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ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE
DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH (MIVPH)

DETECTION AND ANTIMICROBIAL RESISTANCE PROFILE OF *SALMONELLA* ISOLATED FROM RAW COW MILK AND ITS PRODUCTS IN BISHOFTU TOWN, CENTRAL ETHIOPIA: ITS IMPLICATION FOR PUBLIC HEALTH

BY

LEMA TEMESGEN GILE

JUNE, 2024

BISHOFTU, ETHIOPIA

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A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Public Health

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BISHOFTU, ETHIOPIA

APPROVAL

ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE
DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY AND PUBLIC HEALTH

As MSc research advisors, we certify that we have read and evaluated the thesis prepared under our guidance by **Lema Temesgen Gile** entitled: "**Detection and Antimicrobial Resistance Profile of *Salmonella* Isolated from Cow Milk and its Products in Bishoftu Town, Central Ethiopia: Its Implication for Public Health**." We recommend that it be accepted as fulfilling the thesis requirement for the degree of Master of Science in Veterinary Public Health.

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APPROVAL

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As members of the examining board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by **Lema Temesgen Gile** entitled: "**Detection and Antimicrobial Resistance Profile of *Salmonella* isolated from Cow Milk and its products in Bishoftu Town, Central Ethiopia: Its Implication for Public Health**." And recommend that it be accepted as fulfilling the thesis requirement for the degree of Master of Science in Veterinary Public Health.

Chairperson	Signature	Date
External Examiner	Signature	Date
Internal Examiner	Signature	Date

Final approval and acceptance of the thesis dissertation is contingent upon submitting its final copy to the CGS/FGC through the departmental graduate committee (DGC) of the candidate's major department.

I hereby certify that I have read the revised version of this thesis prepared under my direction and recommend that it be accepted as fulfilling the thesis/dissertation requirement.

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STATEMENT OF AUTHOR

First, I declare that this thesis is my *bona fide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced MSc degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under the rules of the library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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Name: Lema Temesgen Gile Signature: _____

Date of Submission: _____

College of Veterinary Medicine and Agriculture, Bishoftu

BIOGRAPHICAL SKETCH

The author, Lema Temesgen was born to his father Temesgen Gile, and his mother Agitu Muleta. On June 26, 1991, Cari Jarso village located 7 km Western of Sire town of Sibu Sire district, East Wollega Zone, Oromia Region. He attended his elementary education at Sibu Sire Primary School from 1997 to 2005 and secondary education at Sibu Sire Secondary School from 2005 to 2009. Then, he joined Addis Ababa University College of Veterinary Medicine and Agriculture and graduated on July 9, 2015, with a Doctor of Veterinary Medicine Degree. He was employed by East Wollega Zone, Gida Ayena Woreda Livestock, and Fisher's Office on Type B Veterinary Clinic as head of the clinic. He served the Woreda for 4 years and transferred to Sibu Sire Woreda and served the Woreda for 2 years as a clinician until he joined the Postgraduate Program Addis Ababa University College of Veterinary Medicine and Agriculture to pursue his MSc degree in Veterinary Public Health in June 2024.

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LIST OF ABBREVIATIONS

AHI	Animal Health Institute
AMC	Amoxicillin-Clavulanic acid
AMP	Ampicillin,
AMR	Antimicrobial resistance
AMU	Antimicrobial use or antimicrobial usage
AST	Antimicrobial susceptibility test
AX	Amoxicillin
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CN	Gentamicin
CVMA	College of Veterinary Medicine and Agriculture
FBD	Foodborne diseases
H2S	Hydrogen sulphide
MDR	Multidrug-resistant
NTS	Non-typhoidal Salmonella
RVS	Rappaport-Vassiliadis with soya
TE	Tetracycline
TMP-SXT	Trimethoprim-Sulfamethoxazole
TSI	Triple Sugar Iron (TSI) test
VP	Voges Proskauer
XLD	Xylose lysine deoxycholate agar

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ABSTRACT

Salmonella is a significant foodborne pathogen, with milk and milk products commonly implicated in its transmission. However, limited information is available regarding the direct link between antimicrobial use (AMU), dairy hygiene practices, and antimicrobial resistance (AMR) in *Salmonella* strains isolated from dairy products in Bishoftu town. Cross-sectional research was done from October 2023 to April 2024 to assess dairy farmers' antimicrobial usage (AMU) and hygiene practices and the occurrence of antimicrobial resistance (AMR) profiles of *Salmonella* isolated from raw cow milk and its products. Two hundred samples were collected from dairy farms, milk vendors, and restaurants and analyzed using standard microbiological methods. Using the OmniLog system, *Salmonella enterica* was successfully identified. Then, the antimicrobial susceptibility was evaluated using the Kirby-Bauer disc diffusion technique. A structured questionnaire was also used to assess the milk value chain's knowledge, attitude, and practices (KAP) regarding AMU, AMR, and hygiene practices. Data were analyzed using STATA version 14.2. Overall, 2% (n = 4) of the samples tested positive for *S. enterica*. of the 4 isolates 3 were identified in dairy farm samples, whereas 1 were isolated from milk vendors. However, no *Salmonella* was identified in cheese or yogurt samples obtained from the restaurants. Regarding the AMR profile, *S. enterica* isolates were resistant to amoxicillin (75%), streptomycin (75%), and tetracycline (50%). Resistant to two or more antimicrobials were identified in 75% of the isolates. Among 41 dairy farmers interviewed it was found that most of the respondents had sufficient knowledge (78%), desired attitudes (90%), and good practices (76%) regarding AMU and AMR. However, 36% of dairy farms had poor hygienic practices. In conclusion, the current investigation indicated contamination of cow milk and its products with *S. enterica*. Therefore, appropriate control measures, including awareness creation among personnel and improving hygienic practices at the milk value chains is recommended to mitigate cross-contamination.

Keywords: *AMU and AMR, KAP assessment, Milk vendors, and S. enterica*

1. INTRODUCTION

Foodborne illnesses seriously threaten global public health, safety, and the economy. Every year, an estimated 600 million infections and 420,000 fatalities occur because of food-borne diseases (Havelaar *et al*., 2015). *Salmonella species*, prominent among foodborne pathogens, ranks as the third largest cause of mortality from diarrheal diseases globally. (Ferrari *et al*., 2019). It is responsible for an estimated 115 million human infections and 370,000 fatalities every year (Qin *et al*., 2022). The World Health Organization (WHO) estimates that around one in ten individuals become sick from foodborne Salmonella infections each year, resulting in the loss of millions of healthy life years (Lee and Yoon, 2021; WHO, 2022). This problem is widespread, affecting countries globally, but developing countries face challenges due to inadequate food safety regulations, poor food handling practices, and limited financial resources (Fufa *et al*., 2017; Ali *et al*., 2022; Bedassa *et al*., 2023).

Salmonella species are gram-negative, rod-shaped bacteria from the Enterobacteriaceae family (WHO, 2022) and consist of two species, *Salmonella bongori* and *Salmonella enterica*, according to the White-Kauffmann system. This categorization is based on the surface structures (lipopolysaccharides, flagella, and capsular polysaccharides). The species *Salmonella enterica* has six subspecies: enterica, salamae, arizonae, diarizonae, houtenae, and indica, with around 2600 serovars (Vinueza, 2017; Ferrari *et al*., 2019). Among these, the subspecies *Salmonella enterica* is responsible for about 1500 serovars, of which 99% might cause infections in animals and humans (Ballal *et al*., 2016). *Salmonella enterica* is classified into two classes based on clinical features of human infections: Typhoidal Salmonella is specific to humans and causes typhoid fever, but Nontyphoidal Salmonella (NTS) has a wide range of hosts and causes several illnesses other than typhoid fever (Fanta, 2021). NTS serotypes are the leading cause of bacterial diarrhea and invasive infections, posing a significant risk to young children, the elderly, and those with weakened immune systems in developing countries (Andoh *et al*., 2017).

Salmonella, a ubiquitous bacterium, poses significant public health concerns due to its ability to infect various animals and contaminate the environment. It is commonly found in the digestive systems of both domestic and wild animals (Ehuwa *et al*., 2021) and shed in their feces, facilitating its widespread presence in animal waste, sewage, and contaminated materials (Pal *et al*., 2015; Abrar *et al*., 2020). As a result, milk and dairy products are susceptible to contamination by infected animals or cross-contamination with fecal-containing pathogens during processing (Teklemariam *et al*., 2023). When humans consume contaminated food, they can develop salmonellosis, a diarrheal illness that can range from mild to severe, including abdominal cramps, fever, nausea, and vomiting. In extreme circumstances, *Salmonellosis* can cause dehydration and even death (Pal *et al*., 2015).

Milk is considered a highly nutritious food, but it can also be a vehicle for microbial hazards, particularly in developing countries where the hygiene and sanitation practices of dairy farms are inadequate (Kashima *et al*., 2013). The bacteria that are associated with raw milk include *Escherichia coli O157:H7, Salmonella enterica, Listeria monocytogenes, Campylobacter spp*., and *Staphylococcus aureus* (Verra *et al*., 2015). These can induce severe gastroenteritis in humans (WHO, 2015). Infections with *Salmonella species* are particularly significant because they cause bacteremia in adults and children in developing countries (Andoh *et al*., 2017).

Antimicrobial resistance (AMR) has emerged as a significant worldwide public health problem, posing substantial challenges to effectively treating bacterial infections (Sobur *et al*., 2019). *Salmonella*, a common foodborne pathogen, is among the bacteria that have developed resistance to various antibiotics. The inappropriate use of antibiotics in livestock particularly, dairy farms has contributed to the rise of antimicrobial-resistant (AMR) Salmonella which can increase human health risks (Endrias Zewdu and Cornelius 2009; Fufa *et al*., 2017). Some MDR *Salmonella* infections in humans have been connected to exposure to dairy farms or contaminated dairy products (Abrar *et* al., 2020). *Salmonellosis*, a costly disease affecting dairy producers, can lead to limited treatment options, prolonged illnesses, decreased milk yield, increased healthcare costs, and potentially fatal outcomes.

Farmers must be aware of *Salmonella's* presence in seemingly healthy cows, as it poses significant food safety concerns (Fufa *et al*., 2017; Bedhasa *et al*., 2022).

In nations with low and middle incomes, such as Ethiopia, there is a growing demand for animal protein, leading to the routine use of antibiotics for growth-promoting, therapeutic, and preventative reasons in livestock production (Van Boeckel *et al*., 2015). Improper antibiotic use in livestock, particularly on dairy farms, along with inadequate waste management practices, can result in the release of resistant pathogens into the environment. This practice poses a significant risk as it can contribute to the emergence of antibiotic-resistant pathogens. This may lead to the development of antibiotic-resistant commensal organisms in livestock, posing a threat to public health (Pandey *et al.,*2024).

Besides improper antibiotic use, the habit of consuming raw milk or unsafe food, cross-contamination, improper food storage, poor personal hygiene practices, inadequate cooling and reheating of food items, and a prolonged time lapse between preparing and consuming food items have been reported as contributing factors to an outbreak of salmonellosis in human (Vanga and Raghavan, 2018). This suggests that milk and dairy products could be a source of *Salmonella* in Ethiopia in general and may be particularly significant in the central part of Ethiopia, where consumption of milk and milk products is high. Therefore, it is important to isolate pathogenic organisms, identify relevant risk factors, and regularly assess their AMR profiles.

In Ethiopia, despite multiple studies identifying *Salmonella* in milk and dairy products, including evidence of its prevalence among raw milk consumers (Tesfaw *et al*., 2013; Ejo *et al*., 2016; Beyene *et al*., 2016; Fufa *et al*., 2017), there remains a critical gap in understanding the direct relationship between antimicrobial use (AMU), dairy hygiene practices, and the development of antimicrobial resistance (AMR) in *Salmonella* isolated from dairy products in the current study area. These gaps need for systematic surveillance and comprehensive investigation along the entire farm-to-fork continuum, encompassing routine examination of raw milk and milk product samples from restaurants, milk vendors, and dairy farms. Addressing these gaps is crucial to safeguarding consumer health,

mitigating foodborne illnesses, and minimizing both direct and indirect economic losses associated with contaminated dairy products in Ethiopia.

1.1 Objectives

General objective

- To assess antimicrobial usage (AMU) and hygienic practices of dairy farmers and the occurrence of antimicrobial resistance (AMR) profile of *Salmonella* isolated from raw cow milk and its products in Bishoftu town.

Specific objectives

- To isolate *Salmonella* from milk and milk products.
- To evaluate the AMR profile of *Salmonella* isolated from milk and other dairy products.
- To assess dairy farmers' AMU and hygienic practices in Bishoftu dairy farms.

2. LITERATURE REVIEW

2.1 Historical Background, Taxonomy, and Characteristics of *Salmonella*

At first, *Salmonella* was discovered and isolated from the intestine of pigs infected with classical swine fever by Theobald Smith in 1855(Eng *et al*., 2015). The bacterial strain was named after Dr Daniel Elmer Salmon, an American pathologist who worked with Smith (Vinueza, 2017). The *Salmonella* classification has been debated over time. According to the latest nomenclatures, the genus *Salmonella* consists of only two species based on differences in their 16S rRNA sequence analysis: *Salmonella enterica* and *Salmonella bongori* (Yada, 2023). Based on biochemical properties and genomic relatedness, *Salmonella enterica* is further classified into six subspecies (Greeshma *et al*., 2023). Roman numbers are used to identify these subspecies: I: *S. enterica subsp. enterica*, II*: S. enterica subsp. salamae,* IIIa: *S. enterica subsp. arizonae*, IIIb: *S. enterica subsp. diarizonae,* IV: *S. enterica subsp. houtenae* and VI: *S. enterica subsp. indica*. Among all the subspecies of Salmonella, *S. enterica subsp. enterica* (I) is found predominantly in mammals and accounts for approximately 99% of Salmonella infections in humans and warm-blooded animals. In contrast, the other five Salmonella subspecies and *Salmonella bongori* are found mainly in the environment and also in cold-blooded animals, and hence are less common in humans (Eng *et al*., 2015; Hassen, 2020).

In addition, the Kauffman and White system classifies *Salmonella* into serotypes based on three major antigenic determinants: somatic (O), capsular (K), and flagellar (H) (Vinueza, 2017). The O antigen is located on the outer cell membrane and is heat stable, forming part of the bacterial lipopolysaccharide (LPS). *Salmonella* serotypes can express multiple O antigens. The H antigens located in the flagella are heat-labile and activate the host immune response. Most *Salmonella species* have two flagellar protein genes, allowing them to be diphasic (expressing one protein at a time). Phase I H antigens determine immunological identity, while phase II antigens are non-specific. The K antigens are rarely found in most *Salmonella* serotypes and are heat-sensitive polysaccharides located on the bacterial

capsular surface. The virulence. Vi antigens are a subtype of K antigen found only in serotypes Paratyphi C, Dublin, and Typhi (Jajere, 2019).

Salmonella was a rod-shaped, motile, facultatively anaerobic, gram-negative, non-spore-forming bacterium that belonged to the Enterobacteriaceae family. Most of its strains are motile owing to peritrichous flagella (Figure 1), except *Salmonella gallinarum* and *Salmonella pullorum* which do not have flagella (Fanta, 2021), and ferment glucose with acid and gas production. Certain strains are adept at forming protective biofilms that shield them from various environmental challenges and antimicrobial treatments. These biofilms provide a defense mechanism against acidic conditions, dehydration, the host immune system's attacks, and antimicrobial agents (Abdullah *et al*., 2017).

Salmonella bacteria thrive best at temperatures between 35°C and 37°C, a pH of around 6.5 to 7.5, and a water activity of 0.84 to 0.94. However, they can still grow to some extent within a temperature range of 5°C to 45°C and a pH range of approximately 4.0 to 9.0 (Ruvalcaba Gómez *et al.,* 2022). *Salmonella* infections typically cause acute gastroenterit is, which is self-limiting in healthy individuals. There are over 2600 different serotypes of *Salmonella* (Elnekave *et al*.,2020), with the majority belonging to the subspecies *Salmonella enterica*. While over 200 serotypes can potentially cause illness in humans, *Salmonella enteritidis* and *Salmonella typhimurium* are the two most common serotypes causing human salmonellosis (Gong *et al*., 2022).

Figure 1: *Salmonella* bacteria with peritrichous flagella.

Source: (Rahman *et al*., 2018).

2.2 Epidemiological Distribution of *Salmonella*

2.2.1 Host range and geographical distribution

Salmonella is a versatile pathogen found in diverse hosts, including humans, animals (reptiles, rodents, birds, and amphibians), and contaminated environments (Gong *et al*., 2022). Some *Salmonella* strains are specific to certain hosts, while others are adaptable to multiple species. Host-specific strains, such as *Salmonella typhi* and *Salmonella paratyphi* in humans and *Salmonella gallinarum* and *Salmonella pullorum* in poultry, only infect one host. Host-adapted strains, like *Salmonella dublin* in cattle and *Salmonella. choleraesuis* in pigs, primarily target a specific host but can also cause illness in other animals. Non-host-specific strains, such as *Salmonella enteritidis* and *Salmonella typhimurium* can cause an asymptomatic carrier state in a wide range of hosts, including humans. These strains are also responsible for a broader geographic spread compared to host-adapted strains (Jajere, 2019).

Salmonella is found worldwide but appears to be most prevalent in areas of intensive animal husbandry, especially dairy and poultry production (Pal *et al*., 2015), with different serovars being more common in different regions. For example: *Salmonella typhimurium* is the most common serovar globally and is found in both developed and developing countries (Ramatla *et al*., 2022). *Salmonella enteritidis* is more common in developed countries, where it is often associated with contaminated eggs. In contrast, *Salmonella typhi* is primarily found in developing countries, where it is a major cause of typhoid fever (Eng *et al*., 2015).

2.2.2 Sources and route of transmission

Salmonella bacteria are commonly found in both domesticated and wild animals. They are especially prevalent in food animals, including poultry, pigs, and cattle, as well as in pets such as cats, dogs, birds, and reptiles like turtles (Fazza *et al*., 2021). *Salmonella* bacteria primarily reside within the intestines of animals and humans, excreting the bacteria through their feces. Foods of animal origin, especially meat, poultry, eggs, milk, and milk products, are the main sources of infection for people. These foods also contribute significantly to the main cause of human Salmonellosis (Hassen, 2020). Certain serotypes are limited to a specific animal reservoir, while others can spread across species and cause diseases in humans, either through direct contact or via food (zoonosis) (Kahsay *et al*., 2023). Animal feces are more significant than human waste, and it's worth noting that animal hides and poultry products may become contaminated from this source (Mohammed *et al*., 2018).

Animal-derived foods, particularly chicken, are a major source of *Salmonella* infections in humans. This is especially the case for foods that might be contaminated by handling them by infected individuals or carriers (Kemal *et al*., 2015 Dairy products (ice cream, cheese, butter, yogurt, and custard), raw and undercooked poultry, beef and pig meat, meat products (burger, luncheon, hash, and sausage), and milk (fresh, raw, fermented, inadequate pasteurized, recontaminated pasteurized, or improper handling) (Abebe *et al*., 2020). Feces containing *Salmonella* have the potential to infect pasture, vegetables, soil, food, and water. Humans and other animals may become infected due to environmental contamination (Rukambile *et al*., 2019).

Salmonella can pass through the entire food chain, from animal feed to primary production and eventually to households and food establishments (Fazza *et al*., 2021). Transmission often occurs when *Salmonella* is introduced into food preparation areas, which can multiply in food due to improper storage temperatures, inadequate cooking, or cross-contamination. Direct contact with infected animals or humans facilitates transmission (Carrasco *et al*., 2012). In humans, Salmonellosis is primarily acquired through the consumption of contaminated food of animal origin, particularly eggs, meat, poultry, and milk (Hoelzer *et al*., 2011). However, other foods, such as green vegetables contaminated with manure, have also been implicated in *Salmonella* transmission (Figure 2).

Milk is most likely to become contaminated by feces from an animal with clinical Salmonellosis or a healthy carrier animal during the milking process. Infection can also be acquired by direct contact in the hospital with patients, their discharges, and contaminated fomites (Pal *et al*., 2015). The fecal-oral transfer is another method of transmission from person to person. When people come into touch with diseased animals, including pets, human cases can also arise (WHO,2018). These infected animals often do not show signs of disease.

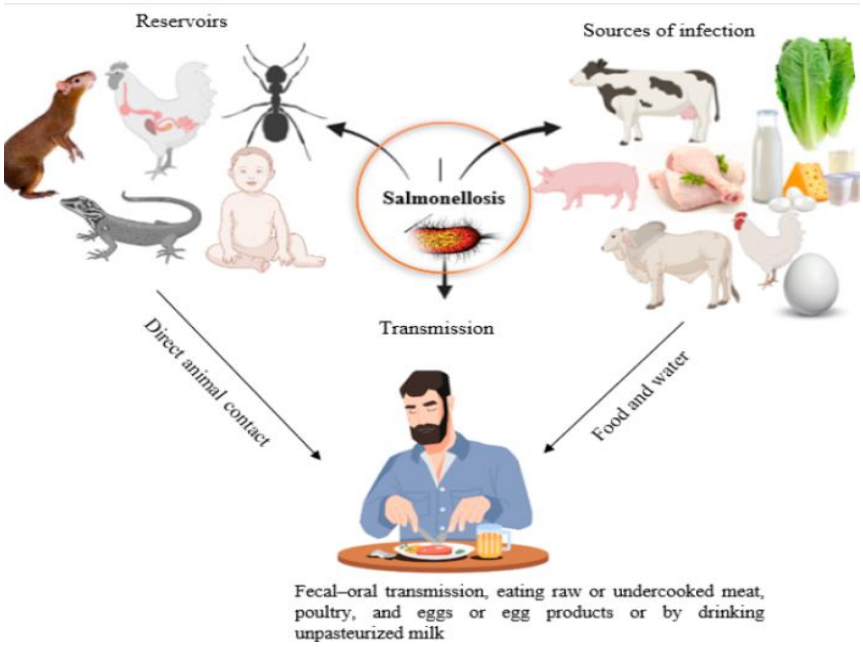


Figure 2**:** Diagram illustrating the main infection source, reservoir, and mode of transmission for human salmonellosis (Teklemariam *et al*., 2023).

Insects, reptiles, humans, and animals can serve as reservoirs for human salmonellosis. Uncooked, undercooked, and/or contaminated foods are familiar sources of infection.

2.2.3 Pathogenesis

Salmonella pathogenesis is a complex process that changes according to the serovars, dosage, age, and immunological condition of the host. It primarily multiplies in the digestive tract, leading to widespread environmental contamination through fecal excretion. The infection is nearly always contracted by ingesting bacteria, typically through contaminated food or water (Rahman *et al*., 2018). Individuals with compromised immune systems, such as children under five years old, and the elderly are more vulnerable to *Salmonella* infections compared to healthy individuals. Almost all strains of *Salmonella* are pathogenic as they can invade, replicate, and survive in human host cells, resulting in potentially fatal disease (Eng *et al*., 2015).

Upon entering the digestive tract via contaminated sources, *Salmonella* bypasses the epithelial cells lining the intestinal mucosa, caeca, tonsils, and Peyer's patches. The fimbriae and flagella on *Salmonella's* surface aid in initial contact with enterocytes or M cells, enabling colonization of the gastrointestinal tract (GIT) (Eng *et al.*, 2015). After attaching to the intestinal epithelium, S*almonella* utilizes its type three secretion system to inject toxins and other effector proteins into intestinal cells. These effector proteins, delivered through the type three secretion system, trigger gastroenteritis, activate secretory pathways, promote inflammation, and disrupt ion balance, leading to the development of diarrhea in the host. Additional significant effector proteins enable the engulfment of *Salmonella* cells by the host cell membrane, forming *Salmonella*-containing vacuoles. Ordinarily, the host cell's immune response triggers the fusion of phagolysosomes to eliminate intracellular bacteria. However, *Salmonella*-containing vacuoles play a crucial role in intracellular survival and proliferation within macrophages by evading phagolysosomal destruction (Kabir, 2018).

Within macrophages, *Salmonella* employs its *Salmonella* pathogenicity island type three secretion system to release effector proteins into the *Salmonella*-containing vacuoles, facilitating the bacterium's escape from the vacuole. Moreover, the *Salmonella* pathogenicity island type three secretion system prevents the fusion of Salmonella-containing vacuoles with lysosomes. Once *Salmonella* survives within macrophages and dendritic cells, it can reach primary multiplication sites like the liver and spleen, which are rich in reticuloendothelial tissue. The ability of *Salmonella* to persist in splenic macrophages contributes to its delayed clearance from the host (Foley *et al.*, 2013).

2.4 Clinical Signs, Diagnosis and Treatment

The most common clinical manifestation of human Salmonellosis is self-limiting gastroenteritis, which usually goes away in two to seven days and is marked by diarrhea, stomach pain, fever, headache, nausea, and vomiting. Systemic bacteremia is a serious and sometimes fatal illness that can develop from an infection in susceptible groups, such as small children and the elderly (Galan *et al*., 2023). Animals frequently get subclinical infections, in contrast to clinical infections. Animal populations may become intermittent or chronic carriers of bacteria if the bacteria are allowed to spread unnoticed. There are several ways that infection can spread, such as through direct contact with infected people, ingestion of contaminated feed or environmental sources, contact with contaminated equipment, ingestion of contaminated water or feces during farm practices, and possible involvement of arthropods as vectors (Galan *et al*., 2023).

The diagnosis was based on the isolation of the organism from either tissue obtained aseptically from environmental samples, milk, blood, rectal swabs, or the carcass. Placenta, vaginal, and fetal stomach swabs, as well as egg contents in the case of poultry, must all be cultured in cases of infection of the reproductive organs. However, in clinically normal carrier animals, diagnosing salmonellosis is more challenging. Therefore, serotyping is very important since there are many different Salmonella serovars and identifying the infection source and the path of transmission is a crucial precondition (Gudina *et al*., 2017: Guyassa and Dima, 2022).

Another method for identifying *Salmonella* is to isolate the pathogen from tissues that are aseptically removed during a necropsy, from ambient samples obtained from the dairy farm, and from animal housing. Although a provisional diagnosis may be made, the clinical indications and findings during the postmortem examination were not specific to salmonellosis, even though serological testing helped identify affected herds. When culture of the placenta, fetal stomach contents, vaginal swabs, and, in the case of poultry, embryonated eggs were required (Pal *et al*., 2020).

Bacteriological culture methods remain the "gold standard" for isolating and identifying *Salmonella*. Traditional culture techniques have formed the foundation for developing supplementary tools to determine the causes of microbial diseases and investigate the clinical and biological characteristics of emerging bacterial diseases. The traditional isolation of *Salmonella species* involves a nonselective pre-enrichment step, followed by a selective enrichment step, and finally plating onto selective media. Nonselective pre-enrichment media provide nutrients to restore sub-lethally injured *Salmonella* cells while suppressing the growth of competing microorganisms. Similarly, selective media contain inhibitors that restrict the growth of non-Salmonella species while allowing Salmonella to thrive (Lee *et al.,* 2015).

According to ISO 6579-1:2017, buffered peptone water (BPW) is used for the pre-enrichment of samples whereas, Rappaport Vassiliadis (RVS) and Muller-Kauffmann Tetrathionate are commonly used for selective enrichment (Mooijman *et al*., 2019). Biochemical assays, molecular detection, and serological testing can confirm *Salmonella* colonies from selective plating media typically used on XLD and BGA (Ahmed *et al*., 2014). The conventional culture method serves as the basis for the isolation of Salmonella due to the ease of use, reliability of results, high sensitivity and specificity, and lower cost compared to molecular-based technologies. However, the procedure requires multiple subculturing tests and is hence time-consuming compared to molecular diagnostic techniques (Geetha and Palanivel, 2018).

In animal treatment, those suffering from severe dehydration, depression, and anorexia require supportive care with intravenous fluid. Cattle that are mildly or moderately dehydrated could benefit from oral fluid and electrolytes, which are also significantly less expensive than intravenous fluids. Multidrug-resistant organisms restrict the range of effective medications available. Electrolyte therapy was the most crucial treatment since treatment usually included giving energy and water. Since non-typhoidal *Salmonella* is known to be susceptible to antibiotic resistance, it's critical to use the right antibiotic, dosage, and duration of therapy when treating calves with antibiotics to prevent resistance (Davidson *et al*., 2018).

Salmonella-induced gastroenteritis usually resolves on its own in three to seven days, with fever subsiding after seventy-two hours. Therefore, treatment should focus primarily on replacing lost fluids and electrolytes (Pal *et al*., 2020). Antimicrobial therapy is generally not recommended for uncomplicated gastroenteritis or to reduce stool excretion during convalescence. Antimicrobial treatment, however, must need to be taken into consideration for any systemic *Salmonella* infection. In cases of life-threatening bacteremia or localized infections suspected to be caused by nontyphoidal *Salmonella* (NTS), empirical therapy should include a third-generation cephalosporin and a quinolone until susceptibility patterns are known. Amoxicillin can be effective in eradicating long-term carriage of *Salmonella*. Amoxicillin and quinolones have theoretical advantages over other antibiotics due to their high concentrations in bile and superior intracellular penetration (Mashe *et al*., 2021).

2.5 Prevention and Control

To effectively prevent and control *Salmonella* in milk and milk products, a comprehensive approach that encompasses on-farm and post-harvest measures is necessary. Key strategies for the dairy industry in this regard include the implementation of proper hygiene practices such as regular equipment cleaning and sanitization, maintaining clean and dry housing for animals, and practicing good personal hygiene. Additionally, effective biosecurity measures like controlling farm access, quarantining new animals, and preventing contact with wild animals should be implemented. Proper animal husbandry practices that include providing clean water and feed, maintaining proper ventilation, and monitoring animal health, can also help reduce the risk of *Salmonella* infection in farm animals (Holschbach and Peek, 2018).

Working with a veterinarian to implement responsible antimicrobial use practices can help reduce the development of antimicrobial-resistant strains of pathogens including Salmonella. Providing education and training to farmers, farm workers, and other stakeholders on the importance of preventing and controlling pathogens including in the dairy industry can also help promote best practices and improve food safety (Lemma *et al*., 2018).

2.6 Antimicrobial Resistance of *Salmonella Species*

Antibiotic resistance is a major biological risk that poses significant threats to both animals and humans. It increases morbidity and mortality rates, leading to a significant public health concern. In recent years, there has been a growing problem with bacteria that have become resistant to antibiotics (Hleba *et al*., 2011). This means that traditional antibiotic treatments are becoming less effective, making it more difficult to treat infections. Since the first report of a single antibiotic-resistant Salmonella strain in the early 1960s, the incidence of multidrug-resistant (MDR) *Salmonella* has increased globally. Multiple studies have shown that *Salmonella* strains from food animals are often resistant to commonly used antibiotics in humans, such as ampicillin, amoxicillin, tetracycline, oxytetracycline, and streptomycin (Dagnew *et al*., 2020). This resistance poses a significant threat to public health, as foodborne illnesses caused by antibiotic-resistant *Salmonella* can be difficult to treat. The emergence of MDR *Salmonella* clones has further complicated the situation. These clones have expanded globally, causing infections in both humans and animals. The spread of MDR *Salmonella* is facilitated by the interconnectedness of the food animal industry and international travel (Jajere, 2019).

In wealthier nations, regular laboratory testing helps determine the most effective antimicrobial treatment for *Salmonella* infections. However, in low-income areas like Ethiopia, this testing may not be feasible, leading to the risk of ineffective treatment, prolonged illness, and higher death rates. Using antibiotics without proper testing can allow *Salmonella* to persist within the host's cells, potentially causing asymptomatic carriage and contributing to complications and antibiotic resistance development (Maka and Popowska, 2016).

Genotypic analysis of antimicrobial resistance has revealed that specific genes play a crucial role in the development of antimicrobial-resistant *Salmonella* strains. For instance, the genes *tet*A, sul1, cat1, and aph have been identified as responsible for resistance to tetracyclines, sulfonamides, chloramphenicol, and aminoglycosides, respectively (Adesiji *et al*., 2014). These genes encoding antimicrobial resistance in *Salmonella* are often located on mobile genetic elements such as plasmids and variants of *Salmonella* Genomic Islands. These elements facilitate the horizontal transfer of resistance genes, contributing to the spread of antimicrobial resistance among *Salmonella* strains (Carroll *et al*., 2017).

Three recognized techniques are available for performing antimicrobial susceptibility testing: agar dilution, broth dilution, and disk diffusion. The least inhibitory concentration (MIC) of antimicrobial drugs that stop bacterial growth is often found using broth and agar dilution. According to Wiegand et al. (2008), the Kirby Bauer disk method, which uses disk diffusion, provides a straightforward and useful method. Antibiotic-impregnated disks are put on an agar plate with a pure bacterial isolate in the disk diffusion method. The solid culture medium (Mueller Hinton agar) absorbs antimicrobial chemicals as they move from the disks. A zone of growth inhibition will develop around the disks if the bacteria are sensitive to the antibiotics. Bacterial susceptibility and the concentration of the antimicrobial agent closely correlate with the size of the zone of inhibition (Eng *et al*., 2015; Mensah *et al*., 2019).

The extensive use or misuse of antimicrobial agents, including their use as therapeutics, prophylactics, and growth promotions, has led to the emergence of multidrug-resistant (MDR) bacteria. This phenomenon is particularly concerning because many bacteria acquire resistance in animals before being transferred to humans through the food chain (Geresu *et al*., 2021). Consequently, the public health risk of salmonellosis stems from the transfer of resistant strains to humans via the consumption of contaminated food products of animal origin.

2.7 Status of *Salmonella* in Ethiopia

2.9.1 Prevalence

Due to widespread poor food handling and sanitation practices, insufficient food safety laws, weak regulatory frameworks, a lack of financing for buying safer equipment, and a lack of training for food handlers, food-borne illnesses were prevalent in developing nations, including Ethiopia (Abebe *et al*., 2020). According to the National Hygiene and Sanitation Strategy program, over 60% of Ethiopia's illness burden is attributable to inadequate hygiene and sanitation, and over 250,000 children die from diseases connected to sanitation and hygiene each year (Ejo *et al*., 2016).

The outbreak of foodborne illnesses has been linked to several factors, including unsafe sources, contaminated raw food items, improper food storage, poor personal hygiene during food preparation, inadequate cooling and reheating of food, and a prolonged period between food preparation and consumption (Kariuki *et al*., 2017). Research carried out across the nation revealed the unhygienic state of catering facilities and the existence of harmful microorganisms such as *Salmonella, Campylobacter, Staphylococcus aureus,* and *Escherichia coli* (Odo *et al*., 2021).

Even though *Salmonella* populations in different geographical areas or in different hosts and environmental niches may undergo different evolutionary changes due to the centralization of food production and distribution and population movement, it is believed that *Salmonella* strains found in the 22 different countries of the world would be clonally related (Gallagher and McKevitt, 2019). NTS isolates in Ethiopia may have similar phenotypic and genotypic characteristics with isolates elsewhere in the world, and NTS enterica infection in children in Ethiopia was a major health problem and was caused by similar serovars to those reported from elsewhere in Africa: *Salmonella typhimurium* and *Salmonella enteritidis* (Zhang *et al*., 2018). The infection most commonly occurs in countries with poor standards of hygiene in food preparation and handling and where sanitary disposal of sewage was lacking. Studies indicated the widespread occurrence and distribution of Salmonella in Ethiopia (Miller *et al*., 2021).

Table 1: Prevalence of *Salmonella* in Ethiopia

Location	Source of sample	No of sample	Positive sample	Prevalence %	References
Kersa	Milk	100	20	20	(Negash *et al*., 2012).
Somali Re	Raw milk	120	4	3.3	(Wolde *et al*., 2016)
Gondar	Milk	50	3	6	(Mebrat Ejo *et al.,* 2016)
Addis Ababa	Bucket	20	1	5	(Zelalem *et al*., 2017)
Oromia	Raw milk	192	42	21	(Firdie *et al.,* 2020)
Arsi Negelle	Raw milk	86	8	9	(Gebeyehu *et al* 2022)
Amhara	Raw milk	80	11	14	(Zelalem *et al.,* 2017)
Modjo	Dairy farm	266	28	10.5	(Fufa *et al*., 2017)
Addis Ababa	Milk	195	6	3	(Addis Seleka *et al*.,2011)
Hawassa	Raw milk	164	14	8.54	(Gebeyehu *et al* 2022)

2.9.2 Antibiotic Resistance

Indiscriminate use of antimicrobial agents has led to the emergence of multidrug-resistant strains of *Salmonell*a, posing a global public health threat (Waghamare *et al., 2*018). In Ethiopia, studies have shown high levels of antibiotic resistance in *Salmonella* isolated from various sources, including chicken-related samples (Abunna *et al.*, 2016; Eguale, 2018), dairy cattle feces (Eguale *et al*., 2016), food animals and food products (Kebede *et al*., 2016; Geresu *et al*., 2021), and humans (Merera, 2018). This resistance undermines the effectiveness of antibiotics, making it more challenging to treat *Salmonella* infections and potentially leading to antimicrobial failure and treatment failures.

Studies have shown that many *Salmonella* isolates exhibit resistance to commonly used antibiotics such as ampicillin, oxytetracycline, and tetracycline. However, susceptibility testing has revealed that a limited number of isolates are resistant to ciprofloxacin and other quinolones, offering potential treatment options (Eguale *et al*., 2015). Among Salmonella strains, *Salmonella kentucky*, *Salmonella typhimurium*, *Salmonella concord*, and *Salmonella saintpaul* have demonstrated multidrug resistance (MDR), including resistance to multiple antibiotics. Notably, *Salmonella kentucky* strains have been found resistant to ciprofloxacin, despite previous reports of susceptibility. Despite these findings, there is still limited information on the specific genes responsible for antimicrobial resistance in most *Salmonella* serovars (Merera, 2018). Research in Ethiopia has identified several factors contributing to the emergence of antibiotic resistance: including inadequate infection prevention and control measures, overuse and misuse of antibiotics in humans and animals, poor sanitation and hygiene practices, lack of regulatory oversight for antibiotic use, limited access to diagnostic tools and antimicrobial susceptibility testing (Kebede *et al*., 2016b; Asfaw *et al*., 2020; Geresu *et al*., 2021).

2.8 Public Health Risk and Economic Impact of Salmonellosis

2.8. 1 Public Health Risks:

Salmonella is a major global health concern, causing an estimated 200 million to over 1 billion infections annually. Among these infections, 93 million results in gastroenteritis, a common cause of diarrheal illness and is responsible for approximately 155,000 deaths each year. Shockingly, 85% of these illnesses are directly attributed to contaminated food sources, highlighting the critical need for food safety measures to prevent Salmonella infections (He *et al*., 2023). *Salmonella* can be introduced into milk and dairy products through direct contact with contaminated sources within the dairy farm environment, as well as through excretion from the udder of an infected animal (Zeinhom and Abdel, 2014), potentially leading to human infections when these products are consumed. This poses a significant public health risk, as salmonellosis can cause gastroenteritis, severe diarrhea, fever, and, in some cases, more serious complications, particularly in vulnerable populations such as the young, elderly, and immunocompromised individuals (Pal *et al*., 2015).

It is clear that farm workers, calf farmers and their families are susceptible to infection with Salmonella species when clinical outbreaks occur, but the risk of exposure extends well beyond farm workers or veterinarians who come into direct contact with animals during outbreaks. People who have direct contact with the animal, its excrement or its milk are at risk of contracting *Salmonella* infection, which is characterized by asymptomatic shedding of the bacteria. This is also a problem for many other common bovine serovars, including Newport and Typhimurium (Holschbach and Peek, 2018). Globally, antimicrobial-resistant (AMR) *Salmonella* infections are a significant public health concern, causing approximately 700,000 deaths annually. This number is projected to increase dramatically, reaching an estimated 10 million deaths by 2050. The economic impact of AMR *Salmonella* infections is also substantial, with a projected loss of $100 trillion. In the United States alone, AMR affects approximately 2 million individuals each year, resulting in approximately 23,000 deaths (Balbin *et al*., 2020; Dadgostar, 2019).

2.8.2 Economic Impact:

Salmonellosis poses a substantial financial burden on farm animals due to expenses associated with managing clinical cases, such as treatment, diagnostics, laboratory testing, cleaning, disinfection, and prevention measures. Additionally, the detection of the disease within a herd can cause significant concern for producers as identifying infected animals can be challenging (Pal *et al.,* 2020).

The occurrence of human Salmonellosis cases linked to contaminated dairy products can lead to increased healthcare costs, including hospitalization, medical treatment, and potential productivity losses due to illness. In dairy farms, outbreaks of *Salmonella* can lead to decreased milk production, as infected animals may experience illness and reduced milk yield. Additionally, infected animals may require veterinary care, further impacting productivity and costs (Cummings *et al*., 2009). The detection of *Salmonella* in dairy products can lead to trade restrictions and market access issues. Contamination events can prompt recalls, impacting the reputation and marketability of dairy products, both domestically and internationally. Managing and treating *Salmonella* infections in dairy cattle can incur veterinary expenses, including diagnostic testing, treatment regimens, and potential losses due to morbidity and mortality among the herd (Agren *et al*., 2015).

3. MATERIAL AND METHODS

3.2 Study population

The study population was all dairy farms and milk and milk product sellers found in Bishoftu town. A total of 41 dairy farms,14 milk vendors and 28 restaurants selected randomly were included in the study. The study animals were apparently, healthy dairy cows in small-scale, medium-scale, and large-scale dairy farms located in the selected study areas. The study population included crossbreeds and local breeds in small-scale, medium-scale, and large-scale dairy farms. Most of them (85%) were crossbreeds whereas a few were local (15%). Concerning management, (78%) of the herds were managed intensively while (22%) of herds were semi-intensive (Figure 5). The intensively managed cattle were kept indoors and received concentrate feeds in addition to hay and crop residues (such as corn stalks, wheat/barley straw and other leftovers from grain threshing). On the other hand, the semi-intensively managed cattle grazed freely on pasture but received supplementary feed in the morning and evening when they were milked. All cows were hand-milked twice daily, in the morning and evening.

3.3 Study Design, Source of Sample and Sampling Method

A cross-sectional study design was used to assess AMU and hygienic practices of dairy farmers, and the occurrence of AMR *Salmonella* isolated from raw cow milk and milk products in the study area from October 2023 to April 2024.

For this study, raw milk and milk products (cheese and yogurt), floor swap, fecal samples and swabs from milk containers were gathered from various sources (milk vendors, restaurants, and dairy farms). Bulk milk, fecal samples, floor swap and swap from milk containers) in Bishoftu town. In addition, cheese and yogurt samples were collected from restaurants, whereas bulk milk samples were collected from milk vendors.

A stratified random sampling method was used to collect samples from dairy farms. The farms were categorized based on their herd size into three strata; small-scale < 10 animals,

medium-scale 10 to 50 animals, and large-scale >50 animals using the classification made by Megersa and his friends (Megersa *et al*., 2011). A simple random sampling technique was employed to select dairy farms, restaurants and milk vendors. Similarly, milk containers were selected by simple random sampling to collect appropriate raw milk and milk product samples. The milk and milk product samples were clearly labeled with the date of sampling, the type of sample, and the name of the farm and then held in an icebox with ice packs and transported to the Veterinary Public Health (VPH) laboratory of the College of Veterinary medicine and agriculture, Addis Ababa University (AAU-CVMA). In the laboratory, the samples were stored at 4°C for a maximum of 24h until they were transferred into an enrichment medium and inoculated onto a standard bacteriological media. After the isolation of *Salmonella*, the positive isolates were transported to Animal Health Institutes (AHI), Sebeta by standard transporting medium for confirmation.

3.4 Sample Size Determination

A simple random sampling technique was used. The necessary sample size was determined concerning the estimated prevalence of *Salmonella* and the desired minimum precision level, as outlined by Thrusfield (2007). The formula calculating the sample size,

$$N = Z^2 * \frac{P_{exp}\,(1-P_{exp})\,n}{d^2}$$

Where n = required sample size, d = desired absolute precision, and P_{exp}= expected prevalence.

According to (Geletu *et al*., 2022), the expected prevalence of *Salmonella* in this study is 4.8%, and the desired minimum level of precision is 5% at a 95% confidence level, with a z value of 1.96. Therefore, the minimum required sample size was 70. However, to increase the precision of the study, 200 samples were collected, including 41 bulk milk sample from dairy farms, 41 swab samples from milk containers, 21 fecal samples from apparently healthy cows, and 41-floor swab samples from cow environment and 14 cheese samples and 14 yogurt samples from restaurants and 14 raw milk samples and 14 swab samples of milk container from open market milk vendors. The sample collection process was on a

voluntary basis, and the willingness of the owners to provide the samples was considered at the farm level. In contrast, raw milk at milk vendors and cheese and yogurt samples from restaurants were purchased. A structured questionnaire was also used to collect socio-demographic information and potential risk factors contributing to the antimicrobial-resistant profile of *Salmonella* isolated from milk and milk products (ANNEX 14 and 15).

3.5 **Sample collection and transportation**

Samples were obtained from various sources, including dairy cows (bulk milk, swap from milk containers, pooled floor swaps, and feces), raw milk from milk vendors, and cheese and yogurt from restaurants. These samples were collected at the beginning of the day, with the timing arranged in advance with the farmers and milkers. The fecal samples were collected directly from the rectum and placed in a 50 ml universal screw-capped bottle containing 10 ml of peptone water as transport media. After milking, the milk samples were collected aseptically from the bulk tank and placed in a milk container. The raw milk cheese and yogurt samples were purchased and collected in plastic bags.

The swab samples were collected before milking using a sterile wooden cotton swab and placed in a sterile test tube containing 10 ml of buffered peptone water as transport media. All samples were labeled and transported immediately to the Veterinary Public Health Laboratory of the AAU-CVMA for bacterial isolation. Finally, the suspected colony of Salmonella was confirmed at the Animal Health Institute (AHI), Sebata by using the OmniLog system and antimicrobial susceptibility testing (AST) was performed on the isolates.

3.6 Bacteriological Isolation of *Salmonella*

The bacteriological analysis was conducted following the microbiology of the food chain guidelines, specifically, the horizontal method outlined in ISO-6579-1, 2017 (Mooijman *et al*., 2019), for the detection, enumeration, and serotyping of *Salmonella*. The process involved a standard three-stage approach: pre-enrichment, selective enrichment, and selective plating to isolate *Salmonella*. In the pre-enrichment stage, 1ml of the milk sample

was aseptically measured and homogenized with 9 ml of buffered peptone water (HIMEDIA BM020, India), followed by an incubation at 37°C for 24 hours to enhance the recovery of *Salmonella*. Subsequently, in the secondary enrichment step, Rappaport-Vassiliadis with soya (RVS) was brought to room temperature as per the manufacturer's instructions. The mixture from the primary enrichment sample was thoroughly mixed, after which a 0.1 ml aliquot was transferred and added to 10 ml of Rappaport-Vassiliadis with soya (RVS) for further incubation.

The enriched samples were then plated on Xylose Lysine Deoxycholate (XLD) agar, a selective medium for *Salmonella* isolation, adjusted to room temperature as per the manufacturer's instructions. After vortexing the secondary enrichment tubes, the samples were streaked onto XLD agar using a 10µl loop and incubated at 41.5°C for 24-48 hours. Suspect *Salmonella* colonies, characterized by pink coloration with or without black centers on XLD agar, were identified. Three to five typical *Salmonella* colonies were selected, streaked onto nutrient agar, and further incubated at 37°C for 18-24 hours for biochemical identification.

3.7 Biochemical characterization of *Salmonella* isolates

All potential *Salmonella* isolates underwent a series of biochemical tests for identification, including the Triple Sugar Iron (TSI) test, Indole test, Citrate utilization test, Methyl red test, Vogues Proskauer (VP) test, and urease test. Isolates showing characteristics such as red slant (alkaline) with a yellow butt (acid) on TSI, blackening due to hydrogen sulfide (H2S) production, and gas production in the butt, negative Indole test, positive Methyl red test (red broth culture), negative urea hydrolysis (yellow), positive citrate utilization (deep blue slant), and negative Voges-Proskauer (VP) test were identified as positive for Salmonella. Isolates meeting these criteria were then transferred and cultured on Nutrient Agar (NA) for antimicrobial sensitivity testing (Mooijman *et al*., 2019).

3.1 Geographical Description of the Study Area

This study was conducted from October 2023 to April 2024 at dairy farms, milk vendors, and restaurants in Bishoftu town. Bishoftu town was purposefully selected because of its larger potential for dairy cattle density, which may pose a risk of *Salmonella* contamination in dairy products due to cross-contamination along the milk value chain. Bishoftu town is located in the east Showa zone of the Oromia region, situated approximately 45 km southeast of Addis Ababa. The city is situated at 9° North latitude and 40° East longitude, with an altitude of 1850 m above sea level in the central highlands of Ethiopia. The town experiences an annual rainfall of 866 mm, with 84% occurring during the long rainy season from June to September, and the remainder in the short rainy season extending from March to May. The dry season extends from October to February. The mean annual maximum and minimum temperatures in the area are 26°C and 14°C, respectively, with a mean relative humidity of 61.3% (NMSA, 2010).

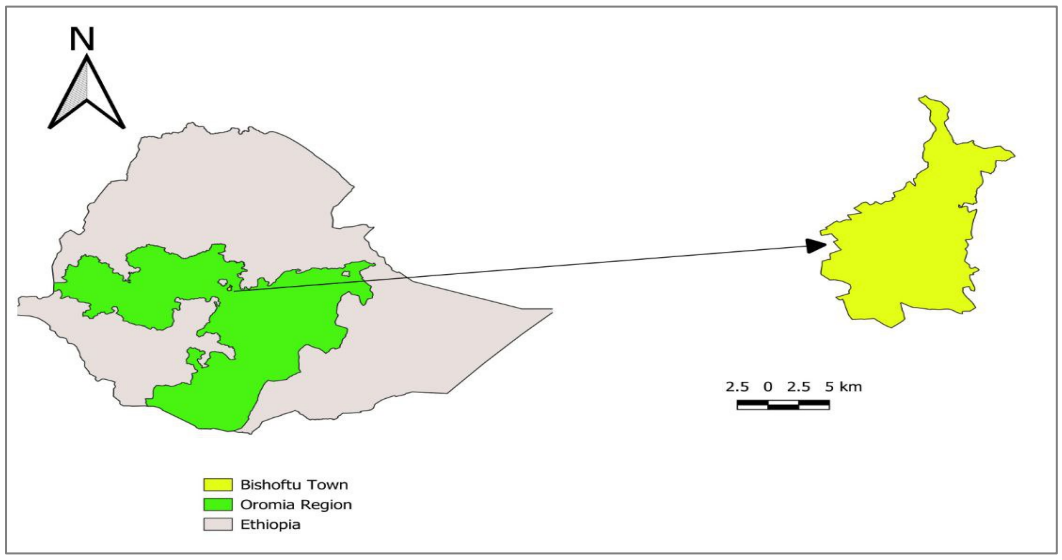


Figure 3**:** Map of study Area (Bishoftu town, Oromia Region, Central highland of Ethiopia

3.8 Identification of *Salmonella* using OmniLog

To identify *Salmonella,* the isolate to be identified was grown on Biolog Universal Growth (BUG) agar medium and then a single colony was suspended in a special "gelling" inoculating fluid (IFA) using inoculazer the recommended cell density. Then, 100 µL of the cell suspension was inoculated into a well of the GEN-III Micro Plate, and the Micro Plate was incubated to allow the phenotypic fingerprint to form. After incubation for 22 hours at 33 ºC the phenotypic fingerprint pattern was read by a combination of the Biolog MicroStation reader. The fingerprint data was imported into Omnilog Data Collection software, which searched an extensive database and made an identification call in seconds. The identification process of *Salmonella* involves four main steps. These steps were isolation of a pure culture on Biolog media, preparation of inoculum, inoculation of Micro Plates and load into the reader, and obtaining of ID results from the printer (BIOLOG, 2010).

3.9 Antimicrobial susceptibility test

The pure isolates of *Salmonella* identified using OmniLog were subjected to AST using the Kirby–Bauer agar disc diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2022). AST of the isolates was performed against eight selected drugs based on the its usage , namely Tetracycline (TE 30µg), Ampicillin (AMP 10µg), Gentamicin (CN 10µg), Trimethoprim-Sulfamethoxazole (TMP-SXT 25µg), Ciprofloxacin (CIP 10µg), Amoxicillin (AX 2µg), Streptomycin (S 10µg), and Amoxicillin-Clavulanic acid (AMC 10µg) (OXOID, UK) based. Refreshed pure isolated colonies from the nutrient agar plates were transferred into tubes containing 5 ml of 0.85% of sterilized saline water. Then, it was measured by a McFarland Densitometer until it achieved 0.5 McFarland turbidity standards. A sterile cotton swab was used to swab the inoculum uniformly over the surface of the Mueller Hinton Agar (Criterion, C6421, USA) plate. The plates were held at room temperature for 3 minutes in a biosafety cabinet to allow drying. Then, antimicrobial disks with the known concentration of antimicrobials were placed on the Muller Hinton Agar plate and were incubated for 22 hr at 37ºC. The diameters of the clear zone of inhibition produced by diffused antimicrobial on lawn-

inoculated bacterial colonies were measured to the nearest mm using a caliper. All eight zones of inhibition against eight antimicrobial agents for each isolate were recorded and compared with standards and interpreted as resistant, intermediate, or susceptible according to a published interpretive chart (CLSI, 2022) (Table 2).

Table 2: Interpretative category and zone diameter breakpoints according to CLSI, 2022

	Types of antimicrobials	Interpretive categories and zone diameter breakpoints, nearest whole mm			
		Disc concentration(μg)	S	I	R
1	Ampicillin (AMP)	10 μg	≥17	14–16	≤13
2	Amoxicillin (AX)	2 μg			
3	Amoxicillin- Clavulanic Acid (AMC)	10 μg	≥18	14–17	≤13
4	Ciprofloxacin (CIP)	10 μg	≥31	21-30	≤20
5	Gentamicin (CN)	10 μg	≥15	13–14	≤12
6	Streptomycin (S)	10 μg	≥15	13–14	≤11
7	Trimethoprim/Sulphamethoxazole (TMP-SXT)	25 μg	≥16	11–15	≤10
8	Tetracycline (TE)	30 μg	≥15	13–14	≤11

3.10 Questionnaire survey

Semi-structured questionnaires were used to assess the antimicrobial usage and hygiene practices of dairy farms. The farm owners, milking personnel, and attendants of selected farms, as well as milk vendor personnel, were personally interviewed through a face-to-face conversation about the way they use antimicrobials and manage farms, milk, and dairy products. The Kobo toolbox data collector tool was used to collect information from farm owners, milkers, farm attendants, and milk seller personnel. A researcher did a direct interview using the local language (Afan Oromo or Amharic). As a result, the hygiene practices adopted in the farms such as house cleaning, udder cleaning, hand washing practices, milking utensils and collecting vessels (buckets), hygiene, and other conditions affecting the hygienic quality of raw milk were assessed.

3.11 Conceptual framework on AMU, Hygienic practices and Salmonella isolates

Antimicrobial agents are frequently employed in dairy farming to manage and treat bacterial infections in cattle. Nonetheless, excessive or incorrect use of these agents can lead to the emergence of antimicrobial resistance (AMR) in bacteria, including *Salmonella* (Gomes *et al*., 2019). Resistant Salmonella strains can survive in the environment, potentially contaminating dairy products and endangering human health. Maintaining hygienic practices is crucial in curbing the spread of *Salmonella* on dairy farms (WHO, 2018). Measures such as regular facility cleaning and disinfection, proper waste disposal, and ensuring clean water sources can lower the incidence of *Salmonella* in cattle. Farmers' commitment to these hygienic practices significantly affects the overall microbial burden in dairy settings, thereby influencing the risk of *Salmonella* contamination (Gomes *et al*., 2019).

The traits of Salmonella isolates, including their antimicrobial susceptibility and genetic composition, indicate the combined effects of antimicrobial use and hygiene practices on the farm. Monitoring *Salmonella* isolates offers insights into how well these practices manage bacterial populations and reduce the risk of antimicrobial resistance. This framework visually demonstrates the synergistic interaction between antimicrobial use and hygiene practices in shaping the presence and characteristics of *Salmonella* isolates on dairy farms (Figure).

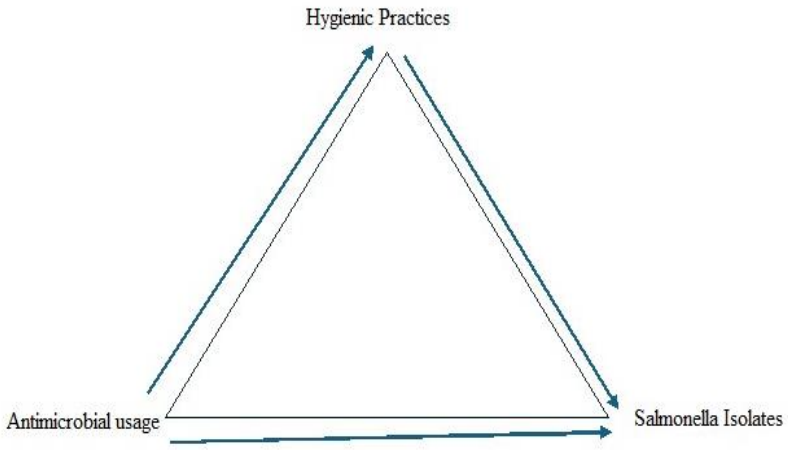


Figure 4: Conceptual framework diagram on AMU, Hygienic practices and Salmonella isolates

3.12. Data Analysis

The raw data generated from the laboratory work was arranged, organized, coded and entered an Excel spreadsheet 2010. Additionally, the KAP survey data gathered through the Kobo Toolbox server was retrieved as Excel files, carefully reviewed for errors, coded, and subsequently imported into the data analysis software. Data were analyzed using Stata/IC version 14.2. The laboratory results of *Salmonella* detected, and their AMR profile were mostly described in proportion.

For the questionnaire survey, descriptive statistics were used to characterize household demographics and farm features. Demographics, knowledge levels, practice levels, and attitudes on AMU and hygiene on dairy farms and milk vendor-related factors were reported as counts and percentages using descriptive statistics. The demographic variables of the visited farms included age category, gender, education level, marital status, herd size, and management type were considered as predictor/ independent variables. Knowledge, attitudes, and practices (KAP) on AMU and AMR were considered as outcome/dependent variables. KAP was assessed by one point if correct and zero if incorrect, whereas attitude was scored by one point for correct responses and zero for

incorrect answers. The total grades per participant were used to determine the mean KAP score, which served as an average for "good" and "poor" knowledge, attitudes, or practices (Hirwa *et al*., 2024). The calculated mean scores for KAP on AMU and AMR were 78%, 90%, and 76%, respectively. Participants with scores more than or equal to the average mean score were considered to have good knowledge, attitude, or practice. Those below the average mean score were regarded as having poor knowledge and attitude.

Responses to questions about the farmers' practice were either "yes vs. no" or multiple choice, with the latter being dichotomized as "correct" and "incorrect." Data were coded by giving one to correct answers and zero to incorrect responses to a specific question or item. The percentages of "appropriate" responses (right answers in the knowledge portion, proper attitude in the attitude question, and using acceptable management methods in the practice section) were determined for each KAP item. The relationship between demographics and KAP levels was investigated using a chi-square test of association between KAP variables (knowledge, attitudes, and practice levels) and possible explanatory independent factors. The demographic factors that showed a significant association during the chi-square were used to perform a multivariable binary logistic regression analysis to identify the key independent variables affecting KAP toward AMR in the study area. The 5% significance level was used to interpret the association results and the 95% confidence intervals for the adjusted odds ratios were used to evaluate the significance and direction of the associations.

3.12 Ethical clearance

This study was granted ethical approval by the College of Veterinary Medicine Animal Research Ethics Committee of Addis Ababa University, with reference number VM/ERC/02/09/16/2024 (ANNEX 18). All procedures were executed by skilled professionals according to the guidelines and regulations established by the university's ethics committee. The welfare and well-being of the animals that participated in this study were ensured throughout the research. Before the commencement of the study, verbal consent was obtained from all farm owners for both the questionnaire interview and the collection of milk and fecal samples from their animals.

3.13 Limitations of study

This study has been subjected to limitations. One limitation is the presence of resource limitations that impeded the comprehensive characterization and identification of antimicrobial resistance (AMR) genes *in Salmonella enterica*. Insufficient availability of materials and equipment, including DNA extraction kits, PCR primers, and DNA sequencing reagents, hindered the execution of necessary procedures.

5. RESULTS

4.1 Growth on solid media and Biochemical test

Bacteriologically, of the 200 samples tested, 13 were initially suspected to be presumptive colonies of *Salmonella* on xylose-lysine-deoxycholate agar (XLD) (ANNEX 8). However, biochemical tests revealed that only six of these samples were found to be positive for Salmonella. These isolates tested positive for citrate and MR tests but were negative for urease, VP, and indole tests. On triple sugar iron agar, *Salmonella* colonies produced hydrogen sulfide, as indicated by the black discoloration of the agar, the formation of bubbles in the agar due to gas, and the red color change in the slant (ANNEX 9).

4.2 OmniLog Identification of *Salmonella*

The six isolates of *Salmonella* identified by biochemical tests subsequently, underwent OmniLog identification, revealing that four of the samples tested were positive for *Salmonella*. All four positive samples were identified as *Salmonella enterica (*ANNEX 10) originating from various sources and sample types including bulk milk, a fecal sample, a floor swap at the farm, and raw milk collected from milk vendors at Bishoftu town.

4.3 Prevalence of *Salmonella* Based on Bacteriological Identification

A total of 200 samples were collected from three separate sources, namely dairy farms, milk vendors, and restaurants, for bacterial examination. Out of these, 2% (4/200) of the samples tested positive for *Salmonella*. Specifically, 2.1% (95% CI: 0.7–6.3), 3/144 of the farm samples and 3.57% (95% CI: 0.44–23.7), and 1/28 of the milk vendor samples were found to have salmonella. However, no *Salmonella* was detected in any of the cheese or yogurt samples collected from the restaurants. In general, of the 4 isolates, three were isolated from samples collected from dairy farms, whereas on were isolated from milk vendors. The study revealed a higher prevalence of *Salmonella* enterica at the farm level in comparison to milk vendors (Table 3).

Table 3: Prevalence of *Salmonella* from milk and milk products in Bishoftu town, Central Ethiopia

Sample source	Sample type	Number of samples examined	*Salmonella* positive	Percentage (%)
Dairy farm	Bulk milk	41	1	2.44
	Fecal sample	21	1	4.67
	Pooled floor swap	41	1	2.44
	Swap of milk container	41	0	0
	Total	144	3	2.1
Milk seller	Raw milk	14	1	7.1
	Swap of milk container	14	0	
	Total	28	1	3.57
Restaurants	Cheese	14	0	0
	Yogurt	14	0	0
	Total	28	0	0
Total		200	4	2
		Pearson chi2(7)	4.6025	
		P-Value	0.708	

4.4 Prevalence of *Salmonella* Based on Farm Category

From the total of 41 dairy farms enrolled in this study (5 small scales, 34 medium scales and 3 large scales), the overall prevalence of *Salmonella* was 7.32%, with most samples testing negative (92.68%). Of these farms, 1 was small and the remaining 2 were medium-sized. From these, 1/41 (2.43%) and 2/41 (4.88%) *Salmonella* isolates were obtained from small-size and medium-size farms respectively; and no *Salmonella* was isolated from large-scale dairy farms. The Pearson chi-squared test revealed 9.65 with a p-value of 0.047. This suggests a statistically significant association between sample type and the occurrence of the pathogen across dairy farms (Table 4).

Table 4: Prevalence of *Salmonella* in small, medium and large-scale dairy farms

Sample type		**Farm size***			**Total**
	Result	**Small scale**	**Medium scale**	**Large scale**	
Bulk milk	Positive	0	1(2.94%)	0%	
	Negative	5(100%)	33(97.06%)	3(100%)	
Swap of milk container	Positive	0	0	0%	
	Negative	5(100%)	34(100%)	3(100%)	
Floor swap	Positive	1(20%)	0	0%	
	Negative	4(80%)	34(100)	3(100%)	
Fecal sample	Positive	0	1(2.94)	0%	
	Negative	5(100%)	33(97.06%)	3(100%)	
Total	Positive	1(20%)	2(5.88)	0%	3(7.32%)
	Negative	4(80%)	32(94.12%)	3(100%)	38(92.68%)
	Pearson chi2(4) = 9.6471				
	P-value = 0.047				

* Number of small-scale farms = 5, medium scale = 34 and large-scale farm = 3

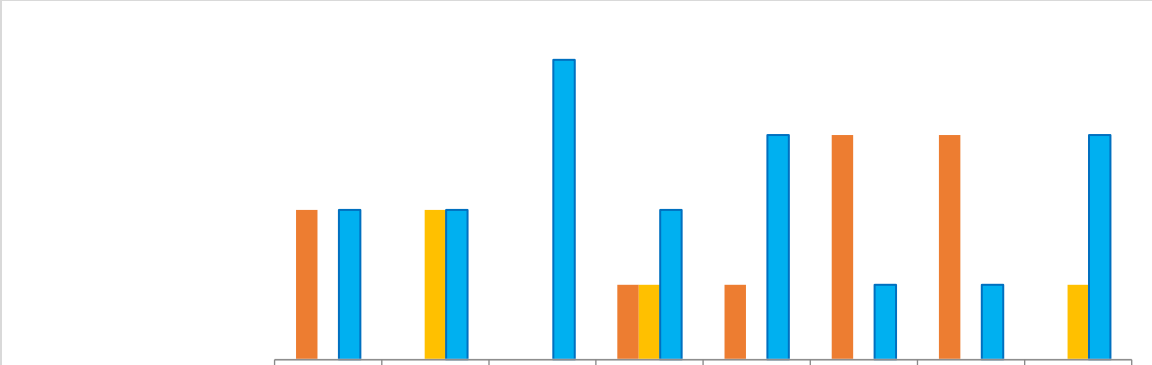
4.5 Antimicrobial Susceptibility Profiles of *Salmonella*

The study revealed that the common antimicrobials used in the farms were oxytetracycline, fixed combinations of penicillin + streptomycin (pen strep), and sulfonamide in 100%, 100% and 65.9% of the farms, respectively. *Salmonella* isolates were subjected to an AST against 8 selected antimicrobial agents. Accordingly, 100%, 75%, and 75% of the isolates were found to be susceptible to gentamicin, amoxicillin-clavulanic acid, and ciprofloxacin, respectively. On the other hand, 75%, 75%, and 50% of the isolates were resistant to amoxicillin, streptomycin, and tetracycline (Figure 4 and Table 5).

Table 5: Antimicrobial susceptibility test result of the salmonella isolates and its status with sample type

	Antibiotics	**Sample type**							
		Bulk milk		Fecal sample		Floor swap		Raw milk from milk vendors	
		Result	Status	Result	Status	Result	Status	Result	Status
1	Tetracycline	10	R	11	R	34	S	30	S
2	Ampicillin	22	S	19	S	16	I	16	I
3	Gentamicin	26	S	20	S	16	S	16	S
4	TMP-SXT	29	S	11	R	30	S	13	I
5	Ciprofloxacin	14	R	42	S	32	S	36	S
6	Amoxicillin	6	R	6	R	8	R	18	S
7	Streptomycin	17	S	10	R	18	R	10	R
8	AMC	20	S	15	I	19	S	18	S

Abbreviations. R=resistant, I=Intermedium, S=Susceptible, TMP-SXT= Trimethoprim-Sulfamethoxazole, *AMC*= Amoxicillin-Clavulanic acid



	TE	AMP	CN	TMP-SXT	CIP	AX	S	AMC
Resistant %	50%	0%	0%	25%	25%	75%	75%	0%
Intermediate%	0%	50%	0%	25%	0%	0%	0%	25%
Susceptible%	50%	50%	100%	50%	75%	25%	25%	75%

Figure 5: Antimicrobial susceptibility results of *Salmonella* isolates.

TE = Tetracycline, AMP = Ampicillin, CN = Gentamicin, TMT- SXT = Trimethoprim-Sulfamethoxazole, CIP = Ciprofloxacin, AX = Amoxicillin, S = Streptomycin, AMC = Amoxicillin-Clavulanic acid

Table 6: Antimicrobial susceptibility profile of *Salmonella* isolated from cow milk and its product from the dairy farms and milk vendors in Bishoftu town, central Ethiopia

Antimicrobial Class	Antimicrobials tested	The number of isolates tested	Status of antimicrobial a gent against the isolate		
			R (%)	I (%	S (%)
Tetracycline	Tetracycline	4	50	0	50
B-lactam	Ampicillin	4	0	50	50
	Amoxicillin	4	75	0	25
	AMC	4	0	25	75
Aminoglycosides	Gentamicin	4	0	0	100
	Streptomycin	4	75	0	25
Sulfonamides	TMP-SXT	4	25	25	50
Quinolones	Ciprofloxacin	4	25	0	75

Key: R=resistant, I= Intermediate, S=susceptible, %=percent; TMP-SXT=Trimethoprim-Sulfamethoxazole; AMC=Amoxicillin-Clavulanic acid

4.6 Multidrug Resistance Profile of *Salmonella*

Multidrug resistance (MDR) profile of *Salmonella* isolated from bulk milk samples, fecal samples and floor swap collected from dairy farms and raw milk collected from milk vendors showed 75% (n = 3/4) of the isolates were resistant to more than two classes of antibiotics. *Salmonella* isolates from fecal samples showed high resistance to four classes of antibiotics while from bulk milk the isolates showed resistance to three classes of antibiotics. Additionally, raw milk collected from milk vendors showed resistance to a minimum of two classes of antibiotics as shown in Table 5.

Table 7: Multidrug resistance profile of *Salmonella*

Antibiotics	Source of MDR	Frequency	Number of antibiotic classes	Percentage
AX, CIP, TE	Bulk milk	1	3	25%
AX, TE, S TMP-SXT	Fecal sample	1	4	25%
AX, S	Floor swaps	1	2	25%
S	Raw milk	1	1	
Overall MDR%		**3**		**3 (75%)**

AX, amoxicillin; CIP, ciprofloxacin; TE, Tetracycline; S; Streptomycin; TMP-SXT; Trimethoprim-Sulfamethoxazole; MDR; multidrug resistance

4.7 Sociodemographic Characteristics of the Respondents and Dairy Farms

In this study, a total of 55 respondents showed their willingness to participate (41 dairy farm workers and 14 milk sellers). The survey revealed that more than half (54%) of the respondents were female, and the majority (56%) were aged between 26-35 years of age category. Additionally, the marital status of the respondents indicated that most of them (58%) were married. About 38% of respondents had graduated from university/college and 33% of participants had elementary school education (Figure 5).

In the course of the study, data on demographic characteristics of dairy farms were also collected from a total of 41 farms. Based on the data, it can be observed that the most of dairy farms in the study area had a medium-scale herd size of 11-50 cows (83%) and follow an intensive management system (78%). Crossbreeds are the most common type of cattle breed on these farms (85%). In terms of feed and water hygiene and storage, about 19% of herds were rated 19% rated as poor (Figure5).

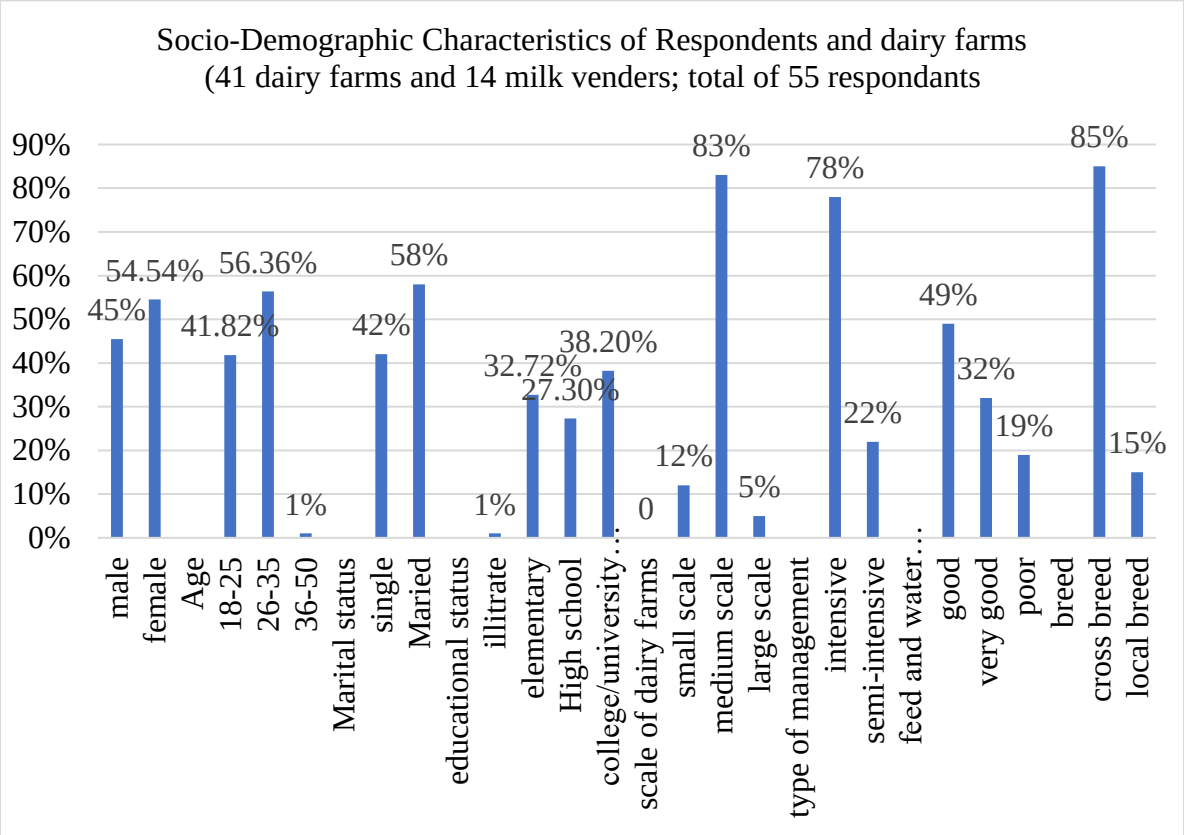


Figure 6: Socio-demographic characteristics of respondents of a dairy farm and milk vendor in Bishoftu Towns, Central Ethiopia.

4.8 Assessment of Dairy Farm Workers Regarding the KAP of AMU and AMR

The results of the average mean score analysis of participants' responses regarding their knowledge of AMU and AMR revealed that most respondents (78%, n = 32/41) demonstrated a good understanding of AMU and AMR. Conversely, 22% of respondents exhibited poor knowledge in this area. Three fourth (75%) of respondents identified antibiotics as effective against bacteria and most (97.6%) of respondents were aware of antibiotic resistance. More than half of participants (63.4%) recognized that antibiotic resistance can result in treatment failure and poor response to treatment and more than half of (53.7%) of participants were aware that an overdose or low-dose course of antibiotics can lead to AMR. Most (80.5%) of respondents understood that incomplete antibiotic courses could lead to antibiotic resistance, (87.8%) were aware of the antibiotic withdrawal period and (80.5%) were aware of antimicrobial side effects (ANNEX 17A).

The adjusted logistic regression analysis output about dairy farm workers' demographic variables and their levels of knowledge indicated a significant association between educational attainment and knowledge of antimicrobial use (AMU) and antimicrobial resistance (AMR). Specifically, respondents who had graduated from college or university were found to be 24.15 times more likely to possess better knowledge in this area compared to those with a primary school education (OR = 24.15, 95% CI: 1.87-311.97). Notably, the analysis revealed a statistically significant difference in knowledge levels based on educational category, while other demographic variables did not show significant associations as their p-values exceeded 0.05 (Table 8).

Regarding the attitude of AMU and AMR, the average mean score of respondents of dairy farm workers revealed that more than (90%) of respondents had a positive attitude toward AMU and AMR while 10% of respondents had a negative attitude toward AMU and AMR. Most (85%) of respondents agreed that consulting a veterinarian before using antimicrobials is necessary. Majority (90%) of respondents disagreed with selling animal products or slaughtering animals during antimicrobial treatment without observing a waiting withdrawal period, while around 10% agreed with this statement. Most (87%) of respondents believed that adequate biosecurity, vaccination, and good management practices help reduce the use of antimicrobials and the majority (85%) of respondents disagreed that the use of antibiotics as growth promoters is necessary for livestock production (ANNEX 17B).

Furthermore, the adjusted logistic regression analysis highlighted those individuals who had attained higher education levels, specifically graduating from college or university, were 6.88 times more likely to have positive attitudes towards AMU and AMR compared to those with primary school education. This association was statistically significant, with an odds ratio of 6.88 and a 95% confidence interval of 1.05-44.99. These results underscore the importance of education in shaping attitudes towards AMU and AMR among dairy farm workers, emphasizing the need for targeted educational interventions to promote responsible antimicrobial practices in the dairy industry (Table 8).

Based on the survey responses of 41 participants regarding antimicrobial usage (AMU) and antimicrobial resistance (AMR) practices in dairy farms, the findings suggest that most respondents (76%) exhibited good practices in AMU and AMR, while a smaller proportion (24%) had poor practices. In terms of obtaining recommendations for antibiotic usage, most (76%) of respondents reported receiving recommendations from veterinarians, while one-fourth of respondents received recommendations from veterinary paraprofessio nals. This indicates that veterinarians play a significant role in guiding the use of antibiotics on dairy farms. Regarding record-keeping, majority (80%) of respondents stated that they always keep a record of antimicrobial usage. This is a positive finding as it demonstrates the awareness and commitment of dairy farm workers to monitor and track the use of

antibiotics. All respondents (100%) reported completing the full course of antibiotics in the last six months. Regarding the disposal of leftover antibiotics, 63% of respondents reported throwing them in the garbage, while 36% mentioned other methods such as burying or burning them (ANNEX 17C).

4.9 Risk factors assessment related to hygienic practices of dairy farm workers and milk vendors

The results of the frequency distribution analysis of dairy farm workers regarding animal house floors indicate that the majority (76%), provided concrete for house floors in enclosed areas. This suggests a strong understanding of the farm's infrastructure. Regarding the frequency of cleaning the barn and/or milking room, 71% of respondents reported regular cleaning, emphasizing the importance of maintaining hygiene standards. Additionally, 61% of respondents reported that the farm has a good drainage system, which is critical for effective waste management and preventing environmental contamination (ANNEX 17 D).

From 14 milk vendor/seller respondents, more than (71%) of the participants obtained milk directly from producers. The survey also showed that the majority (64%) of respondents kept their milk in stainless steel containers, while less than half (35%) of the milk vendors used plastic containers. In addition, more than half (57 %) of respondents cleaned their milk containers with soap and water, while less than half used only water. Among milk vendors, more than (78%) of respondents used pipe water for cleaning utensils. Furthermore, the majority (65%) of respondents reported washing their hands before selling milk (ANNEX 17E).

Table 8: Association of KAP score with demographic characteristics using logistic regression

Independent Variables	**Category**	**Knowledge**		**Attitude**		**Practices**	
		OR (95%CI)	P-value	OR (95%CI)	P-value	OR (95%CI)	P-value
Sex	Female	1.07(0.13-8.44)	0.95	1.52(0.28-8.43)	0.63	1.13(0.22-9.31)	0.88
Marital status	Single	0.55(0.05-6.42)	0.63	1.79(0.21-15.01)	0.53	1.86(0.25-13.87)	0.54
Level of education	High school	2.15(0.13-34.14)	0.58	1.38(0.13-14.35)	0.27	0.07(0.01-1.21)	0.06
	Graduated from college/university	24.15(1.87-311.97)	0.015	6.88(1.05-44.99)	0.04	0.75(0.12-4.66)	0.75
Management type	Semi-intensive	0.22(0.02-2.30)	0.205	1.64(0.19-14.04)	0.65	1.06(0.12-9.31)	0.95
Feed and water storage hygiene	Good	5.93(0.55-63.43)	0.141	2.04(0.28-14.87)	0.48	7.07(0.67-75.06)	0.10
	Poor	16.71(0.75-373.27)	0.076	0.07(0.01-0.94)	0.04	0.45(0.05-3.92)	0.46
Coefficient		0.47	0.47	0.61	0.59	2.55	0.33

5. DISCUSSION

Globally, *Salmonella species* are recognized as prominent foodborne pathogens and rank as the third leading cause of death among diarrheal illnesses in human populations. The primary reservoir of this pathogen is in animals, with transmission to humans predominantly occurring through the consumption of animal-source foods including cow milk and its products (Ferrari *et al*., 2019). Contamination of the environment and along the food chain with bacteria is often attributed to the presence of animal and human wastes that have been contaminated by bacterial pathogens (Abrar *et al.,* 2020).

The result of the present research indicated that the overall prevalence of *Salmonella enterica* based on the OmniLog system was 2% (4/200); of which 2.44%, 0%, 4.76%, and 2.44% were from bulk milk, swab of milk container, fecal sample and floor swab at dairy farms, 3.5% from raw milk and swab of milk container at milk vendors and 0% and 0% cheese and yogurt from restaurant, respectively.

In the present study among the sample types, 2.44% of *Salmonella enterica* were isolated from bulk milk samples at dairy farms which is consistent with the previous research conducted in different locations. Specifically, it aligns closely with the results reported by Liyuwork *et al*. (2013) in Addis Ababa, Ethiopia, and by Van *et al*. (2013) in the United States of America where a prevalence rate of 2.1% and 2.6% were reported respectively. Similarly, the prevalence rate of *Salmonella* isolated from milk samples in Egypt, as reported by Ahmed *et al*. (2014) was 1.5% and in Jigjiga town by Reta *et al*. (2016), was 3.3%, which is within the range of the current study's findings.

Additionally, the prevalence rate reported by Murinda *et al.* (2002) in the USA was 2.24%, further supporting the consistency of the present study's results. However, the prevalence of *Salmonella* isolated from bulk milk in this study is relatively higher than the report of Fufa *et al*. (2018) and Dadi *et al.* (2020) which was 0% and 0.7% at Meki and Sebata town Oromia, Ethiopia respectively. On the other hand, from Dire Daw (18.75%) by Tesfaye *et al.* (2013), Central Ethiopia (10%) by Geletu *et al*. (2023) and reports from Gondor (6.0%) by Ejo *et al.* (2016) are much higher than the current investigation.

The difference in the relative amount of the bacteria present in milk between the current study and previous research carried out in various study areas in Ethiopia could be explained by variations in the potential risk factors contributing to the occurrence of *Salmonella* in milk. Several factors, such as milking procedures, milk handling practices, hygiene and management practices, stocking density, use of contaminated utensils, housing type, animal movement, milking environment, ventilation, and production facilities in different areas, are examples of the main risk factors that influence the occurrence of *Salmonella* (Fufa *et al*., 2017; Gebeyehu *et al*., 2022; Gezahegn *et al., 2023*). Furthermore, methods employed in the research areas may also be a factor in the variation in the relative isolation rate of *Salmonella.*

Even though the current study isolated and identified only 2.44 % of the *Salmonella* from the bulk milk, compared to previous studies, this could pose serious health risks to humans by causing *Salmonellosis* in high-risk populations like newborns, infants, the elderly, and people with immunocompromised, who are susceptible to *Salmonella* infections at a lower infective dose than healthy adults. Because dairy products are frequently consumed in Ethiopia without being properly boiled (Gelatu *et al*.,2022), it is a source of *Salmonella* infection.

In the current study, an isolation rate of 4.76% for *Salmonella enterica* was recorded in fecal samples. This finding is consistent with prior research conducted by Geletu *et a*l. (2023), who reported a similar prevalence rate of 4.7% in central Ethiopia. Additionally, the observed prevalence aligns with the results documented by Gezahegn *et al*. (2023) in the Bedele and Nekemte districts of western Ethiopia, where a prevalence rate of 2.97% was reported. Factors that could explain this consistency include the possibility that common problems with animal husbandry practices, sanitation, and hygiene could have an impact on the observed prevalence rates irrespective of geographical location.

In this study, the fecal prevalence of *Salmonella* was found to be lower than that reported in previous studies conducted by Abunna *et al*. (2017) in Modjo town, Ethiopia, who documented a higher prevalence rate of 7.7% and 12% prevalence rate which was reported by Khan *et al*. (2021) in the Republic of Korea. Additionally, our results were lower than

those reported by Hailu *et al*. (2015) in Northern Ethiopia. The observed differences in prevalence rates between our study and previous research can be due to various factors, including variations in sampling methods, duration of sampling period, environmental conditions, animal management practices, animal husbandry, biosecurity measures, sanitation protocols and geographical variability. Additionally, variations in laboratory techniques and procedures can affect the accuracy and comparability of prevalence estimates across studies.

The prevalence rate of *Salmonella* isolated from floor swabs in the present study was 2.44%. This finding is consistent with previous studies by Gezahagn *et al*. (2023) in the town of Bedelle and Nekemte in western Ethiopia and by Gelatu *et al*. (2022) in central Ethiopia, where the prevalence rate in dairy farms was reported to be 5% in both studies. The consistency of prevalence rates in these studies could be attributed to similar environmental conditions, management practices and biosecurity measures applied on dairy farms in these regions. Factors such as poor hygiene, animal overcrowding, and inadequate cleaning and disinfection protocols could contribute to the presence of *Salmonella* on dairy farm floors.

The average prevalence rate of *Salmonella* isolated from raw milk at milk vendors in the present study was found to be 3.5%, which agrees with the report of Tusa *et al*. (2024) in Asella Town Oromia, Ethiopia with a prevalence rate of 3.3% and the finding of Jassim et al. (2020) in Iraq with the prevalence rate of 3%. However, the prevalence rate of the present finding was much lower than the report of Tesfay *et al*. (2013), Ahamed *et al.* (2020) in Bangladesh and the finding of Anukampa *et al*. (2017) in India which was 41.7%, 45%, and 7.4% respectively.

The prevalence of *Salmonella* in raw milk varies across different milk vendors due to various factors. These include study design, sampling techniques, geographic locations, hygiene practices, and storage conditions. Larger sample sizes and advanced detection methods can yield higher prevalence rates. The prevalence of *Salmonella* in raw milk can fluctuate depending on regional and local practices, environmental factors, animal health, and farm practices. The variation in hygiene practices during milk production, handling,

and storage can increase the risk of bacterial contamination. Inadequate sanitation, equipment cleaning, and improper storage conditions can also increase the risk. The health status of dairy animals and the presence of infectious diseases can also impact the prevalence of *Salmonella* in raw milk. Cross-contamination during milk handling and processing can introduce *Salmonella* from external sources.

In the present study, no *Salmonella* was isolated from cheese and yogurt samples, which agreed with the findings of Ejo *et al*. (2016) and Tesfaw *et al*. (2013), who reported no *Salmonella species* found in cheese and yogurt. The absence of *Salmonella* in cheese and yogurt samples can be attributed to several factors. Proper storage and handling practices, including adequate refrigeration and hygienic handling, help to prevent contamination after processing. The sensitivity of the sampling and detection methods can also influence the absence of *Salmonella.*

In this study, an attempt was made to evaluate and compare the isolation rate of *Salmonella* in dairy farms of different herd sizes, namely small, medium, and large. The result showed out of 41 dairy farms (3 large-scale, 34 medium-scale and 5 small-scale dairy farms) the total prevalence rate of *Salmonella* was 7.32%. Our results showed that the isolation rate of *Salmonella* was significantly comparable between small and medium-sized farms. However, in this cross-sectional study, there was no *Salmonella* isolated from large-scale dairy farms. Several factors could contribute to this study's lack of *Salmonella* isolation from large dairy farms. Some possible reasons could be Strict biosecurity measures: Large dairy farms may have stricter biosecurity protocols in place to prevent the introduction and spread of pathogens, including *Salmonella,* as compared to small-scale dairy farms. Management practices, such as regular cleaning and disinfecting facilities, can help reduce the spread of *Salmonella*.

Antimicrobial resistance is a growing worldwide issue in human and veterinary health, affecting both developed and developing countries. The growing use of antimicrobial drugs in food animal production and humans was a significant contributor to the establishment of bacterial resistance (Gebremedhin *et al*., 2021). In the current investigation, *Salmonella* isolates (n = 4) were evaluated against eight frequently used antimicrobials using CLSI-2022 guidance. Antimicrobial susceptibility testing revealed 75%, 75%, and 50% resistance to amoxicillin, streptomycin, and tetracycline respectively. In comparison, 100% sensitivity to gentamycin was identified, followed by 75%, 75%, 50%, and 50% sensitivity to amoxicillin-clavulanic acid, ciprofloxacin, tetracycline, and ampicillin, respectively.

The current findings revealed that 75% of the isolates were resistant to two or more classes of antibiotics, which was lower than the report of Fesseha *et al*. (2020), who documented the MDR rate of 96.4% from selected dairy farms in Hawasa town. However, these findings were higher than those previously reported by Tesfaw *et al*. (2013), who documented a 50% MDR of *Salmonella* isolate. The possible reasons for the high AMR level of *Salmonella* might be due to the increasing rate of irrational use of antimicrobials in dairy farms, frequent usage both in livestock and public health, use of counterfeit drugs in animal husbandry (Farhan *et al*., 2024), self-medication due to easy access to antimicrobials without prescription in the public health sector, and administration of subtherapeutic doses.

The current investigation found 75% amoxicillin resistance, which was greater than the findings of Beyene *et al*. (2016) and Fesseha *et al*. (2020) in Asella and Hawasa Town, Ethiopia, who reported resistance rates of 58.3% and 25%, respectively. The observed high resistance to streptomycin is not surprising, as these antimicrobials are commonly used in all farms to manage bacterial infection. The streptomycin resistance in the current study is consistent with previous results in Addis Abeba, as reported by Zewdu and Cornelius (2009), who recorded a resistance rate of 75% among food items and personnel. However, the results of our research's resistance rate were lower than those reported by Ketema *et al*. (2018) and Obaidat and Stringer (2019), which were 80% and 89.3%, respectively.

On the other hand, our data suggested a greater resistance rate than the studies by Abra *et al*. (2020), Gelatu *et al*. (2022), and Beyene *et al*. (2016) who documented a 60%, 46% and 41.7% resistance rate respectively. The resistance profile towards tetracycline was 50%, which is comparable with the findings of Mulaw (2017), (52.8%) among lactating cows in dairy farms in Bahir Dar Town, Ethiopia. However, it is interesting to note that the tetracycline resistance rate found in the current study exceeds that of Xu *et al*. (2018) in the United States, which was 28% lower than the report of Fesseha *et al*. (2020), who recorded a resistant rate of 96.4%. This difference in resistance rates might be due to the increasing use of inappropriate antimicrobials on dairy farms. Such methods provide selection pressure, which increases the survival and growth of bacterial strains containing resistance genes (Fesseha *et al*., 2020). As a result, such action may contribute to the variations in resistance profiles found among studies. The growing frequency of antibiotic resistance highlights the critical need for extensive antimicrobial management procedures to prevent the emergence and spread of resistant bacterial strains.

The present results showed that *Salmonella* isolates were susceptible to gentamycin and with a susceptibility rate of 100%. This was consistent with the reports of Tesfaw *et al.* (2013), Abunna *et al*. (2017) and Beyene *et al*. (2020) who documented a resistant rate of 100% but, higher than 73.3% and 75% reported by Addis *et al*. (2011) and Tadesse and Anbessa, respectively. Additionally, the susceptibility rate of ciprofloxacin was 75% which was lower than the report of 83.3% documented by Addis *et al*. (2011). The variation in ciprofloxacin effectiveness in Ethiopian dairy farming might be related to drug type, different bacterial strains, resistance gene evolution, and limited use in Ethiopian animal production.

The Misuse of antimicrobials in livestock may lead to the emergence and spread of pathogens that are harmful to human, animal and environmental health (Hirwa *et al*., 2024). One of the major contributors to the rise of AMR is antibiotic misuse (Gebeyehu *et al*., 2021), which is linked to an antimicrobial knowledge gap. To address the growing threat of antimicrobial resistance (AMR), it is essential to assess the knowledge, attitude, and practices of dairy farmers regarding antimicrobial use (AMU) and antimicrobial resistance (AMR). As part of this cross-sectional study in Bishoftu town, 41 participants completed a

structured questionnaire to assess their knowledge, attitudes, and practices (KAP) regarding AMU and AMR.

In the present findings, about 78% of respondents had a good knowledge of antimicrobial usage and antimicrobial resistance, which was in line with the reports of Pham *et al*. (2019) in Vietnam. On the other hand, a high proportion of livestock keepers in Ethiopia, which was reported by Gemeda *et al*. (2020), were not knowledgeable about AMU and AMR. This difference could be a result of the different AMU and AMR awareness levels of the livestock producers in different localities (Gebeyehu *et al*., 2021).

In the present study, regarding attitude toward antimicrobial usage and antimicrobial resistance, about 90% of respondents had a positive attitude toward antimicrobial usage and antimicrobial resistance. This finding disagreed with the report of Gebayo *et al*. (2021) in which 85.29% of farmers had a negative attitude toward AMU and AMR. This discrepancy may be due to different levels of knowledge and awareness about antimicrobial resistance, which could lead to different attitudes toward AMU and AMR.

In the present finding, 10% of respondents agreed with the statement that selling animal products or slaughtering animals during antimicrobial treatment without waiting for the withdrawal period is acceptable, which agreed with the report by Hossain *et al.* (2022) in Bangladesh who documented 25.5% of farmers follow the withdrawal period of antibiotics. This practice can lead to the presence of antimicrobial residues in food products, which can pose a risk to human health. It is important to continue to educate farmers about the importance of observing waiting withdrawal periods after antimicrobial treatment.

It is also concerning that in the present study, 14% of respondents agreed that the use of antibiotics as growth promoters is necessary for livestock production. The use of antimicrobials as growth promoters is a major contributor to the development and spread of antimicrobial resistance. Even though the dairy farm workers in the study area had relatively a good attitude toward AMU and AMR there is still room for improvement, particularly in the areas of observing waiting withdrawal periods and avoiding the use of antimicrobials as growth promoters.

In the current study, regarding the practices of AMU and AMR, it was found that most respondents (76%) stated that antibiotics were recommended by a veterinarian. Additionally, 80% of dairy farm workers were reported to maintain proper records of antibiotic usage, consistent with previous research in the USA by Green *et al*. (2010). The study also noted an increase in keeping records on antimicrobial usage, possibly due to a growing awareness among dairy farm workers about the importance of record-keeping.

In this study, it is encouraging that all dairy farm workers (100%) did not add antimicrobials to animal feed, which agreed with the findings of Hossain *et al*. (2022) in Bangladesh who reported 98.1% of farmers did not add antimicrobials to animal feeds, suggesting the use of appropriate practices. Additionally, all respondents completed the course of antibiotics in the last six months on their farms. This finding disagrees with the report in Ghana in which a higher percentage of farmers (63%) tended not to complete antibiotic courses. The variation in antibiotic use practices among dairy farms in different regions can be attributed to various factors. These include awareness and education about proper antibiotic use, access to veterinary services and the regulatory environment.

Regarding the hygienic practices of dairy farms and milk vendors, the survey result showed that 60% of respondents had good hygienic practices. However, the type of housing, barn cleaning, drainage system, unhygienic milking methods, type of milk container, type of water used for washing hands and milk equipment were identified as risk factors for the occurrence of *Salmonella* in raw cow milk. This finding was in line with the finding of Bedassa (2021) which identified the source of water used for washing milking equipment and, type of milk container as the most risk factors for the occurrence of *Salmonella.*

6.CONLUSSION AND RECOMMENDATIONS

In conclusion, the present study revealed the occurrence of contamination of cow milk and its products with *Salmonella enterica* along the milk value chain at farms and milk vendors. The isolations of the bacteria from bulk milk, fecal samples, and floor swabs of the cow environment were found to be the potential sources of milk contamination at the farms and milk vendors. The presence of *Salmonella enterica* in bulk milk at the farm level and milk vendors indicates that there was cross-contamination of milk possibly because of Salmonella shedding in cattle feces, poor animal hygiene and housing conditions, contact with contaminated water or feed, fecal contamination of milking equipment or milk storage tanks, unsanitary milking practice, poor hygiene of milk handlers/vendors, improper storage and incomplete or improper antibiotic treatment. This study also revealed an inadequate KAP on AMU and AMR, contributing to the AMR profile of *Salmonella*. The study has also revealed the possibility of a public health risk posed due to *Salmonella enterica* in the study area. In general, the assessment of AMR profile of *Salmonella* in dairy farms is critical for safeguarding public health, ensuring food safety, minimizing economic losses, and promoting the overall well-being of animals, the environment, and humans. By understanding the prevalence and dynamics of *Salmonella* in dairy environments, proactive measures can be taken to prevent contamination, reduce risks, and protect both the dairy industry and consumers. Based on the above conclusion, the following are recommended:

- Creating public awareness about good milk handling practices, milk-borne diseases, and their prevention for dairy farmers and consumers should be implemented.
- Monitoring and rational use of drugs before use should be implemented by dairy farmers, veterinarians, and Regulatory agencies to prevent the spread of AMR and ensure the safety of animal-source food.
- Investigating and designing cost-effective preventive and control options to reduce milk contamination by *Salmonella* and other food-borne pathogens to increase consumer confidence and safeguard public health.

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8. ANNEXES

ANNEX 1: Sample Record sheet

No	Sample code	Collection date	Collection site	Sample type	Presumptive result	Remark

ANNEX 2: Biochemical test record sheet

Code	tests	Indole test	Methyl red	Vogues Proskauer	Citrate	TSI	Lysine	Urease test
	Salmonella spp							
	Results	**-ve**	**+ve**	**-ve**	**+ve**	**+ve**	**+ve**	**-ve**

ANNEX 3 : Procedures for preparation of different media

1. **Buffered Peptone Water (BPW) (CM0509, UK)**

20 grams of dehydrated medium was added to 1 liter of distilled water. Mixed well and distributed into final containers. Then, it was Sterilized by autoclaving at 121°C for 15 minutes.

Composition (gram/liter): Peptone 10.0, Sodium chloride 5.0, Disodium phosphate 3.5, Potassium dihydrogen phosphate 1.5, pH 7.2 ± 0.2 at 25°C.

2. **Rappaport Vassiliadis Soya Broth (RVS Broth) media (HIMEDIA, M1491-500G, India)**

Preparation:

27.11 grams of dehydrated medium was suspended in 1000 ml of distilled water. It was heated to dissolve the medium completely. Then, it was sterilized by autoclaving at 115°C for 15 minutes and dispensed into tubes.

Composition (gram/liter): Papaic digest of soya bean meal 4.5, Sodium chloride 8.0, Potassium dihydrogen phosphate 0.6, Potassium phosphate 0.4, Magnesium chloride. hexahydrate 29.0, Malachite green 0.036, Final pH (at 25°C) 5.2±0.2.

3. **Xylose-Lysine Deoxycholate Agar (XLD) Agar media (HIMEDIA, M031-500G, India)**

Preparation:58.68 grams were suspended in 1000 ml of distilled water. Next, it was heated with frequent agitation until the medium boiled. Then it was transferred immediately to a water bath at 45-50 °C. Mixed well and poured in to sterile petri plates.

Composition (gram/liter): Yeast extract 3.0, L-Lysine 5.0, Lactose 7.5, Sucrose 7.5, Xylose 3.5, Sodium chloride 5.0, Sodium deoxycholate 2.5, Sodium thiosulphate 6.8, Ferric ammonium citrate 0.8, Phenol red 0.08, Agar 15.0, Final pH (at 25°C) 7.4±0.2.

4. Nutrient agar (Criterion, C6461, USA)

Preparation: 20.5gm of the dehydrated culture media was suspended in 1 liter of distilled water. Heated to boiled and mixed to be dissolved completely. Then, the medium was sterilized in the autoclave at 121°C for 15 minutes.

Composition (gram/liter): Pancreatic digest of gelatin 5.0, Beef extract 3.0 and Agar 12.5 and Final pH (at 25°C) 6.8±0.2.

5. Tiples Sugar Iron (TSI) Agar (Criterion, USA)

Preparation: 65.0 grams the medium was weighed and combined to 1 liter of deionized water. Mixed well thoroughly and boiled to dissolve completely. Sterilized by autoclaving at 121°C for 15 minutes. After autoclaving, slant tubes and allowed to cool, and final pH 7.3 ± 0.2 at 25°.

Composition (gram/liter): Agar 12.0 gram, Casein peptone 15.0 gram, lactose 10.0 gram, sucrose 10.0 gram sodium chloride 5.0 gram, animal tissue peptone 5.0 gram, yeast extract 3.0 gram, beef extract 3.0 gram, dextrose 1.0 gram, ferric ammonium citrate 0.5 gram, sodium thiosulfate 0.3 gram, phenol red 24.0 gram.

6. Simmons Citrate Agar (Biolife, Italy)

Preparation: 23.2 grams was suspended in 1-liter cold distilled water. Then, it was heated to boil with frequent agitation to dissolve completely. Distributed into tubes and sterilized by autoclaving at 121 ºC for 15 minutes and cooled in a slant position.

Composition (gram/liter): Ammonium dihydrogen phosphate 0.2, Sodium ammonium phosphate 0.8, Sodium chloride 5, Sodium citrate 2, Magnesium sulfate 0.2, Brom thymol blue 0.08, Agar 15.

7. Methyl-Red and Voges-Proskauer (MR-VP, Oxoid, CM 0043, UK)

Preparation: 17 grams of the medium was weighed and added to 1 liter of distilled water. Mixed well, distributed into final containers and sterilized by autoclaving at 121°C for 15 minutes.

Composition (gram/liter): Peptone 7.0, Glucose 5.0, Phosphate buffer 5.0 and Final pH 6.9 ± 0.2 (at 25°C).

8. Urea agar base (Oxoid, CM0053, UK)

Preparation: 2.4 grams was suspended in 95 ml of distilled water and dissolved completely by boiling. Then, it was sterilized by autoclaving at 115 ºC for 20 minutes and cooled to 50°C and 5 ml of sterile 40% Urea Solution SR0020 was introduced aseptically. Mixed well and 10 ml amounts were distributed into sterile containers and allowed to set in the slope position.

Composition (gram/liter): Peptone 1.0 gram, glucose 1.0 gram, sodium chloride 5.0 gram, di-sodium phosphate 1.2 gram, potassium dihydrogen phosphate 0.8 gram, phenol red 0.012 gram, agar 15.0 gram.

9. Mueller Hinton Agar (Criterion, C6421, USA)

Preparation: 38.04 grams of the medium was combined with one liter of deionized water and stirred to mix thoroughly. Then, it was dissolved completely by boiling and autoclaving at 121oC for 15 minutes. Final pH: 7.3 + 0.1 at 25 ºC.

Composition (gram/liter): Agar 17.0 grams, casein acid hydrolysate 17.5 grams, beef extract 2.0 grams, starch 1.5 gram

ANNEX 4: Procedures for the identification of Salmonella by Omnilog Micro station reader

Preparing of Samples

1) **Isolation in a Pure Culture**
2) **Culturing of *Salmonella* suspected sample on Biolog Universal Growth media** (BUG) for 24 hours at 37 ºC
3) **Inocula Selection**: Protocol A of inoculating fluid-A (IF-A) and Inoculum Density of 90-98% T was selected. Before starting, Gen III Micro Plates and Inoculating Fluid-A (IF-A) were pre-warmed at room temperature.

4) Preparing of inocula:

- For each inocula preparation, blank the turbidimeter with the uninoculated inoculating fluid tube (wiped clean of dirt and fingerprints) by adjusting the 100% transmittance adjustment knob so that the meter reads 100%T.
- The target cell density should be in the range of 90-98%T for Protocol A.
- The cotton-tipped Inoculators were used to touch gently the top of a colony surface of the BUG agar plate.
- The swab shaft at the end was grasped, and held vertically; touch it to the cell growth.
- The Inoculators were inserted into the inoculating fluid tube and emulsified the organisms into the solution Read the turbidity in the turbidimeter
- This resulted in an approximate % transmittance range of 90 to 98 for Protocols A
- The GEN III Microplates was inoculated with the suspension

5) Inoculating protocol

- The side of a Microplate with the organism was labeled.
- The cell suspension was poured into a multichannel pipette reservoir.
- Eight sterile tips were firmly attached to the 8-channel repeating pipetter.
- The tips were filled with the suspension.
- All GEN III Micro Plate wells were filled with 100 µL/well.
- The GEN III Micro Plate is covered with its lid.

Incubating the GEN III Micro Plates

- The Micro Plate was incubated at 33 ºC for 22 hours

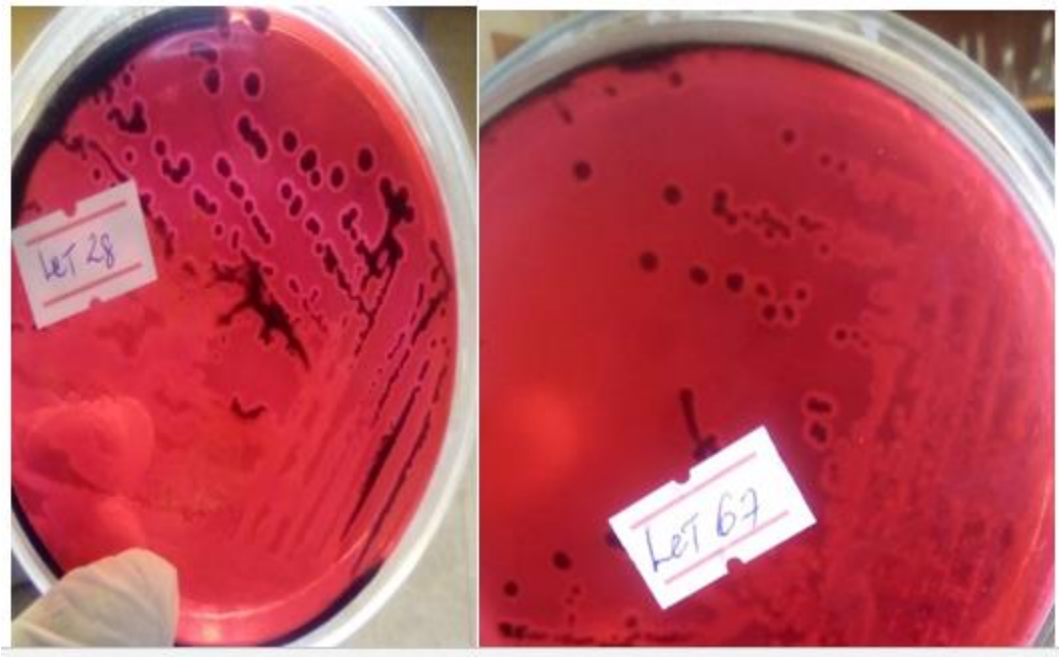
Loading and Reading Micro Plates

After microplates were inoculated, the information was entered into Biolog's OmniLog Data Collection software. This information concerns organizing plate data in batches, managing files, and printing results. Then Microplates where you can be loaded into the Omnilog incubator/reader and re

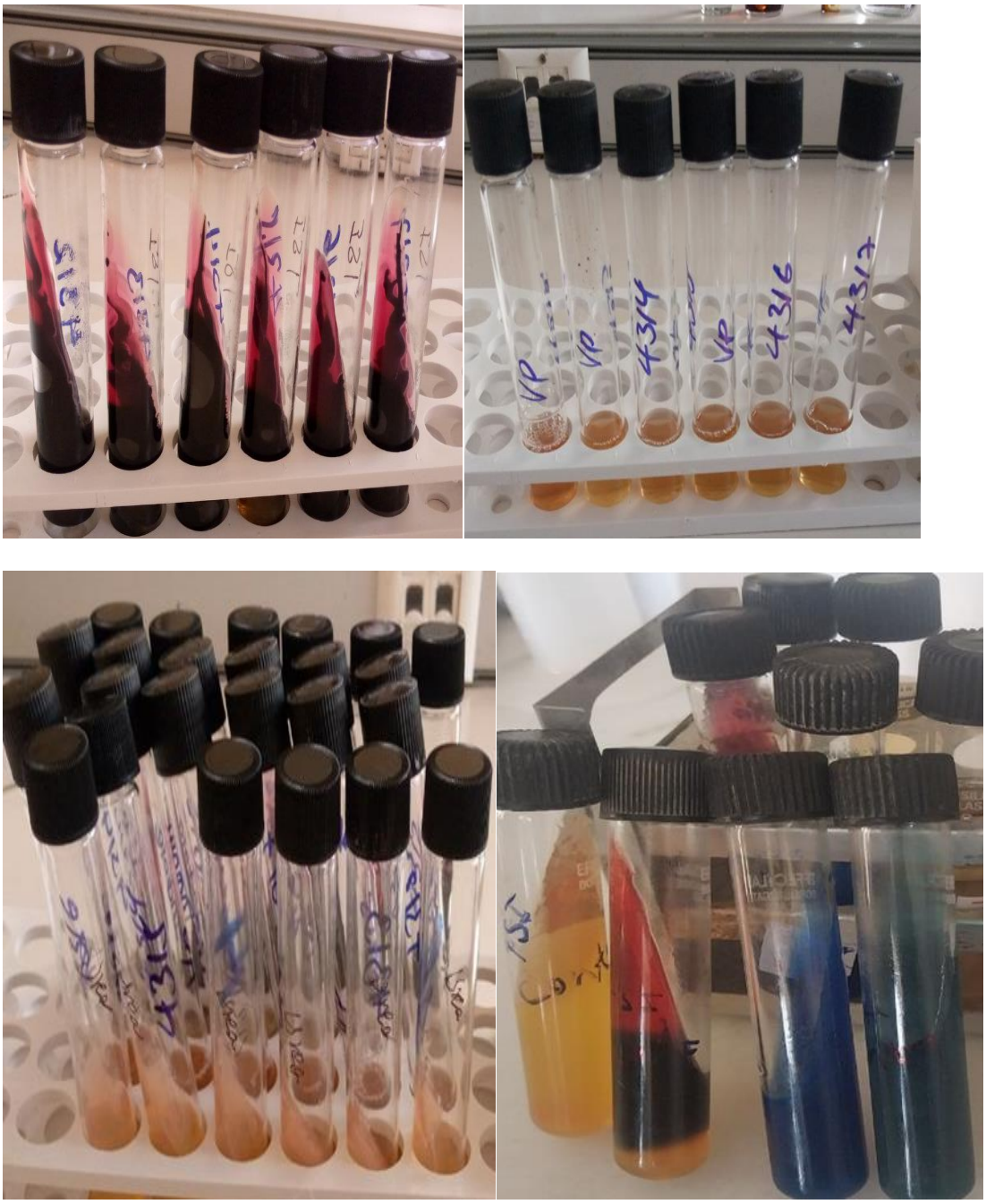
ANNEX 5: Media for non-selective pre-enrichments and selective Enrichment

ANNEX 6 : Media and Reagents used for Biochemical confirmation

ANNEX 7 : Pictures taken during sample collection and laboratory work

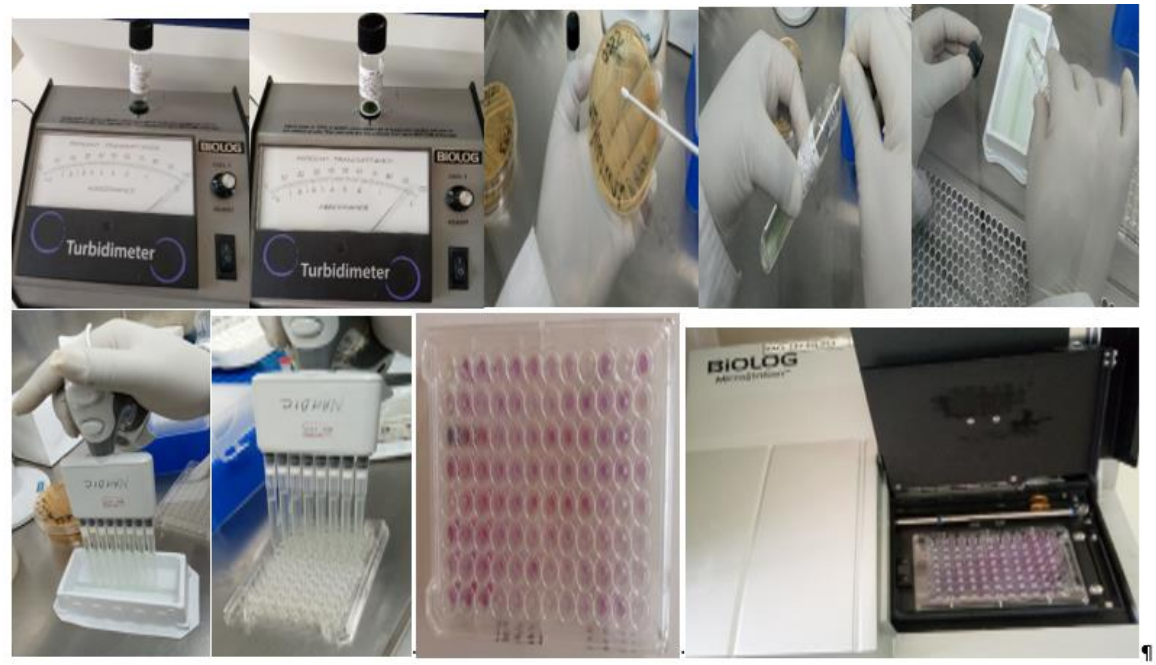


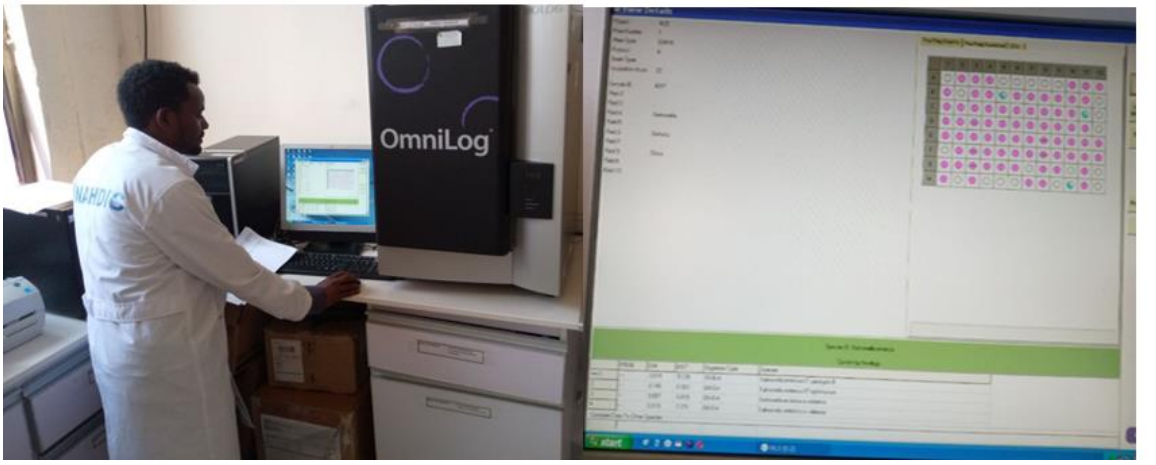
ANNEX 8 : Growth of *Salmonella enterica* on XLD Agar



ANNEX 9 : Biochemical test result of *Salmonella enterica*

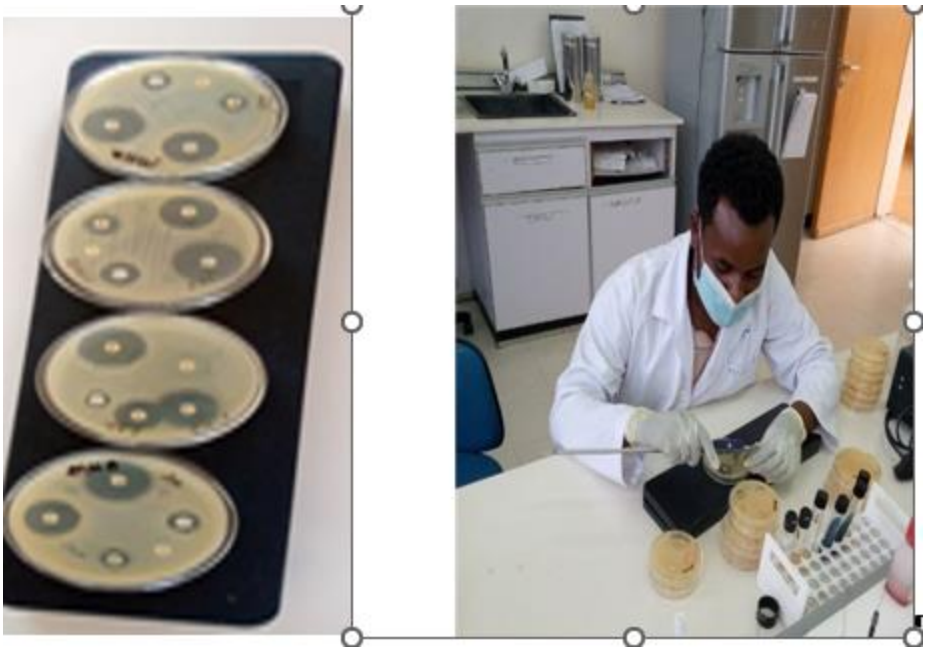
ANNEX 10: Activities performed during identification of *Salmonella enterica by Omnilog system and result*





ANNEX 11: Figure showing Preparation of Antibiotics disc and McFarland measurement

ANNEX 12: Culturing *Salmonella Enterica* on MHA and placing of antibiotics disc on Oxide dispenser



ANNEX 13: Picture showing Measurements zone of inhibition of antibiotics

ANNEX 14: Question Regarding Knowledge and Practices of Dairy Farmers on AMU and AMR Resistance

Hello, how are you? I am Lema Temesgen Gile. I am a postgraduate student a t Addis Ababa University College of Veterinary Medicine and Agriculture, Department of Veterinary Microbiology, Immunology and Public Health. Now I am conducting a search on "**Detection and Antimicrobial Resistant Profile of *Salmonella* Isolated from Raw Cow Milk and Its Products in Bishoftu Town, Central Ethiopia: Its Public Health Implication**". I would appreciate your participation in this study. The objective of the study is to assess the AMU and Hygienic practices of dairy farmers, to isolate salmonella from milk and milk products, to assess risk factors for the occurrence of salmonella species in raw milk and milk products and to evaluate the AMU-resistant salmonella isolated from raw cow milk and dairy products in the study area. To effectively attain the research objective, I request your help. For your participation in the study, no payment will be granted or has no any special privilege to you. All information you give will be kept confidential and won't be accessible to any third party; your name won't be registered on the question sheet, so you will not be identified for any reason. You have the right not to participate from the beginning, or you may stop participating at any time after starting the participation. However, your honest answers to these questions will help me in better understanding of Antimicrobial Usage and Hygienic Practices of Dairy farmers and will eventually help in designing and implementing appropriate interventions to alleviate related problems. If you are willing to participate in the study, you are requested to go through the set of questionnaires and complete the same for 30 minutes.

Will you be willing to participate? Yes ⬭ No ⬭

If you are pleased to participate in the study, please visit the next question.

For any further question, please contact the principal investigator.

Principal investigator name: Lema Temesgen, contact phone number 0912013940

Informed consent: I have read this form or it has been read to me in the language I understand all conditions stated above. Therefore, I am willing to participate in this study.

1. GENERELA INFORMATION

Farm ID or No: ____

Date_____

1.1 Farm name_____ Address_____

1.2. Scale and type of dairy farm: _____ Government_____Private_____

1.3. Herd size_____ Breed: local_____ cross _____ exotic_____

1.4 Feed and water hygiene and storage: A. Excellent B. very good C. Good D. Fair Poor

2. SOCIO-DEMOGRAPHIC CHARACTERISTICS

2.1 Gender A. Male B. Female

2.2 Age_____

2.3 level of education A. Elementary B. High school C.BA degree

2.4 Marital status A. Married B. Single

2.5 Years of farming? _____

2.6 Farm type? A. Only dairy B. Dairy and beef C. Dairy and sheep D. Dairy, sheep, and beef

2.7 Role in the farm? A. Owner B. Manger C. Attendant D. Milker

3. Question Regarding Knowledge of Dairy Farm Workers on AMU and Resistance

3.1. What are antibiotics? _____

Antibiotics are effective against? A. Bacteria B. Virus C. Parasite

3.2 Have you heard about antimicrobial resistance?

A) Yes B) No.

3.3. Have you used antibiotics on your farm last six months?

A. Yes B. No

3.4. How many times did you use it? A. Once B. Twice C. three times D. more than three times

3.5) Do you know an incomplete antibiotic course may lead to antibiotic resistance?

A) Yes B) No).

3.6) Do you know an overdose/low-dose course may lead to antibiotic resistance?

A) Yes B) No

3.7) Have you heard about a withdrawal period of antibiotics?

A) Yes B) No.

3.8) Do you know antimicrobials have some side effects?

A) Yes B) No

4. **Questions regarding Attitudes among Dairy Farm Workers on AMU and Resistance.**

4.1 Do you agree that consulting a Veterinarian before using antimicrobials is necessary?

A. Yes B. No

4.2 Do you agree to sell animal products or slaughter animals during antimicrobial treatment or without maintaining a withdrawal period to reduce the cost of animals?

A)Yes B) No

4.3 Do you agree to alter the doses without consulting the prescribers to get a better response?

A) Yes B) No

4.4 Do you think the use of antimicrobials may be reduced by maintaining proper biosecurity, vaccination, and good management practices?

A) Yes` B) NO

4.4. Do you stop antimicrobial treatment once animals feel better?

A) Yes B) No

4.6 Do you agree to alter the doses without consulting the prescribers to get a better response?

A. Yes B. No).

4.7. Is the use of antibiotics as growth promoters necessary in livestock production?

A) Yes B) No

5) Question Regarding practices among Dairy farmers on AMU and Resistance

5.1) Who recommended you antibiotics? A. Veterinarian B. other farmers C. shopkeepers/representative of the pharmaceutical company D. veterinary paraprofessional/village doctor/ E. quack/self.

5.2) Do you keep a record of using antimicrobials? A. Always B. most frequently C. sometimes D. Rarely E. Never F.do not know).

5.3) Did you complete the antibiotic course the last time? A. Yes B. No.

5.4) Number of antibiotics used at a time on your farm? A. Single B. Combined C. both D.do not know.

5.5) Do you follow the Withdrawal period? A. Yes B. No.

5.6) Do you add antibiotics to the feed of animals? A. Yes B. No).

5.7) Where do you store drugs? A. Storeroom B. Refrigerator C. bedroom/ D. others.

5.8) Do you follow the exact prescription of a veterinarian when purchasing the antibiotics? A. Yes, Always B. Yes, sometimes influenced by medicine seller/ others).

5.9) What do you do with leftover antibiotics? A. Keep for further use B. throw in the garbage C. Give them to other farmers for use D. bury in the ground/burn).

5.10) Do you read the prospectus before using antimicrobials? (Yes/No**).**

ANNEX 15: Questionnaire format for Dairy farm Workers on Hygienic practices

1. If your animals are enclosed, what type of animal house floor is in? (Only single choice) a) Covered with manure b) Concrete c) Earthed floor Others (specify)_____

2. How often the barn and/or the milking room are/is cleaned? A. Twice a day B. Once a day Once per two days D. Others (specify)_____

3. Where do milking? A. In barn B. In milking room C. Anywhere specify_____

4. How do you milk your cows? A. By hand milking B. By milking machine

5. When do you wash your hands? A. Before and after milking B. Between milking C. Only before milking D. Only after milking E. Not at all

6. When do you use teat bathe and towel? A. Before milking B. After milking C. Between milking D. before and after milking E. Don't use any dip and towel

7. Do you sell raw milk to customers? A. Everyday B. Sometimes C. Never

8. When you sell? A. Every morning B. Afternoon C. Evening

9. Have you a transportation facility to deliver milk for your customer? A. YES B.NO

10. Where does the milk go? A. To household consumption B. To restaurants

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

A. Warm water B. Cold water C. Both warm and cold water D. Detergents

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

13. Is there any practice of record keeping? A. Yes B. No

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

Thank you for your time!

ANNEX 16: Questionnaire format for milk sellers' Hygienic practice

1. From where do you collect milk? A. Direct from producer B. from other collectors

2. What type of milk container is used for milk storage? A. plastic B. stainless steel

3. How do you keep milk containers? Washing with: A. wash with soap and water

B. washing with water only

4. Where is the source of water for cleaning utensils? A) tab water B) pipe water

5. Do you wash your hands before selling milk? A. yes B. no

6. Where do you Store milk before selling? A. store in refrigerator B. store at room temperature

7. Do you add any preservatives to the milk to extend its shelf life? A. Yes B. No

8. If yes for Q6 which preservative, do you use? A. Hydrogen peroxide B. Formalin C. Glucose powder D. Antibiotics like Oxytetracycline

9. What type of milk will be provided for markets? A. Fresh or raw milk B. Boiled milk

10. On average, for how much period does the milk stay in your shop center for selling purposes? A. One day B. two days C 3 days D. one week

ANNEX 17: Table shows the assessment of Dairy farm workers on KAP of AMU and AMR and hygienic practices of dairy farms and milk vendors

ANNEX 17A: Table 8: Assessment of Dairy Farm Workers' Knowledge regarding AMU and AMR

Knowledge parameters	No. respondents	Correct answer	%	**p-value**
What are antibiotics**?**	41	30	73%	0.000
Have you heard of Antibiotic Resistance?	41	40	97.56%	
What do you know about antibiotic resistance?	41	26	63.41%	0.075
Do you know an overdose/low-dose course may lead to antibiotic resistance	41	22	53.65%	0.790
Do you know an incomplete antibiotic course may lead to antibiotic resistance?	41	33	80.48%	0.005
Have you heard about the withdrawal period of antibiotics?	41	36	87.8%	0.048
Do you know antimicrobials have some side effects?	41	36	87.8%	0.964
Average mean score (=78%)				
Good>78%			32(78%)	
Poor<78%			9(22%)	

ANNEX 17 B: Table: Assessment of dairy farm workers' attitude to AMU and AMR

Attitude parameters	No. respondents	Correct answer	%	**p-value**
Do you agree that consulting a veterinarian before using antimicrobials is necessary?	41	35	85%	0.000
Do you agree to sell animal products or slaughter animals during antimicrobial treatment or without maintaining a withdrawal period to reduce the cost of animal	41	37	90%	0.003
Do you agree to alter the doses without consulting the prescribers to get a better response?	41	41	100%	
Do you think the use of antimicrobials may be reduced by maintaining proper biosecurity, vaccination, and good management practices?	41	37	90%	0.003
Is the use of antibiotics as growth promoters necessary in livestock production?	41	35	85%	0.000
Average mean score(=90%)				
Positive attitude toward AMU and AMR (≥90%)			37(90%)	
Negative attitude toward AMU and AMR (<90%)			4(10%)	

ANNEX 17 C: Table 1: practices of dairy farm workers regarding antimicrobial usage and antimicrobial resistance

The practice of AMU and AMR parameters	No. respondents	Correct answer	%	**p-value**
Who recommended antibiotics for your animal?	41	31	76	0.000
Do you keep a record of antimicrobials?	41	33	80%	0.000
Do you complete the course of antibiotics in the last six months	41	41	100%	0.000
Number of antibiotics used at a time on your farms?	41	39	95%	0.387
Do you follow the withdrawal period?	41	41	100%	
Where do you store drugs?	41	4	10%	0.232
Do you add antibiotics to feed of your animals?	41	41	100%	
Do you follow the exact prescription of veterinarians when you purchase antibiotics?	41	30	73%	0.223
What do you do with leftover antimicrobials?	41	15	36	
Do you read the prospectus before using antimicrobials?	41	37	90	0.976
Average mean score (=76%)				
Good attitude $\geq$ 76%			31(76%	
Poor attitude $\leq$ 76%			10(24%)	

ANNEX 17D: Table 2: Assessment of risk factors associated with hygienic practices of dairy farms at Bishoftu town, Central Ethiopia

Hygienic Practice	Category	%	P. value
If your animals are enclosed, what type of animal house floor is in?	Covered with manure	24%	0.021
	Concrete	76%	
How often the barn and/or the milking room are/is cleaned?	daily	73%	0.001
	Once per two days	27%	
Having a good drainage system	Yes	61%	0.000
	No	39%	
Where does cow milk?	In barn	79%	0.129
	In milking room	21%	
When do you wash your hands?	Before and after milking	95%	0.070
	Only before milking	5%	
	Between milking	-	
How do you keep milk containers and milking buckets? Washing with	Warm water	-	0.005
	Cold water	-	
	Both warm water and cold water	100%	
Where is the source of water for cleaning utensils?	Tab water	29%	0.823
	Pipe	71%	
What type of milk container is used for milk storage?	plastic	27%	0.609
	stainless steel	73%	
When do you use a towel	Only before milking	43%	0.812
	Before and after milking	57%	
	Don't use	-	
Have you a transportation facility to deliver milk for your customers?	Yes	10%	0.092
	No	90%	
Average mean score (=64%)			
Good practices (>64%)		26(64%)	
Bad practices (<64%)		15(36%)	

ANNEX 17E: Table 3: Frequency distribution of hygienic practices of milk vendors at Bishoftu town, Central Ethiopia (n=14)

Hygienic Practice	Category	%
From where do you collect milk?	Direct from producer	71
	From other collectors	29
What type of milk container is used for milk storage?	plastic	36
	stainless steel	64
How do you keep milk containers?	wash with soap and water	57
	washing with water only	43
Where is the source of water for cleaning utensils?	tab water	21
	pipe water	79
Do you wash your hands before selling milk?	Yes	64
	No	36
Where do you store milk before selling?	in refrigerator	43
	At room temperature	57
Do you add any preservatives to the milk to extend its shelf life?	Yes	-
	No	100
What type of milk will be provided for markets	Fresh or raw milk	100
	Boiled milk	-
On average, for how much period does the milk stay in your shop center for selling purposes	One day	43
	Two days	57
	One week	-
	Two weeks	-

ANNEX 18: Ethical clearance

Ethical clearance certificate

Certificate Ref. No: VM/ERC/02/09/16/2024

Name of Applicant: **Lema Temesgen Gile (DVM, MSc student)**

Address: Department of Microbiology, Immunology and Veterinary Public Health, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Detection and antimicrobial resistance profile of Salmonella isolated from raw cow milk and its products in Bishoftu town, central Ethiopia: its implication for public health*

Date of application:	**December, 2023**
Nature of the project:	**Field investigation and questionnaire survey**
Target animal species:	**Cattle**
Number of animals involved:	**21**
Study area:	**Bishoftu, Ethiopia**

Minutes No. and date of review: **VM/ERC/02/16/024, 26/03/2024**

The Institutional Animal Care and Use Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University has reviewed the above research project and unanimously approved the application of **Lema Temesgen Gile.**

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

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

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Please quote Our Ref. No. When replying

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