JIMMA UNIVERSITY

COLLEGE OF AGRICULTURE AND VETERINARY MEDICINE

SCHOOL OF VETERINARY MEDICINE

ISOLATION, MOLECULAR DETECTION, ANTIBIOGRAM PROFILES AND ASSOCIATED RISK FACTORS OF ESCHERICHIA COLI O157: H7 FROM RAW MILK OF APPARENTLY HEALTHY DAIRY COWS IN JIMMA TOWN, SOUTHWEST OROMIA, ETHIOPIA

MVPH THESIS

BY

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College of Agriculture and Veterinary Medicine

School of Veterinary Medicine

Isolation, Molecular Detection, Antibiogram Profiles and Associated Risk Factors of *Escherichia Coli* O157: H7 in Raw Milk of apparently healthy Dairy Cow in Jimma Town, Southwest Oromia, Ethiopia

By:

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DEDICATION

I dedicate my Thesis work to my parents, my father Abdisa Lemu and my mother Kibitu Legese as well as to all of my siblings, who have always been my sources of my happiness, strength, and spiritual arm. I appreciate your help in realizing this dream for me.

STATEMENT OF AUTHOR

I first state that this thesis is entirely my own work and that all references and materials used in its creation have been properly cited. This thesis is deposited in the University Library to be made accessible in accordance with library policies. It was submitted to fulfill a portion of the requirements for an MSc in Veterinary Public Health at Jimma University College of Agriculture and Veterinary Medicine. I thus declare that this thesis has not been submitted for consideration for any academic degree, diploma, or certificate to any other institution anywhere. Short quotes from this thesis are acceptable as long as the source is properly cited and no additional permission is needed. Request for permission to fully credit a copy of this document, in whole or in part, may be made to Jimma University's School of Graduate Studies and/ or School of Veterinary Medicine. In all other instances, however, permission should be obtained from the author.

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BIOGRAPHICAL SKETCH

The author, Walde Abdisa, was born on December 9, 1987 G.C. from his father, Abdisa Lemu, and his mother, Kibitu Legese, in Sibo 01 Kebele, Bure District of Ilu Aba Bor Zone, and Oromia Regional State, Ethiopia.

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After his graduation, he was hired at the Yayo District Livestock and Fishery Office on August 8, 2007 (G.C.). After working for four years, he joined Mettu University in 2011 G.C. and gained his BA in Economics on July 25, 2015; at a moment, he joined Jimma University in 2015 G.C. and gained his BVSc in Veterinary Science on March 26, 2019 G.C. From that moment, he was assigned as a veterinary clinician in the Yayo town veterinary clinic, and he has been proficiently serving the community for the past 14 years. Finally, after he took and passed the entrance examination of postgraduate program, he joined again Jimma University College of Agriculture and Veterinary Medicine (JUCAVM) to follow his Master (MSc) degree in Veterinary Public Health at the School of Veterinary Medicine in December 2021. In conclusion, after completing his class course and research work, today he is here to defend his MSc thesis.

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LIST OF ABBREVIATIONS AND ACRONYMS

ATJKD	Adami Tulu Jido Kombolcha district
AMR	Antimicrobial Resistance
BPW	Buffered Peptone Saline Water
CFU	Colony Forming Units
DAEC	Diffusely Adherent *E. coli*
DNA	Deoxyribo Nucleic Acid
dNTP's	Dinucleotedtriphosphate
eaeA	intimin
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISAs	Enzyme-Linked Immunosorbent Assays
EMB	Eosin Methylene Blue agar
EPEC	Enteropathogenic *E. coli*
ERB	Ethical Research Board
ESRD	End-Stage Renal Disease
ETEC	Enterotoxigenic *E. coli*
HACCP	Hazard Analysis Critical Control Points
HC	Hemorrhagic Colitis
hlyA	Enterohemolysin
HUS	Hemolytic Uremic Syndrome
JUCAVM	Jimma University College of Agriculture and Veterinary Medicine
LEE	Locus for Enterocyte Effacement
LPS	Lipopolysaccharide
MDR	Multi-Drug Resistant
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
R-PCR	Real-Time PCR
ssDNA	Dabble strand DNA
STEC	Shiga toxin-producing *E. coli*
stx1 and *stx2*	Shiga toxins one and two
TSB	Tryptone Soya Broth
TTP	Thrombotic Thrombocytopenic Purpura
USA	United State of America
VTEC	Vero toxigenic *E. coli*
WHO	World Health Organization
X^2	Chi-square
μl	Microliter

ABSTRACT

Escherichia coli O157:H7, a common foodborne pathogen in developing nations, is found in raw cow milk. Raw milk facilitates the growth of bacteria due to its nutrient-rich properties, which make it ideal for bacterial growth. The current study aims to determine the prevalence of *E. coli* O157:H7, identify associated risk factors, detect specific virulence genes of *E. coli* O157:H7, and assess the antimicrobial resistance profile in raw cow milk from Jimma Town from March to November, 2023. A simple random sampling technique was used for sample collection. A total of 384 raw cow milk samples were collected from 63 dairy cow farms and subjected to bacteriological, PCR, and disc diffusion methods. A structured questionnaire survey was employed to assess the risk factors. Among the cultured samples, 59 isolates (15.36%) exhibited phenotypic characteristics of *E. coli*. While PCR analysis confirmed 12(3.13%) isolates were positive for pathogenic genes: 5/59 (8.47%) for *stx1* and *eaeA* genes and 7/59 (11.86%) for the *stx2* gene. Based on a multivariable logistic regression analysis the associated risk factors, herd size (P = 0.004, OR = 4.95), type of floor (P = 0.001, OR = 5.76), parity (P = 0.049, OR = 2.31), age (P = 0.004, OR = 3.51), hand washing practices (P = 0.03, OR = 2.18) and udder hygiene (P = 0.001, OR = 3.12) were statistically significant with *E. coli*. Those confirmed gene isolates were susceptible to Sulfamethoxazole/Trimethoprim (83.3%), chloramphenicol (91.7%), Gentamycin (91.7%), and resistance to Erythromycin (100%), Penicillin-G (100%), Streptomycin (83.3%), Oxytetracycline (58.3%), and 75% of the isolates developed multiple antimicrobial resistance. Generally, *E. coli* O157:H7 is the major contaminant of the raw cow milk in the current study area. Therefore, awareness on hygienic milk handling and avoiding the consumption of raw milk, rational antimicrobial usage and surveillance of phenotypic and genotypic profiles of pathogenic *E. coli* should be carried out to reduce financial loss and health issues.

Keywords: *Antimicrobial, E. coli O157:H7, Jimma town, PCR, risk factor, virulence gene*

1. INTRODUCTION

1.1. Background

Milk or dairy products are nutritious foods that are consumed daily by billions of peoples in our world. It contains beneficial dietary components such as proteins, fats, minerals, and vitamins (Henchion *et al*., 2021; Rozenberg *et al*., 2016). A large number of farm families and workers, as well as a growing number of the general public continue to drink raw and untreated milk (Robinson *et al*., 2019). But raw milk is considered a high-risk food as it is highly nutritious and serves as an ideal medium for bacterial growth. The common predisposing factors for milk contamination by microorganisms are cows, the milking environment, milk handlers with poor personal hygiene, contaminated milking equipment, milk transportation, and water (Lye *et al*., 2013; Alaru *et al*., 2022; Ntuli *et al*., 2023).

Raw cow milk and milk products have been implicated in the transmission of multiple bacterial pathogens such as *Escherichia coli, Salmonella typhimurium*, *Staphylococcus aureus, Listeria monocytogenes, Yersinia enterocolitica, Campylobacter jejuni, Shigella* spp., *Streptococcus* spp., *Brucella spp.* and *Pseudomonas spp.* (Yohannes, 2018; Khasapan e, 2019).

Therefore, *Escherichia coli* is gram negative, facultative anaerobic, rod-shaped and highly motile bacteria that belong to the family Enterobacteriaceae, genus Escherichia and Species *coli* and a normal inhabitant of the intestines of animals and humans (Virpari *et al*., 2013; Beher *et al*., 2022). The majority of *E. coli* strains are nonpathogenic, but a few are very pathogenic. Based on the epidemiological, clinical, and pathogenic characteristics, *E. coli* is classified into different pathotypes: Shiga toxin-producing *E. coli*, enterotoxigenic *E. coli*, enteropathogenic *E. coli,* enteroaggregative *E. coli,* and enteroinvasive *E. coli* (Rúgeles *et al*., 2010). Most of them are causing watery and bloody diarrhea in both animals and humans due to various virulence; *E. coli* O157:H7 has been linked to life-threatening diseases such as hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura in man (Rahman *et al*., 2017).

Strains of pathogenic *E. coli* that are characterized by their ability to produce Shiga toxins are referred to as Shiga toxin-producing *E. coli*. Which are an important cause of foodborne disease and infections have been associated with a wide range of human clinical illnesses ranging from mild non-bloody diarrhoea to bloody diarrhoea and hemolytic

uremic syndrome (HUS) which often includes kidney failure. A high proportion of patients are hospitalized, some develop end-stage renal disease (ESRD) and some die (WHO, 2019). In susceptible individuals, the infectious dose of *Escherichia coli* O157:H7 might be as low as 10–100 cells (Williams *et al*., 2017; Rahal *et al*., 2012). Enterohaemorrhagic *E. coli* isolates: Shiga toxins (*stx1* and *stx2*), *intimin* (*eaeA*) and *enterohemolysin (hlyA)* are virulence factors that play a key role in the development of above mentioned disorders (Baranzoni *et al*., 2016; Bruyand *et al*., 2019). Ruminants, especially cattle, are the main reservoirs for the highly virulent STEC O157:H7 strain (Oporto *et al*., 2019).

Humans may acquire STEC/VTEC infections primarily from consumption of undercooked meat, raw milk, dairy products, and water contaminated with faeces of animals (Akinjogunla *et al*., 2020). Hence, the detection of *E. coli* O157:H7 from milk and milk products is an indicator of a possible public health risk. The source of *E. coli* O157: H7 in milk and milk products could be the mammary gland, unhygienic milk handling, and milk processing, and marketing (Dehkordi *et al*., 2014; Sarba *et al*., 2023). To recognize and distinguish between the various types of pathogenic *E. coli* a wide variety of methods are employed.

The presence of virulence characteristics in laboratory can be identified by culturing on specific growth media, assessing biochemical tests, serotyping and screening methods. However, these conventional methods are not rapid and reliable enough to distinguish such pathogenic strains which lead to the application of the molecular diagnostic technique for more effective detection and characterization of *E. coli* O157:H7(Manning, 2010). Molecular based approaches are uniquely favorable because of their sensitivity, selectivity, and quick findings (Parsons *et al*., 2016). Generally, PCR is considered to be the most sensitive mean of determining of *E. Coli* O157:H7 in milk samples (Zhou *et al*., 2017).

Antimicrobials are used in the food production process to enhance feed efficiency, improve growth, and prevent and control disease in animals used for food production (O'Connor *et al*., 2020). In order to reduce morbidity, mortality, and the financial impact of bacterial infections, antibiotics have long been used in both human and veterinary medicine (CDC, 2005; Jerab *et al*., 2022). The use of these antibiotics at low doses for long periods of time to feed animals can result in the selection and spread of antibiotic

resistance to other microbes in the food chain (Lima *et al*., 2017). The increased prevalence of resistant bacteria is associated with the indiscriminate and expanding use of antibiotics (CDC, 2005). These days, one of the most pressing issues facing public health is antimicrobial resistance (CDC, 2019). Therefore, raw milk can also facilitate the transmission of antibiotic resistance genes to the human gastrointestinal tract (Tark *et al*., 2017).

The fast development of global market for food industries is followed by, the emerging of several food borne pathogen which revealed a serious health hazard for both developed and developing countries (Beneduce *et al*., 2007). The consumption of raw milk and its derivatives is common in Ethiopia, which is not safe for consumers from a health point of view as it may lead to the transmission of various diseases (Abebe *et al.*, 2020). Even though milk from a healthy udder contains a few bacteria, it picks up many more by the time it leaves the teat of the animal, depending on the hygienic level exercised during milking. These microorganisms are indicators of both the manner of handling milk from milking until consumption and the quality of the milk (Shunda *et al*., 2013).

1.2. Statement of Problems

Globally, dairy products have been linked to approximately 4 % of food-borne illnesses; this prevalence rate is higher in developing countries. The annual economic impact crosses $4 billion USD (de Klerk and Robinson, 2022). When found in milk, *E. coli* is a common milk-borne infection that indicates fecal contamination. Some people prefer to drink unpasteurized milk despite the well-documented risk of enteric infection (Asfaw *et al*., 2022). It is a risky microorganism due to the great zoonotic importance of some of its strains, such as *E. coli* O157:H7 (Surendran, 2017). Generally, foodborne *E. coli* O157:H7 is estimated to cause 2.8 million acute illnesses each year worldwide (Majowicz *et al*., 2014).

In Ethiopia, *E. coli* is a common problem on dairy farms, resulting in financial losses for dairy products. As a result, effective prevention and control of the diseases are necessary. However, control of the diseases is a difficult task since antimicrobial agents, which were the most powerful tool to control them, have become unsuccessful because of the development of drug-resistant *E. coli (*Disassa *et al*., 2017; Mesele *et al*., 2023; Abebe *et al*., 2023). In the present time, AMR, both in human and veterinary medicine, has reached

alarming levels in most parts of the world and has been recognized as a significant emerging threat to global public health and food security (Almansour *et al*., 2023). Moreover, the likelihood that milk and milk products may act as a vehicle for antibiotic-resistant bacterial genes has become a concern for the food industry and a public health issue (Liu *et al*., 2020).

Several studies reported from different parts of Ethiopia indicated that pathogenic *E. coli* O157:H7 isolated from bovine raw milk with a prevalence range of between 1.5% and 14.3% reported by Gudisa *et al*. (2022) and Gugsa *et al*. (2022), respectively, developed high levels of resistance to Tetracycline, Amoxicillin, ampicillin, penicillin-G, Streptomycin, Gentamicin, and Cefotaxime (Ababu *et al*., 2020; Disassa *et al*., 2017; Abebe *et al*., 2023; Dejene *et al*., 2022; Sarba *et al*., 2023; Gudisa *et al*., 2022). But there is no published material on prevalence, antimicrobial resistance, and associated risk factors of *E. coli* O157:H7 occurrence from raw milk of dairy cow farms in Jimma town. Therefore, the study aims to gain a better understanding of the prevalence, molecular characterization of the virulence gene, antimicrobial resistance, and risk factors associated with *E. coli* O157:H7 in the raw milk supply in Jimma Town. This information can be used to develop strategies for reducing the risk of *E. coli* O157:H7 contamination in the milk chain and protecting public health. Therefore, the present study was designed with the following objectives:

1.3. Objectives of the study

- To assess the prevalence of *E. coli* and *E. coli* O157:H7 in Raw Milk of apparently healthy Dairy Cow.
- To estimate potential associated risk factors with *E. coli* in raw cow milk.
- To characterize molecularly *E. coli* O157:H7 strain of specific virulence genes (*stx1, stx2*, *eaeA* and *hlyA gene*) from raw cow milk of dairy farm.
- To determine Antimicrobial resistance profile of *E. coli* O157:H7 isolates

2. LITERATURE REVIEW

2.1. Historical Background

Theodor Escherich, a Bavarian doctor, first described *Escherichia coli* on 14 July 1885 in a lecture to the Society for Morphology and Physiology in Munich. Over 15 months, Escherich observed and isolated 19 different bacteria in Otto von Bollinger's bacteriology laboratory (Escherich, 1988; Foster-Nyarko and Pallen, 2022). But at the time *E. coli* was known as Bacterium coli commune until 1919 when it was renamed to honour Theodor Escherich who firstly isolated and described it. This name was proposed by Castellani and Chalmers in 1919 to honor Escherich, and was officially adopted in 1958 by the Judicial Commission of the International Committee on Systematic Bacteriology. It was subsequently included in the Approved Lists of Bacterial Names in 1980 (Castellani and Chalmers, 1919; Skerman *et al*., 1980; Martinson and Walk, 2020).

E. coli O157:H7 was first detected as a pathogen affecting humans in 1982 after a hemorrhagic colitis outbreak in the United States, and has since been a consistent source of diarrheal illness. In 1993, a multistate outbreak of O157 linked to undercooked beef patties from a fast-food chain exposed O157 as an important source of foodborne illness and initiated more thorough public health action plans such as recalls and food safety information (Rangel *et al*., 2005). After the first outbreak in 1982, *E. coli* O157:H7 has become the most widely known EHEC strain. Reports have shown that an *E. coli* O157:H7 strain that was involved in an outbreak of Hemorrhagic colitis in the United States produced Shiga toxins (Saxena, *et al*., 2015).

2.2. Etiology

Escherichia coli is one of the bacteria that is commonly linked to foodborne illness. It is a Gram-negative, motile, non-spore forming, flagellate, rod shaped, and facultative anaerobi c bacterium belonging to the class Gammaproteobacteria, order of Enterobacterales, family Enterobacteriaceae Genus Escherichia and Species *coli*. Most *E. coli* strains are harmless; however, the *E. coli* O157 strain is a significant foodborne pathogen with a low infective threshold and high resistance to treatment (Wei *et al*., 2018). It is short rods of approximately 1.1 -1.5 x 2.0-6.0µm (Ihorimbere, 2020) with flagella (peritrichous), ferments lactose, with a number of serovars (160 antigenic types O, 56 types H, 80 types K/Vi). *E. coli* has several types of strains from the strains that produce the shiga toxin that

have been referred to as Verotoxin-producing *E. coli* (VTEC), Shiga-toxigenic *E. coli* (STEC), and enterohaemorrhagic *E. coli (*EHEC). O157:H7 is the most common antigenic type of these bacteria, followed by O111 and O26 (Constable *et al*., 2017).

Sequence analysis of the *E. coli* genome was first reported in 1997. Since then, more than 4800 *E. coli* genomes have been sequenced (Jan *et al*., 2009). *Escherichia coli* can replicate in every 20 minutes. This rapid growth makes it suitable for observations of the evolution of microorganisms and research on long-term experimental evolution of more than 50,000 generations (Tenaillon *et al*., 2016).

The genome of *E. coli* is often shaped by lateral gene transfer (Odhong' *et al*., 2019). The pan-genome of *E. coli* contains more than 18000 different genes while a single cell typically carries between 4000 and 5000 genes and only around 3000 is shared within different isolates. This results in high genetic diversity among *E. coli* (Poirel *et al*., 2018), which leads to phenotypic diversity and various levels and ways *E. coli* can be harmful to its hosts. While *E. coli* is mostly seen as a commensal, many types of pathogenic *E. coli* have been described. The variety of *E. coli* led to the need for subspecies classification schemes and several of them have been developed until these days; while early studies used serology method and genotyping, whole-genome sequencing approach is commonly used nowadays (Martinson and Walk, 2020). Using whole-genome sequencing greatly helps to understand the core and accessory genomes of pathogenic and nonpathogenic *E. coli* isolates deeper (Poirel *et al*., 2018). It also allows to perform large scale comparison which can lead to improvements in some long recognized schemes (Abram, 2021).

2.3. Epidemiology

2.3.1. Geographical distribution

Over 250 bacterial species are reported to cause foodborne illnesses in humans among which *E. coli* is considered to be the cause of most of these illnesses (de Jesús Cortés Sánchez and de la Paz Salgado Cruz, 2017). Among the range of zoonotic foodborne *E. coli* has been associated with milk and some of dairy products (Abebe *et al.,* 2014). *E. coli* O157:H7 infections occur worldwide and have also been reported on all continents except Antarctica (CFSPH, 2009). *E. coli* O157:H7-related diarrheal cases have been reported from a number of African countries, including South Africa, Swaziland, Central African

Republic, Kenya, Ethiopia, Uganda, Gabon, Nigeria, and Ivory Coast (Mashood *et al*., 2006).

Many outbreaks of *E. coli* O157:H7 infections were caused by consumption of unpasteurized (raw) milk or dairy products manufactured using raw milk (Doyle *et al*., 2020). It has been found that the epidemiology of each pathogenic strain and species of *E. coli* varies. These harmful organisms were discovered in numerous animal reservoirs, where they spread both inside and to other animals (Croxen *et al*., 2013). In 2015, the data from the World Health Organization revealed that approximately 600 million people fall ill every year from food contaminated with pathogenic bacteria and their toxins, 420,000 of which die (Haddad *et al*., 2018). Among these food-borne pathogens, *E. coli* plays an important part; however its pathogenic potential is not limited (Odhong' *et al*., 2019).

Pathogenic intestinal *E. coli* caused 8 to 10% of cases of diarrhea in children and *E. coli* O157: H7 causes 2,801,000 serious illnesses each year, with a global incidence rate of 43.1 cases per 100,000 person years that includes all of Africa. The annual mortality rate in Africa, Asia, and America is around 4.6 to 6 million people (Lupindu, 2017). *E. coli* O157: H7 are toxigenic strains that cause life threating syndromes and resulted in an estimated 74,000 cases and 61 deaths annually in USA as a result of outbreaks arising from consumption of contaminated cattle products, especially raw milk and meat (Bedasa *et al.,* 2018).

Isolation of the O157:H7 strain that produces Shiga toxin in humans, animals, and food together with the environment has been documented all around the African continent. The first human infection was detected and documented as early as 1990 in the city of Johannesburg, South Africa (Beyi *et al*., 2017). However, in Central Africa during 1996, pathogenic bacteria were isolated from people with hemorrhagic colitis a resulting in mortality. STEC O157: H7 isolation in people was documented in 1998 after the development of bloody diarrhoea in Cameroon (Havelaar *et al*., 2015).

In East Africa, pathogen isolation was reported in Ethiopia, Kenya, and Tanzania. Ethiopia ranks second only to Nigeria in the outbreak of zoonotic diseases on the African continent (Carattoli *et al*., 2014). Ethiopia is in sub-Saharan Africa and is facing the world’s largest outbreak of food poisoning. *E. coli* O157 is the leading cause of food poisoning that threatens to prolong lifespan in Ethiopia (Beyi *et al*., 2017).

2.3.2. Reservoir of *E. coli* O157:H7

E. coli is a species of bacteria naturally occurring in digestive tracts of warm blooded mammals. The major reservoirs are healthy domesticated ruminants, primarily cattle and, to a lesser extent, sheep and possibly goats (Lennon, 2020). Feces from cattle, sheep, goats, pigs, dogs, deer, horses, cats, rodents, fish, bats, and avian species can all be identified by the O157:H7 strain. Cattle and sheep are both the primary etiological agents of O157:H7 infection in humans as well as the main reservoirs of the O157:H7 serotype strain of *E. coli* 103, 104 (Ferens and Hovde, 2011).

2.3.3. Growth characteristic of *E. coli* and inactivation

The well-known Shiga toxin-producing bacterium *E. coli* O157:H7 is a member of the Enterobacteriaceae family. They are reasonably tolerant to different extreme conditions like; minimum pH for growth, heating, irradiation, antimicrobials, ruminant gastrointestinal tract fluids, and even cool nutrient-dilute water as well (Vickers, 2017). *E. coli* is a typical mesophile growing from 7-10 ^{0}C up to 50^{0}C with an optimum around 37 ^{0}C (Adams and Moss, 2008; Xia *et al*., 2010), and it can grow both aerobically and anaerobically and be motile or non-motile depending on flagella presence (Croxen *et al*., 2013). 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. *E. 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 (Ghimire, 2018). Although some ETEC strains growing at temperatures as low as 4 °C have been reported. It shows no marked heat resistance, with a D value at 60 ^{0}C of the order of 0.1 min, and can survive refrigerated or frozen storage for extended periods. A near neutral pH is optimal for growth, but growth is possible down to pH 4.4 under otherwise optimal conditions. The minimum aw for growth is 0.95 (Adams and Moss, 2008).

2.4. Pathogenic *Escherichia coli*

The pathogenicity of *E. coli* is dependent on the control and interaction between a number of virulence factors, and it is regulated by environmental parameters such as host species, host health status, interaction with other bacteria species (Clermont *et al.,* 2011). On the other hand, *E. coli* is a large and diverse genus of bacteria of the family Enterobacteriaceae typically found in the lower intestine of a variety of warm-blooded species, including cattle and humans (Hassan *et al*., 2021). *E. coli* with virulence genes belong within the "pathogenic form" defined by the disease they cause, but individual strains can have virulence attributes in excess of one pathotype. The pathogenic group of *E. coli* are divided into six groups on the basis of their virulence properties such as enterotoxigenic (ETEC, causative agent of diarrhea in humans, pigs, sheeps, goats, cattle, dogs and horses), enteropathogenic (EPEC, causative agent of diarrhoea in humans, rabbits, dogs, cats and horses), enteroinvasive (EIEC, found only in humans), verotoxigenic (VTEC, found in pigs, cattle, dogs and cats), enterohaemorrhagic (EHEC, found in human, cattle, and goats) and enteroaggregative *E. coli* (EAggEC) which found only in human (Xia *et al*., 2010; Croxen *et al*., 2013). Typical virulence factors were associated with these individual pathotypes and allowed them to be distinguished from commensal *E. coli*. These characteristic virulence factors play a major role in the pathogenesis of *E. coli* strains that carry them (Köhler and Dobrindt, 2011). For example, a common pathogenesis mechanism related to carriage of a chromosomally encoded pathogenicity island which enables them to adhere to intestinal epithelial cells and to cause lesions is shared between EPEC and EHEC (Odhong' *et al*., 2019). ExPEC commonly carry certain virulence associated genes which encode production of toxins, hemolysins, serum resistance, adhesins, capsules, invasins, proteases, lipopolysaccharides or are related to iron acquisition (Azam *et al*., 2020).

Escherichia coli O157:H7 was introduced into the cattle population by the means of feed. Grain- fed cattle seem to have a significantly higher level of *E. coli* O157:H7 compared to grass fed cattle. A grain diet turns a cattle's rumen into an ideal habitat for *E. coli* O157:H7 while the lethal strain of *E. coli* cannot survive for long periods of time on a grass fed rumen. Not only is there an increased number of *E. coli* O157:H7 in rumens of grain-fed cattle, but also in the tons of manure they produce each year. Approximately a billion tons of contaminated manure is produced, by agricultural animals, each year and

often ends up in locations other than pastures, causing bacteria to travel to other habitats, animals, and environmental resources. Ultimately, this brings the pathogen closer to humans and finished food products with a chance of increasing infection rates in the human population (Pollan, 2006; Franz *et al*., 2005).

In humans, after ingestion of the *E. coli* O157:H7 strain, the bacteria travel through the gut and attach to the inside surface of the large intestine. The bacteria produce toxins that destroy the cells lining the colon and cause inflammation of the intestine. The toxins may be absorbed and spread to other areas of the body through the blood stream. The toxins can damage cells lining blood vessels (endothelium), and this damage can lead to bleeding, blockage of vessels by thrombosis, and profound inflammation. This can happen not only in the intestine, but also to the small blood vessels of other organs, such as the kidney or brain, causing severe pathophysiology. The incubation period for infection with *E. coli* O157, i.e., the period between ingesting the organism and the onset of symptoms, is between 2 to 12 days and most commonly around three or four days (Tarr *et al*., 2005).

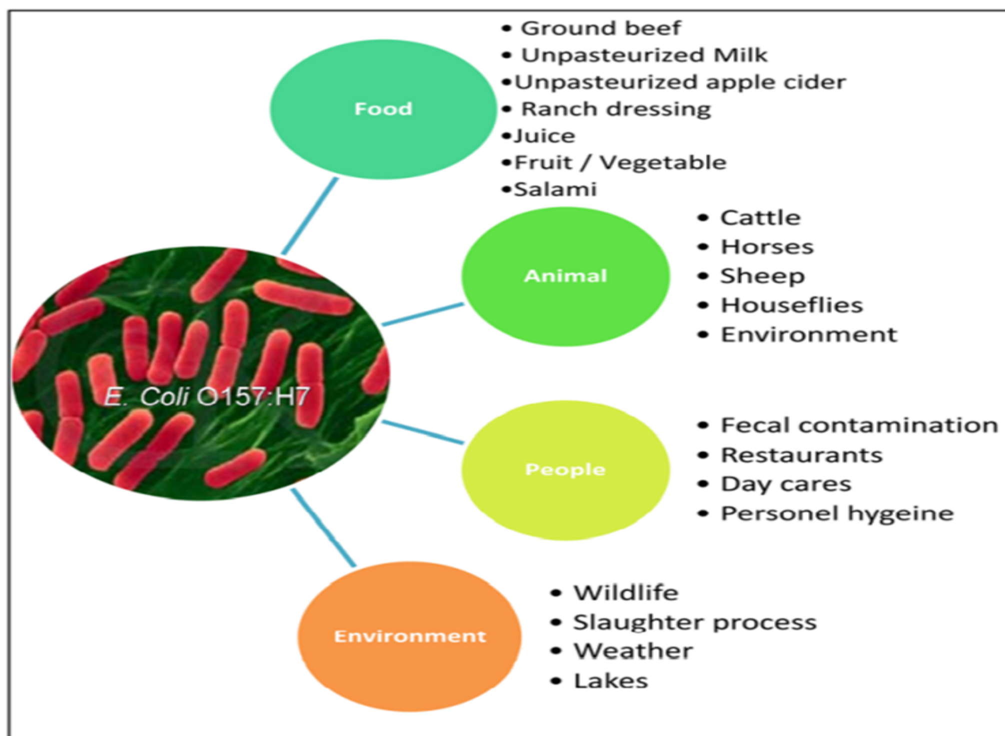

Figure 1: Possible routes of how *E. coli* O157:H7 can enter the human.

Source: Page, 2009

2.5. Virulence Factors

It has been increasingly clear that a variety of distinctively harmful strains of *E. coli* exist. Pathotypes are divisions of pathogenic *E. coli* strains based on their virulence genes (Di Pietro *et al*., 2011). The most prevalent *E. coli* serotypes recovered from mastitis milk are O55, O111, 0124, 0119, 0114, 026, O157, and O44 (Helmy *et al*., 2011).

E. coli pathovars, such as enteropathogenic *E. coli* (EPEC), and Shiga toxigenic *E. coli* (STEC) have been observed in dairy herds (Debroy *et al*., 2018), milk (Van Kessel *et al*., 2011) and other dairy products (Solomakos *et al*., 2009) with a unique set of virulence and colonization factors encoded in the chromosome or in episomal structures (Rúgeles *et al*., 2010). Although there is a large variability in the nucleotide sequences of these genes, in general, these pathotypes encode genes for certain virulence factors that are linked to the attachment and secretion of hemolysins and enterotoxins (Li *et al*., 2018).

2.6. Economic factor

Various strains of *E. coli* infections lead to diseases such abdominal sepsis, urinary tract infections, blood poisoning diarrhoea, haemolytic uremic syndrome, and hemorrhagic colitis, which reduce animal production, especially in the chicken industry(Arshad *et al*., 2006). It is an opportunistic pathogen responsible for causing clinical bovine mastitis (Hinthong *et al*., 2017), causing losses and affecting animal welfare. In clinical bovine mastitis, the signals range from mild to severe, such as pain, fever and swelling, changes in rumination rate, hydration and behavior and there can be animal death. Besides, there are changes in the milk, with the occurrence of flakes and clots, altered color and consistency (Adkins and Middleton, 2018).

Considering the source of the agent, mastitis can also be classified as contagious, where the infection is mostly spread between animals and by milking equipment, or environmental, when it is brought on by ubiquitous environmental pathogens that are typically found in bedding material, dust, flies, and feces, such as *E. coli* (Klaas and Zadoks, 2018). The *E. coli* infection is a disease of economic importance because of medical and outbreak control, and productivity loss (Lu and Breidt, 2015). Due to *E. coli* infection milk, meat and wool production could decline dramatically (Aklilu *et al*., 2013).

2.7. Shiga Toxin Producing (STEC)

Shiga toxins are named after the Japanese microbiologist Kiyoshi Shiga who in 1898 described the bacteria Shiga dysenteriae (Lampel *et al*., 2018). Shiga toxin producing *E. coli* can also be referred to as Verocytotoxin producing *E. coli* (VTEC) for its toxigenic effect to Vero cells (Xia *et al*., 2010). It is a food borne pathogen that causes human gastrointestinal infections across the globe, leading to kidney failure or even death in severe cases (Ray and Singh, 2022).

Shiga-toxin producing *E. coli* (STEC) and Enterotoxigenic *E. coli* (ETEC) were associated with several life-threatening food-borne outbreaks worldwide (Majowicz *et al*., 2014). The term enterohemorrhagic *E. coli* is applied to those STEC serotypes that have the same clinical, epidemiological and pathogenetic features associated with the prototype strain *E. coli* O157:H7. The high virulence of STEC strains O157:H7 is not only dependent on the virulence factors but partially also on the pathogen's ability to survive environmental stress conditions, such as resistance to low pH levels found in the gastrointestinal tract, which contributes to its extremely low infectious dose of 10–100 bacteria or lower (Viazis and Diez-Gonzalez, 2011).

STEC produces cytotoxins encoded by stx1 and stx2 genes. These cytotoxins are associated with serious human illnesses as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) that usually end up with fatal consequences. STEC causes around 3 million cases of acute illness and over 200 deaths each year (Majowicz *et al*., 2014). One of the largest HUS outbreaks ever recorded was caused by a newly identified *E. coli* strain that produces the Shiga toxin, which was found in Germany. The perpetrator belonged to serotype 104:H4 which contained the virulence factors of typical EAEC and a Stx-2 producing pro-phage, but lacked the LEE pathogenicity island. This discovery has led to the emergence of a new pathotype for which the name Entero-Aggregative-Haemorrhagic *E. coli* has been suggested (Brzuszkiewicz *et al*., 2011). This bacterium produces a toxin structurally and antigenically identical to *E. coli* produced Stx1. Shiga toxins are an AB5 toxin type consisting of a monomeric, enzymatically active A subunit non-covalently linked to a pentameric B subunit responsible for binding to the glycosphingolipid globotriaosylceramide (Gb3, also known as CD77 or Pk blood group antigen), a specific receptor on the cell surface (Johannes and Römer, 2010).

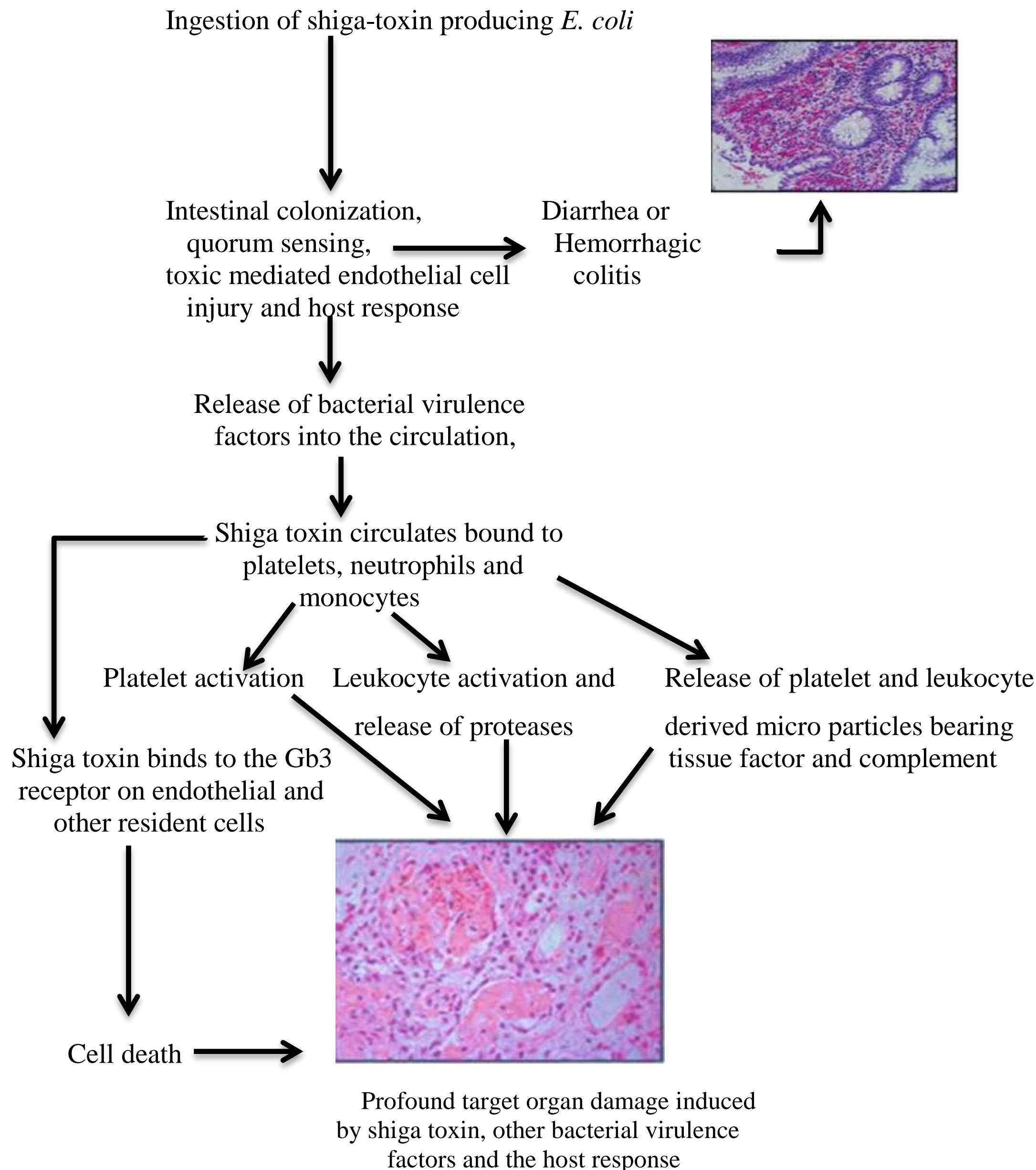

Figure 2: Schematic presentation of the pathogenesis of Shiga toxin-induced disease.

Source: Karpman, 2012

2.8. Modes of Transmission

Milk is an animal-based food that has the potential to act as a reservoir for the spread of infectious bacterial diseases. The presence of *E. coli* bacteria in raw milk is often reported with regard to sources of food-borne disease (Odenthal *et al*., 2016).

Human infection with *E. coli* O157:H7 occur primarily transmitted through contaminated foods and it is classified as a food-borne pathogen. Various contaminated foods can transmit this pathogen including unpasteurized dairy products; raw milk, different meat products, ground beef, vegetables, unpasteurized fruit juices, water, and direct contact

with ruminant feces are the main origin of significant outbreaks around the world (Heiman *et al*., 2015).

The infectious dose of *E. coli* O157:H7 can be as low as 10 cells. Consumption of contaminated food, mainly undercooked ground beef and unpasteurized milk, is the primary source of *E. coli* O157:H7 infection. The main asymptomatic reservoirs for this pathogen are thought to be cattle. Carried in their gut, feces, and milk, cattle can contain this Shiga toxin-producing *E. coli* in ranges from 102 to 105 CFU/g. Ground meat should be cooked to 165°F in order to ensure that *E. coli* O157:H7 has been killed (Marder *et al*., 2018).

EPEC can be transferred through contaminated foods such as vegetables, cheese, tuna fish, potato, macaroni salads, and untreated water and through toys, rubber nipples and fomites among children. EAEC can be transmitted through food. The method of transmission of DAEC is not yet identified (Meng and Schroeder, 2007). Transmission of pathogenic *E. coli* infection from person to person may occur because of unhygienic measures. It can spread within community like families and close contacts through oral route especially among children (Vickers, 2017).

2.9. Clinical Sign

Escherichia coli is a bacterium that lives in both human and animal intestines. The majorities of *E. coli* strains are regarded as typical gut flora and are harmless. But some strains, like *E. coli* O157:H7, have the ability to produce toxins that can cause severe illness (Wang *et al*., 2014). Symptoms of *E. coli* O157:H7 infection includes diarrhoea, abdominal pain, headache, nausea and vomiting. Typically, the infection starts with one to three days of non-bloody diarrhoea that may be preceded by bouts of severe abdominal pain. In up to 90% of cases the diarrhoea becomes bloody and the patient often suffers severe abdominal cramps (Tarr *et al*., 2005). It is well known to cause diarrhea; hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), a serious long-term complication that primarily affects children and can cause kidney failure and death. In adults can present with thrombotic thrombocytopenic purpura (TTP), a severe and often fatal condition similar to hemolytic uremic syndrome (Manning, 2010).

2.10. Antibiotic Resistance

Antibiotic resistance is undoubtedly one of the most unfortunate problems the public health sector has to face. Worldwide, the incidence of resistant bacteria is rapidly increasing, even in nations where the use of antibiotics is strictly regulated. Multi-drug resistant (MDR) bacteria, which may be resistant to three or more antibiotic families and have few treatment options available for potential illnesses caused by them, are particularly concerning. Because of the potentially difficult and extended nature of the therapy, this phenomenon puts human health and lives at peril and increases expenditures (Saputra *et al.*, 2017). The WHO sees fight with antibiotic resistance as one of its priorities as it predicts that in 2050, there will be more deaths caused by infections related to resistant bacteria than by cancer and cardiovascular diseases combined based on the expected increase of resistance prevalence (https://www.who.int/).

A major public health issue worldwide is the rise in bacterial antibiotic resistance. Antibiotic-resistant bacteria have the potential to cause more serious infections than susceptible bacteria, and they have been linked to longer hospital stays, longer disease duration, higher mortality, and considerable economic effect since they drive up healthcare expenses (Patel *et al.*, 2020). It was estimated that 700,000 people die each year from infections with antibiotic resistant bacteria and, sadly, one third of deaths is represented by children of 5 years' age or younger (Patel *et al.*, 2020).

Moreover, bacteria which carry antibiotic resistance determinants are not limited to clinical settings. They can be found in food-producing animals, companion and wild animals as well as in the environment (Poirel *et al.*, 2018) and they are able to circulate between the different niches which makes the whole situation even more challenging to manage and monitor (Sumudumali *et al.*, 2021). Therefore, the resistance monitoring should not be limited only in relation to humans but utilize the One Health approach (Van Puyvelde *et al.*, 2018). *E. coli* represents one of the main reservoirs of antibiotic resistance genes which may contribute to failure of treatment in veterinary and human medicine and it can pass them horizontally even to different bacterial species (Poirel *et al.*, 2018).

2.11. Diagnosis

2.11.1. Clinical diagnosis

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

2.11.2. Isolation

The refrigerated samples were thawed at room temperature for 4 to 6 hours before processing the specimen. 1mL of each milk sample was added into 9mL (1: 9 ratio) of buffered peptone water (HiMedia Laboratories, India) in a test tube, homogenized for 2 minutes, and incubated at 35- 37°C for 20-24 hours for enrichment to increase recovery of the organisms (WOAH, 2016). The culture was then inoculated onto MacConkey agar (Blulux laboratories, USA) for isolation of *E. coli* and incubated aerobically at 37 °C for 24 hrs. The plates were observed for the growth of suspect *E. coli* colonies (smooth, circular, pink colonies). Specimens from the isolated colonies were picked and sub-cultured on Eosin Methylene Blue (EMB) agar (HiMedia Laboratories, India) and incubated at 37 °C for 24 hrs. Bacterial colonies which show the typical characteristic metallic sheen were assumed as *E. coli* (Gudisa *et al*., 2022). Simultaneously, a specimen from the same colony was picked, stained with Gram’s stain, and examined for stain and morphological characteristics using bright-field microscopy. Suspected colonies of *E. coli* (pinkish color appearance on MacConkey agar, metallic sheen on EMB, and Gram-negative rod-shaped) were then inoculated on nutrient agar and incubated aerobically at 37 °C for 24 hrs for the biochemical test.

Detection of *E. coli* O157: H7 is dependent on distinguishing the pathogenic serotypes from normal fecal flora containing commensal strains of *E. coli* (Battisti *et al*., 2006; Woynshet, 2014). Fortunately, *E. coli* O157:H7 has two unusual biochemical markers; delayed fermentation of D-sorbitol and lack of β-D-glucuronidase activity, which help to phenotypically separate *E. coli* O157: H7 isolates from nonpathogenic *E. coli* strains. One of these markers (delayed sorbitol fermentation) enables to develop of several selective media (Sorbitol-MacConkey) which aid in the initial recognition of suspicious colonies isolated from bloody stools (Woynshet, 2014).

E. coli O157 is identified by culture on selective indicator media (Sorbitol MacConkey or the same agar containing cefixime and tellurite) (Pennington, 2010). SMAC containing Cefixime and Tellurite (CT-SMAC) provides highly selective recovery of *E. coli* O157:H7 from other *E. coli* and enteric bacteria. Currently, CT-SMAC is widely used to isolate *E. coli* O157:H7 followed by PCR or latex agglutination confirmation. However, the use of CT-SMAC is not recommended for detection of non-O157 EHEC because most non-O157 EHECs that produce Shiga toxins behave physiologically the same as other commensal *E. coli* strains (Abreham *et al*., 2019). Latex agglutination test is often used for the rapid identification of *E. coli* O157:H7. The test is best used in conjunction with Sorbitol MacConkey Agar. A positive result is indicated by agglutination with the test reagent, whilst the control reagent should appear milky and smooth (Al-Dragy and Baqer, 2014).

Immunoassays and PCR technology have led to more rapid detection of *E. coli* in stools, food, and water. Techniques included in this category are PCR and DNA based techniques, immunomagnetic separation, and enzyme-linked immunosorbent assays (ELISAs) (Bavaro, 2009).

2.11.3. Molecular detection

PCR phase

Many public health laboratories utilize PCR tests to detect the stx1 and stx2 genes in order to diagnose and confirm STEC infection. These assays can differentiate between stx1 and stx2, depending on the primers that are used (Zaki and El-Adrosy, 2007). There are three phases of PCR: these are Denaturation, Annealing and Elongation, which are repeated in each cycle of the PCR. Denaturation: when a double stranded DNA molecule is sufficiently heated (94°C), the hydrogen bonds holding together the double helix are

disrupted and the molecule separates or denatures into single strands. This allows the primers access to the single stranded DNA templates, Annealing: the reaction mixture is cooled (about 50°C) to allow primers to select and bind(hybridize) to their complementary positions on the ssDNA template molecules, which is the exact sequence of nucleotides that flank the area of interest, and Elongation: when the ssDNA/primer solution is heated to 72°C, the heat stable polymerase reads a template DNA in the 3'-5' direction and synthesis a new complementary template in the 5'-3'direction, using free dNTP's as building blocks in the presence of PCR buffer (Viljoen *et al.*, 2005). With each repetition of this cycle, the target is doubled and after about 30 cycles, the reaction will yield in excess of one million copies of the target DNA fragment, which make it easily detected by conventional methods such as gel electrophoresis due to the DNA (Wittwer and Makrigiorgos, 2018).

Multiplex PCR (m–PCR)

The lack of single markers that give unambiguous identification for O157:H7 is one restriction of single gene PCR tests. This is partly due to the genetic similarity of *E. coli* O157:H7 to the majority of other *E. coli* strains frequently found in the gastrointestinal tract of humans and other animals, as well as the fact that the majority of the virulence-related genes routinely identified in non-O157 *E. coli* isolates are targets for PCR amplification (Levin, 2009). But this problem is solved by Multiplex PCR assay which allows several targets to be co-amplified simultaneously in the same reaction by combining several primer pairs (Deisingh and Thompson, 2004). PCR has been used increasingly for rapid, sensitive, and serotype-specific detection of low levels of *E. coli* O157:H7 in feces, foods, water, and environmental samples. Several serotype specific genes such as rfb, uidA and fliC. Also virulence associated gens like *eaeA, hlyA, stx1* and *stx2* have been used for genotyping and detection of *E. coli* O157:H7 in m-PCR formats (Manning, 2010). The routine multiplex PCR procedure is as follows, first extract the DNA by boiling method. Secondly, prepare the master mix, which contain all the input of PCR apart the template. After preparation, dispense the mix into PCR reaction tube followed by addition of boiled culture (crude DNA extract). Then run the PCR using cycling parameters of the standard time and temperature. After this, the post PCR analysis began by running the PCR product on agarose gel and stained by ethidium bromide followed by trans-illumination (WOAH, 2008).

Real-time PCR

Conventional PCR relies on amplification of the target gene in a thermo cycler, separation of PCR products by gel, followed by visualization and analysis of the resultant electrophoresis patterns, which is a process that can take a number of hours. But the development of real-time PCR, which uses fluorescence to detect the presence of a particular gene in real time, has greatly increased the sensitivity and speed of PCR-based detection methods without the need electrophoresis. Most of the R-PCR assays that have been developed for the identification of *E. coli* O157:H7 are based on the detection of *stx* encoding *Stx*-toxins, *eae*-intimin and O-antigen (Sidari and Caridi, 2011). These RT-PCR assays offer the opportunity to quantify the absolute and relative amounts of *E. coli* O157:H7 in complex sample matrices. There are different modes of RT-PCR among them the most commonly used RT-PCR systems are the TaqMan PCR (Levin, 2009).

2.12. Treatment

The use of antibiotics in the treatment of STEC infection is controversial (Ochoa *et al*., 2007). In vitro data have demonstrated that ciprofloxacin or sub inhibitory concentrations of trimethoprim-sulfamethoxazole induce shiga toxin production by *E. coli* O157:H7. Therefore, treatment is mainly supportive to limit the duration of symptoms and prevent systemic complications (Lim *et al*., 2010). Clear liquids are recommended for persons with diarrhea to prevent dehydration and loss of electrolytes (Dulo, 2014).

2.13. Control and Prevention

People typically contract *E. coli* O157 disease from cattle; hence a key control is to slaughter cattle in a hygienic manner to avoid feces contaminating raw meat. Controls are only required to stop human infection as there is no production losses associated with infection. The effectiveness of few farm management practices as on-farm control measures has been demonstrated. Super-shedding does, however, have a significant theoretical impact on the management of *E. coli* O157 on farms and, indirectly, the risk of human infection. Super-shedding has not yet been considered in cow *E. coli* O157 management strategies. By identifying super-shedding animals, control measures could be directed at reducing or eliminating high levels of faecal excretion and at drastically lowering the organism's prevalence. The identification and removal of hyper shedding cattle, testing of individual animals prior to movement, and care for colonized animals on

the farm are examples of potential strategies. The use of immunization to reduce the likelihood of colonization could become an option in the medium term. By doing this, the risk of *E. coli* O157 infection in animals and exposure to people would be reduced. We are aware of companies that market vaccines targeted at the control of *E. coli* O157 in cattle in North America and of a United Kingdom research project under Defra-LINK (LK0666). There are also published trials validating these vaccines (Thomson *et al*., 2009; Fox *et al*., 2009). Several vaccines directed against the *E. coli* O157 LPS antigen (Ahmed *et al*., 2006) or Stx epitopes (Gao *et al*., 2009) have been validated in murine models and phase 2 studies, but none so far has proven its efficiency in reducing the risk of EHEC infection in humans (Szu and Ahmed, 2014). A vaccine for cattle has been developed to aid in the reduction of *E. coli* O157:H7 shedding in cattle. Cattle are immunized with the vaccine to protect them from the proteins that are expressed on the surface of *E. coli* O157:H7 cells. These proteins act as a receptor in intestinal walls, allowing the bacteria to colonize and the vaccine has been shown to reduce shedding (Smith, 2014).

The transmission of *E. coli* O157:H7 from animal to human could be prevented by washing hands with soap and running water for 20 seconds before eating or drinking (Ziemer *et al*., 2010). Implementing intervention measures from farm to table will be essential for an efficient control program to significantly reduce *E. coli* O157:H7 infections. A farm's promising intervention strategies include bacteriophage, competitive exclusion bacteria, and focused animal management techniques that target prevalent contamination spots. Customers can help install intervention controls in food processing and handling. Unfortunately, a lot of consumers consume high-risk foods, handle and store food incorrectly, and disregard warnings about known-to-be-unsafe foods (WHO, 2022). Generally, hazard analysis critical control points (HACCP) could be implemented to avoid the transmission of *E. coli* O157:H7 from animal to human. HACCP is the universally accepted food safety management system (Pennington, 2010).

2.14. Public Health Importance

Foodborne pathogens are the leading causes of foodborne human illness and death in the world (Agüeria *et al*., 2018). However, most cases occur in developing countries due to insufficient food management procedures, lack of public awareness, lack or inadequacy of hygiene services, insufficient food safety legislation, ineffective regulatory disciplines and lack of financial resources (WHO, 2019). This is a major global public health concern for humans and livestock. There is a great concern about the proper implementation of hygiene protocol in livestock production systems (Caprioli *et al*., 2014).

Shiga toxin-producing *E. coli* O157:H7 is a significant public health concern, causing severe, sometimes life-threatening, human illness (Niu *et al*., 2009) and major public health concern in North America, Europe, and other areas of the world (Lim *et al*., 2010). O157:H7 strains carrying *stx2* gene along with enterohaemolysin gene are potentially dangerous to human health. *Stx2* producing strains appear to be more commonly responsible for serious complications such as HUS than those only *Stx1* producing (Kiranmayi *et al*., 2010).

E. coli O157:H7 is associated with outbreaks and sporadic cases of hemorrhagic colitis and hemolytic uremic syndrome and other enteric infections all over the world especially in children under 5 years of age (Fernandez and Padola, 2012). The diseases caused by *E. coli* O157:H7 showed much higher hospitalization and fatality rates (Lim *et al*., 2010).

2.15. Status of *E. coli* in Ethiopia

Food-borne diseases are a major concern throughout the world. This is an important problem in developing countries that lack the application of high sanitation management during the collection and processing of cow's milk. *E. coli* contamination found in raw milk may be caused by cross-contamination of milk with impurities or the lack of hygienic measures during milk collection and processing (Tanzin *et al*., 2016). Many studies have been conducted in Ethiopia to identify *E. coli* O157:H7 and the antimicrobial susceptibility profile of the isolates from raw milk and different sample.

TABLE 1: Prevalence of *E. coli* O157:H7 isolated from milk and different sample type in different parts of Ethiopia.

Study area	Sample type	Prevalence %	Author
ATJKD	water, milk, manure, and feces	4.7%	Mesele *et al*., 2023
Modjo town	faeces	4.7%	Mersha *et al*., 2010
	skin swabs	8.7%	
	carcasses before washing	8.1%	
	carcasses after washing	8.7%	
	water samples	4.2%	
Gojo and Shukute	Raw milk	1.5%	Gudisa *et al.*, 2022
Asosa	Raw milk	2.9%	Disassa *et al*., 2017
Bishoftu	Raw milk, yogurt and cheese	3.5%	Bedasa *et al*., 2018
Asella town	Raw milk	8.9%	Abunna *et al*., 2018
Holeta	cottage cheese (Ayib)	2.2 %	Melkamsew *et al*., 2012
Ada Berga	cottage cheese (Ayib)	3.6%	
Holeta, Ambo & Bako	Udder milk, cottage cheese &yoghurt	0.2%	Sarba *et al*., 2023
Holeta	Raw milk	5.2%	Ababu *et al*., 2020
Haramaya University	cattle carcasses	2.65%	Taye *et al*., 2013
Jimma town	Carcass swab	2.77%	Shumi *et al*., 2021
	Meat at butcher shop	1.38%	
	Utensil at slaughter house	1.38%	
Jimma town	Feces	53.5%	Ali *et al*., 2021
Mojo	Raw milk	4.2%	Welde *et al*., 2020
Mekelle	Raw milk, yogurt, and meat	14.3%	Gugsa *et al*., 2022
Dessie and kombolcha	udder Milk	9.6%,	Abebe *et al*., 2023
	tank milk	16.7%	
	beef swab	4%	
	carcass swab	5.6%	
Jimma town	Bovine carcass & Cecal content	9.3% & 7.3%	Haile *et al*., 2017

3. MATERIALS AND METHODS

3.1. Study Area

The study was carried out in Jimma Zone, south-western Oromia, Ethiopia, at a dairy farm in Jimma town. Jimma town is the town of the Jimma zone, which is 352 km from Addis Ababa, the capital city of Ethiopia. Geographically located at 7°41′N latitude, 36°50′E longitude, and an average altitude of 1,780 meters above sea level, it is commonly characterized by warm weather with mean annual maximum and minimum temperatures of 30°C and 14°C, respectively (Alemu *et al*., 2011). The climate of the area is a tropical humid climate characterized by humid tropical with bimodal heavy rainfall that ranges from 1200-2000mm per annual with short and main seasons occurring from mid-February to May and June to September, respectively (GOR, 2006). Jimma Zone is bordered on the south by the southwest Ethiopia region, the northwest by the Buno Bedelle zone, on the north by East Wollega, and on the northeast by the southwest Shoa zone; part of the boundary is with the east by the Gibe River and the central Ethiopia region. It is divided into 21 districts. Jimma town, one of the districts of Jimma Zone, is bordered by four districts, namely in the south by Seka Chekorsa district, in the north by Manna district, in the east by Qarsa district, and in the west by Dedo district.

The Jimma zone has one of the largest livestock populations in Ethiopia. With its livestock population estimated at 2,560,207 heads of cattle, 859,914 heads of sheep, 570,387 heads of goats, 68,664 heads of horses, 21,395 heads of mules, 191,054 heads of donkeys, 2,235,702 heads of poultry, and 532,728 beehives (CSA, 2021).

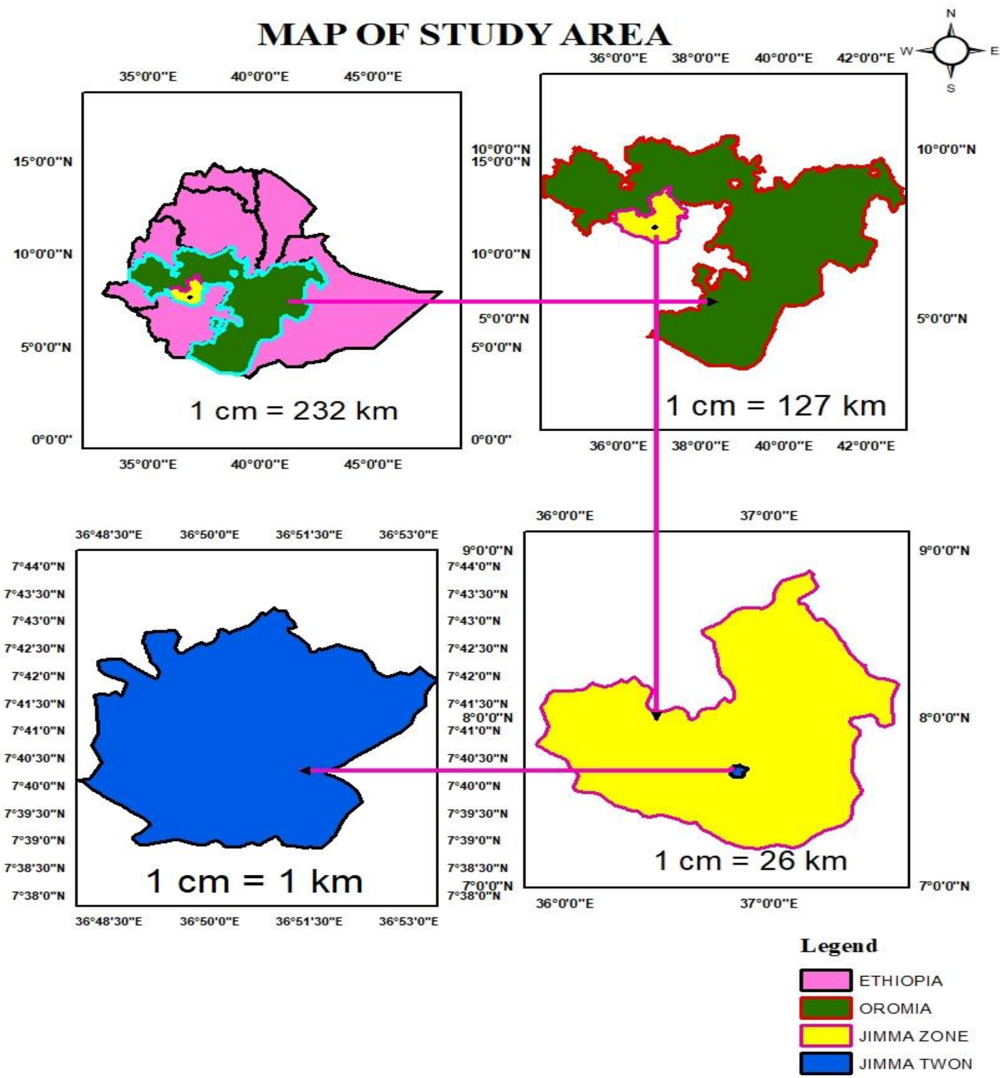

Figure 3: Map of Study Area

Source: GIS (Arc Map)

3.2. Study Design and period

A cross-sectional study design was used for the collection of samples from May 2023 to November 2023 in order to address the objectives of the study.

3.3.The Study Population

The target populations in this study are all apparently health-lactating cows from dairy farms in Jimma town. The dairy animals are kept under intensive and semi-intensive conditions, and two breeds of cattle (cross breed and local breed) were included in the study. The sampling unit was individual lactating cows within a farm under study. Accordingly, total of n=384(348 cross breeds and 36 Local breeds) lactating cows were included in the study.

3.4. Sample Size Determination

The sample size for lactating cows was determined according to Thrusfield (2005) using a 95% confidence interval and a 5% absolute level of precision. The prevalence of the selected bacteria is not known from the raw milk sample in dairy farm level in the study area. Hence, the sample size calculated using the formula given here below was 384.

$$n = (Z\alpha/2)^2 * \frac{Pexp\ (1 - Pexp)}{d^2} = (1.96)^2 * \frac{0.5(1-0.5)}{(0.05)^2} = 384$$

Where **n** = required sample size, ***Pexp*** = Expected prevalence, **d** = Desired absolute precision **5%** with **95%** confidence of level and ***Zα/2*** = statistic for a level of confidence, which is **1.96**. In Jimma town dairy farm, there is no previous study on this title from raw cow milk. So, the expected prevalence should be **50%** with a sample size of **384**.

3.5. Inclusion criteria

Selected dairy farms in the study area were chosen, and lactating cows from those farms were randomly chosen.

3.6. Exclusion criteria

The farms that have ≤ 2 animals, do not have full information, non-lactating, mastitis lactating cows and unselected lactating cows in them, as well as dairy farm owners who were unwilling to cooperate and participate in the study, were not included.

3.7. Study Methodology

3.7.1. Questionnaire survey

Before sample collection, all information related to each sample was gathered by using semi structured questionnaire. Accordingly, information regarding hygienic practices of milking, washing hand during period of milking, washing udder, farm cleaning period, type of floor, and storage knowledge regarding contamination of milk with *E. coli*, and the associated risk factors were collected. And, also using face-to-face interview of the farm owners or handlers all information about lactated cow such as age of lactating cow, parity, herd size, previous exposure of mastitis were recorded and including the management of the farm by using closed ended questionnaire translated to local language (Annex 1).

3.7.2. Sampling techniques

In order to determine the total number of dairy farms, farm size, farming system, number of lactation cows, and sanitary condition record, a baseline survey was first carried out. For the purpose of this study and easy data analysis, the herd size was categorized as small farm if the number of cows is (≤5), medium (between 6 and 10 cows), and large ≥ 11 cows (ILRI, 1996). This is due to the fact that, in the Ethiopian context, farms with ≤5 cows are small-scale farms that are run as side businesses. According to data obtained from the district livestock and fishery agency, the district has 196 dairy farms. That means 60 small, 96 medium, and 33 large dairy farms; the left seven (7) farms don’t have full information. After dividing all farms based on population size, only one-third of all farms were included in this study. Accordingly, a total of 384 lactating cows were sampled from 63 selected dairy farms. So, when divided proportionally, 63 farms (20 small farms, 32 medium farms, and 11 large farms) were included. To take the appropriate sample size three (3), six (6), and twelve (12) lactating cows were sampled from small, medium, and large farms respectively. That means this study considers 60 samples from a small farm, 192 samples from a medium farm, and 132 samples from a large farm as individual lactating cows. The farm and individual lactating cows were selected using a simple random sampling method (Annex 2).

The age of the animals was determined from birth records and dentition characteristics and categorized as young (≥3 to 6 years), adults (>6 to ≤10 years), and old (>10). Parity were categorized as few (with ≤3 calves), moderate (4–7 calves), and many (>7 calves)

(Soulsby, 1962; Payne, 1990). The hygienic status of the leg and udder were No contamination, slightly dirty (2-10% of the area covered in dirty), moderately dirty (10-30% of the area covered in dirty) and Very dirty (>30% areas completely covered in dirty) (Schreiner and Ruegg, 2003) (Annex 4).

A simple random sampling technique was applied to collect samples. The udder and teats were thoroughly cleaned and dried before sampling; each teat was rubbed gently with cotton swabs moisturized with 70% ethyl alcohol. A sterile universal bottle with tight-fitting cups was used. A milk sample was collected directly from all quarters of the selected individual milking cow teats. Strict foremilk (first jets) was discharged to reduce the number of contaminants in the teat canal. About 25 mL of raw udder milk was collected aseptically from a cow's teats (composite milk from all functional teats).

All collected samples were tagged by name of farm, cow ID, and date of sampling. Finally, the samples were transported to the JUCAVM School of veterinary medicine microbiology and veterinary public health laboratory using an ice box in the cold chain for microbiological analysis. On arrival, the samples were stored in the refrigerator at 4°C for 24 hours. Until being processed for isolation and identification of *E. coli,* as described by Quinn *et al*. (2002).

3.8. Isolation and Identification of Bacteria

The isolation and identification of *E. coli* O157:H7 was performed using techniques recommended by Quinn *et al*. (2002). One milliliter of each milk sample was added into nine milliliters (1: 9 ratios) of buffered peptone water (BPW) in a test tube, homogenized for 2 minutes, and incubated at 37°C for 20-24 hrs. for enrichment to increase recovery of the organisms. All pre-enriched samples were inoculated on MacConkey agar (SRL. Pvt. Ltd., Mumbai, India) at 37°C for 24 hrs. Typical colonies on MacConkey agar (pink, due to their ability to ferment lactose) were transferred to Eosin Methylene-Blue (EMB) agar (SRL. Pvt. Ltd., Mumbai, India). The colonies with metallic sheen on EMB agar at 37°C for 24 hrs. which is typical feature of *E. coli* and stained using gram stain and observed for their staining and morphological characteristics. Gram-stained films of suspected *E. coli* colonies showing Gram negative, road shape, and non-spore forming bacilli (Annex 6).

Pure cultures of a single colony from Eosin Methylene-Blue agars were placed onto a nutrient agar plate for confirmatory biochemical testing. In order to validate the presence of *E. coli* in the test samples, confirmatory biochemical tests such as Motility- Indole -lysine test , Methyl red, Voges-Proskauer, and Simeon Citrate tests and Triple sugar Iron test were done to confirm the presence of *E. coli* in the test samples. According to Quinn *et al.* (2002), colonies were classified as *E. coli* positive if they showed positive results for Motility-Indole-lysine agar in the indole test, positive results for methyl red (red ring), negative results for Simeon citrate utilization (green slant), negative results for the Voges-Proskauer test. The Triple Sugar Iron (TSI) test was conducted in accordance with Swanson *et al.* (1992); the test produced positive results yellow color and gas production without H_2S (Annex 7).

Isolates of presumptive *E. coli* for all biochemical tests were cultured on nutrient agar at 37°C for 18hrs to 24hrs. Picked a fresh pure colony from nutrient agar for preservation and inoculated with 20% glycerol with brain heart infusion broth (HI-MEDIA, India) and kept at -24 °C for further testing.

3.9. Molecular Identification

Pure colonies of bacteria that exhibited typical *E. coli* characteristics were picked for molecular detection after a series of tests including bacterial culture, gram staining, and biochemical testing. Preserved all positive samples of *E. coli* at -24 ^{o}c after being inoculated in Brain Heart Infusion (BHI) broth (HI-MEDIA, India) with 20% glycerol until transported. All samples that were preserved with brain heart infusion were transported to the National Agricultural Biotechnology Research Center (NABRC), of the Animal Biotechnology Research Program at Holeta town using an ice box under the cold chain for molecular detection and antibiotic susceptibility testing.

3.9.1. Genomic DNA extraction of *E. coli* O157:H7

In order to perform genomic DNA extraction, all presumed isolates were cultured in BHI agar (^{TM}MEDIA) at 37ºC for 18-24 hour. The method of thermally lysing suspicious strains of cells was used to extract DNA. Using a 10 µl loop, one colony was picked up and re-suspended in 100 µl of nuclease-free water by swirling the loop in a micro centrifuge tube, and the tube was vortexes for 30 seconds. After that, the tube was placed in a heated thermal block and left for ten minutes at 95°C to 100°C. The tube was

centrifuged for five minutes at 13000 rpm in a mini centrifuge after being cooled for two minutes at 4°c. After centrifugation, the 50 µl of supernatant was transferred carefully into a new micro centrifuge tube. The supernatant was used as template DNA. Subsequently, Sambrook and Russell, (2001) and Amin *et al*. (2017) reported using up to 5 µl of the collected supernatant per 50 µl PCR reaction or up to 2.5 µl per 25 µl PCR reaction after extraction, the purity and concentration of DNA were measured at 260nm by optical density using a Nanodrop ND-2000 spectrophotometer (Nanodrop Technologies, Rock land, DE, USA). The ratio of OD260/OD280 in the range of (1.8+0.2) for pure DNA indicates the quality of the DNA solution, which was kept at -20°C until additional analysis.

3.9.2. Molecular confirmation of *E. coli* O157:H7 with gene amplification

The molecular confirmation was carried out with polymerase chain reaction (PCR) (Biorad Thermal Cycler T100TM, USA) that used a specific primer against a *stx1* (180bp), *stx2* (255bp), *hlyA* (534bp) and *eae*A (384bp) amplicon size. The primers, which were synthesized by Sigma-Aldrich (Bon, Germany) and PCR master mix (2X DreamTaq Green master mixes, Lithuania) were commercially available and used to set up the PCR reaction. Details information of each primer sequence, specific target genes amplicon sizes, and PCR condition were summarized in table 2. The lyophilized primers were reconstituted using Nuclease free sterilized water to obtain 100 µM stock solutions and final diluted to a working concentration. Each PCR reaction mixture was done with 25µl final volume containing 12.5µl of 2X DreamTaq Green master mix, 1µl of primer each (F and R), 2.5µl of DNA template, and the final volume of the reaction was completed to 25µl using 8µl of Nuclease free water (Annex 9).

Table 2: The sequences of primer, Amplicon size, and PCR thermal cycling conditions used to detected virulence genes of *E. coli* O157:H7 isolate

Target gene	Oligonucleotide sequence(5′…..3′)	Amplicon size (bp)	PCR thermal cycle condition						Reference
			Initial Denaturation	Denaturation	Annealing	Extention	Cycle	Final extansion	
Stx1	**F**:ATAAATCGCCATTCGTTGACTAC **R:**AGAACGCCCACTGAGATCATC	180	95^oc 5minute	95^oc 30sec	62^oc 30sec	72^oc 30sec	35	72^oc 7minute	Paton and Paton, 1998
Stx2	**F**: TTAACCACACCCACGGCAGT **R**: GCTCTGGATGCATCTCTGGT	255	95^oc 2minute	94^oc 60sec	55^oc 2minut	72^oc 2minute	35	72^oc 5minute	Paton and Paton, 1998
eaeA	**F:**GACCCGGCACAAGCATAAGC **R**:CCACCTGCAGCAACAAGAGG	384	95^oc 1minute	95^oc 1minute	62^oc 2minute	72^oc 90sec	35	72^oc 5min	Paton and Paton, 1998
hlyA	**R**= CCACCTGCAGCAACAAGAGG **F**= GCATCATCAAGCGTACGTTCC	534	95◦C, 10 min	95^OC 1minute	64^oC 2minute	72^oc 90sec	35	72^oc 5min	Paton and Paton, 1998

3.9.3. Agarose gel electrophoresis analysis of the PCR products

The PCR products (amplicon) were separated by 1.5% (w/v) Agarose gel electrophoresis, which was prepared from 1X Tris Acetate EDTA buffer dyed with gel red, in order to evaluate the final result. For the purpose of confirming the amplicon size, the first well was loaded with the molecular weight marker (Bio Basic 100 bp ladder). *E. coli* ATCC-25922 was used as positive control and Nuclease free water as a negative control were used as quality controls. Each PCR product (8µl) was loaded into a different well of the gel's pre-made wells. The gel was then electrophoresed for 60 minutes at 100V. Lastly, utilizing bench-top UV light and BioDoc-Tran illuminator gel imaging systems (Cambridge, UK), band sizes of PCR products were seen and recorded in a gel documentation system (Annex 9).

3.10. Antimicrobial Susceptibility Patterns

After molecular confirmation, ten antimicrobial disks were subjected to an in vitro antimicrobial susceptibility test utilizing the Kirby Breuer disk diffusion method, as recommended by clinical and laboratory standards organizations (CLSI, 2020) on Difco ^{TM} Muller Hilton agar(Becton Dickinson and company, 7 Loveton circle, sparks, MD 21152 USA). All antimicrobial discs used in this study with their concentrations given in parenthesis were, Kanamycin (K)(30µg), Amikacin (AK) (30µg), Erthromycin (E)(15µg), penicillin G, Sulfamethoxazole /Trimethoprim (SXT) (25µg), Gentamycin (GEN) (10µg), Chloramphenicol(C) (30µg), Streptomycin (S) (10µg), Tetracycline(TE) (30µg), Oxytetracycline (OT) (30µg). Finally, the result of the test was interpreted as resistance (R), intermediate (I) and susceptible(S) based on a standard zone of the interpretive table (CLSI, 2020) (Table 3). Multiple drug resistance was defined as the ability of an isolate to withstand at least three different classes of antibiotics (MDR) (Founou *et al*., 2016).

Table 3: *E. coli* O157: H7 Antimicrobials Zone of Inhibition Interpretation

Antimicrobial drug classes	Antimicrobial disk	Conc. (µg)	Zone of inhibition R	I	S
	Kanamycine (K)	30	≤13	14-18	≥19
Aminoglycoside	Amikacin (AK)	30	≤14	15-16	≥17
	Streptomycin (S)	10	≤11	12-14	≥15
	Gentamycin(GN)	10	≤12	13–14	>15
Potentiated sulfonamides	Sulfamethoxazole/ Trimethoprim(SXT)	25	≤10	11-15	≥16
Beta-lactam	Penicillin-G(P)	10	≤28	-	≥29
Phenicols	Chloramphenicol(C)	30	≤12	13–17	≥18
Macrolide	Erythromycin (EM)	15	≤13	14-22	≥23
Tetracycline	Tetracycline (TE)	30	≤11	12–14	>15
	Oxytetracycline(OT)	30	≤11	12–14	≥15

Conc. = Concentrations, **R**= Resistance **I**= Intermediate **S**= susceptible

3.11. Statistical Analysis

All data obtained from the investigations (recorded histories of the animals and laboratory results) were coded and entered in Microsoft Excel 2010 spreadsheet and then analyzed using IBM SPSS version 25.0 (SPSS, 2017). The agent isolation rates were expressed as a percentage with a 95% confidence interval by dividing the total number of animals positive for isolates by the total number of animals examined. The association between the dependent variables (outcome) and independent variables (herd size, type of floor, Age, Parity, udder hygiene, Hand wash, Management system, Farm cleaning period, Previous exposure to mastitis, and Housing system) was initially assessed using univariable logistic regression to compute the odds ratio associated with potential risk factors. The Multivariab le logistic regression model included variables with a $p < 0.25$ in the univariable analyses (Thrusfield, 2008). In the final model was assessed for goodness-of-fit using the Hosmer-Lemeshow x^2 test. The model was assumed of fitting the data if the Hosmer-Lemeshow x^2 test was insignificant ($p > 0.05$) (Hosmer and Lemeshow, 1980). For all statistical analyses odd ratios and 95% confidence interval and a *P*-value of less than 0.05 was considered as statistically significant.

The assessment of antimicrobial susceptibility was conducted by comparing the zone of inhibition of each drug with 0.5 McFarland turbidities. Regarding the molecular detection's the banding patterns of individual strains were scored based on the presence or absence of the bands with the appropriate base pairs.

3.12. Ethical Considerations

Ethics approval and consent to participate in this research were approved by Jimma University and the JUCAVM institutional ethical review board animal research ethical committee with reference number **21A/Mar/2023**. This protocol was presented to the initial and continuing review boards. All methods were performed by skilled experts with relevant guidelines and regulations listed by the ethical committee of the university. The safety, welfare, and wellbeing of the study animals were secured during the study. Informed consent for study participation was obtained from all farm owners for a questionnaire interview and to collect raw milk samples from the dairy cows. Since the study involves domestic animals, ethical approval for the study was obtained from the Ethical Research Board (ERB) of the JUCAVM for domestic animals.

4. RESULTS

4.1. Prevalence of *E. coli* and *E. coli* O157:H7

The overall prevalence of *E. coli* was 59 (15.36%; 95% CI: 12.10-19.31) milk samples were contaminated with *E. coli*, out of 384 raw milk samples collected from apparently healthy dairy cows and processed. Of which 12 samples were *E. coli* O157:H7 strain. Finally, the prevalence of *E. coli* O157:H7 was 3.13% (95% CI: 1.80-5.38) based on detailed bacteriol ogical and PCR detection.

Table 4: The overall prevalence of *E. coli* and *E. coli* O157:H7

Isolated bacteria	Number of sample processed	Positive	Prevalence %
Escherichia coli	384	59	15.36
Escherichia coli O157:H7	384	12	3.13

4.2. The association between *E. coli* and potential risk factors

In the current study, 10 potential risk factors variables considered in univariable logistic regression analysis of their statistically relation with the contamination of *E. coli.* While, housing system (OR = 1.25, 95% CI: 0.655–2.387, P = 0.499), previous exposure to mastitis (OR = 1.40, 95% CI: 0.751–2.599, P = 0.291), and management system (OR = 1.16, 95% CI: 0.619–2.170, P = 0.645) were not statistically significant (p>0.05) during screening univariable logistic regression and did not satisfy the criteria to be included in the multivariable analysis after checking for co-linearity and *P*-value (p < 0.25) (Table 5). Those risk factors having (p > 0.25) on the initial univariate logistic regression analysis were withdrawn from further analysis.

Table 5: Univariate logistic regression analysis

Risk factors	Categories	No. of examination	No. of positive	Proportion (%)	Univariate analysis			
					P-value	**COR**	95% CI	
Herd size	Small	60	8	13.33	**1**			
	Medium	192	19	9.89	0.454	**0.71**	0.295	1.725
	Large	132	32	24.24	0.089	**2.08**	0.894	4.838
Type of floor	Good concrete	133	13	9.77	**1**			
	Bad concrete	154	19	12.34	0.492	**1.31**	0.615	2.742
	Soil	97	27	27.84	0.001	**3.56**	1.726	7.346
Age	Young	128	12	9.38	**1**			
	Adult	169	23	13.61	0.265	**1.52**	0.727	3.190
	Old	87	24	27.59	0.001	**3.68**	1.726	7.857
Parity	Few	144	16	11.11	**1**			
	Moderate	163	22	13.50	0.527	**1.25**	0.628	2.481
	Many	77	21	27.27	0.003	**3.00**	1.457	6.177
Housing system	Separated pen	105	14	13.33	**1**			
	Grouped barn	279	45	16.13	0.499	**1.25**	0.655	2.387
Hand wash	Before and after milking	219	23	10.50	**1**			
	After milking only	165	36	21.82	0.003	**2.38**	1.347	4.199
Udder hygiene	Washing with soap and drying	237	20	8.44	**1**			
	Washing only in water	147	39	26.53	0.000	**3.92**	2.180	7.043
FCP	Two times in day	173	38	21.97	**1**			
	Once in day	111	21	18.92	0.220	**1.44**	0.803	2.592
PEM	No	294	42	14.29	**1**			
	Yes	90	17	18.88	0.291	**1.40**	0.751	2.599
Management system	Intensive	289	43	14.88	**1**			
	Semi-intensive	95	16	16.84	0.645	**1.16**	0.619	2.170

COR- crude odd ratio, **AOR**- Adjusted odd ratio, **CI**- Confidence interval, **1**- Reference, **FCP**- Farm cleaning period **PEM**- Previous exposure to mastitis

On the other hand, in the present study, multiple logistic regression analysis revealed that herd size, type of floor, age, parity, hand washing practice, udder hygiene, and farm cleaning period have statistically significant associations with the isolation of *E. coli* and satisfy the criteria of (p< 0.25) (Table 6).

The occurrence of *E. coli* in the study area was mostly dependent on herd size, large farms with more than 11 herd sizes having a greater prevalence (24.24%) than farms with 6–10 herd sizes (9.89%) and less than 6 herd sizes (13.33%). The odds of isolating the isolates were 4.95 (95% CI=1.651-14.86, P = 0.004) higher in large herd sizes compared to medium and small herd sizes.

The percentage of isolation was highest in soil-type floor farms at 27.84%, compared to 12.34% in bad concrete and 9.77% in good concrete farms. The lactating dairy cow in the soil floor types farm was statistically significant (OR = 5.76, 95% CI: 2.085–15.928, P = 0.001) and six times more likely to be affected by *E. coli* than lactating dairy cows living in bad concrete farms and good concrete farms. Highest prevalence of isolate *E. coli* was found in lactating cows of ages greater than ten years (27.59%) when compared with lactating cows of adult age greater than six to less than or equal to ten years (13.61%) and young greater than or equal to three to six years old (9.38%). The odds of isolation of the isolates were 3.51 (95% CI = 1.486–8.280, P = 0.004) higher in aged lactating cows than in adults and young's.

The study found that the isolation rate of the agents was statistically significant ($p<0.05$) when compared to the parity number of lactating dairy cows. The highest isolation rate was observed in cows that gave birth to many calves (more than seven) (27.27%), followed by cows that gave birth to four to seven calves (13.50%), and the lowest prevalence was found in cows that gave birth to few calves (less than or equal to three) (11.11%). The odds of isolation of the isolates were 2.31 (95% CI = 1.002–5.339, P = 0.049) higher in many than medium and few parity's.

Hygienic practices such as hand washing during milking and udder washing also have a significant role in the highest prevalence of *E. coli*. In this study, the prevalence of *E. coli* is higher (21.82%) in no-washing-hand practice during the milking period than 10.50% in washing-hand before and after milking practice. The odds of isolation of the isolates were 2.18 (95% CI = 1.064–4.448, P = 0.033) higher in no-washing-hand practice than washing hands before and after milking practice. The isolation of *E. coli* isolates is higher in those who wash only in water (26.53%) udder when compared to those who wash with soap and dry their cow's udder during the milking period (8.44%). The odds of isolation of the isolates were 3.12 (95% CI = 1.548–6.307, P = 0.001) higher in washing only in water than washing with soap and drying. In the present study, there was no statistically significant difference in farm cleaning period (OR = 0.78, 95% CI: 0.354–1.731, P = 0.546) in isolation, though the highest percentage was found in the once-a-day cleaning period on the farm. Besides, the Hosmer-Lemeshow goodness-of-fit test suggested that the model fit the data (x^2 = 4.85; p = 0.77).

Table 6: Multivariate logistic regression analysis of the association between occurrences of *E. coli* and risk factors

Risk factors	Categories	No. of examination	No. of positive	Proportion (%)	Multivariate analysis			
					P-value	AOR	95% CI	
Herd size	Small	60	8	13.33	**1**			
	Medium	192	19	9.89	0.967	**0.98**	0.333	2.87
	Large	132	32	24.24	0.004	**4.95**	1.651	14.86
Type of floor	Good concrete	133	13	9.77	**1**			
	Bad concrete	154	19	12.34	0.933	**1.04**	0.424	2.545
	Soil	97	27	27.84	0.001	**5.76**	2.085	15.929
Age	Young	128	12	9.38	**1**			
	Adult	169	23	13.61	0.327	**1.51**	0.661	3.463
	Old	87	24	27.59	0.004	**3.51**	1.486	8.280
Parity	Few	144	16	11.11	**1**			
	Moderate	163	22	13.50	0.791	**1.12**	0.498	2.500
	Many	77	21	27.27	0.049	**2.31**	1.002	5.339
Hand wash	Before and after milking	219	23	10.50	**1**			
	After milking only	165	36	21.82	0.033	**2.18**	1.064	4.448
Udder hygiene	Washing with soap and drying	237	20	8.44	**1**			
	Washing only in water	147	39	26.53	0.001	**3.12**	1.548	6.307
FCP	Two times in day	173	38	21.97	**1**			
	Once in day	111	21	18.92	0.546	**0.78**	0.354	1.731

AOR- Adjusted odd ratio, **CI**- Confidence interval, **1**- Reference, **FCP**- Farm cleaning period

4.3. Genotypic detection of *E. coli* O157:H7strain

In this study from the total of fifty nine (59) phenotypically identified *E. coli,* twelve (12) isolates were molecularly confirmed as *E. coli* O157:H7. Accordingly, for the detection of specific virulent genes, conventional PCR was applied. The result shows, 5/59 (8.47%) showed both an amplicon size of (180bp) *stx1* gene, and (384bp) *eaeA* gene and 7/59(11.86%) showed an amplicon size of (255bp) *stx2* gene was confirmed but no one detected enterohemolysin (*hlyA*) gene results (Figure 3).

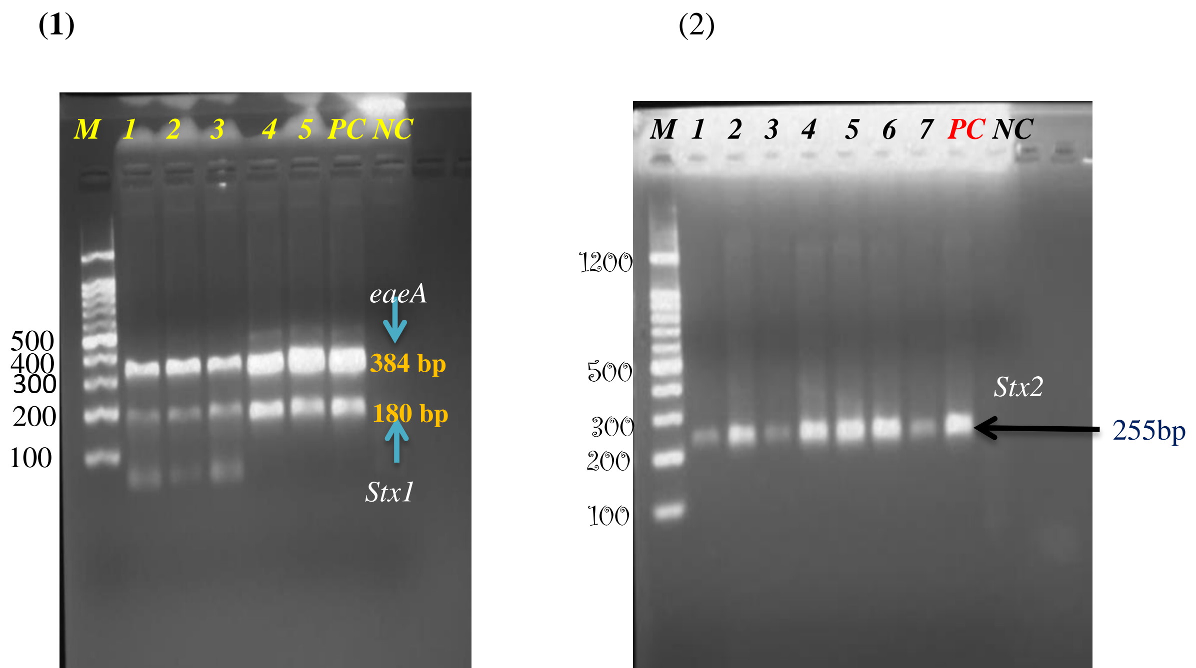

Figure 4: The BioDoc-Transilluminator gel imaging system photographs the results of Agarose gel electrophoresis of the (1) *stx1* (180bp) gene and the *eaeA* (384bp), (2) *stx2* (255bp) gene in the *E. coli* O157:H7 strain isolated from raw cow milk, respectively. Lane M = 100bp DNA ladder; PC= positive control; 1, 2, 3…7 positives samples; NC = negative control.

4.4. Antimicrobial Susceptibility test

The Antimicrobial susceptibility profile of 12(twelve) *E. coli* O157:H7 isolates was tested against 10 (ten) antimicrobial drugs after molecular detection. Sulfamethoxazole/ trimethoprim (83.3%), chloramphenicol (91.7%), and Gentamycin (91.7%) potentiated susceptibility for these bacteria. However, high resistance was found to Penicillin-G

(100%), Erythromycin (100%), Streptomycin (83.3%), and Oxytetracycline (58.3%), demonstrating a variety of microbiological resistances as indicated in Table 7.

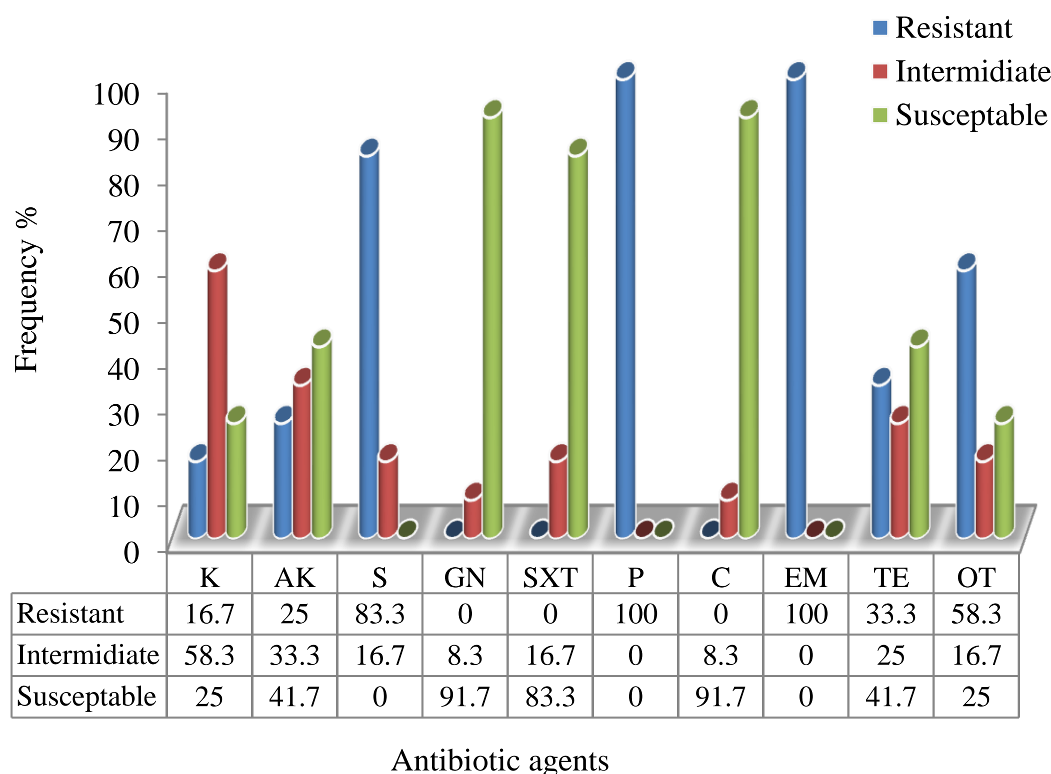

Figure 5: Percent of resistance of 12 bacterial isolates to different antimicrobials

Table 7: Antimicrobial susceptibility profile of *E. coli* O157:H7strains (N=12)

Antimicrobial drug classes	Antimicrobial disk	Conc. (µg)	*E. coli* (N=12)		
			R (%)	I (%)	S (%)
	Kanamycine (K)	30	2(16.7)	7(58.3)	3(25)
Aminoglycoside	Amikacin (AK)	30	3(25)	4(33.3)	5(41.7)
	Streptomycin (S)	10	10(83.3)	2(16.7)	0
	Gentamycin(GN)	10	0	1(8.3)	11(91.7)
Potentiated sulfonamides	Sulfamethoxazole/ Trimethoprim(SXT)	25	0	2(16.7)	10(83.3)
Beta-lactam	Penicillin-G(P)	10	12(100)	0	0
Phenicols	Chloramphenicol(C)	30	0	1(8.3)	10(91.7)
Macrolide	Erythromycin (EM)	15	12(100)	0	0
Tetracycline	Tetracycline (TE)	30	4(33.3)	3(25)	5(41.7)
	Oxytetracycline(OT)	30	7(58.3)	2(16.7)	3(25)

Conc. = concentrations, **R**= Resistance **I**= Intermediate **S**= susceptible

4.4.1. Prevalence of multiple antimicrobial resistant *E. coli* O157:H7

E. coli O157:H7 isolates demonstrated multiple antimicrobial resistance (MAR), which is defined as the name implies resistance of the isolate to three or more antimicrobial classes. Nine (75%) of the twelve *E. coli* O157:H7 isolates that were tested against antimicrobial resistance discs showed multiple antimicrobial resistance. 33.3%, 33.3%, and 8.3% of the MAR *E. coli* O157:H7 isolates were resistant to three, four, and five antimicrobial classes, respectively.

Table 8: The list of MAR patterns of *E. coli* O157:H7 isolates (N=12)

Resistance category	Resistance pattern	Isolate No (%)	Sub-total (%)
Against two	EM-15/P-10, S-10	2(16.7)	3(25)
	EM-15/P-10, TE-30	1(8.3)	
Against three	EM-15/P-10, OT-30, S-10	1(8.3)	4(33.3)
	EM-15/P-10, TE-30, S-10	2(16.7)	
	EM-15/P-10, TE -30, OT-30	1(8.3)	
Against four	EM-15/P-10, OT-30, S-10, K-10	1(8.3)	4(33.3)
	EM-15/P-10, OT-30, S-10, AK-30	2(16.7)	
	EM-15/P-10, TE-30, OT-30, AK-30	1(8.3)	
Against five	EM-15/P-10, OT-30, S-10, K-10, AK-30	1(8.3)	1(8.3)

5. DISCUSSION

The current study showed 3.13% (CI: 1.80-5.38) of *E. coli* O157:H7 prevalence, which is nearly similar with the investigations of Ivbade *et al*. (2014); Ghali-Mohammed *et al*.(2023); Welde *et al*. (2020); Ababu *et al*. (2020); Al-Zogibi *et al*. (2015) and Ullah *et al*. (2022) who reported 2% from Ogun State, Nigeria, 2.3% from Kwara State, Nageria, 4.2% from Mojo town, Ethiopia, 5.2% from Holeta town, 4.26% from Saudi Arabia, and 5.0% from Khyber Pakhtunkhwa, Pakistan respectively. However, it was higher than the 1.5% reports of Gudisa *et al.* (2022) from Gojo and Shukute, Ethiopia, 0.66% reports of İnat *et al*. (2017) from Turkey and the 1% reports of Amer and Soliman (2004) from Egypt. On contract, this result was lower than the findings of Lye *et al*. (2013); Abunna *et al*. (2018); Abebe *et al*. (2023), and Ariyanti *et al*. (2022) who reported 8.75% from Malaysia, 8.9% from in and around Asella, Oromia Ethiopia, 9.6% from Dessie and Kombolcha, Ethiopia and 65.38% from Indonesia respectively. Variations could be to blame for the disparity in *E. coli* O157:H7 prevalence between the investigations due to variations in the agro-ecology of the study area, farm husbandry practices, season of sample collected, herd size, sample size, and veterinary service coverage.

In the present investigation, many factors were analyzed for associations with *E. coli* in this study, yet relatively few were significant in the final model. The odds of finding *E. coli* in cow milk were 4.95 times higher in large herds (P = 0.004, AOR = 4.95, CI = 1.651–14.86) than in small herds. Large herds have increased probabilities of *E. coli* prevalence, which is likely associated to high humidity, poor ventilation, and high stocking density. Besides, this indicates a lack of knowledge in the community regarding animal husbandry, and the large size herd can affect the health of animals. This finding is corroborating with Ababu *et al*. (2020). Also, the study indicated that Type of floor had a significant (P = 0.001, AOR = 5.76, CI=2.085-15.929) impact on the occurrence of *E. coli*. The odds ratio of *E. coli* occurrence in farms where floor type (soil) was 5.76 times higher risk when compared with farms with concrete floors. This is in agreement with Mesele *et al*. (2023). Since the floor (soil) is one of the potential sources of microorganisms, particularly *E. coli* is environmental pathogens.

Age was also found to be significantly (P = 0.004, AOR = 3.51, CI = 1.486-7.280) associated with the occurrence of an *E. coli* infection. The study found 3.51 higher in animals of old age cows (>10) more likely infection than in younger cows; this finding

was coinciding with the reports of Ababu *et al*. (2020) and Yohannes, (2018). The number of parities was one factor in the occurrence of *E. coli* and has been found to be significantly associated with different parity numbers. The highest percentages of *E. coli* isolates were isolated from cows with a parity number above seven (P = 0.049, AOR = 2.31, CI = 1.002–5.339) when compared to those with a parity number greater than or equal to four to less than or equal to seven (moderate), and less than or equal to three (few). This is in agreement with Ababu *et al*. (2020); Shubisa *et al*. (2022) and Yohannes, (2018). Both may have to do with an animal's immune system's ability to defend against pathogens and the stresses of multiple parturition, which ultimately down-regulate their immunity. Additionally, immunity typically declines with age, making an animal more vulnerable to infection with *Escherichia coli* (Rezaei *et al*., 2013; Sharf *et al*., 2009).

The prevalence of *E. coli* was 3.12 times higher in cows with dirty udders than in cows with cleaned udders. Dirty udders contribute to a favorable condition for the multiplication of both contagious and environmental pathogens. Udder hygiene was also found to be statistically significant (P = 0.001, AOR = 3.12, CI = 1.548- 6.307) with the incidence of *E. coli*. This is similar to the report of Ababu *et al*. (2020). Besides, Hand washing practice also had a significant impact (P = 0.03, AOR = 2.18, CI = 1.064- 4.448) on the occurrence of *E. coli*. The odds ratio of *E. coli* occurrence in farms where hand washing is practiced only before milking was 2.18 times higher when compared with farms where before and after milking hand washing is experienced. This is in agreement with Mesele *et al*. (2023) and Abunna *et al*. (2018).

Another part of present investigation was specialized to determine *E. coli's* virulence genes; all positive *E. coli* samples during biochemical test were tested to find four specific virulence genes of the *E. coli* O157:H7 strains (*stx1*, *stx2*, *eaeA* gene and *hlyA* gene) by applying convectional PCR. Therefore, four primers (*stx1* 180bp, s*tx2* 255bp, *eaeA* 384bp and *hlyA* 534bp) were used in this study. From the virulence gene detected, 11.86% (7/59) of isolates carried the *stx2* gene. The finding agreed with the reports of Ivbade *et al*. (2014); Vanitha *et al*. (2018); Ombarak *et al*. (2016), and Abbas *et al*. (2017) who found that 60%, 90.91%, 0.9%, and 14.28% reported the *stx2* gene from raw cow milk, respectively.

On the other hand, both of *stx1* and *intimin* (*eaeA*) gene was also detected in 8.47% (5/59) of isolates carried in this study similarly, the investigation of the *stx1* and *eaeA* gene in

raw cow milk was in agreement with the reports of Liu *et al*., (2021); Huasai *et al.* (2012) and Ivbade *et al*. (2014) who found that (9%, 4.5%), (2.3%, 22.11%), and (20%, 50%), respectively. In general, the disparity in the percentage of virulence genes isolated between the studies of the other owner may be due to differences in the sample sizes examined, the sensitivity and specificity of the diagnostic tools used, and the technical efficiency of the investigations. But the *hlyA* gene was not confirmed in this study.

In recent times, antimicrobial resistance has grown to be a global issue, especially in developing countries. This has led to a significant reduction in treatment choices due to the obvious transmission of resistant pathogenic strains to humans through food. The results of the in vitro antimicrobial susceptibility assay conducted on *E. coli* O157:H7 isolates showed that, in accordance with a previous study, the isolates were most susceptible to gentamycin (91.7%). It was similar with the report of Shubisa *et al*. (2022) (100%); Ababu *et al*. (2020) (100%); Igbinosa and Chiadika, (2021) (100%); Mumed *et al*. (2022) (93.75%); Gugsa *et al*. (2022) (91.7%); Ibrahim *et al*. (2022) (88.2%); Abebe *et al*. (2023) (88.0%) but the present result was higher than the findings of Sarba *et al*. (2023)(50%). The 83.3% isolates were susceptible to sulfamethoxazole/trimethoprim, which was similar with the reports of Abebe *et al*. (2023) (100%); Ababu *et al*. (2020) (54.54%); Gugsa *et al*. (2022) (79.2%); Shubisa *et al*. (2022) (83.87%) and chloramphenicol (91.7%) also susceptible with the isolates which agreed Nobili *et al*. (2016) (100%); Mumed *et al*. (2022) (100%); Elmonir *et al*. (2018)(100%).Conversely, the isolates were less susceptible to kanamycin (25%), amikacin (25%), Tetracycline (41.7%) and oxytetracycline (25%) in this study. The highest level of susceptibility of *E. coli* O157:H7 to antimicrobials indicat ed above is likely because those antibiotics are either less available or not present at all in the dairy farm in the study area. Therefore, the likelihood of the development of antimicrobial resistance has decreased due to less frequent antibiotic usage.

On contrary, the finding revealed that ≥50% of *E. coli* O157:H7 isolates were found to be highly resistant to Erythromycin (100%), and penicillin-G (100%) which was in agreement with the reports of Abebe *et al*. (2023) (92%, 100%); Ibrahim *et al*. (2022)(100 %,76.5%); Igbinosa and Chiadika,(2021) (89.5 %, 100%); Gugsa *et al*. (2022) (91.7%, 54.2%) respectively. In addition, Streptomycin, and oxytetracycline are followed by 83.3% and 58.3% respectively. The 83.3% isolates were resistant to Streptomycin similar with the reports of Dejene *et al*. (2022) (70.37%) and Mumed *et al*. (2022) (87.5%); but

was higher than Elmonir *et al*. (2018) (31.3%) report. This study also revealed that 58.3% of the isolates showed resistance against oxytetracycline which line with the report of Igbinosa and Chiadika, (2021) (78.9%), but higher than the reports of Ullah *et al*. (2022) (31%). The Variation in the alarming degree of *E. coli* O157:H7 resistance to the above antimicrobials may be related to antimicrobial dosages, redundancy in the use of the same medication for infection treatment and prevention in the study area, as well as the use of the same medication for control and prevention.

Shiga toxins *Stx1*, *Stx2*, and intimin (*eaeA*), which can be generated by STEC strains, are the most important virulence factors in *E. coli* strains found in milk and dairy products. Moreover, multiple investigations have shown that STEC pathogens carry the MAR characteristic (Momtaz *et al.,* 2012). The multiple-antimicrobial resistance (MAR) *E. coli* O157:H7 (75%) isolated in this finding was in agreement with the study of Sarba *et al*. (2023); Gugsa *et al*. (2022) and Mumed *et al*. (2022), who reported 88.1%, 75% and 37.5% of MAR of *E. coli* O157:H7 isolate from central Ethiopia, Mekelle, Haramaya, and Harar town, respectively.

6. CONCLUSION AND RECOMENDATIONS

The current investigation revealed that as *E. coli* O157:H7 was the major contaminant in the raw milk of dairy cattle in Jimma town. That indicates public health issue since milk can spread food-borne illnesses. It has been suggested that the following predisposing risk factors encourage the onset and transmission of the disease: herd size, floor type, age, parity, udder hygiene and hand washing practices during milking. The findings of this study indicate that the isolation of *E. coli* O157:H7 harboring virulence genes such as *stx1, stx2*, and Intiman (*eaeA)* from raw milk of an apparently healthy cow. Additionally, antimicrobial susceptibility test of molecularly confirmed isolates was performed, Penicillin-G, Erythromycin, Streptomycin, and Oxytetracycline were completely inactive an timicrobials against these agents while, highly susceptible antimicrobials against these isol ates were noted to be Sulfamethoxazole/trimethoprim, chloramphenicol, and Gentamycin. Similarly, antimicrobial test data revealed the presence of MAR isolates of *E. coli* O157: H7. Therefore, based on the above conclusion, the following recommendations were forwa rded:

- Public awareness should be raised by stakeholders on hygienic milk handling and the avoidance of raw milk consumption,
- The government must make a policy on proper use of antimicrobial drug, handling, and storage.
- To restrict the spread of virulent strains and antimicrobial-resistant strains of *E. coli* from animals to humans, it is beneficial for interdisciplinary experts to apply a one health approach.
- The molecular detection of the rest of the pathogenic strain and virulence gene, resistance gene detection of *E. coli,* and the further genomic sequence of the isolates need to be carried out to effectively compact the MAR *E. coli* infection.

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8. ANNEXES

ANNEX 1: Questionnaire Format

A. ***General information***

1. Owners Name: ____________________ Dairy Farm name: ______________
2. Address__________________________ Date of sample Collection_________________

B. ***Farm status and Housing information***

1. What is management system of the farm? A) Intensive B) Semi Intensive
2. What is the Herd size of the farms? A) Small (≤5) B) Medium (6-10) C) Large (≥11)
3. What type of floor is there in the farm? A) Good concrete B) Bad concrete C) Soil
4. How often the barn and/or the milking room are/is cleaned? A) Two time per day B) Ones in day
5. Feed & water hygiene and storage: A) Excellent B) Very good C) Good D) Fair E) Poor
6. What is the Housing system? A) Separate Pen B) Group (shared) barn
7. Is there bedding material? A) Yes B) No

C. History of each sampled cow

1. ID No.___________ Parity: A. few (with ≤3 calves) B. moderate (4–7 calves) C. many (>7 calves)
2. How old is the cow? (Age of cow in years) A) 3-5 (Young) B) 6-8 (Adults) C) ≥9 (Old)
3. What is lactation stage of the cow? A) Early (1-3) B) Medium (4-6) C) Late (≥7)

D. Physical inspection of the udder, teat and milk

1. What is udder & leg hygiene of Cow? A) Slightly dirty B) Moderately dirty C)Very dirty
2. Is there any inflammatory sign (Heat and swelling) on the udder and teat? A) Yes B) No
3. Is there lesion on the udder/ teat? A) Yes, C) No, it is absent
4. Is there previous exposure to mastitis problem in the farm? A) Yes B) No
5. Is there any blindness of teat canal? A) Yes B) No
6. Is there any tick infestation of teat? A) Yes, it is present B) No it is absent
7. What is gross milk quality? A) Normal B) watery C) Clots/flakes D) blood tinged/pus

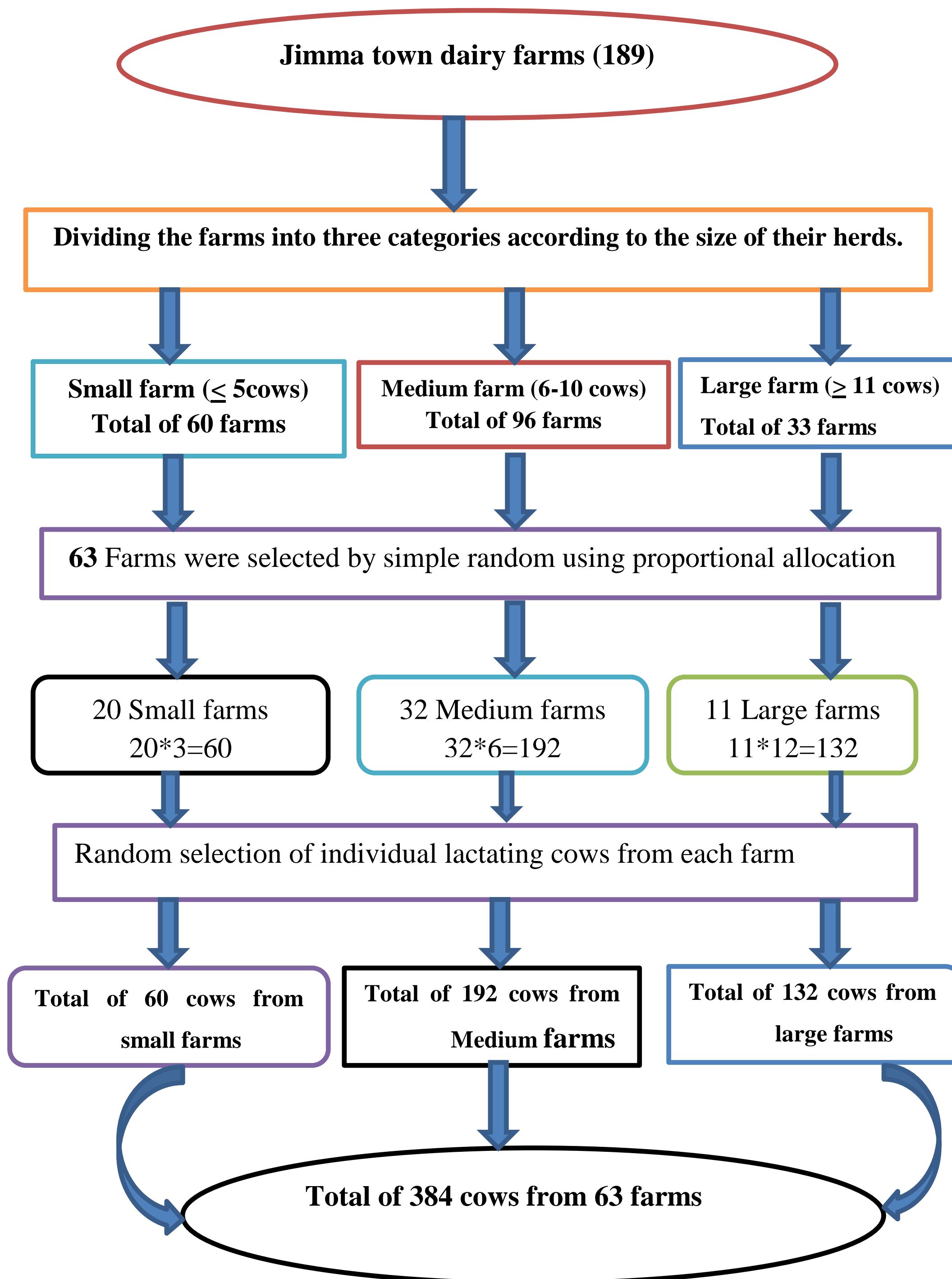
E. Status of hygienic milking practice

1. Do you wash your hand during milking by detergent? A) Yes B) No
2. Do you wash the udder? A) Washing and drying by towel B) Washing only by water
3. Where do cows milked? a) In barn b) In milking rooms
4. With what detergent do you wash your hand? a) With soap b) Without soap
5. What type of udder washing do you follow? a) Washing &drying b) washing only
6. Do you drink fresh raw milk? A. No B. Yes

F. ANTIMICROBIAL RESISTANCE RELATED QUESTIONS

1. Did your cow treated by any drugs? A. Yes B. No
2. Did your cow recovered after treatment? A. Yes B. No
3. Did you know the name of drug you use it? A. Yes B. No

Annex 2: A simplified diagram of the sampling technique.



Annex 3: Format used for recording data in the field

I: Format for collecting data on farm visits

No	Cow ID	Breed of cow	Management systems	Parity	Age	Farm cleaning period	Previous exposure to mastitis	Herd size	type of floor	Housing systems	wash your hand during milking	Hygiene of udder	cow treated by any drugs	cow recovered after treatment cow
1														
2														
3														
4														

II: The laboratory data sheet for *E. coli* O157:H7 bacteriology and molecular detection

Cow ID	Sample Code	Colony characteristic		Gram staining	Methyl red	VP	TSI	SC	PCR	Remark
		MaCc	EMB							
1										
2										
3										

MaCc= MacConkey agar, EMB= Eosin methyl blue, VP= Voges-Proskauer, TSI= Triple sugar iron agar, SC= Simon citrate agar, PCR= Polymerase Chain Reaction

Annex 4: Categories of different risk factors

No	Risk factors	Categories	References
1	Age	Young adults (>3 to 6 years)	
		Adults (>6 to ≤10 years)	Soulsby,
		Old (>10)	1962; Payne,
2	Parity	Few (with ≤3 calves)	1990.
		Moderate (4–7 calves)	
		Many (>7 calves)	
3	Leg and udder hygiene score	No contamination Slightly dirty(2-10% of the area covered in dirty) Moderately dirty(10-30% of the area covered in dirty) Very dirty(>30% areas completely covered in dirty)	Schreiner and Ruegg, 2003
4	Herd size	Small farm (≤5 cows) Medium (6-10cows) Large ≥ 11 cows	ILRI, 1996

Annex 5: Media preparation

MacConkey agar: MacConkey agar REF (76875(MM011)) composed of 17 g/l of peptic digest animal tissue, 10 gm/l of lactose, 5 gm/l of sodium chloride, 0.03 gm/l of neutral red and 13.5 g/l of agar was prepared according to the manufacturer's instructions where 50.03 gm of the powder was dissolved in 1000 ml of distilled water. The solution was heated to dissolve and sterilized by autoclaving at 121 ºC for 15 minutes. Before use the media was cooled to 45°C and poured onto sterile Petri dishes. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility and to dry the condensed vapor on the plate cover.

Eosin methyl blue (EMB) agar: Eosin methyl blue (HIMEDIA® HiMedia Laboratories Pvt.Ltd., India) containing; 10.00 g/l Peptic digest of animal tissue, 2.00g/l Di potassium phosphate, 10.00g/l Lactose, 0.40g/l Eosin-Y, 0.065g/l Methylene blue, 15.00g/l Agar was prepared according to the manufacturer's instructions. Briefly, 37.46g of the powder

was dissolved in 1000ml of distilled water. The solution was boiled to dissolve completely and sterilized by autoclaving at 121 ºC for 15 minutes. Cool to 45-50 ^{o}c and shake the medium in order to oxidize the methylene blue (i.e. restore its blue color) and to suspend the precipitate which is an essential part of this medium. Before use the media was cooled to 45°C and poured onto sterile Petri dishes. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility and to dry the condensed vapor on the plate cover.

Nutrient agar: Nutrient agar (HIMEDIA® HiMedia Laboratories Pvt.Ltd., India) containing; 5.00 g/l of peptone, 1.50 g/l HiM peptone B#, 1.50 g/l of yeast extract, 5.00 g/l of sodium chloride and 15.00 g/l of agar was prepared according to the manufacturer's instructions. Briefly, 28.0 g of the powder was dissolved in 1000ml of distilled water. The solution was boiled to dissolve completely and sterilized by autoclaving at 121 ºC for 15 minutes. Before use, the media was cooled up to 45-50 ºC poured onto sterile Petri dishes. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility and to dry the condensed vapor on the plate cover.

Annex 6: Gram staining Procedure

Pure colony of *E. coli* was taken from nutrient agar, then colony was streaked on slide and drop of water was added to it and emulsified. Then, air dry and pass through the Bunsen flame to two to three times taking care not overheat the smear. Then, gram staining chemicals was diffused on it sequentially. First, crystal violate (primer stain) was diffused on slide for one minute. Then, it washed by water. Then, mordant iodine was diffused on it for one minute. Then, it is washed by tap water. Then, decolorize with Gram's decolorizer solution 95% acetone alcohol for 15-20 second until the blue dye no longer flows from the smear and gently wash smear with tape water. Then, safranin solution or carbolfusschin (counter stain) was diffused on it for one minute. Then, it was washing off the red safranin solution with water. Then, it is air dried and examined under microscope by oil immersion at 100x magnification power. Finally, we had appreciated rod shapes with pink color of *E. coli* (Quinn *et al*., 2004).

Annex 7: Biochemical tests (Difco. Difco manual, 10th edn. Difco Laboratories, Detroit, MI. Cowan ST. 1984.)

Catalase test: Colonies that demonstrate the Gram's reaction identical with the E coli species was further tested for the presence of catalase enzyme. Pure colonies of the isolates were picked from the nutrient agar using a sterile loop and mixed with a drop of hydrogen peroxide (H_2 O) on a clean microscope glass slide. Positive reaction indicated by the liberation of bubbles of oxygen within few seconds and those with negative reaction did not produce bubbles the catalase positive isolates were considered as *E. coli.*

Indole Test: Tryptone Broth was prepared and about 6ml of it dispensed in test tubes using a sterile pipette. The composition of the medium casein enzymic hydrlysate 10gr, and NaCl 5gr. Then, sterile loops was used to pick a well-isolated colony of bacteria and inoculated into test tubes, thereafter, the tubes was incubated at 37ºC for 48 hours. After incubation period, 0.5 ml of Kovac's Indole Reagent (Loba Chemie Pvt. Ltd, Lot LM01131303) was added to the inoculated test tubes. The tubes was subjected to gentle shaking and examined for red color in the surface layer within 10 minutes (Cheesbrough, 2005). A red ring on top of the tube indicated indole positive reaction.

Methyl red test: The standard buffered glucose MR-VP broth used for the MR and VP tests was modified by substituting sodium chloride (NaCl) for di-potassium phosphate and adding 1% agar. The composition of the medium per liter was as follows: proteose peptone, 7 g; glucose, 5 g; NaCl, 5 g; agar, 10 g. The medium was dissolved by steaming and sterilized at 121°C for 15 min. Final pH was 6.3. For performance of the MR test, 5 drops of MR indicator solution were added to bacterial growth on the agar surface.

Voges-Proskauer: The VP test for the production of acetylmethylcarbinol was performed on the same modified (unbuffered) medium used for the MR test. Growth on the agar surface was flooded with 0.6 ml of 1-naphthol (5% in absolute ethyl alcohol) followed by 0.2 ml of creatine KOH reagent. The creatine-KOH reagent was stored at 3°C for a maximum of 21 days.

Citrate agar test: Citrate utilization was determined on conventional Simmons citrate agar. The medium was dissolved by steaming and sterilized at 121°C for 15 min. No reagents were used for this test. After inoculated pure colon incubated for 48hrs.

TSI (Triple sugar Iron Agar):

Annex 8: Media preparation for the refresh the sample preserve under -24°c for PCR

EMB procedure of preparation follow depend on **Annex IV**

Brain Heart Infusion Agar (BHI Agar): Brain Heart Infusion agar (TITAN BIOTECH LTD. MEDIA®, A-902,RIICO Industrial Area, Phase-III, Bhiwadi-301019, Rajasthan, India) containing; 12.50g/ltr. Calf brain infusion solids, 15.0g/ltr. Agar, 10.0g/ltr. Proteose peptone, 5.0g/ltr. Beef heart infusion solids, 5.0g/ltr. Sodium chloride, 2.50g/ltr. Disodium phosphate, 2.0g/ltr. Dextrose and PH 7.4$\pm$ 0.2 at 25°c of agar was prepared according to the manufacturer's instructions. Briefly, 52.0 gm of the powder was dissolved in 1000ml of distilled water. Gently heated to dissolve the medum completely. Sterilize by autoclaving at 15psi (121°c) for 15 minutes. Before use, the media was cooled up to 45-50 ºC poured onto sterile Petri plates. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility and to dry the condensed vapor on the plate cover.

Annex 9: Conventional PCR Reaction Protocol and its Master Mix Preparation

A. Extraction of bacterial genomic DNA by using thermally lysing suspicious strains of cells method.

1. Sample Preparation on BHI agar (inoculated with *E. coli* O157:H7 isolated and incubated for 24 hr)
2. Using a 10 µl loop, one colony was picked up and re-suspended in 100 µl of nuclease-free water by swirling the loop in an micro centrifuge tube
3. The tube was vortexes for 30 seconds.
4. The tube was placed in a heated thermal block and left for ten minutes at 95°C to 100°C.
5. The tube was centrifuged for five minutes at 13000 rpm in a mini centrifuge after being cooled for two minutes at 4°c.
6. After centrifugation, the 50 µl of supernatant was transferred carefully into a fresh new tube (carefully avoid the pellet). The supernatant was used as template DNA.
7. Measure DNA quantity by UV absorption at A260 (1.0 OD unit is equivalent of 50 µg). Assess genomic DNA quality by an analytical 0.7% agarose gel. The length of genomic DNA is around 50 kb
8. Store the supernatant with extracted DNA at -20 ^{o}c for as long as needed. PCR confirmation may be done at a later time.

9. Use up to 5 µl of the collected supernatant per 50 µl PCR reaction or up to 2 µl per 20 µl reaction.

   B. Protocol for master mix reaction preparation

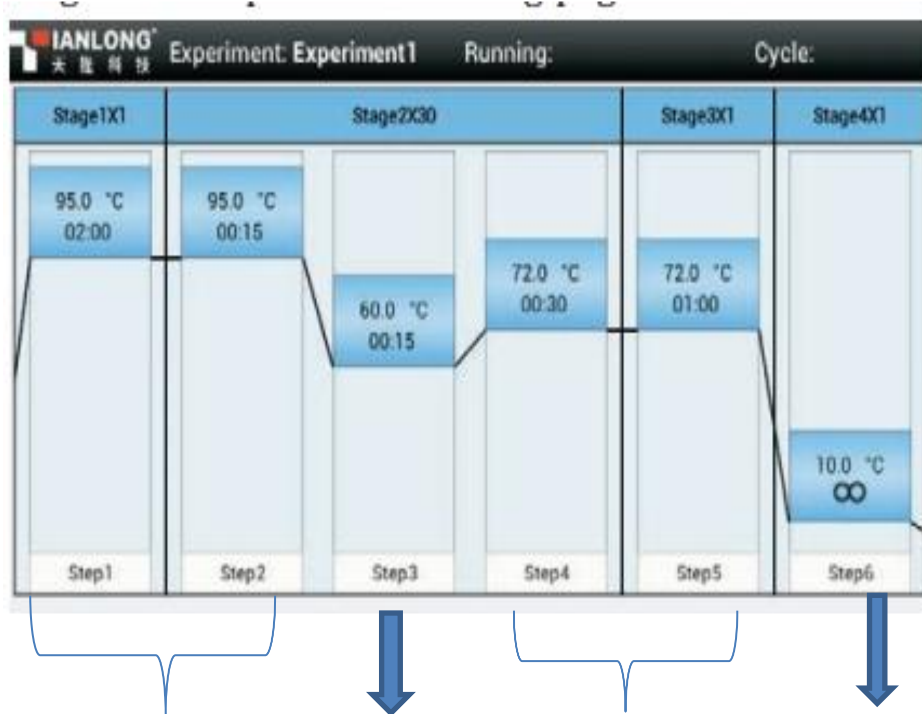
1. Use ice to thaw the frozen reaction components, including the master mixture.
2. Centrifuge after fully mixing by inverting tubes multiple times, and then set aside on ice.
3. Prepare (on ice) adequate master mix for n-reactions, incorporating all necessary ingredients (apart from the DNA template) and one additional reaction to account for any pipetting errors.

Master Mix preparation methods

Components	Final concentration	Volume for one reaction (µl)	Volume for (n) reaction (µl)
2X Gotaq PCR Master Mix	1X	12.5	
Primer F	0.5µl	1	
Primer R	0.5 µl	1	
Nuclease free water		8	
Total volume		22.5	

4. Before spinning down the mixture, combine the master mix by repeatedly inverting the tubes
5. Carefully transfer 22.5µl of the master mixes into each individual strip tube, making sure the mix reaches the tubes' bottoms.
6. Fill each tube with 2.5µl of the extracted DNA template.

C. The cycling program of PCR reactions



Denaturing/melting Annealing Elongation/extension step Cooling

D. TAE buffer (50 x, 0.04M, pH 8.5)

Prepare 600mL of dH_2O in a suitable container. Add 242g of Tris free base to the solution. Add 18.61g of Disodium EDTA to the solution. Add 57.1g of Glacial Acetic Acid to the solution. Adjust the pH 8.5 using 1M NaOH. Add d H2Ounity volume is 1 litter. Store the 50x stock solution in the fridge. Use 1 x TAE buffer for running gel electrophoresis and for preparation of the gel. Change the buffer in gel electrophoresis once a week or as soon as you see impurities floating in the chamber.

E. Prepare 1.5% Agarose gel

Small gel (10cm x 7.5cm x 2cm, 50ml): Mix 0.75g of Agarose with 50ml of 1x TAE buffer in a glass flask. Medium gel (14cm x 10cm x 4cm, 100ml): Mix 1.5g of Agarose with 100ml of 1x TAE buffer in a glass flask. Close the flask (not tightly, to allow for the steam release), lightly swirl the suspension and microwave for 30s, carefully swirl the flask (wear the heat- protective gloves), microwave for another 30s, carefully swirl again (wear the heat- protective gloves) and microwave for another 15s,

there should be no visible particles of agarose floating in the suspension prior, if there are some, continue boiling the suspension.

Let the gel cool down for approximately 3min and the pour in the cast with the well comp placed into the comb holders. Let the gel solidify for 30 min (may differ depending on size) prior to transferring into electrophoresis chamber.

F. Agarose gel electrophoresis analysis of the PCR products
    1. At the PCR program, load 2 µl of loading buffer with gel red for 5 µl of amplified PCR product on 1.5% Agarose gel (1.5g of Agarose in 100ml of 1XTAE).
    2. Load 4 µl ladder plus 2 µl loading dye in the 1st well
    3. Load 8 µl positive controls second well.
    4. Load 8 µl of DNA molecular weight marker starting into the third wells.
    5. Load 8 µl Negative controls at the end into last well.
    6. Run electrophoresis at 100 volts for 60 minutes.
    7. Amplified fragment sizes were 180bp, 255bp, 384bp and 534bp for *stx1, sxt2, eaeA* and *hlyA* genes, respectively for this study.

G. Gel capture use the gel documentation system to snap the gel
   The size of the PCR product were determined by UV illumination

Annex 10: Muller Hilton agar concentrations used for the antimicrobial sensitivity testing

Difco TM Muller Hilton agar (Becton Dickinson and company, 7 Loveton circle, sparks, MD 21152 USA) Ingredients gm/liter: Beef extract powder 2.0g, dehydrated infusion 300 g, Acid digest Casein 17.50 g, Starch 1.50g, Agar 17.0g, PH at 7.3 ± 0.1 Preparations: Add 38 gram to 1000ml of distilled water. The solution was boiled to dissolve completely and sterilized by autoclaving at 121 ºC for 15 minutes. Cool to 45-50 ^{o}c and shake the medium in order to oxidize the methylene blue (i.e. restore its blue color) and to suspend the precipitate which is an essential part of this medium. Before use the media was cooled to 45°C and poured onto sterile Petri dishes. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility and to dry the condensed vapor on the plate cover.

ANNEX 11: The procedure Disk Diffusion Method

1. At least 4-5 pure colonies of the isolates were picked from the nutrient agar and homogenized in a tube containing 4-5 mL of sterile saline solution

2. The turbidity of the suspension was adjusted to the density of 0.5 McFarland standards by adding a sterile saline solution or more colonies to standardize the size of the inoculum.

3. A sterile cotton swab was immersed in the suspension, 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.

4. The streaking procedure was repeated three or more times and the plate were rotated at approximately 60^0ceach time to ensure an even distribution of inoculum over the Mueller-Hinton agar.

5. The plates were held at room temperature for 15 minutes to allow drying of the flood.

6. Antimicrobial discs were distributed evenly on the MH plate so that they were placed 24mm gap between each other and 15mm from the edge of the plates to prevent overlapping of the inhibition zones. This is equivalent to 6 discs per standard 90mm petri plate.

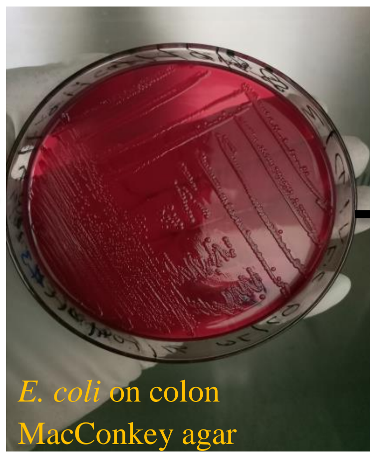
7. After the plate's dried, antibiotic disks were placed on the inoculated plates using sterile forceps were used to distribute and gently press down the disks into MH agar to insure complete contact with the agar surface.

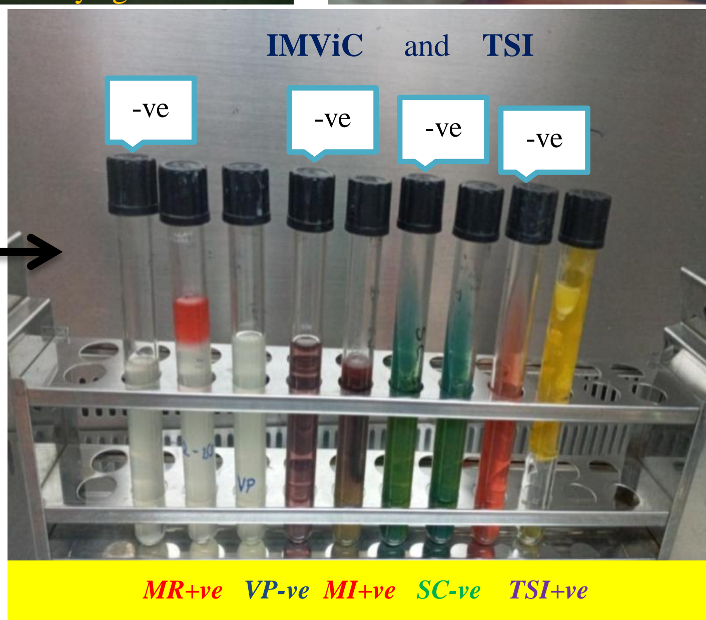
8. The plates were allowed to stand for 30 minutes for the diffusion of the active substance of the agents and incubated at 37^0c for 24hours in an upside-down position.

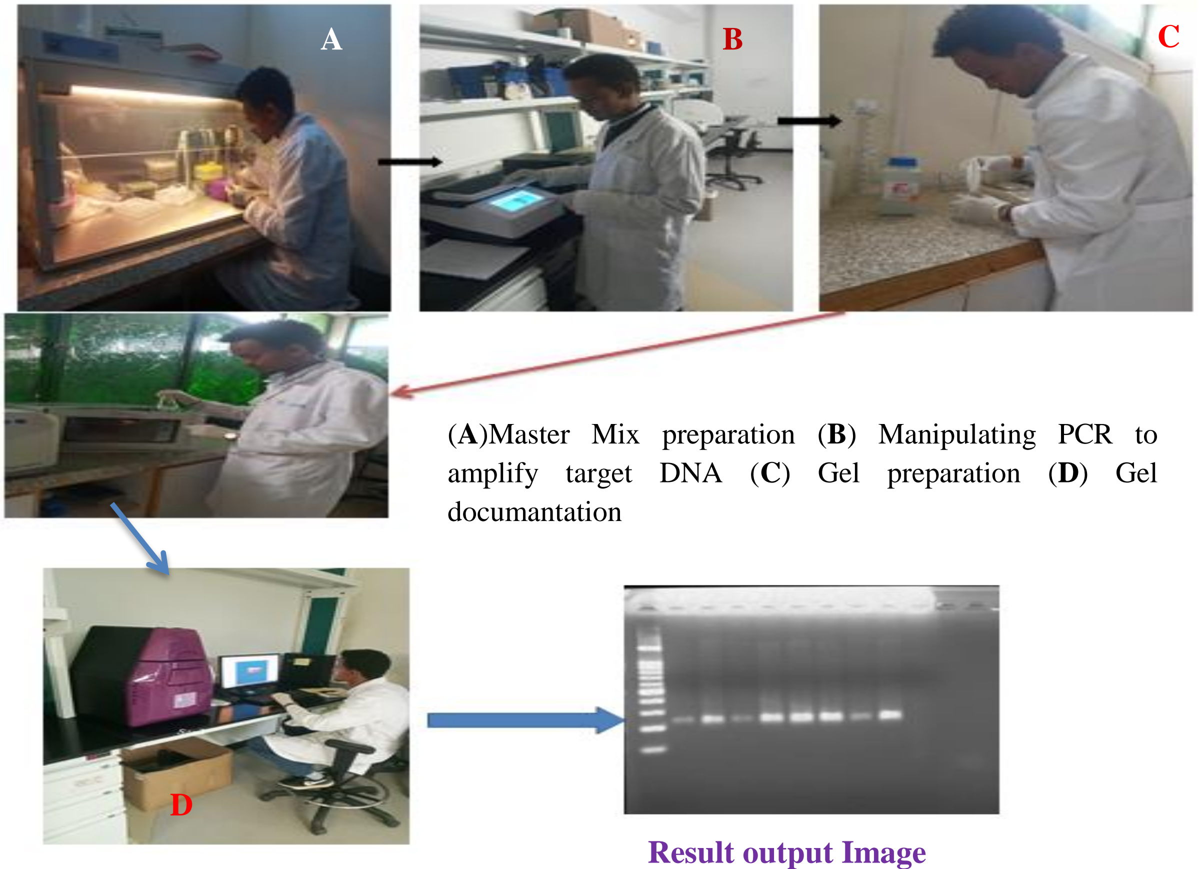
9. Finally, after overnight incubation, the diameters of the zone of inhibition around each disc were measured, with the help of caliper (a ruler) which was held on the black of the inverted MH plate.

10. The result of isolates were interpreted as susceptible (S), intermediate (I), and resistant (R) based on a standard zone of the interpretative chart presented in table 6.

Annex 12: Miscellaneous Images of Lab. Work Done Inside the Study







(**A**)Master Mix preparation (**B**) Manipulating PCR to amplify target DNA (**C**) Gel preparation (**D**) Gel documantation

Photo: Work flow in the molecular Laboratory

Photo: Zone of inhibition measurement using different AM discs