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ISOLATION, IDENTIFICATION, ANTIMICROBIAL PROFILE AND MOLECULAR CHARACTERIZATION OF ENTEROHAEMORRHAGIC E. COLI O157:H7 ISOLATED FROM RUMINANTS SLAUGHTERED AT DEBRE ZEIT ELFORA EXPORT ABATTOIR AND ADDIS ABABA ABATTOIRS ENTERPRISE

MSc Thesis

By

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Department of Microbiology, Immunology and Veterinary Public health

MSc program in Veterinary Microbiology

October, 2015

College of Veterinary Medicine and Agriculture, Bishoftu

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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 Veterinary Microbiology

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And recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Veterinary Microbiology

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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 M.Sc. 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.

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LIST OF ABBREVIATIONS

AE	Attaching and effacing lesions (*eae*A)
Aw	Water activity
BPW	Buffered pepton water
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
Cpds	Compounds
CO_2	Carbon dioxide
CR-SMAC	Cefixime Rhamnose Sorbitol MacConkey agar
CSA	Central statistical agency
DNA	Deoxyrobinucleic acid
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
EDEC	Oedema disease *E. coli*
EHEC	Enterohemorrhagic *Escherichia coli*
Ehly	Enterohemolysin
EIEC	Enteroinvasive *Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EMB agar	Eosin methylene blue agar
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GIT	Gastrointestinal tract
HACCP	Hazard Analysis Critical Control Point
HC	Hemorrhagic colitis
HUS	Hemolytic uremic syndrome
IMS	Immunomagnetic seperation
ISO	International standardization office

LPS	Lipopolysaccharides
LT	Heat liable toxin
m.a.s.l	meter above sea level
MAR index	Multi antimicrobial resistance index
MDR	Multi antimicrobial resistance
ml	Mill liter
mm	Mill meter
Nacl	Sodium chloride
NSF	Non-Sorbitol Fermenting
OIE	Office International des Epizooties
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RT-PCR	Real time
SLT	Shiga like toxin
SMAC	Sorbitol MacConkey agar
Spp	Species
SPSS	Statistical Package for Social Science
ST	Heat stable toxon
STEC	Shiga-toxigenic *Escherichia coli*
Stx	Shiga toxin gene
TLRs	Toll like receptors
TSB	Tryptose Soya Broth
TTP	Thrombotic thrombocytopaenic purpura
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
VT	Verocytotoxin
VTEC	Verotoxin-producing *E. coli*
WHO	World Health Organization
µg	Microgram

Isolation, identification, antimicrobial profile and molecular characterization of enterohaemorrhagic *E. coli* O157:H7 isolated from ruminants slaughtered at Debre Zeit ELFORA export abattoir and Addis Ababa Abattoirs Enterprise

ABSTRACT

A cross sectional study was conducted from December 2014 to June 2015 on apparently healthy ruminants slaughtered at Debre Zeit ELFORA export abattoir and Addis Ababa Abattoirs Enterprise in order to determine the prevalence of *E. coli* with special emphasis on *E. coli* O157:H7 and to assess its antimicrobial resistance pattern. The study was also aimed to molecularly characterize the shiga toxin producing *E. coli* strains using multiplex PCR technique. 635 carcass swab samples were collected and processed according to the standard to isolate and identify the pathogen that lead to an overall prevalence of 12.9% (*E. coli*) and 5.5 % (*E. coli* O157:H7). The prevalence of *E. coli* O157:H7 recorded had a statistical difference among animals species and age groups ($P<0.05$). However, the result showed that the prevalence of *E. coli* O157:H7 among animals' origin was not found to be statistically significant ($P<0.05$). The prevalence of *E. coli* O157:H7 was higher in the Addis Ababa abattoir enterprise than that of the privately owned abattoir. Furthermore, *E. coli* isolates were subjected to the *in vitro* antimicrobial sensitivity test and the result indicated the presence of different degrees of susceptibility and resistance to most of the antibiotics. The degree of susceptibility ranges from 14.03% up to 85.75% and resistance it is between 5.75% and 48.6%. Out of the 35 *E. coli* O157:H7 isolates, only 5 (5.7%) of them showed the presence of multidrug resistance. Analysis of multiplex PCR assay in detecting the virulence genes revealed that 3 out of 20 (15%) were positive in which only one isolate were found to contain both *Stx1* and *Stx2* genes. In conclusion, the presence of *E. coli* O157:H7 in carcasses that developed resistance can be considered as potential pathogens to the society having the habit of eating uncooked meats. Therefore, strict measures like practicing proper hygiene in abattoirs and antibiogram test to select effective drugs should be implemented.

Keywords: *Addis Ababa Abattoirs Enterprise, Antimicrobial susceptibility test, Contamination, E. coli O157:H7, ELFORA export abattoir, PCR, Prevalence, Ruminants*

1. INTRODUCTION

Food safety is one of the leading issues for the agricultural industry including livestock production sector (Barry and Richard, 2011). Some data from both developing and developed countries indicate that at least 10% of the population may experience a food borne disease (Cohen *et al*., 2006). This indicates that microbial food-borne illness still remains a global concern despite the extensive scientific progress and technological developments achieved in recent years especially in developed countries (Pal, 2012). Such problems are usually occurred because of the prevailing poor food handling and sanitation practices, inadequate food safety laws, weak regulatory system, lack of financial resources to invest in safer equipment and lack of education for food-handlers (Haileselassie *et al*., 2013). Numerous epidemiological reports have implicated food of animal origin as the major vehicles associated with illness caused by food borne pathogens, such as *Escherichia coli (E. coli)*, Salmonella, Shigella and Campylobacter for human infection and this problem is highly aggravated in the developing world (Zhao *et al*., 2001; Humphrey and Jorgensen, 2006; Pal, 2007).

Both domestic and wild animals are the sources of *E. coli* O157, but the major animal carriers are healthy domesticated ruminants, primarily cattle and, to lesser extent, sheep, and possibly goat (Sima *et al*., 2009; Kiranmayi *et al*., 2010; Rahimi *et al*., 2012). Sporadic cases and outbreaks of human diseases caused by *E. coli* O157 have been linked to ground beef, raw milk, meat and dairy products, vegetables, unpasteurized fruit juices and water (Sima *et al*., 2009).

According to Pal (2007), *E. coli* O157:H7 is one of the most significant food-borne pathogens that has gained increased attention in recent years. This bacterium is often termed as an enterohaemorrhagic *E. coli* (EHEC) and causes food borne illness. Typical illness as a result of an *E. coli* O157:H7 infection can be life threatening, and susceptible individuals show a range of symptoms including haemolytic colitis, hemolytic uremic syndrome, and thrombocytopenia purpura (Sima *et al*., 2000; Chileshe and Ateba, 2013). It has been recognized that some diarrheagenic strains of *E. coli* produce toxins that have

an irreversible cytopathic effect on cultured Vero cells. Such verocytotoxigenic *E. coli* (VTEC) have been shown to belong to over 100 different serotypes (Johnson *et al*., 1996; Paton and Paton, 1998). They are also described as Shiga toxin-producing *E. coli* (STEC) due to the similarity demonstrated between verocytotoxins (VT) and Shiga toxins (Stx) of *Shigella dysenteriae. E. coli* O157:H7 is the predominant and most virulent serotype in a pathogenic subset of VTEC, designated EHEC. Other non-O157 serotypes, including O26:H11, O104:H21, O111:H and O145:H has been associated with occasional outbreaks of human disease (O'Brien and Laveck, 1983).

The major source of carcass contamination is contact with the skin during hide removal or contamination by spillage of stomach contents during evisceration (Humphrey and Jorgensen, 2006; Mersha *et al*., 2009). Moreover, during hide stripping, some bacteria originating from the animal hide become suspended in the abattoir atmosphere. This contaminated air may come into contact with food products including carcasses, containers, equipment and other food contact surfaces during processing, where they may adhere strongly (Sutton, 2004).

Possible contamination of edible carcass tissue is the most significant challenge to food safety, and the extent and nature of such contamination are related to the *E. coli* O157:H7 status of the pre slaughter animal, and any processes which distribute the organism within or between carcasses during dressing operations (McEvoy *et al*., 2003).

Antimicrobial resistance has emerged in the past years as a major global problem and many programs have been set up for its surveillance in human and veterinary medicine. (Lanz *et al*., 2003). However, little attention has been paid to the resistance in specific animal pathogens (Lanz *et al*., 2003; Taluo *et al*., 2008). Use of antimicrobial agents in agriculture, over prescribing by physicians, and misuse by patients have been identified as the three main causes of antimicrobial resistance (Doughari, 2012). Some antimicrobia ls, such as quinolons, trimethoprim, and furozolidone, were shown to induce toxin gene expression and should be avoided in treating patients with potential confirmed EHEC

infections (Demissie, 2005). It is believed that this situation is further complicated by the potential of drug resistant bacteria to transfer their resistance determinants to resident constituents of the human microflora and other pathogenic bacteria (Olatoye *et al*., 2012). Some studies have been conducted in Ethiopia in order to determine the proportion, and antimicrobial susceptibility of *E. coli* O157:H7 in faeces, skin swabs and carcasses swabs of sheep, goat and cattle in different cities of the country including Addis Ababa 10.2% (Bekele *et al*., 2014), Debre Zeit and Modjo 4.2% (Hiko *et al*., 2008), Dire Dawa 15.89% (Mohammed *et al*., 2014), and Mekelle 5% (Balcha *et al*., 2014). A report by Demissie (2005) indicated that *E. coli* O157:H7 from human stool and minced beef samples with the rate 5.7% and 4.9% respectively in Debre Zeit and Addis Ababa Ethiopia. Hiko *et al.* (2008) isolated *E. coli* O157:H7 from beef, sheep meat and goat meat with the prevalence rate, 8%, 2.5%, and 2% respectively. However, there is only one previously conducted research to detect the various shiga toxin producing *E. coli* strains.

Despite the widely presence of the problem, there is still lack of compressive survey of such food borne pathogens at abattoirs having different standards (variation in hygienic practices during pre and post slaughter of animals, infrastructures used for slaughtering activities and knowledge and skills of the persons) by considering the different susceptible animal species using various bacteriological and molecular techniques.

General objective

- ✓ To assess the level of carcass contamination with different strains of Enterohaemorrhagic *E. coli* O157:H7 in ruminants slaughtered at two abattoirs with contrasting standards (Export vs. Municipal)

Specific objectives

- ✓ To find out the prevalence of ruminants' carcass contamination with different strains of *E. coli* in apparently healthy ruminants slaughtered at both abattoirs
- ✓ To isolate and identify *E. coli* O157:H7 from ruminants' carcass
- ✓ To determine the antimicrobial susceptibility pattern of *E. coli* isolates
- ✓ To detect shiga toxins 1 and 2 producing *E. coli* O157:H7 strains using multiplex PCR

2. LITERATURE REVIEW

2.1. General overview of *E. coli* as a pathogen

E. coli are Gram-negative, facultative anaerobic, rod-shaped and highly motile bacteria. They are often classified under enterobacteriacea known to be normal inhabitants of the gastrointestinal tract of both animals and human beings but only some strains of *E. coli* have become highly adapted to cause diarrhoea and a range of extra-intestinal diseases (Kubitschek, 1990). *E. coli* were first isolated by a German paediatrician, Theodore Esherich, in 1884 from faeces of human neonates (Khan and Steiner, 2002). The first confirmed isolation of *E. coli* O157:H7 in the United States of America was in 1975 from a Californian woman with bloody diarrhoea, while the first reported isolation of the pathogen from cattle was in Argentina in 1977 (Fernandez, 2008). It was since 1982 that EHEC have been recognized as an important aetiological agent of diarrhoeal diseases in man and animals. *E coli* O157 was described as a rare serotype (Karmali *et al*., 1983). Studies conducted between 1983 and 1985 in the United States and Canada, have linked EHEC infection to haemorrhagic colitis (HC) and it had a close relation with the classical form of haemolytic uraemic syndrome (HUS) (Karmali *et al*., 1985). As a result of these and other studies, Orskov *et al.* (1987) re-examined isolates of *E coli* belonging to the O157 serogroup that had been submitted to the International Escherichia and Klebsiella Centre. Three isolates were found that had the H7 antigen (Orskov *et al*., 1987). These three isolates were from the faeces of one animal out of a batch of 39 calves with colibacillosis in Argentina. Orskov and colleagues (1987) also speculated that cattle might be the reservoir for these organisms.

2.2. Taxonomic classification *E. coli*

The comparative analysis of 5S and 16S ribosomal RNA sequences suggest that *Escherichia* and *Salmonella* diverged from a common ancestor between 120 and 160

million years ago, which coincides with the origin of mammals. *Escherichia* and *Shigella* have been historically separated into different genera within the *Enterobacteriaceae* family. DNA sequence analysis of their genomes reveals a high degree of sequence similarity and suggests that they should be considered a single species (Ochman & Wilson, 1987). There are several types of *E. coli* strains but these strain types can be divided into six groups or pathotypes based on the mechanism they cause disease: Enteropathogenic *E. coli* (EPEC), Attaching and effacing *E. coli* (A/EEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), EHEC and Enteroaggregative *E. coli* (EAEC). *E. coli* strains that produce the Stx toxins have been referred to as Vero Toxin-producing *E. coli* (VTEC), shiga-toxigenic *E. coli* (STEC) and enterohaemorrhagic *E. coli* (EHEC) (Karmali, 1989; Nataro & Kaper 1998).

Scientific classification of *E. coli* is under:

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Family: Enterobacteriaceae

Genus: *Escherichia*

Species: *E.coli*

Source: Nataro & Kaper (1998)

EPEC organisms are a significant cause of infant diarrhea in developing nations. Enteropathogenic *E. coli* (EPEC) were historically recognized on the basis of serotypes such as O55:H6 and O127:H6. EPEC are an established etiological agent of human infantile diarrhea. In developing countries, Enteropathogenic *E. coli* (EPEC) is one of the most common pathogens (Fagundes & Scaletsky, 2000).

EIEC organisms often cause a broad spectrum of human's diseases. They are biochemically, genetically and pathogenetically closely related to *Shigella* species. Both characteristically cause an invasive inflammatory colitis, but either may also elicit a

watery diarrhea syndrome indistinguishable from that caused by other *E. coli* pathogens. (Nataro & Kaper, 1998).

ETEC strains are a major cause of secretory diarrhea in both humans and animals (Bern *et al*., 1992). ETEC produce toxins which are heat-labile (LT) and/or heat-stable (STa and STb) that are also causing diarrhea. It is known to be a frequent cause of diarrhea in both humans and animals.

EAEC strains are defined by their distinctive adherence pattern on HEp-2 cells in culture (Nataro & Kaper, 1998). The essential element of the aggregative phenotype is the stacked brick pattern by lying side-by-side with an appreciable distinction of where one bacterium begins and another ends. (Nataro & Kaper, 1998).

Enterohaemorrhagic *E. coli* (EHEC) strains are implicated in food-borne diseases principally due to ingestion of uncooked minced meat and raw milk. These strains produce shiga like toxin 1 (*Stx1*), shiga-like toxin 2 (*Stx2*). Serotype EHEC O157:H7 is the prototype of increasing importance and is associated with hemorrhagic colitis, bloody diarrhea and the hemolytic uremic syndrome (Pickering *et al*., 1994; Cornick *et al*., 2000).

2. 3. Cell structure and physiology of *E. coli*

E. coli is the head of the large bacterial family, *Enterobacteriaceae*, the enteric bacteria, which are facultative anaerobic and nonperforming bacilli having about 2μm long and 0.5μm in diameter with a cell volume of 0.6 to 0.7μm3 (Kubitschek, 1990). They are approximately 0.5 μm in diameter and 1.0–3.0 μm in length. Within the periplasm is a single layer of peptidoglycan. The peptidoglycan has a typical subunit structure where the *N*-acetylmuramic acid is linked by an amide bond to a peptide consisting of L-alanine, D-glutamic acid, *meso*-diaminopimelic acid and D-alanine. *E. coli* are commonly motile in liquid by means of peritrichous flagella. *E. coli* are commonly fimbriated. The type 1 pili

are the most common and are expressed in a phase switch on or off manner that leads to piliated and nonpiliated states (Eisenstein, 1987). One of the traits commonly encoded on the larger genetic islands of the different pathotypes of *E. coli* are additional pili (chaperone-usher and type IV pili families and non-pili adhesions (Schreiber & Donnenberg, 2002).

Among *E. coli* isolates, there is considerable variation and many combinations of somatic (O and K) and flagellar (H) antigens. *E. coli* are routinely characterized by serological identification of somatic O, flagellar H and capsular K antigens. However, while some serotypes correlate closely with certain clinical syndromes, differentiation of pathogenic strains from the normal flora depends on the identification of virulence characteristics. Among pathogenic strains, there are few patterns of these antigens and few phylogenetic groupings. For *E. coli*, there are over 150 antigenically unique O-antigens (Whitfield & Valvano, 1993). K type capsular material occurs in two or four forms on the basis of physical, biochemical and genetic criteria (Whitfield & Roberts, 1999).

Over 80 serologically and chemically distinct capsular polysaccharides have been recognized. In addition, a slime layer, colonic acid extracellular polysaccharide, is common to many *E. coli* isolates and can be co-expressed with some K-type capsules. There are 53 H-antigen specificities among *E. coli* (Schreiber & Donnenberg, 2002).

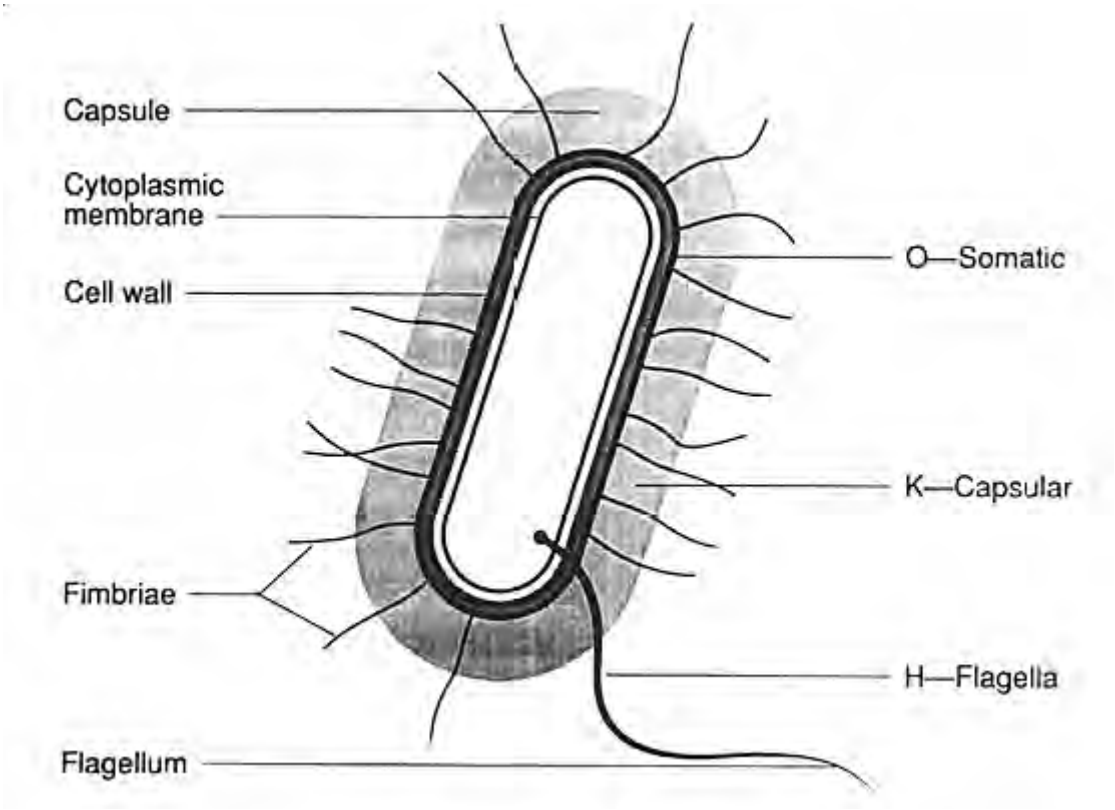

Figure1: schematic presentation of *E. coli* showing its morphology (cell surface antigens)

Source: (Hirsh and Zee, 1999)

2.4. Growth characteristic of *E. coli*

E. coli is a facultative anaerobe. Though most *E. coli* strains are capable of growing over a wide range in temperature (approximately 15–48°C), the growth rate is maximal in the narrow range of 37–42°C. *Escherichia coli* can grow within a pH range of approximately 5.5–8.0 with best growth occurring at neutrality. Some diarrheagenic *E. coli* strains have the ability to tolerate exposure to PH 2.0. Such an acid shock mimics transit through the stomach and induces expression of sets of genes involved in survival and pathogenesis (Fotadar *et al.,* 2005). It is capable of reducing nitrates to nitrites. When growing fermentatively on glucose or other carbohydrates, it produces acid and gas (mainly H_2

and CO_2). By traditional clinical laboratory biochemical tests, *E. coli* is positive for indole production and the methyl red test. Most strains are oxidase, citrate, urease and hydrogen sulfide negative. The classic differential test to primarily separate *E. coli* from *Shigella* and *Salmonella* is the ability of *E. coli* to ferment lactose, which the latter two genera fail to do (Fotadar *et al.*, 2005).

Aside from lactose, most *E. coli* strains can also ferment D-mannitol, D-sorbitol, and Larabinose, maltose, D-xylose, trehalose and Dmannose. There are limited instances where pathogenic strains differ from the commensals in their metabolic abilities. For example, commensal *E. coli* strains generally use sorbitol, but *E. coli* O157:H7 does not. Most diarrheagenic strains cannot utilize D-serine as a carbon and nitrogen source, but uropathogenic and commensal fecal strains can use this enantiomer of serine (Roesch *et al.*, 2003).

2.5. Geographical distribution, Reservoir hosts and ways of transmission of *E. coli* O157:H7

EHEC 0157:H7 infections occur worldwide; infections have been reported on every continent except Antarctica. Other EHEC are probably also widely distributed. Species affected are Ruminants, especially cattle and sheep, are the major reservoirs for EHEC 0157:H7 (Griffin, 1995). This organism can sometimes be found in other mammals including pigs, rabbits, horses, dogs, domestic and other wild birds. In some instances, it is not known whether a species normally serves as a reservoir host or if it is only a temporary carrier. For example, rabbits shedding EHEC O157:H7 have caused outbreaks in humans, but most infected rabbits have been found near farms with infected cattle (Alam and Zurek, 2006).

EHEC O157:H7 are transmitted by the fecal–oral route. They can be spread between animals by direct contact or via water troughs, shared feed, contaminated pastures or other environmental sources. Birds and flies are potential vectors. The organism was

thought to have become aerosolized during high pressure washing of pens, but normal feeding and rooting behavior may have also contributed (Griffin and Tauxe, 1991 Leomil, 2005).

The reservoir hosts and epidemiology may vary with the organism. Ruminants, particularly cattle and sheep, are the most important reservoir hosts for EHEC O157:H7. A small proportion of the cattle in a herd can be responsible for shedding more than 95% of the organisms. These animals, which are called super-shedders, are colonized at the terminal rectum, and can remain infected much longer than other cattle. Super-shedders might also occur among sheep. Animals that are not normal reservoir hosts for EHEC O157:H7 may serve as secondary reservoirs after contact with ruminants. EHEC O157:H7 is mainly transmitted to humans by the consumption of contaminated food and water, or by contact with animals, feces and contaminated soil. Person-to-person transmission can contribute to disease spread during outbreaks; however, humans do not appear to be a maintenance host for this organism (Lee, 2008).

Most food borne outbreaks with *E. coli* O157:H7 are often caused by direct or indirect contact with cattle, drinking (unpasteurized milk), eating uncooked beef, mutton and other animal products. Irrigation water contaminated with feces is an important source of EHEC O157:H7 on vegetables. This organism can attach to plants, and survives well on the surface of a variety of fruits, vegetables and fresh culinary herbs. EHEC O157:H7 can remain viable for long periods in many food products. It can survive for at least nine months in ground beef stored at -20°C (-4°F). It is tolerant of acidity, and remains infectious for weeks to months in acidic foods such as mayonnaise, sausage, apple cider and cheddar at refrigeration temperatures (Leomil, (2005); Nataro and Kaper, (1998)).

According to the WHO Consultation report on the Prevention and Control of Enterohaemorrhagic *E. coli* Infections (1997), there have been three cases of *E coli* O157 identified in Pretoria since 1988. The first case of *E coli* O157:H7 in South Africa associated with hemorrhagic colitis was reported by Browning *et al.* (1990).

2.6. The epidemiological status of *E. coli* O157:H7 infections in Ethiopia

In Ethiopia, there were studies conducted by some researchers to determine the occurrence and proportion of *E. coli* O157:H7 in faeces, skin swabs and carcasses of sheep, goat and cattle in different areas of the country. Based on a retrospective review of culture results of clinical sources (Dessie Regional Health Research) including urine, ear discharge, pus swab from wounds, and eye discharge, 14.2 % *E. coli* O157:H7 was isolated from 446 samples (Kibret and Abera, 2011).

One research conducted in Addis Ababa municipal abattoir showed that the prevalence of *E. coli* O157:H7 as 10.2% (13.3% Beef followed by 9.4% mutton and 7.8% goat meat) (Tizita *et al*., 2014). Similar findings revealed that the prevalence of *E. coli* O157:H7 as a carcass contaminant in sheep of goat and cattle slaughtered at Debre Zeit municipal abattoir and Modjo export abattoir (Hiko *et al*., 2008; Mersha *et al*., 2009).

According to the research conducted in the eastern part of Ethiopia (Dire Dawa and Haramaya), prevalence of *E. coli* O157:H7 as a contaminant of meats of ruminants was recorded from 2.55% up to 30.97% (Taye *et al*., 2013; Dulo, 2014 and Mohammed *et al* 2013). Balcha *et al.* (2014) also reported that prevalence of 62.5% *E. coli* from beef samples collected from Mekele Municipal abattoir and meat retailers.

2.7. Disease causing mechanisms and clinical manifestations

The pathogenicity of *E coli* O157:H7 is a multistep process involving different complex interactions between bacteria and host. The organisms have to survive the acid environment of the stomach to colonize the intestines Colonization of the colon and distal small intestine resulted in the formation of the typical attachment-effacement lesions accompanied by bloody diarrhoea (Nataro and Kaper, 1998). The events of pathogenesis

can be summarized as follows: i) the colonization of the gut, ii) the effect of the virulence factors on the host and iii) disease caused by the virulence factors

Some kinds of *E. coli* cause disease by making a toxin called Shiga toxin. The bacteria that make these toxins are called “Shiga toxin-producing” *E. coli* or STEC for short. One of the major virulence factors attributed to EHEC disease pathogenesis is phage-encoded Shiga toxins. The cytotoxic effects of these toxins were first documented in Vero cells (African green monkey kidney epithelial cells) in 1977 (Konowalchuk *et al*., 1977) and later confirmed in 1983 from a patient with an EHEC O157:H7 infection and HUS. Subsequent studies indicated that multiple *E coli* serotypes elicit the same cytotoxic effects (Karmali *et al*., 1985) and further studies showed that EHEC contains two closely related, but genetically distinct, Shiga toxins encoded by bacteriophages (*Stx*1 and Stx2) (Obrig, 2010). The strain of Shiga toxin-producing *E. coli* O104:H4 that caused a large outbreak in Europe in 2011 was frequently referred to as EHEC. The most commonly identified STEC in North America is *E. coli* O157:H7 (often shortened to *E. coli* O157 or even just “O157”). When you hear news reports about outbreaks of “*E. coli*” infections, they are usually talking about *E. coli* O157 (Karmali, 1989).

In addition to *E. coli* O157, many other kinds (called serogroups) of STEC cause disease. Other *E. coli* serogroups in the STEC group, including *E. coli* O145, are sometimes called "non-O157 STECs." Currently, there are limited public health surveillance data on the occurrence of non-O157 STECs, including STEC O145; many STEC O145 infections may go undiagnosed or unreported (Nataro and Kaper, 1998).

EHEC, especially *E coli* O157:H7 is responsible for a variety of clinical manifestations that vary from asymptomatic infection to mild uncomplicated non-bloody diarrhoea or gross bloody diarrhoea (Nataro and Kaper, 1998). The clinical syndrome which causes bloody diarrhoea is better known as haemorrhagic colitis and is characteristic of EHEC infections The incubation period of EHEC diarrhoea is between 3 and 4 days but could be as long as 5 to 8 days or as short as 1 to 2 days in exceptional cases (Nataro and Kaper, 1998).

Systemic complications of *E coli* O157:H7 include syndromes such as haemolytic Uraemic syndrome (HUS) and thrombotic thrombocytopaenic purpura (TTP) (Nataro and Kaper, 1998). The onset of EHEC infection is firstly characterized by abdominal cramps with little or no diarrhoea, progressing to watery diarrhoea (a frequent early manifestation) and subsequently bloody diarrhoea (Karmali, 1989). Complications such as HUS and TTP are associated with children and the elderly infected with *E coli* O157:H7 (Karmali, 1989).

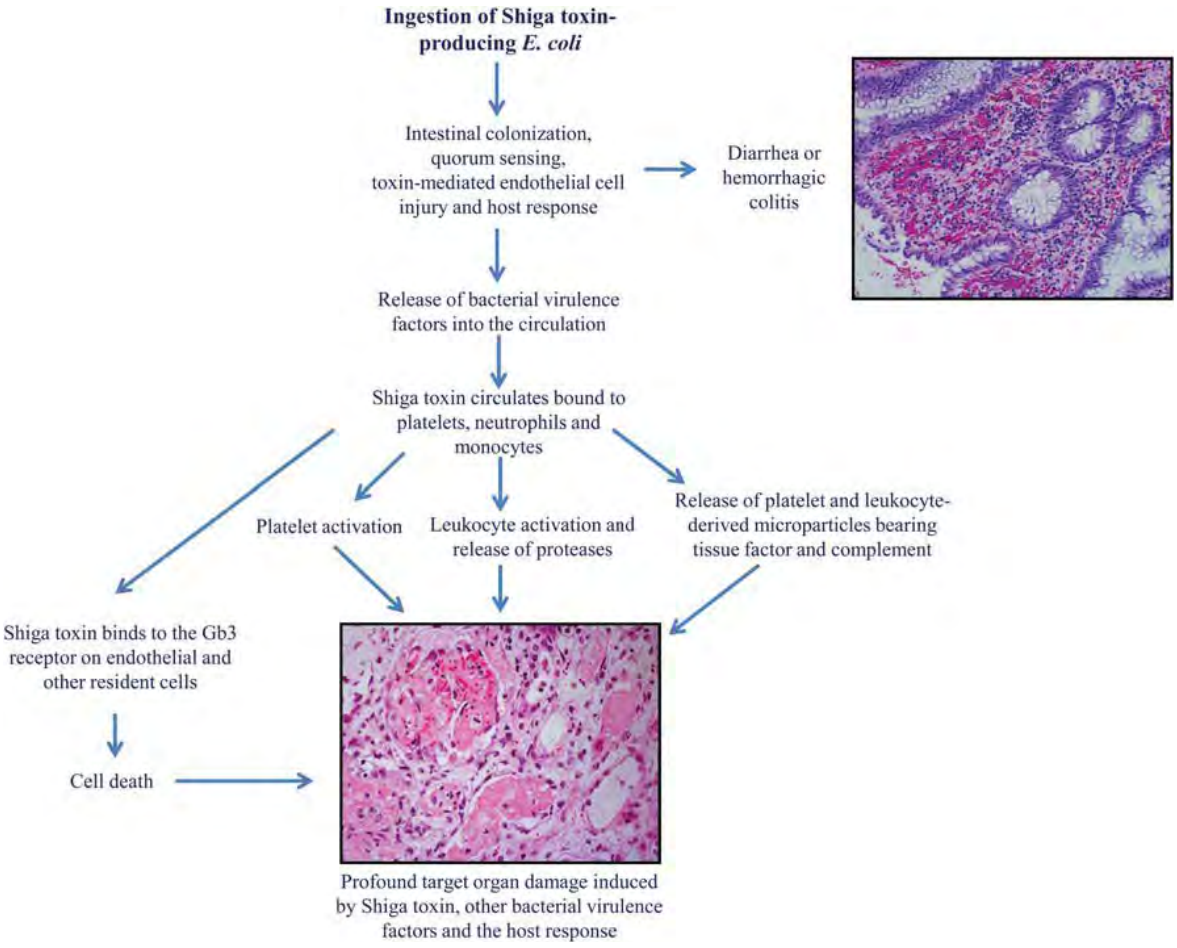

Figure 2: Schematic presentation of the pathogenesis of Shiga toxin-induced disease.

Source: Karpman, 2012

2.8. Host immune response against *E. coli* O15:H7 infection

One of the first lines of host defense against EHEC infection is through the activation of the innate immune system, using pattern recognition receptors to detect pathogen-associated molecular patterns expressed by microbes to elicit a protective antimicrobial immune response (Jones and Neish, 2011).

Of the many host pattern recognition receptors, Toll-like receptors (TLRs) and Nod-like receptors (NLRs) are the principal pathogen recognition receptors involved in recognizing and responding to microbes, including bacterial antigens. TLRs are a family of membrane-bound receptors that recognize specific microbial components such as Gramnegative bacterial lipopolysaccharide (TLR 4) and flagellin (TLR 5) (Trinchieri and Sher, 2007). In contrast, NLRs detect other microbial products, such as peptidoglycan by Nod-2, in the cytoplasm of the host cell. The activation of TLRs and NLRs leads to a general activation of the immune system, resulting in the release of immune mediators and immune cell activation that collectively serves to contain and clear the bacterial infection (Saleh, 2011).

An important signalling cascade that is activated to clear a microbial pathogen is the interferon-gamma (IFNγ) signal transduction pathway). Proinflammatory cytokines, including IFNγ, are secreted into the extracellular environment by macrophages, natural killer T cells, and activated T cells following EHEC O157:H7 infection resulting in the activation of up to 2000 IFNγ-stimulated genes in recipient host cells that together mount the host defense against pathogenic microbes (Zhang *et al*., 2008).

2.9. Diagnostic Tests

2.9.1. Culture and Isolation of Shiga Toxin-Producing E. coli

There are no biochemical characteristics that distinguish the majority of VTEC from other *E. coli,* however, the inability of most strains of *E. coli* O157:H7 to ferment D-sorbitol rapidly and their lack of betaglucuronidase activity can be exploited in the isolation and identification of these organisms. Several methods have been developed for the isolation of *E coli* O157:H7 from food and clinical samples based on the knowledge that sorbitol-fermenting enteric bacteria produce β-glucuronidase (Doyle et al., 1987). A variety of selective and differential plating media have been developed for the isolation of *E coli* O157:H7. The selective medium which is commonly used for experimental and routine screening of samples for *E coli* O157:H7 is Sorbitol MacConkey (SMAC) agar. MacConkey agar containing 1% D-sorbitol instead of lactose is a useful and inexpensive medium on which non-sorbitol-fermenting *E. coli* grow as small, round greyishwhite colonies. Selectivity is improved by the addition of 0.5% rhamnose, and addition of 0.05 mg/litre cefixime (CR-SMAC) inhibits overgrowth by *Proteus* spp. While fewer presumptive colonies require testing on this medium, rhamnose is an expensive supplement.

This medium is used for a number of reasons: i) *E coli* O157:H7 does not ferment sorbitol, and for this reason it is distinguishable as colourless colonies from other *E coli* organisms that do ferment sorbitol ii) it is inexpensive when compared to other *E coli* O157 selective media, and is thus suitable when screening large numbers of samples. However, as many as 20% of *E coli* strains may be sorbitol-negative and other species of enteric bacteria can grow as colourless colonies on SMAC (Biolog website, 1999). SMAC is not useful in screening for other EHEC strains.

CHROM agar O157 is another 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 seperation (IMS) with Dynabeads anti-*E coli* O157. The vast majorities of other bacterial species are inhibited or give blue or colourless colonies (CHROM agar website, 2000)

2.9.2. Immunological assays

Immunological methods are widely used for the detection of VTEC. The methods utilize specific poly- or monoclonal antibodies targeting surface antigens and thus detect specific VTEC sero groups while others detect the toxins produced by VTEC. This shows that specific immunoassays used in detecting *E coli* O157:H7 are directed at the O157 lipo polysacharide (LPS) and H7 flagellar antigens of *E coli* O157:H7 (Nataro and Kaper, 1998). Suspect colonies are directly screened for the O157 antigen from the selective media but the H7 antigen requires passage through motility medium (Nataro and Kaper, 1998). Enzyme-linked immunosorbent assay (ELISA) methods offered significant advantages over tissue culture-based methods for the detection of shiga toxins in patients with suspected Stx infection (Karmali, 1989).

The merits of using immunological techniques include a shorter identification time than using DNA hybridization methods (Karmali, 1989). ELISA kits available to detect *E coli* O157:H7 antigen directly from stool samples are rapid, accurate and easy The ELISA detects the presence of shiga toxin-producing *E coli* (STEC) (or other Stx-producing bacteria) regardless of the sero group.

This method could also be used to detect *Stx*-producing organisms where cell culture facilities are not available. ELISAs are less sensitive than the vero cytotoxicity assay (Downes *et al*., 1989). A number of commercial diagnostic kits are currently available on the market. These include kits for latex agglutination, ELISA and gold-labelled

antibodies. Most of these kits seem to produce similar *Stx*-detection results when compared (Sowers *et al*., 1996).

2.9.3. Molecular Detection of Shiga Toxin-Producing E. coli

The World Health Organization (WHO) has called the rapid identification of virulent O157 STEC a public health priority (WHO, 1998). Recommendations from the CDC suggest that future STEC methods should include an assessment of the potential of the organisms to cause severe disease, possibly by detecting virulence factor genes. Various polymerase chain reaction (PCR) techniques are utilized for detection of virulent toxins and other virulence markers. Currently, there are a range of molecular techniques such as conventional PCR, multiplex PCR as well as Real time PCR (RT-PCR) (Nataro and Kaper, 1998).

PCR can be used on pure or mixed plate or broth cultures, and extracts from food or feces. It can also be used to detect genes in non-viable organisms. As well as its role in diagnosis, PCR has the potential to be used to screen samples for VTEC in epidemiological studies (Nataro and Kaper, 1998)

Amplification of target genes in bacterial DNA extracts from feces is less successful than from pure cultures, and careful preparation of the sample is required to improve sensitivity. Feces contain nonspecific PCR inhibitors and no single method of removing these is ideal. Sensitivity is improved by nonselective enrichment prior to testing, but remains lower than using IMS or the Vero cell cytotoxicity assay. Commercial assays are available (Paton and Paton 1998).

PCR is especially advantageous when dealing with food samples, where a relatively low number of cells are present. The occurrence of real time PCR makes it possible for simultaneous quantification and detection of *E. coli*. Recently, great efforts have been

taken to design methods which can examine a strain for all known *E. coli* virulence genes to assess its virulence potential (Billips *et al*., 2007).

2.10. Control and prevention of *E. coli* O157:H7 infections

Diarrhea caused by various diarrheagenic *E. coli* normally are self-limiting and do not require antibiotic treatment. For infants and children who are at high risk for dehydration, fluid replacement would be given in the case of fluid loss caused by ETEC, EPEC and EAEC (King *et al*., 2003). Although antibiotics have been very useful in treating *E. coli* infections in the past, the increase in antibiotic resistance in *E. coli* make treatment of *E. coli* infections complicated. For example, the emergence of fluoroquinolones resistant and extended-specturm beta-lactamse producing strains have caused great concern due to limited therapeutic options for infections with those *E. coli* strains (Fadda *et al* ., 2005).

Frequent hand washing, especially before eating or preparing food, and good hygiene are important in preventing transmission from animals and their environment. Hand washing facilities should be available in petting zoos and other areas where the public may contact livestock, and eating and drinking should be discouraged at these sites. To protect children and other household members, people who work with animals should keep their work clothing, including shoes, away from the main living areas and launder these items separately. Two children apparently became infected with EHEC O157:H7 after contact with bird (rook) feces, possibly via their father's soiled work shoes or contaminated overalls. After a number of outbreaks associated with camping in the U.K., the Scottish *E. coli* O157 Task Force has recommended that ruminants not be grazed on land for at least three weeks before camping begins (Werber *et al.*, 2008).

Contamination of public water supplies is prevented by standard water treatment procedures. Livestock-should be kept away from private water supplies. Microbiological testing can also be considered. To the extent possible, people should avoid swallowing

water when swimming or playing in lakes, ponds and streams (Scheiring *et al*., 2008). Good hygiene, careful hand-washing and proper disposal of infectious feces can reduce person-to-person transmission. Thorough hand washing is especially important after changing diapers, after using the toilet, and before eating or preparing food. Bed linens, towels and soiled clothing from patients with hemorrhagic colitis should be washed separately, and toilet seats and flush handles should be cleaned appropriately (Seto *et al*., 2007).

Vaccines to control zoonotic VTEC are not available so far. Various approaches to the immunological control of EHEC infections in humans are being explored (Levine, 1998). These are aimed at preventing colonisation, intestinal disease or the serious sequelae of haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura. They include the use of conjugate vaccines (e.g. O157 polysaccharide linked to the B-subunit of VT1 and VT2 as carrier proteins), live-vector vaccines, toxoid vaccine or passive immunisation with hyperimmune globulin or monoclonal antibodies against VT.

However, were an effective vaccine to become available, there is debate about the social, political and economic consequences of widespread vaccination of people against pathogens in their food (OIE, 2008). As animals, mainly cattle, are thought to be the reservoirs of infection for the human population, a novel strategy being explored is to vaccinate cattle in order to reduce colonisation with pathogenic VTEC and thereby reduce contamination of food and the environment (i.e. to make food safer as opposed to protecting people against their food (OIE, 2008). One approach is to use a live, toxin-negative colonising strain as an oral vaccine to induce antibodies against surface components, and another is to deliver colonisation factors, such as intimin, as an edible vaccine in transgenic plants (Gyles, 1998).

The commonly accepted rules of herd management should be followed in animals. For calves, colostrum is important for the prevention of white scours, and for pigs, all unnecessary stress should be avoided during weaning in order to prevent edema (Acha and Szyfres, 2001).

2.11. Antimicrobial resistance

Antimicrobial resistance in *E. coli* has been reported worldwide and increasing rates of resistance among *E. coli* is a growing concern in both developed and developing countries. A rise in bacterial resistance to antibiotics complicates treatment of infections (Erb *et al*., 2007). In Ethiopia, a number of studies have been done on the prevalence and antimicrobial resistance patterns of *E. coli* from various clinical sources (Gebre-Sellassie, 2007). The recent research findings of Taye *et al.* (2013); Hiko *et al.* (2008); Bekele *et al.* (2014) and Mohammed *et al*. (2013) confirmed that *E. coli* O157:H7 have developed already different degrees of resistant against various commonly used antibiotics including erythromycin Amoxycillin-Clavulanic acid, Sulfonamides, Ampicillin and Tetracycline, some strains also developed Multi drug resistant.

From the recent research done by Dulo (2014) from carcass swabs taken from goast slaughtered at Dire Dawa municipal slaughter house showed the presence 100% and 83.3% resistant *E. coli* O157:H7 against Erythromycin and Ampicillin respectively. Such types of drug resistance are believed to be as a result of multiple factors like wide use of antibacterial drugs especially in food animals and transfer of drug resistance carrying plasmid gene among *E. coli* species.

2.12. Economic and public health importance

The CDC estimated the annual disease burden of *E. coli* O157:H7 in the United States to be more than 20,000 infections and with as many as 250 deaths (Boyce *et al*., 1995). WHO is concerned about this organism because bloody diarrhoea is a major cause of morbidity and mortality among children in developing countries in the southern hemisphere, including South Africa (WHO, 1997). The *E. coli* O157:H7 strain has tended to dominate the world literature on EHEC. Infections caused by *E. coli* O157:H7 are now recognized more frequently, which reflects increased interest in the incidence and detection of this organism (Nataro and Kaper, 1998).

According to CDC, the incidence of EHEC 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 EHEC 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 U.S. estimates that EHEC O157:H7 causes approximately 73,000 illnesses, 2,000 hospitalizations, and 50-60 deaths each year.

3. MATERIALS AND METHODS

3.1. Study Location

The study was carried out in both Addis Ababa Abattoirs Enterprise and Debre Zeit ELFORA Export Abattoir from December 2014 to June 2015. Addis Ababa Abattoirs Enterprise is found in Addis Ababa which is the capital city of the Federal Democratic Republic of Ethiopia (CSA, 2008). The enterprise has a total capacity of slaughtering 2000 cattle, 1000 sheep and goats, 100 pigs as well as 10 camels per 8 hours. The enterprise has 35 vehicles for transporting meat to the customers and 6 vehicles for transporting live animals from different nearby markets to the abattoir. The origin of animals come to the abattoir to be slaughtered include Gojam, Wellega, Ginchi, Guder, Kata, Borena, Dessie, Debrebrhan, Sheno, Harar, Adama, Awash, and Arsi (Gudeta, 2012). ELFORA Export abattoir is found in Debre Zeit city which is located 47 kms South-East of Addis Ababa. The geography of the area is marked by creator lakes. It is located at 9°N latitude and 40°E longitude and at an altitude of 1850 m.a.s.l (NMSA, 1999). The livestock production system in the area is both intensive and extensive type (CSA, 2008). In this abattoir, higher number of sheep and goats are often slaughtered on daily basis for export purpose but less number of cattle are slaughtered at irregular intervals for a local consumption only. The abattoir is equipped with lairage, better washing facilities and sewerage system, and well designed slaughtering operations. The personnel working there are also trained and do have the basic knowledge on hygienic management and slaughtering skills (Personal observation).

3.2. Study population

The study population comprised apparently health cattle, sheep and goats brought from different areas of the country and slaughtered at the Debre Zeit ELFORA Export Abattoir and Addis Ababa Abattoirs Enterprise.

3.3. Study design and sample size determination

A cross-sectional study was conducted to determine the prevalence and assess the antimicrobial susceptibility of *E. coli* O157: H7 isolated from beef and small ruminants' carcasses. It was also designed to molecularly characterize the shiga toxin producing *E. coli* O157: H7 strains. Sample size was determined by using the formula given by Thrusfield (2005).

$N=\frac{Z^2 Pexp (1-Pexp)}{d^2}$, in which Pexp was taken as 10.2% (Bekele *et al.*, 2014).

Where, **n**= required sample size

Z= statistic for level of confidence = 1.96

d=desired absolute precision of 0.05

n = 140 but it was multiplied by 4 to maximize the precision of the study. Hence, a total of 635 samples were collected during the study period.

3.4. Sample collection

Six hundred thirty five (635) swab samples were selected using a systematic random technique. The selected carcasses were swabbed using the method described in ISO17604 (2005) by placing sterile template (10 x 10 cm) on specific sites of a carcass. Sterile cotton tipped swab, (2X3 cm) fitted with shaft, was first soaked in an approximately 10 ml of buffered peptone water rubbed first horizontally and then vertically several times on the carcasses. The abdomen (flank), thorax (lateral) and breast (lateral); which are sites with the highest rate of contamination (ISO 17604, 2005); were chosen for sampling using the same swab over all the sites. On completion of the rubbing process, the shaft was broken by pressing it against the inner wall of the test tube and disposed leaving the cotton swab in the test tube. Finally, 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 hrs until being processed for isolation.

3.5. Isolation and Identification of *E. coli*

Up on arrival on the laboratory, all pre-enriched carcass swab samples were subsequently subcultured on Eosin methylene blue (EMB) agar and incubated at 37 °C for 24 hours. Purified suspected *E. coli*-like colonies were identified by examining the morphology and biochemical properties of growing colonies. Colonies showing typical dark red to purple red with metallic sheen were taken as *E. coli* isolates (Holt *et al*., 1994).

3.6. Culture and isolation of *E. coli* O157:H7

Laboratory procedures were conducted according to the general guidelines of (ISO 16654: (2001)). The method is based on enrichment in a selective broth medium (modified Tryptone Soya Broth supplemented with Novobiocin) and plating on a Sorbitol MacConkey and then the application of a rapid screening test for confirmation.

3.6.1. Cultivation and Isolation of E. coli on Sorbitol MacConkey Agar

After selective enrichment was made on modified Tryptone Soya Broth supplemented with Novobiocin, they were cultured in to 1% Sorbitol MacConkey agar and incubated at 37°C for 24 hours. The Non-Sorbitol-Fermenting (NSF) *E. coli* (colorless or pale colonies) were considered as *E. coli* O157:H7 strains where as pinkish colored colonies (Sorbitol-Fermenters) were considered as non O157:H7 *E. coli* strains. NSF isolates were again subjected to Latex *E. coli* O157:H7 agglutination test for confirmation (Annex II).

3.6.2. Confirmatory test for E. coli O157:H7 by latex agglutination test

Rapid Latex Test kit is a rapid latex agglutination test intended for confirmatory identification of *E. coli* serogroup O157 (Non-Sorbiotl Fermenting isolates). This test allows the rapid differentiation of *E. coli* O157 from other *E. coli* serotypes (Biolife, 2010). The test was conducted by just adding one drop of latex suspension and dispensing near the edge of the circle on the reaction card. Then a portion of a typical

colony to be tested was emulsified using a loop in a drop of sterile saline solution near the drop of test latex on the test card. After ensuring a smooth suspension of the bacteria and saline, the test latex was mixed with the suspension and spread to cover the reaction area over the loop. Then, the card was rocked in a circular motion for one minute and examined for agglutination by naked eye. Agglutination of the test latex within one minute was considered as positive result (Biolife, 2010) (Annex III).

3.7. Antimicrobial susceptibility test

The antimicrobial susceptibility test was performed following the standard agar disk diffusion method according to CLSI (2008) using commercially available antimicrobial disks (Annex-VI). Each isolated bacterial colony from pure fresh culture was transferred into a test tube of 5 ml Tryptone Soya Broth (TSB) (Oxoid, England) and incubated at 37°C for 6 hrs. The turbidity of the culture broth was adjusted using sterile saline solution and by adding more isolated colonies to obtain turbidity usually comparable with that of 0.5 McFarland standards (approximately $3x10^8$ CFU per ml). Mueller-Hinton agar (Bacton Dickinson and Company, Cockeysville USA) plates were prepared according to the manufacturer guidelines. A sterile cotton swab was immersed into the suspension and rotated against the side of the tube to remove the excess fluid and then swabbed in three directions uniformly on the surface of Mueller-Hinton agar plates. 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. Following this the diameter of inhibition zone formed around each disk was measured using a black surface, reflected light and transparent ruler by lying it over the plates. The results were classified as sensitive, intermediately resistant, and resistant according to the standardized table supplied by the manufacturer (CLSIFAD, 2012).

3.7.1. Identification of Multi Drug Resistance (MDR) E. coli isolates

Multi Drug Resistance is defined as resistance to at least three of the antimicrobials tested. The Multi Drug Resistance (MDR) character of the isolates was identified by observing the resistance pattern of the isolates to the antibiotics (Rota *et al*., 1996).

3.7.2. Multiple Antibiotic Resistance (MAR) Index

The MAR Index of an isolate is defined as a/b, where 'a' represents the number of antibiotics to which the isolate was resistant and 'b' represents the number of antibiotics tested. The MAR indexes of the isolates were calculated and noted if and only if the isolated bacterium has a MAR Index > 0.2 indicates originate from a situation by which several antibiotics are used at greater frequencies (Jayaraman *et al*., 2012; Subramani and Vignesh, 2012).

3.8. Protocol of Multiplex PCR

3.8.1. DNA extraction

A simple bacterial DNA extraction method was done involving centrifugation and boiling. The bacterial isolates grown at nutrient broth were harvested and centrifuged at 5000g for 10 minutes. The supernatant was discarded. The pellet was then washed with 1.0 ml sterile distilled water and vortexed. The bacterial pellets were lysed by boiling in a water bath at 95^{o}c for 10 minutes and the lysate was then centrifuged. Finally, the supernatant was used as a DNA template for PCR (Jothikumar & Griffiths, 2002).

3.8.2. Multiplex PCR

After extraction of the target DNA, *E. coli* isolates were subjected to PCR in order to detect virulence genes. First, multiplex PCR assay was carried out to detect the presence of stx genes in a 20µl reaction volume in a PCR master mix (Himedia India) containing 1 µl of each primer (*Stx*1 *and Stx*2), 4µl of PCR buffer with 17.5 mmol of MgCl2, 1nl of 0.33 mm of each dNTPS, 1U of Taq polymerase enzyme, sterile distilled water and 5µl of template DNA. A negative control was performed by adding 20 µl of sterile deionized water; a positive control was performed by adding 5 µl of known DNA sample. The amplification of the reaction mixtures was performed in Eppendorf thermal-cycler with a temperature program consisting of the initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 2 minutes, annealing at 55^{o}c for 1 minute and polymerization at 72°C for 1 minute. Final elongation step was done at 72°C for 10 minutes (Labnet; USA). Detailed descriptions of primer gene sequences with their different reaction temperatures and the predicted amplicon length are indicated in table 1.

Table 1: Oligonucleotide sequence of the primers used in multiplex PCR

		PCR condition (35 cycles)			
Primer	**Nucleotide sequence**	**Denaturing**	**Annealing**	**Extension**	**Product size (bp)**
FP-*Stx1*	5'-CAACACTGGATGATCTCAG-3'	94^{o}c, 2 min	55^{o}c, 60s	72^{o}c, 60s	349
RP-*Stx1*	5'-CCCCCTCAACTGCTAATA-3'	94^{o}c, 2 min	55^{o}c, 60s	72^{o}c, 60s	349
FP-*Stx2*	5'-ATCAGTCGTCACTCACTGGT-3'	94^{o}c, 2 min	55^{o}c, 60s	72^{o}c, 60s	110
RP-*Stx2*	5'CTGCTGTCACAGTGACAAA-3'	94^{o}c, 2 min	55^{o}c, 60s	72^{o}c, 60s	110

Key: FP= Forward primer, RP= Reverse primer, *Stx* = Shiga like toxins producing genes

Source: Naidu *et al.*, 2011

3.8.3. Agarose gel electrophoresis

Amplified PCR products were analyzed by gel electrophoresis at 120 volt for 45 minutes in 2% agarose containing Ethidium Bromide using a marker DNA ladder of 100 bp (Himedia; India). The products were visualized with UV illumination and imaged with gel documentation system.

3.9. Data management and analysis

In order to have the desired analysis, the coded data were entered in to MS-Excel and then analyzed using SPSS version 20. Descriptive statistics (determination of proportions) were used to summarize the generated data on the rate of *E. coli* O157: H7 and non O157:H7 enterohaemorrhagic *E. coli* isolation and sensitivity pattern of the *E. coli* O157:H7 isolates. The prevalence was calculated as the number of positive samples were divided by the total number of samples examined multiplied by 100. Difference among and between proportions of the groups with certain determinant factors were determined by chi-square ($\chi 2$) test. A p-value <0.05 was considered to be indicative of a statistical significant difference. Regarding the molecular characterization, the banding patterns of individuals' strains were scored based on the presence or absence of the bands with the appropriate base pairs.

4. RESULTS

4.1. Overall Prevalence of *E. coli* isolates

Out of the total of 635 different samples examined, 82 (12.9%) and 35 (5.51%) were found to be contaminated with *E. coli* and *E. coli* O157:H7 strains, respectively as indicated in table 2.

Table 2: Prevalence of *E. coli* isolated from swabs of meat of ruminants

Total sample examined	**Number of positive *E. coli* and *E .coli* O157:H7**	
	E. coli isolates	***E. coli* O157:H7 strains**
	Number of Positive (%)	Number of Positive (%)
N= 635	82 (12.9%)	35 (5.51%)

4.2. Prevalence of *E. coli* and *E. coli* O157:H7 associated with the risk factors

Once the overall prevalence of *E. coli* was determined, its prevalence related to multiple risk factors was also assessed as described in table 3 in which both animal species and age were found to be have a significant difference on the prevalence of *E. coli* ($P< 0.05$) where as origin of animals did not show any significant difference ($P>0.05$). Predisposing factors like sex and breed couldn't be analyzed because all the samples were taken from the same sex and breed i.e. male and local breed ruminants.

Table 3: Prevalence of *E. coli* and *E. coli* O157:H7 in relation to species, age and origin of animals as potential risk factors

		Prevalence (Number of positive) of *E. coli* isolates	
		E. coli	***E. coli* O157:H7**
Risk factors	**No examined**	**Number of Positive (%)**	**Number of Positive (%)**
Species			
Sheep	192	28 (14.58%)	17 (8.85%)
Goat	192	13 (6.77%)	9 (4.68%)
Cattle	251	41 (16.3)	9 (3.6%)
χ^2		5.14	3.06
p-value		0.031	0. 025
Age			
Young	132	26 (19.7%)	14 (10.6%)
Adult	454	50 (1.1%)	19 (4.2%)
Old	49	6 (12.25%)	2 (4.08%)
$\chi 2$		9.19	10.21
p-value		0.012	0.006
Origin			
Afar	76	3 (3.94%)	1 (1.32%)
Arsi-bale	121	21(17.36%)	11 (9.17%)
Harar	65	5 (7.69%)	3 (4.62%)
Ogaden	51	15 (29.41%)	5 (9.8%)
Yabello	132	12 (9.09%)	1 (0.76%)
Wollo	94	18 (19.15%)	9 (9.57%)
Wolayta	96	8 (8.3%)	5 (5.2%)
$\chi 2$		17.53	16.91
p-value		0.06	0.07

4.3. *In vitro* antimicrobial susceptibility test result of *E. coli* isolates

The result of antimicrobial susceptibility test of 82 *E. coli* and 35 *E. coli* O157:H7 isolates against 8 commercially available antimicrobial agents is summarized in table 4. The antibiogram result of *E. coli* O157:H7 isolated from different raw meat types revealed a varying degree of susceptibility and resistance to the different antimicrobial agents tested. The degree of susceptibility ranges from 12.2% up to 79.3% and 14.025% up to 85.75% in *E. coli* and *E. coli* O157:H7 respectively. Similarly, resistance ranges from 6.1% up to 36.6% in *E. coli* but it is between 5.75% and 48.6% in case of *E. coli* O157:H7.

Table 4: Antimicrobial susceptibility test result of *E. coli* isolates

		Susceptibility and resistance pattern of all *E. coli* isolates					
Antimicrobial agent	**Disc concentration**	***E. coli***			***E. coli* O157:H7**		
		S (%)	**R (%)**	**I (%)**	**S (%)**	**R (%)**	**I (%)**
AMC	20 µg	60 (73.1)	8 (9.6)	14 (17.11)	30 (85.7)	4 (11.42)	1 (2.9)
CRO	30 µg	50 (61)	8 (9.8)	24 (29.24)	21 (60)	3 (8.6)	11 (31.4)
KA	30 µg	62 (75.6)	0 (0)	20 (24.4)	25 (71.4)	0 (0)	10 (28.57)
NA	30 µg	0 (0)	30 (36.6)	52 (63.4)	0 (0)	17 (48.6)	18 (51.4)
TTC	30 µg	65 (79.3)	5 (6.1)	12 (14.64)	27 (77.1)	2 (5.7)	6 (17.14)
S	10 µg	48 (58.5)	1 7(20.7)	17 (20.7)	17 (48.6)	10 (28.6)	8 (22.9)
S-$_3$	300 µg	64 (78.0)	5 (6.1)	13 (15.86)	30 (85.7)	0 (0)	5 (14.3)
W-$_5$	5 µg	10 (12.2)	28 (34.2)	44 (53.7)	5 (14.02)	10 (29)	20 (57.14)

Key: %= Percent, S = Sensitive, I = Intermediate, R = Resistant, Amoxicillin-Clavulanic acid (AMC), Ceftriaxone (CRO), Kanamycin (KA), Nalidixic acid (NA), Streptomycin (S), Sulfonamides (S-$_3$), Trimethoprim (W-$_5$), Oxytetracycline (TTC)

4.4. Antibacterial activity of selected antimicrobials on *E. coli*

The result of the antimicrobial sensitivity test showed that all 82 *E. coli* isolated from the raw meat of ruminants were resistant to the 7 antimicrobials tested except to that Kanamycin as indicated in tables 5 and 6. Most of the isolates showed resistance against two or three antimicrobials while only one isolate showed resistance against four antimicrobials tested. On the other hand, no *E. coli* isolate was found to be resistant against four antimicrobials though most of them showed similar resistance pattern to that of *E. coli* isolates. Regarding the multidrug resistance profile, 18.3% of *E. coli* and 14.3% of *E. coli* O157:H7 isolates were found to be MDR.

Table 5: Antimicrobial resistance profile of *E. coli* isolates (n= 82)

	Antibacterial resistance pattern	**Total No of Resistant *E. coli***
For one	AMC (8), CRO (8),W-5 (28), NA (30), TTC (5), S (17), S-3 (5)	101
For two	AMC W-5 (6), NA CRO (4),W-5 S-3 (3), AMC S-3 (3), NA TTC (3); S W-5 (6) TTC S (2), NA S (2), AMC CRO (2) NA AMC (2), TTC W-5 (2)	35
***For three**	NA S W-5 (5) AMC S-3 W-5(2), AMC S-3 CRO (1), NA TTC S (3), NA TTC W-5 (2), NA S-3 W_{-5} (1),	14
***For four**	NA TTC S W_{-5} (1)	1

*Multidrug resistant = when a single isolate is resistant against more than 2 drugs

Table 6: Antimicrobial resistance patterns of *E. coli* O157 isolates (n= 35).

	Antimicrobial resistance pattern	Total No resistant *E. coli* O157:H7
For one	AMC (4), CRO (3), NA (17), TTC (2), S (10), W-5 (10)	46
	NA CRO (1),W-5 AMC (1), NA S (2), TTC W5 (1)	
For two	NA AMC (1), AMC TTC (1), S W-5 (2)	9
	NA S W-5 (2), AMC NA CRO(1)	
***For three**	NA TTC S (1), AMC NA W-5 (1)	2

*Multidrug resistant = when a single isolate is resistant against more than 2 drugs

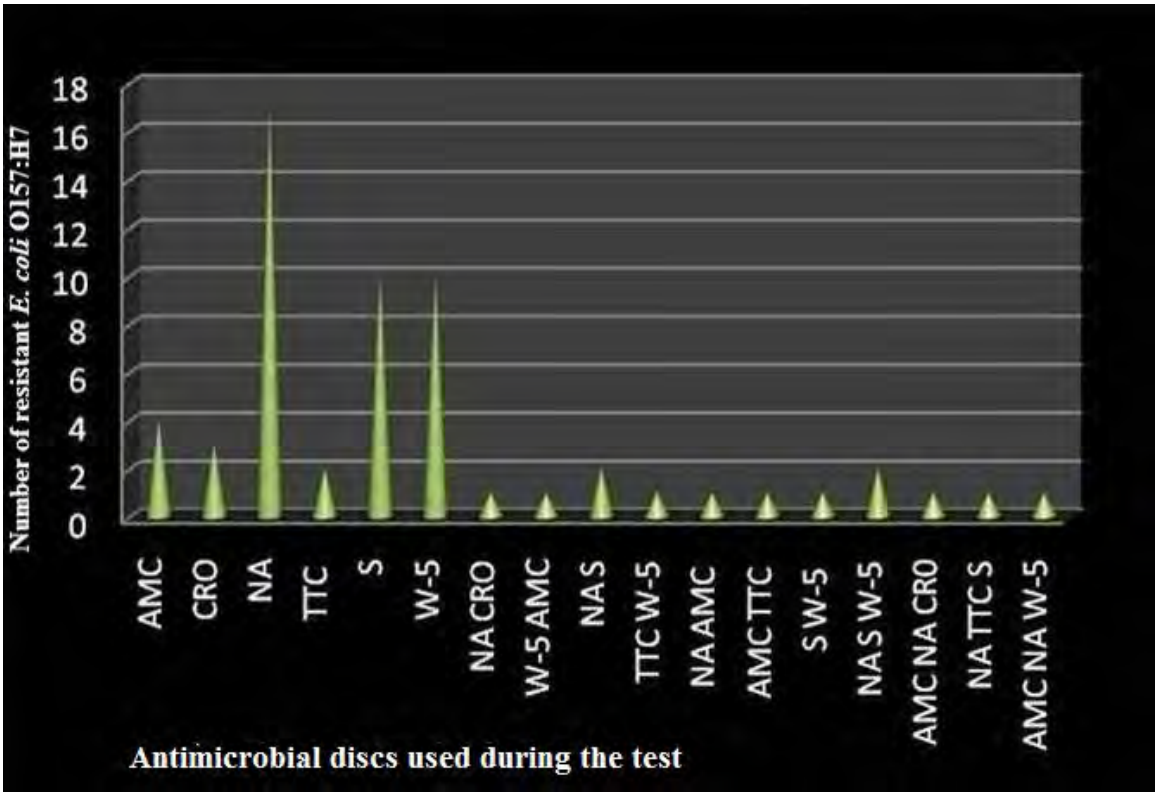

Figure 3: Antimicrobial resistance pattern of *E. coli* O157:H7 isolates

4.5. Multi antibacterial resistance index (MAR) analysis

The analysis of MDR Index indicated that *E. coli* isolates have very high MAR index value (>0.2) as illustrated in figure 4.

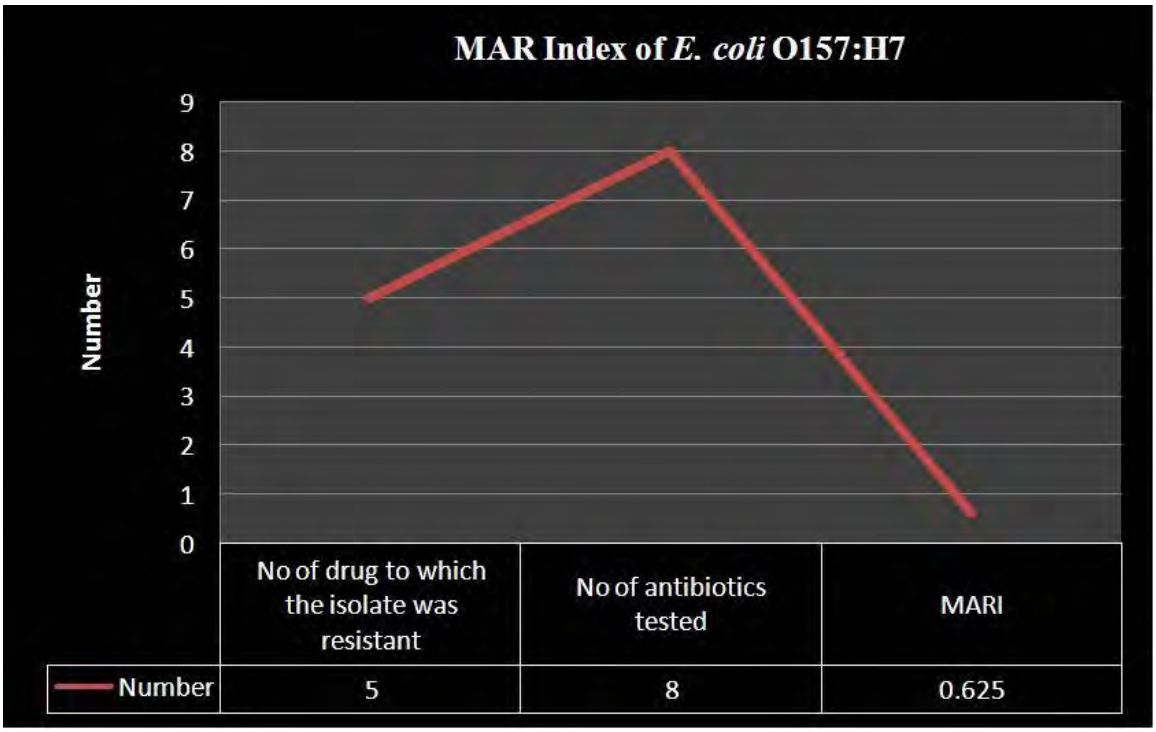

Figure 4: Multi antimicrobial resistance index analysis of *E. coli* O157:H7 isolates

4.6. Molecular characterization of *E. coli* O157:H7 isolates

Multiplex PCR analysis of enterohaemorrhagic *E. coli* O157:H7 strains revealed that out of the 35 *E. coli* O157:H7 isolates, 20 of them were subjected to multiplex PCR in order to detect the shiga toxin producing genes (*Stx1* and *Stx2*) in which only 3 (15%) of them were found to be positive. However, only one of them was found to contain *both Stx1* and *Stx2* genes as illustrated in figure 5.

Figure 5: Multiplex RCR showing the amplification of 349 bp and 110 bp fragment of *Stx*1 and *Stx*2 genes of Enterohaemorrhagic *E. coli* O157:H7 isolated from the swab samples taken from the surface of meat of ruminants.

Key: Lane 1: DNA marker, lanes 1-10: samples (extracts from the isolates), lane 11: negative control and lane 12: known positive control

5. DISCUSSION

Human infections of *E. coli* O157:H7 have mostly been recognized to be from food products with animal origin (Jo *et al*., 2004). Domestic ruminants, mainly cattle, sheep, and goats, have been established as major natural reservoirs for STEC and play a significant role in the epidemiology of human infections (Griffin *et al*., 1991). It is clear that the consumption of raw meat and its products is common in the Ethiopian traditional diet but studies on various food contaminating pathogens like Enterohaemorrhagic *E. coli* remains limited despite the massive feeding habits of raw beef and mutton in the country.

The present study evidenced a considerable presence of *E. coli* and *E. coli* O157:H7 as a contaminant in meats of sheep, goat and cattle slaughtered at both Debre Zeit ELFORA Export Abattoir and Addis Ababa Abattoirs Enterprise. The current study revealed that th e overall prevalence of carcass contamination with *E. coli* and *E. coli* O157:H7 in the slaughtered animals was 12.9% (82/635) and 5.5% (35/635) respectively. The prevalence of *E. coli* O157:H7 is lower than *E. coli* species isolated is in agreement with the reports of Balcha *et al.* (2014) in which prevalence of *E. coli* O157:H7 and *E. coli* was 18% and 62.5% respectively. This result was also in consistent with the report of Taye *et al*. (2013) in which the overall prevalence of carcass contamination with *E. coli* was 30.97% and that of *E. coli* O157:H7 was 2.65%. This situation can possibly indicate that there are lots of meat contaminant *E. coli* species other than *E. coli* O157:H7. The Prevalence of *E. coli* O157:H7 in meats of sheep and goats was 14.58% and 6.7% respectively which significantly lower than that of that of meat of cattle (16.7%) ($P<0.05$). This could be due to difference in hygienic status of abattoir of privately owned and governmental slaughter houses since samples from beef were taken from Debre Zeit ELFORA Export Abattoir but the samples from meats of sheep and goat were collected from Addis Ababa Abattoirs Enterprise. This is supported by the reports of Hiko *et al*. (2008) in which the prevalence of *E. coli* O157:H7 in export and municipal abattoirs was 2.1% and 6.8% respectively. This is also in agreement with Mohammed *et al.* (2014) reported that the prevalence in Dire Dawa ELFORA abattoir and Dire Dawa Municipality abattoir respectively were

17.29% and 24.48%. This marked difference in the prevalence of *E. coli* among the two different abattoirs can also be justified by a number of other reasons including those small ruminants presented for slaughter at Debre Zeit ELFORA export abattoir are allowed to stay starved for 24 hours in a well sanitized lairage with provision of water which has an effect on the reduction of normal intestinal flora and subsequently on meat contamination with *E. coli* from feces. Similarly, Siragusa *et al*. (1995) confirmed that a major source of microbial contamination on the carcasses of small ruminant is feces.

The overall prevalence of *E. coli* O157:H7 in the current study was slightly higher than 4.2% (Hiko *et al.,* 2008) but greatly lower than 10.2% (Bekele *et al.,* 2014). In contrast to the current study, a higher prevalence of *E. coli* O157:H7 were reported from different countries; 9% in India (Luga *et al*., 2007) and 31% in Canada (Doyle and Schoeni, 1987). Such variations may be due to differences in hygienic status abattoir (source of samples), type of samples, method of sampling and culturing techniques. For the presence of prevalence of *E. coli* O157:H7 in meats of ruminants can also be reasons as Okonko *et al*. (2010) indicated the equipment used for each operation in the abattoir, the clothing and hands of personnel and the physical facilities themselves can also be potential sources of microbial contaminations of *E. coli* in cattle meat slaughtered in abattoirs.

Regarding the risk factors associated with the prevalence of *E. coli* O157:H7, Estimates of the prevalence of *E. coli* O157:H7 among animal species and age of animals vary considerably. The prevalence of *E. coli* O157:H7 among animal species showed significant difference ($P<0.05$) in which *E. coli* O157:H7 was recovered at lower prevalence in meat samples obtained from goat (6.77%) and sheep (14.58%) than beef (16.3%). This can be due to the fact that cattle are implicated as the principal reservoir of *E. coli* O157:H7 (Chapman *et al*., 2001).

There was a significant variation ($P<0.05$) among the different age groups of slaughtered ruminants showing a prevalence of 19.7% (young), 1.1% (adult) and 12.25% (old) respectively. This disagrees with the work of Taye *et al.* (2013). In the other side, the difference in prevalence of *E. coli* O157:H7 among animal origin was not statistically

significant (P>0.05) though a higher prevalence was recorded in those ruminants that came from Wollo area (19.15%) than Afar (3.94%). These results are in accord with report of Taye *et al.* (2013). This could be logical since degree of contamination of carcass is more likely affected by hygienic conditions during flaying of the skin than the geographical locations from where animals are brought for slaughter.

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 (Scott *et al*., 2002). In Ethiopia, there have been reports on the drug resistance of *E. coli* O157:H7 isolates from animal derived food products (Hiko *et al*., 2008; Bekele *et al*., 2104, Bula 2014, Mohammed *et al.* 2014, Taye *et al.,* 2013).With regard to the antibiogram of *E. coli* in the current study, 8 different commercially available antimicrobial discs were used and all the 82 *E. coli* isolates subjected to antimicrobial sensitivity test were found to be susceptible to all the antimicrobials except to that of Nalidixic acid but they were resistant against most of the discs except to that Kanamycin. The degree of susceptibility for *E. coli* and *E. coli* O157:H7 isolates ranges from 12.2% up to 79.3% and 14.03% up to 85.75% respectively however resistance ranges from 6.1% up to 36.6% in *E. coli* but it was between 5.75% and 48.6% in case of *E. coli* O157:H7. The absence of resistance against kanamycin is not in agreement with the previous findings of Hiko *et al*. (2008) whose study showed all the isolates were resistant to kanamycin but it agrees with Taye *et al.* (2013) reported that all the *E. coli* isolates were found susceptible to Kanamycin.

Multi Drug Resistance is defined as resistance of an isolate to more than 2 antimicrobials tested (Dominic *et al*., 2005). Multiple drug resistance was also seen in 15 (18.3%) *E. coli* and 5 (14.3%) *E. coli* O157:H7 isolates which is consistent with the findings of Dulo (2014) that showed the presence of multiple antimicrobial resistance of 3 *E. coli* O157 against three (16.5%) and four (33.3%) antibiotics. In the present study, all the 5 *E. coli* O157:H7 isolates were resistant against three drugs. From the mentioned antimicrobials, Nalidixic acid and Trimethprim were found in almost all of the MDR *E. coli* O157:H7 isolates. This finding was supported by Bekel *et al*. (2014); Hiko *et al.* (2008); Adetunji

et al. (2014); Meng *et al*. (1998) and Schroeder *et al*. (2002) who reported the existence of multidrug resistant *E. coli*. This is correlated with Ahemed *et al*. (2006) who noted that multidrug resistant phenotypes have been spread widely among Gram negative bacteria. Furthermore, it is stated that studies in other developing countries have shown the trend in enteric pathogens is toward increasing antimicrobial resistance (Hohe *et al*., 1998). In general, the development of drug resistant *E. coli* strains can be linked to various aspects including the practice of indiscriminate use of antibiotics in food producing animals (Cosgrove and Carmeh, 2003) and due to the selective pressure to rampant use of antibiotics in the animal industry (Mohammed *et al*., 2014). Monitoring of antimicrobial resistance in *E. coli* O157:H7 isolates is valuable for epidemiological uses and for monitoring the increase of antimicrobial resistance among different microbial species (Osaili *et al*., 2013).

The multi antimicrobial resistance index for *E. coli* O157:H7 was analyzed and found to be 0.625 since five antibiotics out of the 8 antimicrobial discs tested were found in the list where the isolates showed MDR. Thus, the result (MAR index >0.2) indicated that the MDR was occurred as a result of enormous and frequent use of antibiotics in food animals (Subramani and Vignesh, 2012)

Shiga toxigenic *Escherichia coli* strains are an important cause of gastrointestinal diseases in humans, particularly since these infections may result in life-threatening sequelae, such as hemolytic uremic-syndrome (Karmali, 1989). The virulence of *E. coli* O157:H7 has mainly been associated with the production of shiga toxins encoded by *stx1* or *stx2* genes (Chahe *et al*., 2006). PCR is a rapid and reliable tool for the molecular-based diagnosis of a variety of infectious diseases (Fredricks and Relman, 1999) and due to its sensitive and specific nature of the assay; it should be applied for further confirmation of the isolated *E. coli* species. So far, there is only one research conducted to detect the shiga toxin producing genes of *E. coli* which were isolated from small ruminants slaughtered at Modjo export abattoir by sending samples to the National Food Institute, Technical University of Denmark and they found that out of the 53 *E. coli* O157:H7 isolates, one isolate from a goat carcass had both *stx1* and *stx2* genes (Mersha *et*

al., 2008). In the current study, out of 20 *E. coli* O157:H7 isolates subjected to Multiplex PCR, only 3 (15%) were found to be positive but only one of them was found to contain both *Stx*1 and *Stx*2 genes. However, the remaining two isolates were found to contain *Stx2* virulence gene only but not *Stx*1. Similar findings were reported by Mersha *et al*. (2009); Adrienne *et al.* (2002); Shahzad *et al*. (2013); Kiranmayi *et al*. (2010) and Sahilah *et al*. (2010). The result form the current study is consistent with the previous reports where virulence genes were detected in only few *E. coli* strains that may be due to the fact that there are occasional strains that have the genes but do not express the toxins (Dontoroua *et al*., 2004; Lenahan *et al*., 2007 and Kaper *et al*., 1998). In general, multiplex PCR analysis is a valuable diagnostic and epidemiological tool and it will also facilitate the analysis of large STEC culture collections (Adrienne *et al.*, 2002).

6. CONCLUSION AND RECOMMENDATIONS

The present study showed a considerable presence of *E. coli* O157:H7 and other *E. coli strains* in sheep goat and cattle slaughtered at Debre Zeit ELFORA Export Abattoir and Addis Ababa Abattoirs Enterprise. In this study, the overall prevalence of *E. coli* O157:H7 that contaminate the meat of cattle was higher that of the prevalence recorded from carcass swab samples of small ruminant origin which can suggest that consumption of raw beef is a potential risk of zoonosis. The prevalence of *E. coli* O157:H7 was higher in samples taken from Addis Ababa Abattoirs Enterprise that can be related to the poor hygienic managements. The occurrence of *E. coli* had significant difference among animas having different age groups however origin of animals as potential predisposing factor was found to be not significantly associated with the occurrence of *E. coli* contamination. Though most of the *E. coli* isolates subjected to antibiotic sensitivity test show different degrees of resistance against the antibiotic discs tested, none of them were found to be susceptible to Nalidixic acid. Multi drug resistance as a result of massive use of some of the commonly available antimicrobials in food animals was also recorded in this work which may pose a threat to human beings by further limiting the therapeutic options. According to the investigation from multiplex PCR, only 3 out of 35 *E. coli* isolates were positive to contain *Stx* virulence genes.

Based on the above concluding remarks, the following recommendations are forwarded:

- Intensive trainings should be given to those personnel working in municipal slaughter houses to ensure the hygienic practices during slaughtering of animals.
- *In vitro* drug sensitivity testing of *E. coli* O157:H7 should be performed so that proper treatments can be instituted for *E. coli* O157:H7 infected patients.
- Further molecular characterization of both *E. coli* O157:H7 and other shiga toxin producing *E. coli* strains should be conducted

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8. ANNEXES

Annex I: Composition and preparation of Medias use for transportation and culture of the bacteria

Table 7: Buffered Peptone Water (BPW) (HIMEDIA, India)

Ingredients	gm/litre
Sodium chloride	5.00gm
Casein enzyme hydrolysate	10.00gm
Disodium hydrogen phosphate	9.00gm
Mono potassium hydrogen phosphate	1.5gm
Final PH at 25 ^{O}C	7.0 ±0.2

Preparations:

Add 20 gram to 1 liter of distilled water. Heat if necessary to dissolve the medium completely. Mix it well and sterilize by autoclaving at 120 OC for 15 minutes.

Annex II: Medias used for the primary isolation of *E. coli* O157:H7

Table 8: Eosin Methylene Blue (EMB) (Oxoid, ® Hampshire, England)

Ingredients	gm/liter
Peptone	10.00 g
Lactose	5.00 g
Dipotassiummono hydrogen phosphate	2.00 g
Mthylene blue	0.06 g
Eosin Y	0.04 g
Agar	13.50 g
PH at 25^{O}C	7.1 ± 0.2

Table 9: MacConkey agar (CMO115, Oxoid)

Formula	grams per litre
Peptone	20.0
Lactose	10.0
Bile salts	No.3 1.5
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.001
Agar	15.0
PH	7.1 (Approximately)

Preparation

51.5 grams of the powder were suspended in a litre of distilled water brought to boil to dissolve completely and dissolved completely and sterilised at (1210C for 15 minutes).The molten agar was cooled to 500c and approximately 20ml poured into a Petri dish (90mm in diameter) and allowed to cool and solidify at room temperature.

Table 10: Sorbitol MacConkey Agar (SMAC) (CM0813 Oxoid, England)

Ingredients	gm/litre
Peptic digest of animal tissue	17.00gm
Proteose peptone	3.00gm
Agar	13.50gm
D- Sorbitol	1.00gm
Bile salts	1.5gm
Sodium chloride	5.00gm
Neutral red	0.03gm
Crystal violet	0.001gm
Final PH at 25^{O}C.	7.1 ± 0.2

Preparations:

Suspend 50 gram of Sorbitol MacConkey Agar in one liter of purified water. Heat with frequent agitation and boil 1 minute to completely dissolve the powder. Autoclave at $121^{O}C$ for 15 minutes. Allow the media to cool to $45\text{-}50^{O}C$ before adding the potassium tellurite and cefixime supplement. Add cefixime 0.05 mg/l and potassium tellurite 2.5 mg/l supplement.

Principle and Interpretation

MacConkey Sorbitol Agar is based on the standard formulation described by Rappaport and Henigh . This medium is recommended for isolation of enteropathogenic *Escherichia coli* O157:H7, which ferments lactose but does not ferment sorbitol, hence produces colourless colonies. This organism has been recognized as a cause of hemorrhagic colitis *E. coli* O157:H7 is a human pathogen associated with hemorrhagic colitis that results from the action of a shiga-like toxin (SLT) MacConkey Sorbitol Agar however should not be solely used to detect pathogenic *E.coli* O157: H7 strains as some nontoxic strains will also not ferment sorbitol. On standard MacConkey Agar containing lactose, this strain is indistinguishable from other lactose-fermenting *E .coli*. In MacConkey Sorbitol Agar Base, lactose is replaced by sorbitol. Unlike most *E. coli* strains, *E. coli* O157:H7 ferments sorbitol slowly or not at all. The growth of *E. coli* O157:H7 on MacConkey Agar with Sorbitol shows colourless colonies and most of the fecal flora ferment sorbitol and appear pink. MacConkey Agar with Sorbitol therefore permits ready recognition of *E. coli* O157:H7. Peptic digest of animal tissue and proteose peptone supply necessary nutrients like nitrogenous and carbonaceous compounds, minerals, vitamins and trace ingredients for the growth of organisms. Crystal violet and bile salt mixture present in the medium inhibit growth of gram-positive bacteria. Sodium chloride maintains osmotic equilibrium. Neutral red is an indicator. D-Sorbitol is the fermentable carbohydrate.

Table 11: Blood Agar (Oxoid, ® Hampshire England)

Ingredients	gm/liter
Proteose peptone	15.00 g
Liver extract	2.50 g
Yeast extract	5.00 g
Sodium chloride	5.00 g
Agar	15.00 g
PH at $25^{O}C$	7.4 ± 0.

Directions:

Suspend 40g in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121oc for 15 minutes. Cool to 45-$50^{O}C$ and add 7% sterile blood. Mix with gentle rotation and pure into sterile dishes or other containers.

Annex III: *E. coli* O157:H7 Latex Test Kit as a confirmatory for *E. coli* O157:H7

Test Principle

The polystyrene latex particles provided in the kit are coupled to antibodies against *E. coli* serotype O157:H7. When the latex particles are mixed on a test card with fresh colonies of *E. coli* O157, the bacteria will bind to the antibody causing the latex particles to agglutinate (positive reaction). Bacteria that are not *E. coli* O157:H7 will not bind to the antibody and will not agglutinate the latex particles (negative reaction).

Test procedure:

1. Dispense one drop (30µL) of sample diluent on to two wells of a clean, dry agglutination slide.

2. Using an inoculating loop, remove several suspected *E. coli* colonies from the sorbitol MacConkey agar plate. Only select colorless colonies whose morphology resembles that of *E. coli.*
3. Emulsify the colonies in the two drops of sample diluent on the test slide to produce a heavy, smooth suspension. Spread the suspension, over the entire surface of the wells.
4. Rock the slide gently for 30 seconds and observe for auto agglutination or clumping. Of the suspensions remain smooth, proceed to section 5. If the suspension is "stringy" or "granular", the sample is unsuitable for testing with *E. coli* O157:H7 Rapid Latex Test Kit since it may give a falsely positive agglutination when latex is added. Here, alternative test method should be used.
5. Gently shake each latex reagent to ensure a homogeneous suspension.
6. Add 1 drop of REAG TEST ECl to one of the bacterial suspensions, and one drop of reagents control to the other. Do not allow the latex dropper to touch the bacterial suspensions.
7. Mix the suspensions with a fresh mixing stick for each combination.
8. Rock the slide gently for two minutes and observe for agglutination. An agglutination reaction is indicated by visible aggregation of the latex particles.
9. Discard the used slides and mixing sticks into a suitable disinfectant.

Table 12: Nutrient Agar (Oxoid, ® Hampshire, England)

Ingredients	**gm/liter**
Peptone digest of animal tissue	5.00 gm
Yeast extract	1.50 gm
Beef extract	1.50 gm
Sodium chloride	5.00 gm
Agar	15.00 gm
PH at 25 ^{O}C	7.4 ± 0.2

Directions:

Powder: suspended 28g in 1liter of distilled water. Boil to dissolve completely. Sterilize by autoclaving at 121 ^{O}C for 15 minutes.

Annex IV: Media and antibiotic discs used during the antimicrobial sensitivity testing with their respective concentration

Table 13: Table Mueller Hinton Agar (Oxoid, ® Hampshire, England)

Ingredients	**gm/liter**
Beef, dehydrated infusion	300 gm
Casein hydrolysate	17.50 gm
Starch	1.50 gm
Agar	17.10 gm
PH at 25 ^{O}C	7.3 ± 0.1

Preparations:

Add 38 gram to 1liter of distilled water. Bring to the boil to dissolve the medium completely. Sterilize by autoclaving at 121^{O}C for 15 minutes.

Table 14: Antibiotic discs used during the antimicrobial susceptibility testing of *E. coli* O157:H7 with their respective concentrations

Antimicrobial Agent	**Symbol**	**Disk Content**	**Zone of inhibition in with its interpretation**		
			S	**I**	**R**
Amoxicillin-Clavulanic acid	AMC	20 µg	≥ 18	14-17	≤ 13
Ceftriaxone	CRO	30 µg	≥ 23	20-22	≤ 19
Kanamycin	KA	30 µg	≥ 18	14-17	≤13
Nalidixic acid	NA	30 µg	≥ 19	14-18	≤ 13
Streptomycin	S	10 µg	≥ 15	12-14	≤ 11

Sulfonamides	S-3	300 µg	≥ 17	13-16	≤ 12
Tetracycline	TTC	30 µg	≥ 15	12-14	≤ 11
Trimethoprim	W-5	5 µg	≥ 16	11-15	≤ 10

Source: CLSI, (2012)

Table 15: Characterization of *E. coli* O157:H7 isolates and non O157:H7 enterohaemorrhagic *E. coli* species

Name of culture media	Identification
*Eosin Methylene Blue (EMB) agar	*Moist circular colonies with dark centers yellow-green metallic sheen
*1% Sorbitol MacConkey Agar (SMAC)	*Rose pink colonies (sorbitol fermenter) – Non O157:H7 *E. coli*
*Agglutination reaction	pale- white (Non sortibtol fermenter (*E. coli* O157:H7) agglutinated for *E. coli* O157:H7
*Blood Agar	Gama hemolysis (non *E. coli* O157:H7)
*Nutrient Agar	Smooth, circular, white to gryish colony

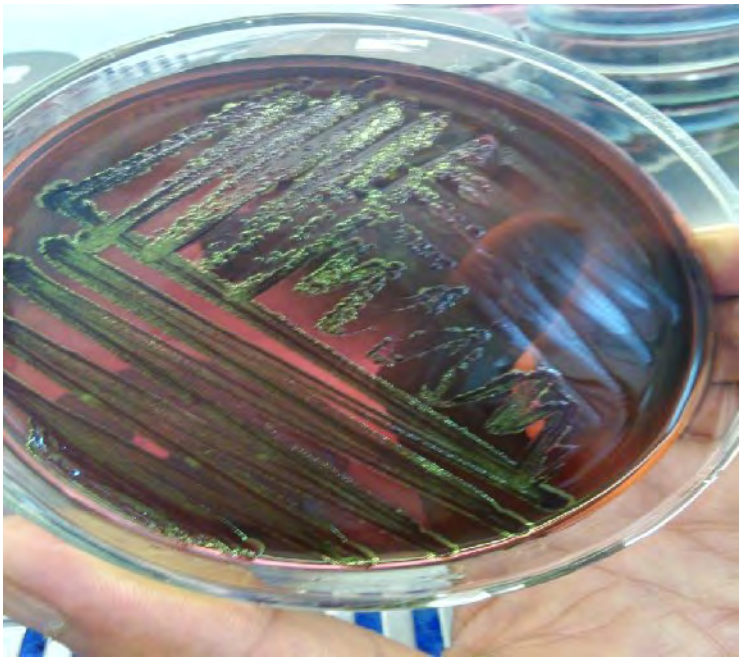

Figure 6: Metallic sheen colonies indicating the growth of *E. coli* on EMB Agar

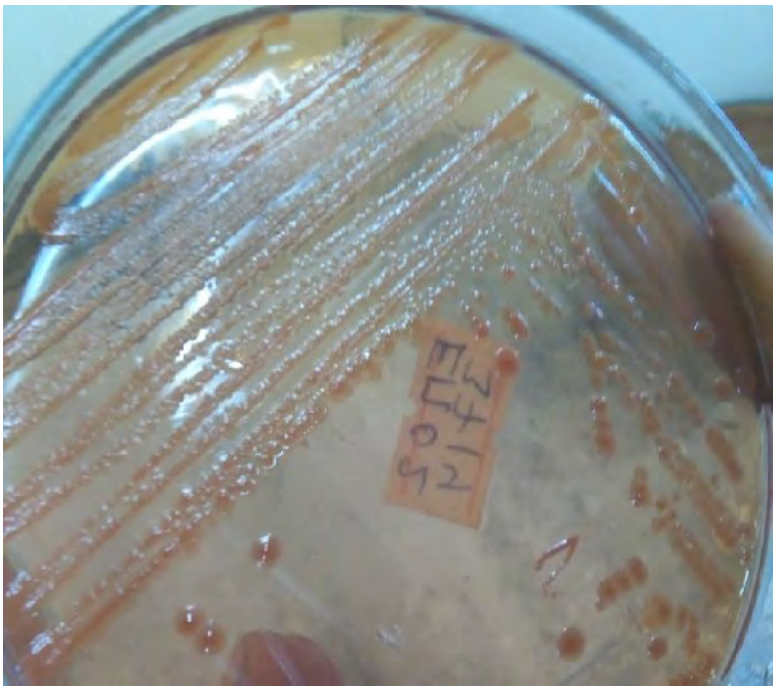

Figure 7: *E. coli* O157 – Sorbitol Non fermenter (pale colonies) grown in sorbitol MacConkey agar medium

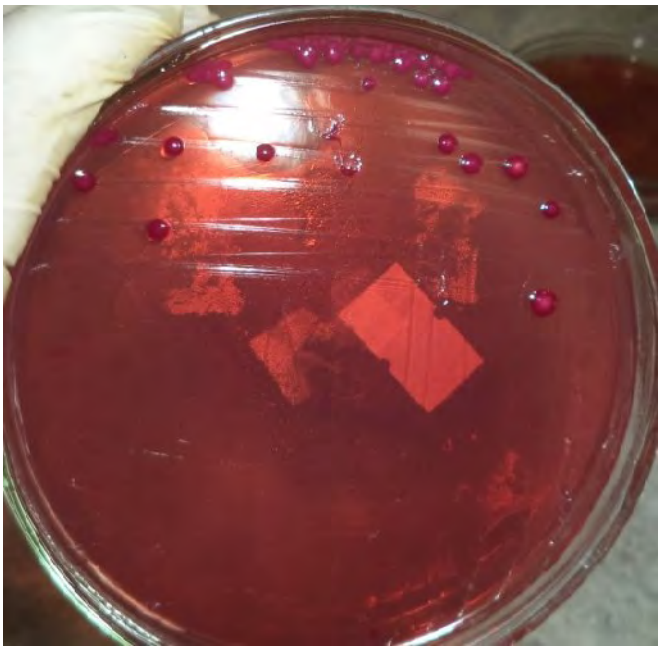

Figure 8: Non *E. coli* O157:H7 - Sorbitol fermenter (pink colonies) grown in Sorbitol MacConkey agar medium

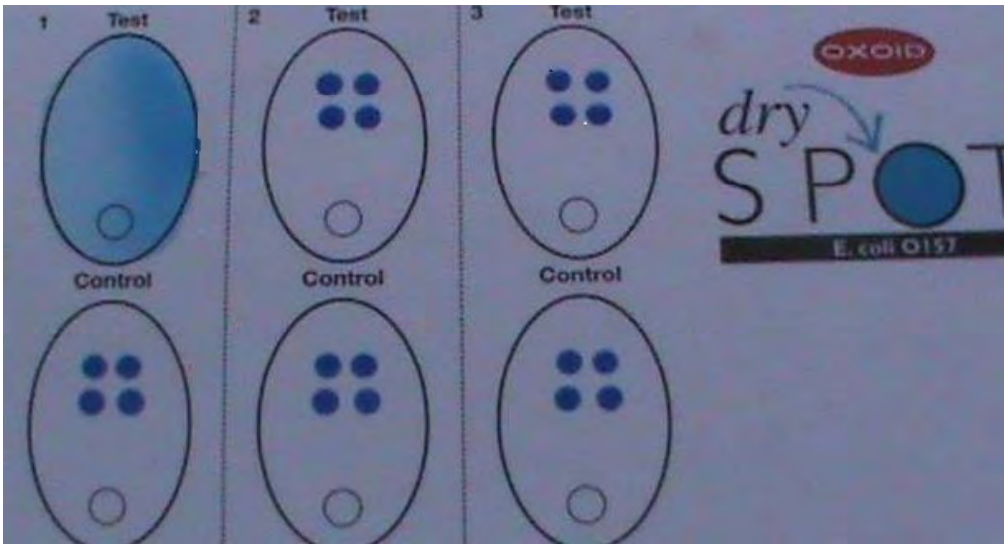

Figure 9: Latex aglutination test for *E. coli* O157:H7

Figure 10: Antimicrobial sensitivity test showing different degrees of zone of inhibition