals involved: 244

Study area: Borena Zone, 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



Please quote Our Ref. No. When replying

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