ISOLATION, MOLECULAR DETECTION AND ANTIMICROBIAL SUSCEPTIBILITY OF *SALMONELLA* FROM COW MILK AND BEEF IN SELECTED DISTRICTS OF SIDAMA REGIONAL STATE, ETHIOPIA

MSc THESIS

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HAWASSA, ETHIOPIA
OCTOBER, 2023

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A Thesis submitted to Faculty of Veterinary Medicine

Hawassa University
College of Natural and Computational Science

In Partial fulfillment of the Requirements for the Degree of Master of Science in Veterinary Microbiology

Hawassa, Ethiopia
October, 2023

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This is to certify that the thesis entitled "Isolation, Molecular detection and Antimicrobial Susceptibility of *Salmonella* from Cow milk and Beef in Selected Districts of Sidama Regional State, Ethiopia" submitted in partial fulfillment of the requirements for the degree of Master's with specialization in Veterinary Microbiology, the graduate program of the Faculty of Veterinary Medicine, has been carried out by Tariku Geinoro under my/our supervision. Therefore, I/we recommend that the student has fulfilled the requirements and hence here by can submit the thesis to the Faculty of Veterinary Medicine.

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We, the under signed, members of the Board of examiners of the final open defense by **Tariku Geinoro** have read and evaluated his/her thesis entitled "Isolation, Molecular detection and Antimicrobial Susceptibility Test of *Salmonella* from Cow milk and Beef in Selected Districts of Sidama Regional State, Ethiopia" and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfillment of the requirements of Master's in **Veterinary Microbiology**.

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ACKNOWLEDGEMENTS

It is my pleasure to express my heartfelt gratitude and appreciation to my advisor, Dr. Getachew Ngusie, for his devotion of precious time and effort to deliver advice on research work and develop the paper.

I have also a great gratitude and thanks to my co-advisor as well as owner of the project on the specified title, Dr. Alemayehu Gebeyehu, for his devotion of precious time and effort to develop the paper. His willingness to guide me and work together is also another favor for me to appreciate him.

His earnest attitude to work hard, support with lacking media, reagents and chemicals and overall encouraging efforts paid by Dr. Gemechu Chala during my research period urge me to extend my profound gratitude.

Last but not least, I forward my gratitude to officers, professionals, farm and dairy cattle owners and butcher men for their willingness to offer the needed sample and other necessary information during sample collection.

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ABBREVIATIONS

AMR	Antimicrobial resistance
CATs	Chloramphenicol Acetyltransferases
CFU	Colony Forming Unit
CSF	Classical Swine Fever
DNA	Deoxyribonucleic Acid
ELFA	Enzyme-linked fluorocent assay
ELISA	Enzyme linked immunosorbent assay
ICG	Immunochromatography strip test
ISO	International Organization for Standardization
LFD	Lateral flow devices
MDR	Multiple Drug Resistance
MKTTn broth	Muller-Kauffman Tetrathionate novobiocin broth
NTS	Non-Typhoid *Salmonella*
PCR	Polymerase Chain reaction
RNA	Ribonucleic Acid
RT-PCR	Reverse transcriptase Polymerase Chain Reaction
RV broth	Rappaport Vassiliadis broth
TBE	Tris-base boric acid Ethylene Diamine Tetra-Acetic acid
TSIA	Triple Sugar Iron Agar
WHO	World Health Organization
XLD agar	Xylose Lysine Deoxycholate agar

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ABSTRACT

Salmonella is one of the most common causes of foodborne diseases and frequently reported for exacerbating increase of multi-drug resistance worldwide. Unhygienic practices throughout food chain and cultural habits of raw animal products contributed for the expansion of the infection and increment in antimicrobial resistance especially in developing world like Ethiopia. A cross-sectional type of study was conducted from December, 2022 to June, 2023. The study was aimed with isolation, molecular detection and determination of antimicrobial susceptibility of *Salmonella* from cow milk and beef in Hawassa, Yirgalem and Wondo Genet districts of Sidama Regional State. A total of 216 samples of which 152 milk and 64 beef samples were collected with purposive and simple random sampling methods for isolation and biochemical identification of *Salmonell*a. Positive isolates obtained from bacteriological and biochemical tests were further confirmed by polymerase chain reaction (PCR). Then, the isolate's antimicrobial susceptibility profile was tested by using disk diffusion method for twelve selected antimicrobials. SPSS windows version 25.0 was used for statistical analysis. Fisher's exact test was the measure of association of isolates with different attributes. Bacteriological and biochemical detection tests revealed that 6.5% (N=14/216) *Salmonella* was isolated from the total sample. However, in molecular detection, only 9 of the 14 isolates were confirmed to be *Salmonella* using PCR test, which was 4.17% of the total. The proportion was 5.38%, 3.23%, and 2.38% in Hawassa, Yirgalem and Wondo Genet districts, respectively. There was no significant variation in prevalence among the districts and between sample types milk (3.9%) and beef (4.7%) ($p > 0.05$). Similarly, no significant ($p > 0.05$) variation was observed in the *Salmonella* isolation rate among retailers (4.84%), households (5.56%) and farms (1.85%) as well as between yoghurt (6.45%) and raw (3.31%) milk. The result of the antimicrobial susceptibility test showed that *Salmonella* isolates were 100% resistant to ampicillin, nalidixic acid, clindamycin and cephalothin while they were above 50% sensitive to ceftriaxone, chloramphenicol and gentamycin. Multi-drug resistance (MDR) was demonstrated in all isolates. Overall, this study showed that *Salmonella* was prevalent in cow milk and beef produced and consumed; and developed MDR which may pose public health concern in the study area. Thus, subsequent regular investigations on serotypes, AMR genes and risk factors as well as rational use of antimicrobials is necessary.

Key words: *Antimicrobial susceptibility, Beef, Isolation, Milk, Molecular detection, Salmonella, Sidama, Ethiopia*

1. INTRODUCTION

1.1. Background

The emergence of zoonotic bacterial infections and the ever-increasing occurrence of antimicrobial-resistant bacteria is a currently burning issue in the globe. *Salmonella* is among the bacteria with high zoonotic potential linked to variety of foods and are known to cause gastroenteritis, enteric fever and septicemia (Mthembu *et al*., 2019; Khan and Rahman, 2022). *Salmonella* is a rod-shaped, Gram-negative, oxidase-negative facultative anaerobe that belongs to the family *Enterobacteriaceae*. It is 0.7-1.5µm×2-5µm in size and motile with peritrichous flagella except *S.* Pullorum and *S.* Gallinarum. Within the genus *Salmonella*, there are two species and several serotypes have been identified and most of these serotypes have the ability to adapt within a variety of animal hosts, including humans (Ryan *et al*., 2017; Pearce *et al*., 2021).

Studies have revealed that *Salmonella* contamination of beef could happen at a number of points, including during slaughter, skinning, evisceration, shipping, preparation for consumption and cutting in retail markets, which can be extremely dangerous to the general public health. Contamination of equipment, utensils, and personal hygiene of food handlers help to spread *Salmonella* (Hussain *et al*., 2020; Han *et al*., 2022). *Salmonella* can also be shed in the milk of asymptomatic carrier dairy cows and can be recovered from the mammary glands and are commonly isolated from dairy products in the marketplace (Abunna *et al*., 2017; Abunna *et al*., 2018; Holschbach and Peek, 2018; Castañeda-Salazar *et al*., 2021).

In Ethiopia, there are a well-liked traditional meal called "*kurt*" (beef cubes) and "*kitfo*" (beef mince) that are often eaten raw or only moderately cooked. The practice of eating raw beef and the presence of *Salmonella* in beef create an environment that is favorable for infection to spread across the community (Wabeto *et al*., 2017; Fegan *et al*., 2022). Similarly, raw milk is available in an open-air retailer shops where consumers purchase either for home consumption consume there in the form of "*ergo*" (spontaneously fermented milk). The poor hygienic practices while handling and milking favored the occurrence of *Salmonella* in milk and dairy products samples from farmers and markets (Abunna *et al*., 2017; Keba *et al*., 2020; Asfaw *et al*., 2022).

The emergence of *Salmonella* with multiple drug resistance to frequently used antimicrobials has become a danger to both the public health and veterinary sectors in many countries like Ethiopia (Jelalu *et al*., 2015; Geletu *et al*., 2022; Usmael *et al*., 2022). The extensive use of the first-line drugs has led to the development of multiple drug resistance (MDR) at a level which pose a serious problem (Wabeto *et al*., 2017). The increase in antimicrobial resistance is due to factors such as misuse, unregulated sales, incomplete use, inappropriate prescription and dispensing practices, poor hygiene practices, spectrum, duration and dose of antimicrobials used (Noto, 2022). The routine practice of the use of antimicrobials to domestic animals as a means of preventing and treating diseases, as well as growth promoter, is another factor in the emergence of antimicrobial-resistant bacteria that are consequently transferred to human (Patel *et al*., 2020).

1.2. Statement of the Problem

Numerous research works have been conducted in Ethiopia over the years that investigate the presence of *Salmonella* in cow milk, beef and feces as well as the emergence of patterns of resistance to various antimicrobials in human and veterinary medicine (Addis *et al*., 2011; Abunna *et al*., 2017; Wabeto *et al*., 2017; Sime, 2021; Gebeyehu *et al*., 2022). Salmonellosis is a significant socioeconomic issue because it is believed to be the primary cause of foodborne illness. It is one the most problematic zoonosis mainly because of the difficulty in controlling and the significant morbidity and mortality rates. Most of the studies done to isolate and identify the agent have been only from samples of abattoir origin and dairy farm origin, for beef and cow milk, respectively. However, butcher houses or beef retailers and milk retailing shops are other considerable areas in the course of food chain, which must be addressed (Han *et al*., 2022; Wójcicki *et al*., 2022). The commonly applied isolation and identification techniques are conventional ones. Thus, reports involving molecular detection of the pathogen are minimal in the current study area and in the country as well. There have been also frequent alarms from the world as well as from the country to take part in minimizing food safety risks and antimicrobial resistance through continuous and periodic evaluation of microbiological and their antimicrobial susceptibility patterns (Asfaw *et al*., 2022; Garg *et al*., 2022). In addition, data concerning the focus of this research are not available for the selected districts of Sidama Regional State (especially Yirgalem and Wondo Genet). Fortunately, they are of the potential areas of the country for milk and beef production and consumption associated with increasing urbanization. Hence, additional research on ascertaining the presence of *Salmonella* in milk and beef; and the profile of its antibiogram is also anticipated to supplement the baseline data in Ethiopia and worldwide.

1.3. Objectives of the Study

1.3.1. General objective

The current study was aimed to isolate and identify *Salmonella* from cow milk and beef samples collected from Hawassa, Yirgalem and Wondo Genet districts of Sidama Regional State using cultural, biochemical, and molecular approaches as well as figuring out the organism's antimicrobial susceptibility profile.

1.3.2. Specific objectives

- Isolation and identification of *Salmonella* from cow milk and beef samples;
- Molecular detection of *Salmonella*;
- Figuring out the antimicrobial susceptibility profile of *Salmonella*

1.4. Research Questions

- What is the proportion of the isolate/s from the collected samples from the study areas?
- What is the variation in proportion of the isolate/s among the sample types and sample sources?
- What is the response of isolate/s to the tested clinically used antimicrobials with standard concentration?

1.5. Significance of the Study

Frequent and periodic surveillance of such a globally concerned pathogen and its antimicrobial susceptibility pattern is paramount crucial in order to safeguard the public (Asfaw *et al*., 2022). The current study areas were known areas where milk and beef are consumed. Thus, implementing bacteriological and molecular detection is essential to generate scientific information about the status of *Salmonella*, which may help to develop control and prevention strategies. Examining bacterial pathogens for antimicrobial susceptibility and detection by molecular methods enable researchers to ascertain whether a drug will be effective in a certain area. This aids in appropriate prescription of the right treatment to patients with a particular bacterial infection, rather than prescription which may lead to the development of AMR.

2. LITERATURE REVIEW

2.1. *Salmonella* Classification and Nomenclature

Salmonella was initially discovered and isolated by Dr. Daniel Elmer Salmon and his co-worker Theobald Smith in 1855 from pigs infected with Hog cholera caused by *Salmonella* Choleraesuis so that given the name *Salmonella* (Bhat *et al*., 2022; Kazmi, 2022). The *Salmonella* species are classified into serovars (serotypes) based on the lipopolysaccharide (O), flagellar protein (H), and sometimes the capsular (Vi) antigens. Accordingly, there are more than 2700 known serovars. Within a serovar, there are strains that differ in virulence (Ryan *et al*., 2017; Pearce *et al*., 2021).

The nomenclature system of *Salmonella* is a complex process. This genus is composed of two main species, *Salmonella enterica* and *Salmonella bongori*. The type species *Salmonella enterica* is further divided into six subspecies on the basis of biochemical properties and genomic relatedness (Pearce *et al*., 2021). The subspecies are denoted by Roman numerals: I. *S. enterica subsp. enterica*; II. *S. enterica subsp. salamae*; III. *S. enterica subsp. arizonae*; IIIa. *S. enterica subsp. diarizonae*; IV. *S. enterica subsp. houtenae*; V. *S. enterica subsp. indica*. Recently, based on genomic data, it has been proposed to extend the number of subspecies within *S. enterica* to include subspecies *londinensis* (VII), *brasiliensis* (VIII), *hibernicus* (IX), *essexiensis* (X) and *reptilium* (XI), while elevating *S. enterica subspecies arizonae* to the species level, *S. arizonae*. The *S. enterica subsp. enterica* (I) is most common subspecies of *Salmonella* and is found to be predominant infections in humans & warm blooded animals. The remaining five subspecies and *S. bongori* are mainly attributed to infections in cold blooded animals and rare in humans (Dieckmann *et al*., 2008; Pearce *et al*., 2021; Sedrakyan *et al*., 2022). To avoid confusion between serotypes and species, the serotype name is not italicized and starts with a capital letter. The genus name is given followed by the word “serotype” or abbreviation “ser.” and then the serotype name, e.g. *Salmonella* serotype or ser. Typhi. Afterward the name may be shortened with the genus name followed directly by the serotype name, e.g. *Salmonella* Typhi or *S.* Typhi (Brown *et al*., 2021).

2.2. Epidemiology

2.2.1. Sources of infection and Mode of transmission

Salmonellosis has been recognized in all countries but appears to be most prevalent in areas of intensive animal husbandry, especially poultry and swine production. It has complex epidemiology largely because there are several distinct serotypes (serovars) with different reservoirs and diverse geographic incidences. Changes in food consumption, production, and distribution have led to an increasing frequency of multistate outbreaks associated with fresh produced and processed foods. They are carried asymptomatically in the intestines or gall bladder of many animals, and are continuously or intermittently shed in the feces. These bacteria are also shed in the feces of animals and humans that are ill. Fomites and mechanical vectors (e.g. insects such as flies) can also spread *Salmonella* (Khan *et al*., 2022; Ramtahal *et al*., 2022).

Salmonella species are mainly transmitted by the fecal-oral route. Animals can become infected by ingestion of contaminated feed (including pastures), drinking water or close contact with an infected animal (including humans). Birds and rodents can spread *Salmonella* to livestock. Carnivores are also infected through meat, eggs and other animal products that are not thoroughly cooked (e.g. raw meat diets). People are often infected when they eat contaminated foods of animal origin like meat (poultry, pork, and beef), egg and dairy products (Ryan *et al*., 2017; Brown *et al*., 2021; Adem, 2022). Aerosol transmission, vertical transmission from dam to fetus and horizontal transmission though coitus are proposed modes of transmission in some animals like bovine and equine (Hanson *et al*., 2016; Holschbach and Peek, 2018).

These bacteria can survive for long periods in the environment and can be isolated from many sources. They can thrive between 8^0C and 45^0C (optimally at 37^0C) and at a pH of 4 to 9. A temperature higher than 70^0C quickly kills them. Pasteurization at 71.1^0C for 15 seconds is sufficient to destroy *Salmonella* in milk (Li *et al*., 2022). *Salmonella ser.* Choleraesuis can survive for at least 3 months in wet swine feces and 13 months in dry swine feces. *Salmonella ser.* Typhimurium has been isolated from cattle feces at 48 days; the bacterium may survive even longer in water (152 days) and soil (231 days) (Abunna *et al*., 2017; Brown *et al*., 2021).

2.2.2. Risk factors

Salmonellosis in animals is mainly associated with contaminated feed, water and environment, which may be attributed to indiscriminate feeding habits. Some animals like dogs and cats are considered to be asymptomatic carriers of the pathogen (Holschbach and Peek, 2018). Working with animals is regarded as occupational hazard for the possibility humans get infected. Consumption of contaminated raw or undercooked animal products (meat, milk, and egg), vegetables and indirect mechanical contact to equipments are also the potential risk areas for humans acquiring infection (Fegan *et al*., 2022).

2.3. Zoonotic Importance

Most of the isolates that cause disease in humans and other mammals belong to *S. enterica subsp. enterica. Salmonella* serovars, sometimes referred to as non-typhoidal *Salmonella*, are potentially zoonotic. The most common serovars infecting humans worldwide are *Salmonella ser.* Typhimurium and *Salmonella ser.* Enteritidis. Some host-adapted serovars such as *S.* Choleraesuis and *S.* Dublin have also been shown to cause serious diseases in humans (Li *et al*., 2022). Highly exposed human groups such as farmers, slaughterhouse workers, cookers and animal product handlers have a higher ratio of resistant *Salmonella* in their feces than the general population (Noto, 2022).

2.4. Pathogenesis and Clinical Aspects

The favorable outcome of a pathogen is based on its capability to enter a host, evade host defense barrier and initiate infection. *Salmonella* has developed deviating schedule to disrupt normal host cellular functions that enable the entry and proliferation inside the host cell (Bhat *et al*., 2022). Different virulence factors that *Salmonella* generates are crucial to its pathogenicity. These include the ability to invade the cell, a perfect lipopolysaccharide coat, to replicate intra-cellularly and capability to secrete toxins (van Asten *et al*., 2005; Beshiru *et al*., 2018; Moxley, 2022; Salim and Motaweq, 2022).

Following entry into the host cell, the bacterium is encased in a membrane compartment called a vacuole, which is composed of the host cell membrane. Under normal circumstances, the presence of a bacterial foreign body would activate the host cell’s immune response, resulting in the fusion of the lysosomes and the secretion of digesting enzymes to degrade the intracellular bacteria. However, *Salmonella* uses the type III secretion system to inject other effector proteins into the vacuole, causing the alteration of the compartment structure. The remodeled vacuole blocks the fusion of the lysosomes, and this permits the intracellular survival and replication of the bacteria within the host cells. The capability of the bacteria to survive within macrophages allows them to be carried in the reticuloendothelial system (RES) (Li *et al*., 2022).

Based on the clinical patterns in human salmonellosis, *Salmonella* strains can be grouped into typhoid *Salmonella* and non-typhoid *Salmonella* (NTS). Non-typhoidal serotypes can be transferred from animal to human and from human-to-human. They usually invade only the gastrointestinal tract and cause salmonellosis, the symptoms of which can be resolved without antimicrobials. In human infections, the four different clinical manifestations are enteric fever (mostly typhoidal serovars), gastroenteritis (NTS), bacteremia and other extra intestinal complications, and chronic carrier state. Diarrhea, cramping, abdominal pain and fever are major symptoms with infective dose being 10^4 colony forming unit (CFU) (Asfaw *et al*., 2022; Li *et al*., 2022).

2.5. Isolation and Identification of *Salmonella*

2.4.1. Culture

Depending on the form of the disease, *Salmonella* may be detected in feces; placenta, fetal tissues and vaginal discharge; blood; or various internal organs at necropsy. Embryonated eggs can be cultured from birds. Foods of animal origin like milk, meat from poultry, swine and bovine are also possible sources of the agent. Intensive methods (pre-enrichment) to detect *Salmonella* can resuscitate stressed organisms and increase the probability that small numbers of organisms can be detected. *Salmonella* grow on many selective and nonselective media (Brown *et al*., 2021). *Salmonella* species can be identified with biochemical tests. Distinctive features are observed in various culture media and tests (Table 1).

Table 1: Characteristic features of *Salmonella* on some culture media and biochemical tests

No	Culture media or Biochemical test	Characteristic feature	Description
1	Gram's stain	Gram-ve bacilli/ Red or pink coloured/	Take Safranin/counter stain/ not Crystal violet
2	Rappaport-Vassiliadis with soya (RVS)	Turbid	Malachite green inhibits many G+ves and enriches *Salmonella*; MgCl inhibits Proteus sp and *E. coli*
3	Muller-Kauffman Tetrathionate novobiocin Broth (MKTTn)	Turbid	Ox bile inhibits growth of G+ves; Sodium thiosulphate pentahydrate sulphur source which is selective agent; Brelliant green inhibits growth of G+ves & some G-ves; and Novobiocin is selective agent ant inhibits Proteus sp
4	MacConkey agar	Colonies are colourless	Lactase –ve; non-lactose fermenter
5	Brelliant green agar	Colonies are red	Brelliant green inhibits growth G+ves & most G-ve bacilli
4	Xylose lysine deoxycholate agar (XLD)	pink colonies with black centers	L-lysine differentiate *Salmonella*; Xylose differentiate Shigella; Sodium deoxycholate inhibits G+ves & Sodium thiosulphate indicates H_2S production
5	Hektoen enteric agar (HE)	Colonies are blue-green with black centers	Bile salt mixture inhibits G+ves; Sodium thiosulphate indicates H_2S production; & Acid fuchsin & Bromothymol blue differentiate non-pathogenic organisms, *Salmonella* & Shigella
6	Urease test	phenol red, be yellow or orange/no change	No urease production, negative reaction
7	Simmons Citrate (Citrate utilization)	Bromothymol blue from green to blue	Sodium carbonate, a byproduct of Krebs cycle raises pH
8	Methyl red test	diffuse red color (+ve)	There is mixed acid fermentation
9	Vagus-Proskauer test	VP-negative, no pink-to-red color throughout bro	No production of non-acidic end product
10	Triple Sugar Iron Agar (TSIA)	Alkaline (red) slants and acid (yellow) butts with blackening of the agar	Red or unchanged slant: lactose and sucrose negative (no fermentation of lactose or sucrose); Yellow butt: glucose positive (glucose fermentation);
11	Indole test	Yellow-brown (-ve)	Absence of Tryptophanase enzyme

Source: (Akter *et al*., 2018; Andrews *et al*., 2018; Moxley, 2022)

2.5.2. Serology

Immunological-based (serology) can identify the somatic (O), flagellar (H) and capsular (Vi) antigens (Ryan *et al*., 2017). A huge improvement to these methods has been made with the introduction of more specific target antibodies and genes. Immunological methods detect unique *Salmonella* molecules using two antibodies; a surface-bound primary antibody to capture the target molecule and a reporter antibody to detect the antibody target complex. The techniques can replace isolation agars, lowering the time to presumptive positive result to one to two days. The immunological assays available for *Salmonella* detection are latex agglutination, lateral flow devices (LFD), enzyme linked immunosorbant assay (ELISA), immunochromatography strip test

(ICG) (Xia *et al*., 2016), immunomagnetic separation (Leon-Velarde *et al*., 2009) and Enzyme-linked fluorescent assay (ELFA).

2.5.3. Molecular methods

Molecular-based assays are the fastest analytical detection methods developed due to the advancement in molecular sciences. These involve the hybridization of short oligonucleotide fragment known as a DNA/RNA probe or primer to detect specific targeted DNA/RNA sequences. Specific primer/probe can be isolated from microorganisms or engineered according to their specific target. Conventional PCR, real-time PCR, multiplex PCR, and reverse transcriptase PCR have all been used to detect *Salmonella* (RT-PCR) (Lin *et al*., 2020). *InvA* gene (Ferretti *et al*., 2001) and 496-bp segment of histidine transport operon gene (Cohen *et al*., 1993) have been mostly used primer targets to detect the highly conserved regions of *Salmonella* genus in the gel electrophoresis technique.

2.6. Prevention and Treatment

In all animals, the risk of clinical salmonellosis can be decreased by good hygiene and minimizing stressful events. Colostrum is important in preventing disease in young animals. Measures to prevent salmonellosis in pets, such as dogs and cats, include those that minimize consumption of raw foods. Biosecurity is the cornerstone of *Salmonella* prevention on the farm. The risk of introducing the bacterium into a herd/flock can be decreased by buying animals or eggs from *Salmonella*–free sources; isolating newly acquired animals; and practicing all-in/all-out herd or flock management, where appropriate. Fecal contamination of feed and water supplies should be prevented (Brown *et al*., 2021). Contaminated buildings and equipment should be cleaned and disinfected, and contaminated material should be disposed off. Livestock vaccines are available for some serovars such as *Salmonella* ser. Dublin, *Salmonella* ser. Typhimurium, *Salmonella* ser. Abortusequi and *Salmonella* ser. Choleraesuis in some countries. There are also two licensed vaccines currently available against *S.* Typhi: the injectable Vi capsular polysaccharide (Vi CPS) vaccine (Typherix or Typhim Vi) and the oral live attenuated vaccine Ty21a (Vivotif) (Kopecko *et al*., 2009; Gayet *et al*., 2017). To decrease the risk of salmonellosis, both food safety practices and the prevention of transmission from animals are important (Brown *et al*., 2021; Li *et al*., 2022).

Severe salmonellosis can be treated with a number of antimicrobials including ampicillin, amoxicillin, gentamicin, trimethoprim/sulfamethoxazole, fluoroquinolones and third generation cephalosporins (e.g. cefotaxime, ceftriaxone). Choice of drugs should be based on susceptibility testing when possible. Unfortunately, resistance to many of the drugs used to treat both human and animal salmonellosis has been increased in recent years (Patel *et al*., 2020; Usmael *et al*., 2022).

2.7. Antimicrobial Resistance Pattern of *Salmonella*

Antimicrobial resistance is the ability of bacteria to resist the bacteriostatic and/or bactericidal effects of one or more antimicrobial agents at clinically achievable concentrations, leading to treatment failure (Noto, 2022). Most identified foodborne bacterial pathogens, including *Salmonella* species, in Ethiopia exhibited high levels of antimicrobial resistance today (Asfaw *et al*., 2022). Inactivation of the antimicrobial agent, decreased permeability, an active efflux pump system, alteration of the target site, and overproduction of the target site in numerous serotypes to overwhelm the antimicrobials utilized are the main mechanisms of antimicrobial resistance in the *Salmonella* species (van Duijkeren *et al*., 2018; Auda *et al*., 2020; Noto, 2022).

Antimicrobial resistant *Salmonella* species counteract the action of different antimicrobials through such a resistance mechanisms. For example, the inactivation of aminoglycosides is mediated by the enzymes adenyl transferases, acetyltransferases, and phosphotransferases. Cleaving β-lactam rings by β-lactamases is the most common resistance mechanism conferred against β-lactams, preventing it from binding to and inactivating bacterial cell wall (Adamczuk *et al*., 2015; Wójcicki *et al*., 2022). Similarly, *Salmonella* resist the effects of phenicols, chloramphenicols, through inactivation with chloramphenicol acetyltransferases (CATs) enzyme. Active efflux is also known to be the most common resistance mechanism observed in *Salmonella* against tetracyclines (Maka and Papowska, 2016). Most of currently available antimicrobials against *Salmonella* have been reported to develop resistance at least with 50% (Table 2). Previously effective drugs may loose their potency with standard concentration because of overdose, underdose and misuse in human and veterinary medicines (Girma, 2015; Noto, 2022).

Table 2: List of antimicrobials reported about last decade for inefficiency against *Salmonella*

R/N	Antimicrobial	Reference
1	Gentamycin	Addis *et al*., 2011; Pritha *et al*., 2020; Sohail *et al*., 2021;
2	Ampicillin	Abunna *et al*., 2018; Banti, 2018; Mthembu *et al*., 2019; Yang *et al*., 2019; Bawa *et al*., 2020; Hussain *et al*., 2020; Geresu and Desta., 2021; Gutema *et al*., 2021; Gebeyehu *et al*., 2022
3	Streptomycin	Addis *et al*. , 2011; Wabeto *et al*., 2017; Mthembu *et al*., 2019; Yang *et al*., 2019; Pritha *et al*., 2020; Geresu *et al*., 2021; Alemu *et al*., 2022; Gebeyehu *et al*., 2022
4	Kanamycin	Banti, 2018; Mthembu *et al*., 2019; Geresu and Desta, 2021;
5	Nitrofurantoine	Wabeto *et al*., 2017; Bawa *et al*., 2020; Geresu and Desta, 2021;
6	Doxycycline	Ghoddusi *et al*., 2015; Sohail *et al*. , 2021;
7	Cefpodoxime	Sohail *et al*. , 2021;
8	Ciprofloxacin	Hussain *et al*., 2020; Pritha *et al*., 2020; Sohail *et al*. , 2021;
9	Enrofloxacin, Colistin sulphate and Erythromycin	Pritha *et al*., 2020;
10	Neomycin	Hussain *et al*., 2020; Alemu *et al*., 2022
11	Cefotaxime	Sohail *et al*. , 2021; Gebeyehu *et al*., 2022
12	Trimethoprim-sulfamethoxazole	Mthembu *et al*., 2019; Yang *et al*., 2019; Bawa *et al*., 2020;
13	Chloramphenicol	Ghoddusi *et al*., 2015; Wabeto *et al*., 2017; Abunna *et al*., 2018; Gutema *et al*., 2021
14	Cefoxitin	Banti, 2018
15	Nalidixic acid	Abunna *et al*., 2018; Banti, 2018; Yang *et al*., 2019;
16	Tetracycline	Wabeto *et al*., 2017; Mthembu *et al*., 2019; Yang *et al*., 2019; Bawa *et al*., 2020; Hussain *et al*., 2020; Pritha *et al*., 2020; Geresu and Desta, 2021; Gutema *et al*., 2021
17	Amoxicillin-Clavulonate	Abunna *et al*., 2018; Banti, 2018; Mthembu *et al*., 2019; Bawa *et al*., 2020; Hussain *et al*., 2020; Worku *et al*., 2022
18	Ceftriazone	Mthembu *et al*., 2019;
19	Florfenicol	Ghoddusi *et al*., 2015;
20	Cephalothin	Bawa *et al*., 2020;
21	Sulfamethoxazole	Gutema *et al*., 2021; Talal *et al*., 2022
22	Clindamycin	Talal *et al*., 2022

2.7. Current Status of *Salmonella* in Ethiopia

The problem is serious in Ethiopia as developing country due to limitations in ensuring optimal hygienic food handling practices and irrational drug use practices (Girma, 2015). A study in Jimma, South West Ethiopia, (Geresu and Desta, 2021) Gondar, Ethiopia (Ejo *et al*., 2016) and Wolita Sodo, South Ethiopia (Wabeto *et al*., 2017) showed high contamination rates of *Salmonella* species isolated from raw beef. In another study conducted in Addis Ababa, Ethiopia, in a selected number of butcher shops also found inadequate meat quality and microbiological safety (Zerabruk *et al*., 2019). Additionally, another study found that cooked meat and fish posed a public health risk in Ethiopia (Bedada *et al*., 2020). A higher percentage of multidrug-resistant isolates were also detected in ready to eat raw meat (beef, mutton and chevon) at Arba Minch town hotels and restaurants, which may result in serious risk of transmission to handlers, consumers and the environment (Tonjo *et al*., 2022).

As a review report, among the pathogenic bacteria *Salmonella* species are one of the most common isolates from milk and dairy products in Ethiopia (Keba *et al*., 2020). Research in the East Wollega Zone of Sibu Sire districts (Adugna and Eshetu, 2021) and Lume and Siraro districts of Oromia state (Amenu *et al*., 2014) showed poor hygienic practices and microbiological quality of milk obtained from farmers and markets. Collectively, a number of review articles (Pieracci *et al*., 2016; Asante *et al*., 2019; Abebe *et al*., 2020 and Cavalerie *et al.*, 2021) and primary research (Mekonnen *et al*., 2021) have identified enormous zoonotic bacterial pathogens, among these were *Salmonella* species which are still a public health problem in Ethiopia.

According to numerous studies, most bacterial species of human, animal, food, and environmental origin are becoming more resistant to antimicrobials (Gemeda *et al*., 2021). This resulted in the loss of valuable antimicrobials. According to a review study, the pooled prevalence of AMR bacteria from live food-producing animals was 20% (Asfaw *et al*., 2022). High estimates of pooled AMR prevalence were found to be 29% for bacteria identified in milk and environmental samples and 28% for meat (Gemeda *et al*., 2021). *Salmonella* species are reported to have higher MDR pattern in samples (meat and meat products, dairy and dairy products, poultry products, and food contact surfaces) (Belina *et al*., 2021). Thus, interactions between humans-animals-ecosystems raise the risk of emerging and re-emerging antimicrobial resistance, which results in fatalities and financial losses each year (Erkyihun *et al*., 2022).

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted in selected districts of Sidama Regional State (Hawassa, Yigalem and Wondo Genet) which supply milk and beef for the big market at Hawassa. They were purposively selected due to their relatively larger potential for cattle population; and milk and beef production. Hawassa is the capital city of the Sidama Region State, located at 275km south of Addis Ababa. Geographically it lies between 703′1.35″ N latitude and 380 29′43.81″ E longitudes at an altitude of 1750m above sea level. The elevation of Dale/Yirgalem district ranges from 1200m above sea level along the shores of Lake Abaya to 3200m at its western most point. Wondo Genet is one of the Woredas located in the Sidama Regional State located in the Great Rift Valley of Ethiopia that extends between $6^0$59″N-70^{0}6″ N and 38^{0}37″-38^{0}43″ E, 1720-2620 above sea level and about 24 kilometers east of Hawassa Town (Argaye, 2022).

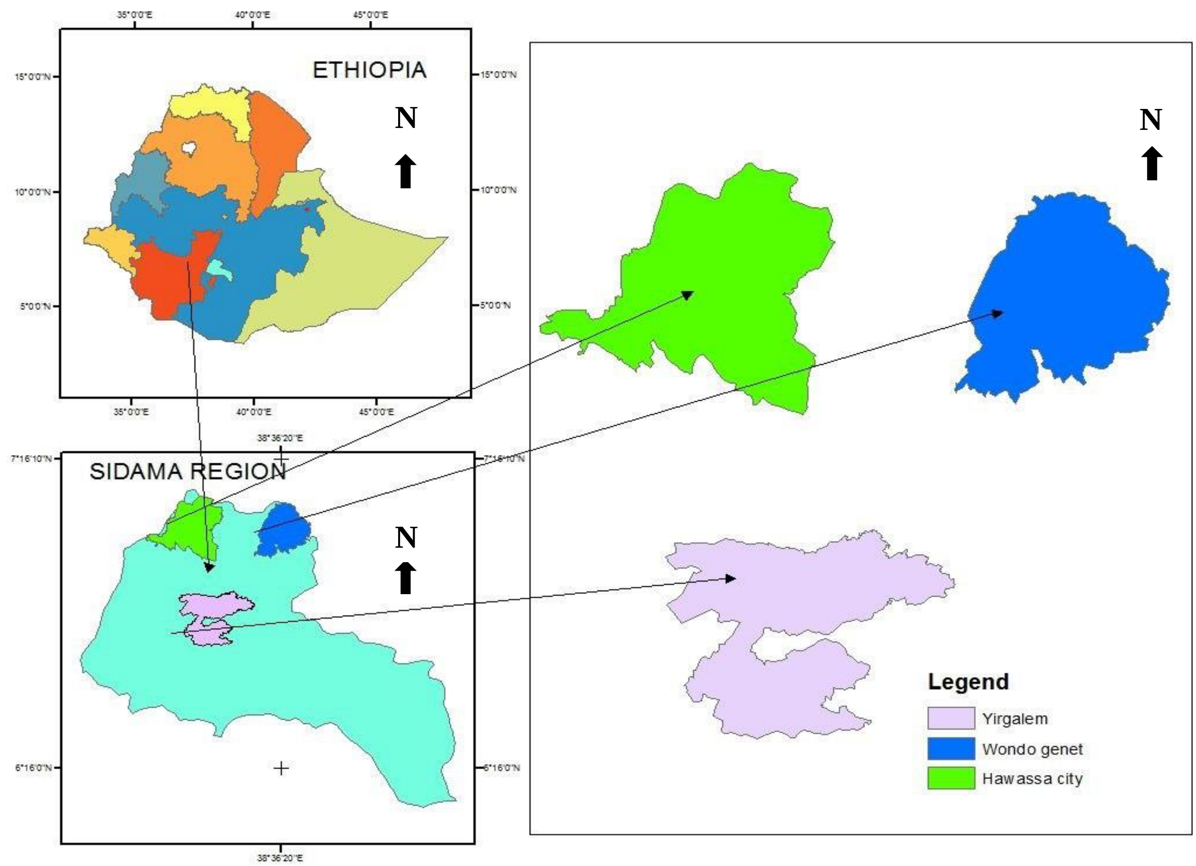


Figure 1: Geographical location of the study area

3.2. Source of Samples

Beef samples for the present study were collected from beef retailer shops (butcher houses), whereas milk samples were collected from milk retailer shops, dairy farms and dairy households found in Hawassa city, Yirgalem and Wondo Genet districts. Dairy farms were farms which were engaged mainly for commercial purpose whereas dairy households were those involved in small-scale dairy farming with main focus on home consumption. For collection of milk and beef samples from retailers, the shops were selected by using simple random sampling technique. Prior to sample collection, a cooperation letter were used to facilitate sample collection. As a response, an animal health technician was assigned who helped during collection of samples.

3.3. Study Design and Sample Size Determination

A cross-sectional study design was applied from December, 2022 to June, 2023 to isolate, detect molecularly, and characterize antimicrobial susceptibility profiles of *Salmonella* species from cow milk and beef samples collected from selected districts of Sidama Regional State. The sample size was determined using prevalence rate of 10.42% for milk and 4.1% for beef from previous studies (Gebeyehu *et al.*, 2022; Worku *et al.*, 2022) with 5% absolute precision and 95% confidence interval using the formula recommended by Thrusfield (2007).

$$n = \frac{1.96^2\, Pexp\, (1 - Pexp),}{d^2}$$

Where, n = sample size; Pexp = expected prevalence and d = desired absolute precision

Accordingly, the sample size being 143 for milk and 61 for beef but 5% was intentionally added to have 152 milk and 64 beef, collectively 216 sample size.

3.4. Methods of Sample Collection and Transportation

3.4.1. Milk sample

The simple random sampling technique was used for dairy farms and milk retailers based on the information, about total number and production potential, obtained from the respective districts' Agricultural office, and Trade and Market Development office. Tabor, Tula, Mehal ketema and Hayk dar sub cities were selected for dairy farms and all sub cities except Tula were selected for milk retailers in Hawassa. The two sub city administrations (Arada and Filwuha) in Yirgalem and six kebeles in and around Wondo Genet town were included for all milk sample collection.

Purposive sampling was applied to select households of all districts with ease of accessibility and willingness of the owners. Samples were collected early in the morning around 6:00 to 8:00 AM immediately after milking by arranging time with the milkers' and owners of the farms. The milk containers/tanks were also accessed with simple random sampling technique. The approximate amount of 25 ml milk sample was collected by using universal bottles with prior agitation according to the International Organization for Standardization guideline (ISO, 6579-1, 2019).

3.4.2. Beef sample

The list of licensed butcher houses were gained from each town's Municipality and Trade and Market Development offices. Seven out of eight sub cities, the two sub city administrations and the six kebeles in Hawassa, Yirgalem and Wondo Genet, respectively, were addressed for the beef sample collection. The simple random sampling technique was used based on the data obtained from the respective districts' offices. Fresh or non-frozen beef was sampled early in the morning from 6:00 to 8:00 AM. The approximate amount of 25g beef sample was collected according to the International Organization for Standardization (ISO, 6579-1, Mooijman, 2019). The beef sample was cut and minced with knife then put in sterile plastic bags by using sterile forceps.

3.4.3. Labeling and transportation

All the collected samples were properly identified by sample type, date of collection and sources, and immediately transported to the laboratory (Hawassa University, Faculty of Veterinary Medicine, Veterinary Microbiology Laboratory) in an ice box with freeze packs under completely sterile conditions for microbiological analysis. In the laboratory, the samples were processed and cultured immediately or sometimes stored at 4°C for a maximum of 24 hours until transferring into enrichment medium and inoculation onto a standard bacteriological media (Mooijman, 2019).

3.5. Laboratory Procedures

3.5.1. Isolation and identification of *Salmonella*

The procedure for isolation of *Salmonella* from milk and beef was according to standard procedures (ISO, 6579-1, Mooijman, 2019). Twenty five milliliters of milk sample was measured and homogenized in to 225 ml of buffered peptone water (Himedia, India). The mixture was incubated at 37^0C for 24 hours. Then 1ml and 0.1ml aliquot was transferred and added to 10 ml of tetrathionate broth (Techno pharmchem, India) and Rappaport Vassiliadis (RVS) broth, respectively. This in turn vortexed and incubated at 41.5°C (RVS) and 37^0C (MKTT) for 24 hours. After incubation, a loop-full of inoculum from the RV broth and/or tetrathionate broth transferred and streaked onto the surface of xylose lysine deoxycholate agar (XLD agar) (Alpha Chemika, India). The plates were incubated at 37°C for 24 hours.

Separately, 25 g of beef sample was measured and homogenized in to 225 ml of buffered peptone water by using stomacher for 30 seconds for each sample. The mixture incubated at 37^0c for 24 hours. Then 0.5ml and 0.1ml aliquot transferred and added to 10ml of MKTTn tetrathionate broth and RVS broth, respectively. This again vortexed and incubated at 42°C (RVS) and 37^0C (MKTT) for 24 hours. After incubation, a loop-full of inoculum from the RV broth and/or tetrathionate broth was transferred and streaked onto the surface of xylose lysine deoxycholate agar (XLD agar). The plates were incubated at 42°C for 24 hours.

After proper incubation, the two (milk and beef) samples plates were examined for the presence of suspected *Salmonella* colonies, which were detected as red with a black center on XLD agar. Suspected *Salmonella* species then streaked on nutrient agar and conformation done by standard metabolic and biochemical procedures using Triple Sugar Iron Agar (TSI) (Himedia, India), Simmons Citrate Agar (Himedia, India), Lysine Iron Agar (LIA) (Himedia, India), Urea Agar (Himedia, India), and Sulfide Indole Motility (SIM) (Himedia, India) broth tests. Colonies that produced alkaline (red) slant with acid (yellow) butt on TSI with hydrogen sulphide production; negative for urea hydrolysis (no color change or yellow) on urea agar; positive for sulphide production (blackening), negative for tryptophan utilization (indole test) (yellow-brown ring) and with or without motility on SIM; positive for citrate utilization (bright blue) on SC and positive for Lysine decarboxylation (purple slant/purple butt) on LIA were considered to be *Salmonella* positive isolates (ISO, 6579-1, Mooijman, 2019). The identified *Salmonella* isolates were picked from each positive sample and stored as 25% (v/v) glycerol stocks at -20^0C for further characterization.

3.5.2. Molecular detection of *Salmonella*

DNA extraction: Genomic DNA extraction from *Salmonella* isolates were done by boiling and chilling as described by Ramadan *et al*. (2016). Two milliliter of aliquot from the overnight nutrient broth were taken into an Eppendorf tube and centrifuged at 10,000 rpm for 10 minutes at 4^0C. The supernatant was discarded and the pellet obtained at the bottom. The pelleted cells obtained finally were re-suspended in 100µl of nuclease free water, kept in a boiling at 100^0C for 10 minutes, and then immediately chilled on crushed ice for 20 minutes. Then the samples were centrifuged at 10,000 rpm for five minutes and supernatants were stored at -20 °C for further use.

DNA quality and concentration*:* The DNA quality and concentration were detected using UV spectrophotometer (JENWAY Spectrophotometer, 6705). The extracted DNA samples were diluted by 1x TE buffer with ratios 10:990 ul in 1.5 ml Eppendorf tube and mixed gently. Then the solution was transferred in to cuvette having maximum volume of 1400µL. The absorbance reading at 260nm, 280nm and 260/280 nm were measured to assess DNA purity and concentration. Thus, the extracted DNA was considered as pure if the ratio of absorbance 260/280 was between 1.7 and 2; and the concentration was determined by multiplying the ratio by fifty.

PCR amplification: All isolates shown specific biochemical characteristics of *Salmonella* were further confirmed by using genus-specific PCR as described by Cohen *et al*. (1993). This was based on the amplification of a 496-bp segment of histidine transport operon gene: 5'ACTGGCGTTATCCCTTTCTCTGGTG3' and 5'ATGTTGTCCTGCCCCTGGTAAGAGA3', forward and reverse, respectively. This is highly conserved among species of *Salmonella*. *Salmonella enterica* serovar Typhimurium ATCC 13311 was a reference strain used as a positive control during PCR. Nuclease free water was used as negative control. PCR reaction mix (20 µl) consisting of 8 µl nuclease-free water, 1 µl of the template DNA, 0.5 µl of each of primers (reverse and forward), and 10 µl of PCR premix (hot tart Taq Master Mix, QIAGEN, USA) were mixed in PCR tubes. The mixture was then amplified using a thermal cycler with an initial denaturation (4 minutes at 95º C) followed by 30 cycles of denaturation [(30 seconds at 95º C), annealing (30 second at 60º C) and elongation (45 seconds at 72º C)] and final extension for 5 minutes at 72º C (Elkenany *et al*., 2019).

Electrophoresis of PCR products: Each reaction mixture was analyzed by gel electrophoresis through 1.5% (w/v) agarose in 1xTBE buffer for 1:10 hours and visualized by ultraviolet transillumination after staining with ethidium bromide (1µg/ml). DNA bands were photographed using an ultraviolet transilluminator (BTS-20), and a100bp DNA ladder was used as a molecular size marker. Gel electrophoresis used to be carried out at 120 volts for 60 minutes considered beneath under an ultraviolet transilluminator (Nyabundi *et al*., 2017).

3.5.3. Antimicrobial Susceptibility Testing

The antimicrobial susceptibility tests of the *Salmonella* isolates were performed using Kibry-Bauer disk diffusion test on Muller Hinton agar (Hudzicki, 2009). Pure colonies from nutrient agar taken with a wire loop, transferred to a tube containing 5 ml of saline water, and emulsified. The emulsified broth culture incubated at 37°C as far as it reached the 0.5 McFarland turbidity standards. Sterile cotton head swab soaked into the emulsified broth and the bacteria swabbed evenly over the surface of Muller Hinton agar (Himedia, India) plate. The plates kept at room temperature for 15 minutes to allow drying. Discs with known concentration of antimicrobials then carefully placed on the plates (15-20mm apart from each other) and the plates be incubated for 24 hours at 37°C. Each isolate of *Salmonella* was tested for a series of twelve common antimicrobials which are commonly used and available in the market. Ampicillin (AMP-10µg), cephalothin (CF-30µg), ceftriaxone (CTR-30µg), chloramphenicol (C-30µg), gentamycin (CN-10µg), streptomycin (S-10µg), clindamycin (CD-20µg), nalidixic acid (NA-30µg), ciprofloxacin (CIP-5µg), nitrofurantoin (F/M-300µg), oxytetracycline (T-30µg) and trimethoprim-sulphamethaxazole (SXT-23.75µg). That is, the zone of inhibition was measured with caliper and interpreted as susceptible, intermediate, or resistant categories assigned on the basis of standard given (CLSI, 2022).

Multidrug resistance (MDR) was determined by ascertaining the drug class of each test antimicrobial and registering those isolates with resistance to three or more classes (Sweeney *et al*., 2018). Multiple drug resistance (MDR) indexes were calculated as described by Saba *et al* (2011). MDRI= a/b where “a” is the total number of antimicrobials to which an organism is resistant and “b” is the total number of antimicrobials against which the organisms were tested.

3.6. Data Management and Analysis

Data describing *Salmonella* isolates along with districts, sample sources, sample types and detection techniques were classified, filtered, coded and entered in to Microsoft Excel® 2016. The data then exported to SPSS windows version 25.0 (SPSS) for appropriate statistical analysis. The proportion of result and resistance of isolates to drugs were determined by using descriptive statistics. Fisher’s exact test was used to measure the association between the sample sources and presence of *Salmonella* isolates. Association between sample type and presence of the isolate was also measured. Effects were reported as statistically significant if p-value less than 5% ($p < 0.05$).

4. RESULTS

4.1. Overall *Salmonella* Isolation in Association with Districts

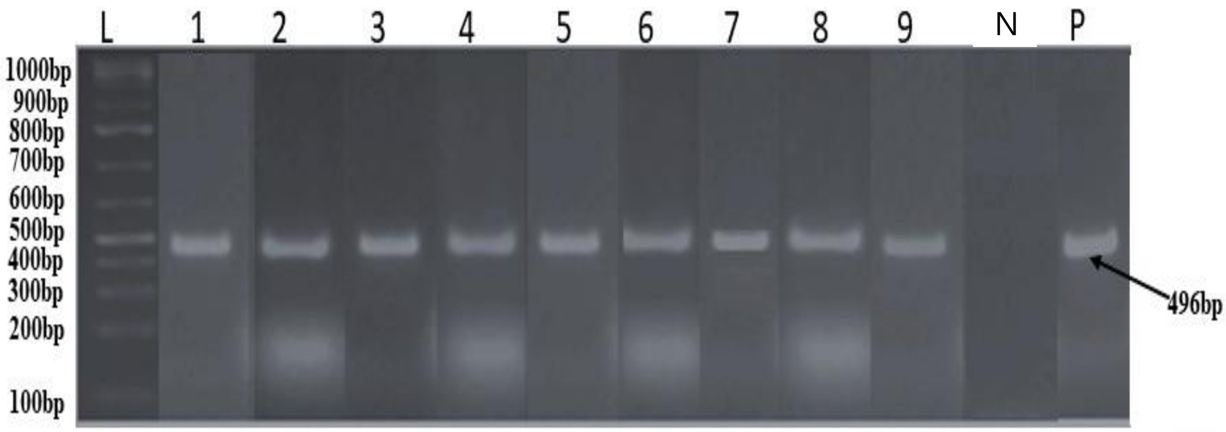
During the study period, one hundred fifty two milk and sixty four beef, a total of two hundred sixteen samples were collected from three districts. Out of these samples examined, 112 (51.9%), 62 (28.7%) and 42 (19.4%) were collected from Hawassa, Yirgalem and Wondo Genet, respectively. Only fourteen (14) (6.5%) isolates showed typical biochemical properties of *Salmonella* (Table 3).

Table 3: Overall isolation rate of *Salmonella* and its association with districts

District	Sample examined	Positives	Isolation rate (%)	p-value
Hawassa	112	11	9.8	
Yirgalem	62	2	3.2	0.18
Wondo Genet	42	1	2.4	
Total	216	14	6.5	

4.2. Molecular Confirmation Results of Salmonella Isolates

From these nine (9/216, 4.17%) isolates were further confirmed by PCR amplification (Figure 2). Out of the 9 isolates, 6 (66.7%), 2 (22.2%) and 1 (11.1%) were from samples collected from Hawassa, Yirgalem and Wondo Genet districts, respectively.



Note: Lanes: L= Ladder, 1-9= *Salmonella* positives, N=Negative control and P=Positive control

Figure 2: Agarose gel image of 496-bp segment of histidine transport operon gene of *Salmonella* isolates

4.3. *Salmonella* Isolation Rate in Association with Sample Type

It was observed that out of the total 152 milk samples collected during the study period, 6 (3.9%) were found to be positives for *Salmonella*. On the other hand, from the 64 beef samples collected, 3 (4.7%) were found to be positive for *Salmonella* (p = 0.73, Table 4).

Table 4: *Salmonella* isolation from milk and beef samples

Sample type	Sample examined	Positives	Isolation rate (%)	p-value
Milk	152	6	3.9	
Beef	64	3	4.7	0.73
Total	216	9	4.17	

4.4. *Salmonella* Isolation Rate in Beef in Association with Districts

Out of 64 beef samples examined, 36 (56.3%), 16 (25.0%) and 12 (18.8%) were collected from Hawassa, Yirgalem and Wondo Genet, respectively. The isolation rate of *Salmonella* was relatively higher in samples collected from Yirgalem district (6.3%) than from Hawassa (5.6%) and Wondo Genet (0.0%) (Table 5).

Table 5: *Salmonella* isolation rate from beef in relation to districts

District	Sample examined	Positives	Isolation rate (%)
Hawassa	36	2	5.6
Yirgalem	16	1	6.3
Wondo Genet	12	0	0.0
Total	64	3	4.7

4.5. *Salmonella* in Milk in Association with Sources

A total of one hundred fifty two (152) milk samples were collected from the three different sources, 62 (40.8%) from retailers, 54 (35.5%) dairy farms and 36 (23.7%) households. Out of the total milk sample eight (5.3%) isolates showed typical biochemical properties of *Salmonella*. From these, six (6/152, 3.9%) isolates were further confirmed by PCR amplification. Out of the 6 isolates, 2 (33.3%), 3 (50%) and 1 (16.7%) were from milk samples collected from households, retailers and farms, respectively (p = 0.66, Table 6).

Table 6: *Salmonella* isolation from milk sample and its association with sources

Sources	Sample examined	Positives	Isolation rate (%)	p-value
Households	36	2	5.56	
Retailers	62	3	4.84	0.66
Farms	54	1	1.85	
Total	152	6	3.9	

4.6. *Salmonella* in raw milk and yoghurt

It was observed that out of the total 121 raw milk samples collected during the study period, 5 (4.13%) were found to be positives for *Salmonella*. On the other hand, from the 31 yoghurt samples collected, 1 (3.22%) were found to be positive for *Salmonella*. Though the overall isolation rate of *Salmonella* was higher from yoghurt, the difference was not statistically significant (p = 1.00, Table 7).

Table 7: *Salmonella* isolation rate from raw milk and yoghurt samples

Milk type	Sample examined	Positives	Isolation rate (%)	p-value
Raw milk	121	5	4.13	
Yoghurt	31	1	3.22	1.00
Total	152	6	3.9	

4.7. Antimicrobial Susceptibility Profile of *Salmonella* Isolates

Salmonella isolates were 100% resistant to ampicillin, nalidixic acid, clindamycin and cephalothin while they were 88.9%, 55.6% and 66.7% sensitive to ceftriaxone, chloramphenicol and gentamycin, respectively (Figure 3).

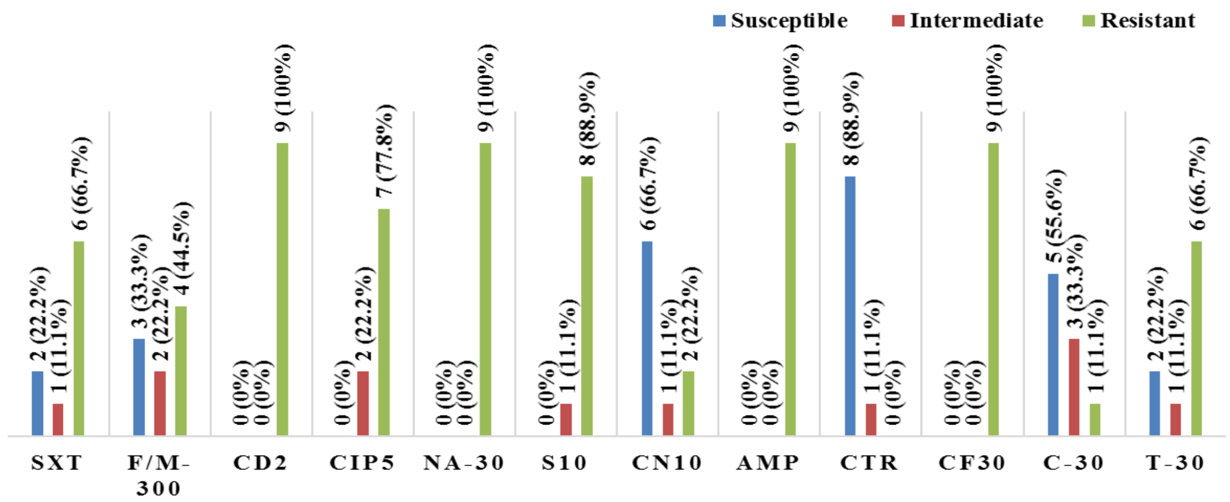


Figure 3: Antimicrobial susceptibility profile of *Salmonella* isolates

4.8. Multidrug Resistance Pattern and MDR Indexes

Multi-drug resistance (MDR) at least for five antimicrobial classes was demonstrated in all isolates. Five different antimicrobial resistance patterns were observed (Table 8). From the 9 isolates tested for antimicrobial susceptibility 1, 2, 4 and 2 showed resistance to five, seven, eight and nine antimicrobial classes, respectively. The MDR indexes of the isolates obtained from this study ranged from 0.42 to 0.83, inclusive. The average MDR index value give 0.66.

Table 8: MDR pattern of *Salmonella* isolates

No of AMR	Resistance pattern	No of AMC	No of isolates (%)
Six	CD2, CIP5, NA-30, AMP, S10, CF30	5	1 (11.1)
Eight	SXT, F/M-300, CD2, CIP5, NA-30, AMP, S10, CF30	7	2 (22.2)
	SXT, CD2, CIP5, NA-30, AMP, S10, CF30, T-30		
Nine	F/M-300, CD2, CIP5, NA-30, AMP, S10, CF30, C-30, T-30		
	SXT, CD2, CIP5, NA-30, AMP, S10, CF30, C-30, T-30		
Ten	SXT, F/M-300, CD2, CIP5, NA-30, CN10, AMP, S10, CF30, T-30	8	4 (44.4)
	SXT, F/M-300, CD2, CIP5, NA-30, AMP, S10, CF30, CTR, T-30		
Eleven	SXT, F/M-300, CD2, CIP5, NA-30, AMP, S10, CF30, C-30, CTR, T-30	9	2 (22.2)
	SXT, F/M300, CD2, CIP5, NA-30, CN10, AMP, S10, CF30, C-30, T30		

Key: SXT=Sulfamethazone Trimethoprim, F/M-300=Nitrofurantoin, CD2c=Clindamycin, CIP5=Ciprofloxacin, NA-30=Nalidixic acid, CN10=Gentamycin, AMP=Ampicillin, S10=Streptomycin, CF30=Cephalothin, C-30=Chloramphenicol, CTR=Ceftriaxone, T-30=Oxytetracycline

5. DISCUSSION

Foods of animal origin has been considered to be the major sources of foodborne salmonellosis. Hence, routine detection of *Salmonella* in foods become an important part of public health programs. The current cross-sectional bacteriological study was conducted to isolate and identify *Salmonella* from cow milk and beef collected from Hawassa city, Wondo Genet and Yirgalem districts. Even though, 6.5% was identified by typical biochemical properties, the PCR test confirmed overall isolation rate of *Salmonella* in current study was 4.17% from which 66.7% and 33.3% were of milk samples and beef samples, respectively.

The presence of a 496-bp segment of histidine transport operon gene in *Salmonella* serovars and its absence from the other bacteria proved it as a genetic marker for the identification of *Salmonella* (Cohen *et al*., 1993; Ejo *et al*., 2016 and Alemu *et al*., 2022). In this study, the gene was amplified from 9 (64.29%) out of the 14 *Salmonella* isolates. The remaining five isolates, which were biochemically, identified as *Salmonella* were excluded by polymerase chain reaction method. The possible justification for this is, in the conventional bacterial isolation and biochemical characterization, *Salmonella* might be confused with other related *Enterobacteriaceae*. However, PCR is a highly sensitive test to detect the 496-base pair segment of histidine transport operon gene which is highly specific for *Salmonella* but not for other related bacteria.

The isolation rate of *Salmonella* in the two sample types were compared among districts. Here, the isolation rate of *Salmonella* from Hawassa (5.38%), Yirgalem (3.23%) and Wondo Genet (2.38%) was comparable without significant statistical association. The possible reason for the absence of variation among districts was supposed to be due to their similar hygiene and management practices, housing conditions, milking and milk handling practices. Similar finding and/or explanation was supported in the research works from Hawassa, Dale and Arsi negele districts (Gebeyehu *et al*., 2022).

The isolation rate of *Salmonella* was relatively higher in samples collected from beef (4.7%) than from milk (3.9%); however, the difference was not statistically significant ($p = 0.80$) (Tables 4 and 5). The isolation rate in beef was comparable with the findings of 4.1% reported from Hawassa (Worku *et al*., 2022), Ethiopia, 4.64% reported from Addis Ababa, Ethiopia (Ketema *et al*., 2018), but higher than the reports from Turkey (0.7%) (Cetin *et al*., 2020), Gondar, Ethiopia (3.1%)

(Garedew *et al*., 2016) and Dire Dawa retailer shops (1%) (Mengistu *et al*., 2017). However, this result was lower than the isolation of 6.08% from export abattoirs in East Shewa (Alemu *et al*., 2022), 12% from raw beef (Ejo *et al*., 2016) and 17.3% from minced beef (Garedew *et al*., 2015) in Gondar town. This difference might be owing to poor personnel hygiene in the abattoirs, butcher houses and equipments (viz. knives, table and balance). There was no statistically significant variation in isolation rates from the three districts of sample collection. This might be due to comparable hygienic, beef handling and management conditions among the areas.

The proportion of *Salmonella* isolates in milk (3.9%) in the current study corroborates with the findings of 3.63% *Salmonella* isolates in cow milk from Iran (Tajbakhsh *et al*., 2012) but lower than the findings of Shayka *et al*. (2020) from Bangladesh (28.88%), Gebeyehu *et al*. (2022) (10.42%) and Asefa *et al*. (2023) (9.3%) from Southern Ethiopia. On the other hand, it was higher than the reports of Murinda *et al*. (2002) (2.24%) in bulk tank milk from Tennessee, USA. As stated by Asefa *et al*. (2023), impure environmental conditions, unclean milkers' hands and milking equipments and poor udder preparation might expose milk to bacterial contamination.

The study revealed that the milk sample sources: households, retailers and dairy farms were not significantly associated with the isolation rate of *Salmonella* 5.56%, 4.84% and 1.85%, respectively. *Salmonella* was isolated regardless of milk sample source. The absence of variation among sample sources could be attributed to their similar hygiene and management practices, housing conditions, milking and milk handling practices. Such congruent isolation of the organism irrespective of milk sample source was in correspondence with the report of Gebeyehu *et al*. (2022), who compared dairy farms and households in Southern Ethiopia.

Raw milk and yoghurt samples were further assessed for the isolation rate determination. Accordingly, the isolation rate of *Salmonella* in raw milk was 4.13% whereas that of yoghurt was 3.22% without statistically significant association. This finding was lower than the findings of Asfaw *et al*. (2023) who reported 13.33% and 6.67% isolation rates in raw milk and yoghurt, respectively. The hygienic status of milk handling and processing could be possible reason for the discrepancies of the findings.

The twelve antimicrobials tested were from nine different classes: penicillins (ampicillin), tetracyclines (oxytetracycline), phenicols (chloramphenicol), cephems (ceftriaxone and cephalothin), aminoglycosides (streptomycin and gentamycin), quinolones and fluoroquinolones (ciprofloxacin and nalidixic acid), lincomycins (clindamycin), folate pathway antagonists (sulfamethoxazole trimethoprim) and Nitrofuran (nitrofurantoin). The augmented score of antimicrobial resistance in *Salmonella* has become a public health issue. In this study, 100% resistance to ampicillin was reported in number of studies such as Fadlalla *et al*. (2012), Abunna *et al*. (2018), Gutema *et al*. (2021) and Asfaw *et al*. (2023). The higher percentage of resistance for nalidixic acid, clindamycin, oxytetracycline and trimethoprim-sulphamethoxazole was comparable with the findings of Abunna *et al*. (2018), Yang *et al*. (2019) and Bawa *et al*. (2020). Higher resistance to cephalothin was also reported by Bawa *et al*. (2020). In contrast, the isolates were 88.9%, 55.6% and 66.7% sensitive to ceftriaxone, chloramphenicol and gentamycin, respectively. Finding in agreement with susceptibility profile for chloramphenicol and gentamycin was reported by Talal *et al*. (2022). Similarly, the highest susceptibility profile for ceftriaxone was in line with the report of Alemu *et al*. (2022). This might be because they were not widely used in Ethiopia for animal treatment.

All the nine (100%) *Salmonella* isolates showed resistance to five or more antimicrobial classes tested. This finding can indicate that misuse of antimicrobial agents in animal production and treatment failure as a result of drug resistance, food poisoning outbreaks would be difficult to treat. This finding was in harmony with studies in Ethiopia by Fadlalla *et al*. (2012), Abunna *et al*. (2018) and Gebeyehu *et al*. (2022) who reported 100% resistance of all *Salmonella* isolated from raw milk in Sebeta, Addis Ababa and Southern Ethiopia, respectively. However, this finding was higher than the percentage of isolates showed MDR in previous studies in Ethiopia such as (Birhanu *et al*., 2003) (36.4%), (Wassie *et al*., 2006) (52.5%), and (Sefinew and Bayleyegn, 2012) (31.8%). This might be due to the increasing rate of inappropriate utilization of antimicrobials in the animal production systems, which favor selection pressure that increased the advantage of sustaining strains of bacteria having resistance genes (Fadlalla *et al*., 2012).

Two isolates were resistant to eleven antimicrobials with different resistance pattern and the other two *Salmonella* isolates showed resistance to ten with different resistance pattern. Consecutively, two other isolates were also found resistant to nine antimicrobials with different resistance pattern: Seven *Salmonella* isolates were resistant to three antimicrobial of two with similar resistance pattern whereas only one isolate was resistant to six antimicrobials. The highest multiple antimicrobial resistance was seen in the pattern (CD2, CIP5, NA-30, S10, AMP and CF30) where nine isolates showed resistance to them. MDR indexes of the isolates obtained from this study ranges from 0.42 to 0.83, inclusively, with average value 0.66. MDR index values greater than 0.2

indicates unwise use of antimicrobials (NARMS, 2019). As a result, the current study confirmed cow milk and beef as potential sources of MDR, and is a potential public health issue in the study area.

6. CONCLUSION AND RECOMMENDATIONS

The present study revealed detection of *Salmonella* in cow milk and beef samples from selected districts of Sidama Regional State. This indicated that *Salmonella* become important contaminants of cow milk and beef irrespective of the source, districts and types of samples. The study further determined that the isolated *Salmonella* had developed varying degrees of resistance to commonly used antimicrobials such as ampicillin, streptomycin, oxytetracycline, ceftriaxone and gentamycin. It was identified that all of the isolates were resistant to two or more antimicrobials. Thus, the high percentage of MDR isolates recovered indicate the likely importance of cow milk and beef as sources of MDR *Salmonella* infections and a grave public health concern.

Therefore, in view of the above conclusion the following outlooks are forwarded.

- Regular antimicrobial susceptibility testing together with assessment of rational use of antimicrobials should be conducted.
- Further research on determining serotypes of *Salmonella* isolates, ascertaining AMR genes in the isolates and detailed epidemiological studies should be conducted.

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8. ANNEXES

Annex I: *Salmonella* on XLD agar

Figure 4: *Salmonella* on XLD

Annex II: Biochemical test results

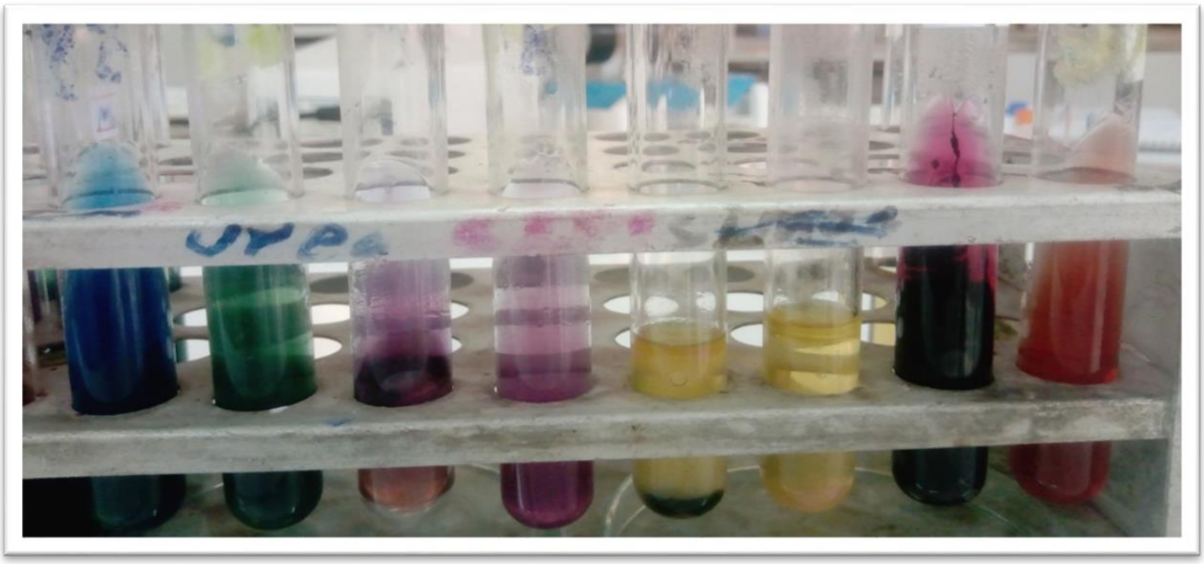

Figure 5: Biochemical test results (SC, LIA, SIM & TSI from left to right) with respective –ve controls at the right of each

Annex III: Some of urease negative isolates

Figure 6: Some of urease negative isolates

Annex IV: AST results of some *Salmonella* isolates

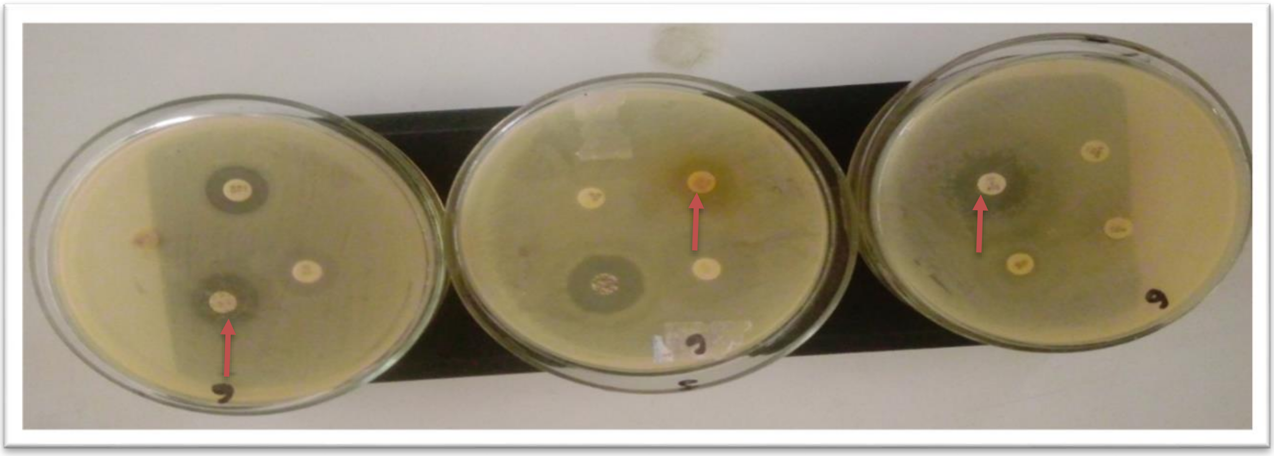

Figure 7: AST results of some *Salmonella* isolates

Annex V: Antimicrobials tested and their standard zone of inhibition

No	Antimicrobial agent	Disk potency	Zone of inhibition (diameter in mm)		
			Resistant	Intermediate	Sensitive
1	Ampicillin	10µg	≤ 13	14-16	≥ 17
2	Ceftriaxone	30µg	≤ 19	20-22	≥ 23
3	Cephalothin	30µg	≤ 14	15-17	≥ 18
4	Oxytetracyclin	30µg	≤ 11	12-14	≥ 15
5	Streptomycin	10µg	≤ 11	12-14	≥ 15
6	Nitrofurantoin	300µg	≤ 14	15-16	≥ 17
7	Clindamycin	2µg	≤ 10	11-13	≥ 14
8	Nalidixic acid	30µg	≤ 13	14-18	≥ 19
9	Ciprofloxacin	5µg	≤ 20	21-30	≥ 31
10	Trimethoprim-sulfamethoxazole	23.75µg	≤ 10	11-15	≥ 16
11	Chloramphenicol	30µg	≤ 12	13-17	≥ 18
12	Gentamycin	10µg	≤ 12	13-14	≥ 15

(CLSI, 2022)

Annex VI: Laboratory work record and report format

Date	No	Sample ID	Tests and results													Remark
			XLD		TSIA		Citrate		Urease		LIA		SIM			
			+ve	-ve	Slant	Butt	+ve	-ve	+ve	-ve	Slant	Butt	Sulfide	Indole	Motility	

Annex VII: Sample collection format

District and/or kebele	Sample type	Sample source	Sample code	Sign	Date

Annex VIII: Antimicrobial Susceptibility Test record format

No	Sample code	Antimicrobials and readings of inhibition zone											
		SXT	**F/M-300**	**CD2c**	**CIP5**	**NA-30**	**CN10**	**AMP**	**S10**	**CF30**	**C-30**	**CTR**	**T-30**
1	**C-1**												
	Remark												
2	**C-2**												
	Remark												
3	**C-3**												
	Remark												
4	**C-4**												
	Remark												
5	**C-5**												
	Remark												
6	**C-6**												
	Remark												
7	**C-7**												
	Remark												
8	**C-8**												
	Remark												
9	**C-9**												
	Remark												

Annex IX: Media (BPW, RVS, MKTTn, XLD agar, MH agar) preparation and biochemical tests procedures

Buffered Peptone Water

A non-selective pre-enrichment medium for increasing recovery of injured *Salmonella* species from foods prior to selective enrichment and isolation.

Composition: Peptone (10 g), Sodium chloride (5 g), Disodium hydrogen phosphate (9 g), Potassium dihydrogen phosphate (1.5 g)

Preparation of the media

1. Suspend 20.00 grams 1000ml in distilled water.
2. Shake it well and boil to dissolve the medium completely.
3. Dispense in tubes/universal bottles.
4. Sterilize by autoclaving at 15 lbs pressure (121^0C) for 15 minutes.

Modified Rappaport Vassiliadis Soya broth

It is *Salmonella* enrichment broth from food and environmental specimens.

Composition: Soya peptone (5 g), Sodium chloride (8 g), Monopotassium phosphate (1.6 g), Magnesium chloride hexahydrate (40 g), Malachite green (0.04 g)

Preparation of the media

1. Suspend 30.07 grams 1000ml in distilled water.
2. Boil with frequent agitation to dissolve the medium completely.
3. Dispense in tubes.
4. Sterilize by autoclaving at 15 lbs pressure (121^0C) for 15 minutes.

Muller-Kauffman Tetrathionate novobiocin Broth (MKTTn, Techno pharmchem, India)

It is recommended media *Salmonella* enrichment from food and environmental specimens.

Composition: Peptone (2.5 g), Casein enzymic hydrolysate (2.5 g), Bile salts (1 g), Calcium Carbonate (10 g), Sodium thiosulphate (30 g).

Preparation of the media

1. Suspend 46 grams 1000ml in distilled water.
2. Boil to dissolve the medium completely.
3. Cool below 45^0C and add 20 ml of iodine solution (iodine 6 g and potassium iodide 5 g in 20 ml of distilled water).
4. Add 10 ml of 0.1% brilliant green solution.
5. Mix well and dispense in tubes in 10 ml quantities.

XLD Agar (Alpha Chemika)

This test is a microbiological test for selective isolation and enumeration of *Salmonella Typhi* and other *Salmonella* species.

Composition: Xylose (3.5 g), L-Lysine (5 g), Lactose monohydrate, Sucrose (7.5 g), Sodium chloride (5 g), Yeast extract (3 g), Phenol red (0.08 g), Sodium deoxycholate (2.5 g), Sodium thiosulphate (6.8 g), Ferric ammonium citrate (0.8 g) and Agar (13.5 g).

Preparation of the media

1. Suspend 56.64 grams 1000ml in distilled water.
2. Boil with frequent agitation to dissolve the medium completely.
3. Transfer immediately to a water bath at 45-50^0C.
4. Mix well and pour in to sterile petri-dishes.

Muller Hinton Agar (MHA, Himedia India)

This test is a microbiological test for non-selective growth of bacteria and antimicrobial susceptibility testing.

Composition: Meat infusion (2 g), Casein acid hydrolysate (17.5 g), Starch (1.5 g), Agar (17 g).

Preparation of the media

1. Suspend 38 grams 1000ml in distilled water.
2. Boil to dissolve the medium completely.
3. Sterilize by autoclaving at 15 lbs pressure (121^0C) for 15 minutes and cool to 45-50^0C.
4. Mix well and pour in to sterile petri-dishes.

TSI Agar (Himedia, India)

The Triple Sugar Iron (TSI) test is a microbiological test named for its ability to test a microorganism's ability to ferment sugars (glucose, lactose and sucrose) and to produce hydrogen sulfide.

Composition: Enzymatic digest of casein (5 g), enzymatic digest of animal tissue (5 g), yeast enriched peptone (10 g), dextrose (1 g), lactose (10 g) sucrose (10 g), ferric ammonium citrate (0.2 g), NaCl (5 g), sodium thiosulfate (0.3 g), phenol red (0.025 g), agar (13.5 g), per 1000 ml and pH 7.3.

Preparation of the media

1. Suspend 64.52 grams 1000ml in distilled water.
2. Boil to dissolve the medium completely.
3. Dispense in tubes.
4. Sterilize by autoclaving at 15 lbs pressure (121^0C) for 15 minute and allow the tubes to cool in sloping/inclined position.

Procedure of TSI test

1. With a straight inoculation needle, touch the top of a well-isolated colony.
2. Inoculate TSI by first stabbing through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant.

3. Leave the cap on loosely and incubate the tube at 35°-37°C in ambient air for 18 to 24 hours.
4. Examine the reaction of medium.

Possible results for *Salmonella*:

- An alkaline/acid (red slant/yellow butt) reaction: It is indicative of dextrose/glucose fermentation only.
- Blackening of the medium: Occurs in the presence of H_2S
- Gas production: Bubbles or cracks in the agar indicate the production of gas (formation of $CO_2$and H_2)

SIM Broth

SIM (Sulfide, Indole, and Motility) medium is useful for the differentiation of gram-negative enteric bacilli. SIM test helps to isolate the organisms on the basis of sulfide production, indole formation, and motility.

Composition: Ingredients for 100 ml of distilled water- Pancreatic digest of casein (2.0 g), Peptic digest of animal tissue (0.61 g), Ferrous ammonium sulfate (0.02g), Sodium thiosulfate (0.02g), Agar (0.35gm) and pH 7.3 +/- 0.2 at 25°C.

Preparation of the media

1. Suspend 36.23 grams 1000ml in distilled water.
2. Boil to dissolve the medium completely.
3. Dispense in tubes.
4. Sterilize by autoclaving at 15 lbs pressure (121^0C) for 15 minute and allow the tubes to cool in an upright position.

Procedure of SIM Test

1. Take pure colonies from an 18-24-hour old culture on a solid medium.
2. Inoculate the SIM Medium by stabbing the center of the medium to a depth of half an inch.
3. Incubate the inoculated medium aerobically at 37°C for 18-24 hours.
4. Observe for hydrogen sulfide production and motility of the test organism.

5. Only apply Kovac's reagent (three drops) after reading the result of H_2S and motility reaction to the surface of the medium.
6. Observe the development of a pink-to-red color.

Possible results for *Salmonella:*

- Positive H_2S test: blackening of the medium
- Positive motility test: a diffuse zone of growth flaring from the line of inoculation
- Indole negative test: A yellow color denotes a negative indole test after the addition of Kovac's reagent

Simmons Citrate Agar (Himedia, India)

Simmons Citrate Agar is an agar medium used for the differentiation of *Enterobacteriaceae* based on the utilization of citrate as the sole source of carbon.

Composition: Ingredients per liter of deionized water: Sodium Chloride (NaCl) (5 g), Sodium Citrate (dehydrate) (2 g), Ammonium Dihydrogen Phosphate (1 g), Dipotassium Phosphate (1 g), Magnesium Sulfate (heptahydrate) (0.2 g), Bromothymol Blue (0.08 g), Agar (15 g) and Bromothymol Blue

Preparation of the media

1. Suspend 24.28 grams 1000ml in distilled water.
2. Boil to dissolve the medium completely.
3. Dispense in tubes.
4. Sterilize by autoclaving at 15 lbs pressure (121^0C) for 15 minute and allow the tubes to cool in sloping/inclined position.

Procedure of SC test

1. With a straight inoculation needle, touch the top of a well-isolated colony.
2. Inoculate SC by first stabbing through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant.
3. Leave the cap on loosely and incubate the tube at 35°-37°C in ambient air for 18 to 24 hours.

4. Examine the reaction of medium.

Possible result for *Salmonella:*

Positive growth (i.e. citrate utilization) produces an alkaline reaction and changes the color of the medium from green to bright blue.

LIA

Lysine iron agar or LIA is a differential media used to distinguish bacteria that are able to decarboxylate lysine and/or produce hydrogen sulfide from those that cannot. Lysine deamination is an aerobic process which occurs on the slant of the media. Lysine decarboxylation is an anaerobic process which occurs in the butt of the media.

Composition: Peptic digest of animal tissue (5 g), yeast extract (3 g), dextrose (1 g), L-lysine (10 g), ferric ammonium citrate (0.5 g), sodium thiosulfate (0.04 g), bromocresol purple (0.02 g), agar (15 g), per 1000 ml, pH 6.7.

Preparation of the media

1. Suspend 34.56 grams 1000ml in distilled water.
2. Boil to dissolve the medium completely.
3. Dispense in tubes.
4. Sterilize by autoclaving at 15 lbs pressure (121^0C) for 15 minute and allow the tubes to cool in sloping/inclined position.

Procedure of the test

1. The medium is tubed, sterilized and slanted so that a short slant and deep butt are formed.
2. With a straight inoculating needle, inoculate LIA by twice stabbing through the center of the medium to the bottom of the tube and then streaking the slant.
3. Cap the tube tightly and incubate at 35°-37°C in ambient air for 18 to 24 hours.
4. Examine at 18-24 and 40-48 hours for growth and color changes in tube butt and slant, and for blackening at the apex of slant.

Possible results for *Salmonella*

Lysine Decarboxylation (detected in butt):

- Positive Test: Purple slant/purple butt (alkaline), the butt reaction may be masked by H_2S production

H_2S Production:

- Positive Test: Black precipitate
- Gas production: demonstrated by the presence of bubbles or cracks in the medium

Urea Agar

This test is used to differentiate organisms based on their ability to hydrolyze urea with the enzyme urease.

Composition: Sodium Chloride (5 g), Monopotassium Phosphate (2 g), Peptone (1 g), Dextrose (1 g), Phenol Red (0.012 g) and Agar (15 g).

Preparation of the media

1. Suspend 24.51 grams in 950ml distilled water.
2. Boil to dissolve the medium completely.
3. Sterilize by autoclaving at 15 lbs pressure (121^{0}C) for 15 minute.
4. Cool to 50^{0}C and add 50 ml of sterile 40% urea solution and mix well.
5. Dispense in tubes and allow the tubes to set in sloping/inclined position.

Procedure of Urease Test

1. Streak the surface of a urea agar slant with a portion of a well-isolated colony or inoculate slant with 1 to 2 drops from an overnight brain-heart infusion broth culture.
2. Leave the cap on loosely and incubate the tube at 35°-37°C in ambient air for 48 hours to 7 days.
3. Examine for the development of a pink color for as long as 7 days.

Possible result for *Salmonella*

Negative Reaction; that is, no urease production: No color change or yellowish color.

Annex X: PCR procedure and Gel electrophoresis

Components of (PCR)

- DNA template (the sample DNA that contains the target sequence to amplify)
- Deoxyribonucleoside triphosphates (dNTPs)
- PCR buffer
- Primers (forward and reverse)
- Taq polymerase

Procedure

1. Mix the above components starting with highest volume.
2. Set up the program on the thermocycler for the temperature and time adjustment in each steps.
3. Add the sample to the thermocycler.
4. Start on the program.
5. Take out the result, finally.

Gel preparation and Electrophoresis

1x TBE buffer preparation

- ✓ 10.8gm of Tris-base and 5.5gm of boric acid was dissolved in 805ml distilled water.
- ✓ 4ml of 0.5M EDTA at P^H = 8.0 was added to the above mixture
- ✓ Finally, the volume was adjusted to 1L by adding distilled water.

Preparation and loading of a 1.5% agarose gel.

- ➢ 4.5gm of agarose was added to a 500ml Pyrex flask containing 250ml of 1xTBE buffer
- ➢ Boiled in microwave until the agarose was fully dissolved.
- ➢ 1µl of Ethidium bromide was added to the dissolved agarose at 45^{o}C and dissolved by slowly shaking the flask not to form air bubble.
- ➢ Then it was poured on to the 25cm gel making plate
- ➢ Allowed to solidify to form a gel cast for about 40 minutes.
- ➢ The gel cast was placed into the gel doc after removing the combs and then overlaid with 1xTBE buffer until the gel cast is fully covered.
- ➢ The gel was loaded with the amplified PCR product along with the loading dye and 100bp DNA ladder → allowed to run for 1:10 hour.
- ➢ Finally, the DNA bands were photographed.

9. BIOGRAPHY

The author, Tariku Geinoro Alleyo, was born in 1992 G.C. from his father Geinoro Alleyo and his mother Selfame Eniferaw in Lalo kebele, Durame town, Central Ethiopia Regional State, Ethiopia. He attended his elementary education (1-4) in Teza Gerba Primary School and Durame Kutir 1 Primary School (5-8). Consecutively, he attended his high school and preparatory program in Durame Secondary and Preparatory School. Having completed his preparatory program in 2007 G.C., he joined Addis Ababa University College of Veterinary Medicine and Agriculture in 2008 G.C. and awarded with Degree of Doctor of Veterinary Medicine (DVM) in 2014 G.C. with very great distinction. After graduation he got employment in Kedida Gamela Woreda Livestock and Fishery Office as veterinary clinician since June, 2015 G.C. and worked for about one and half years. Then after, he worked as veterinary drug institutions inspector in Ethiopian Veterinary Drug and Feed Administration and Control Authority since October 1, 2017 G.C. Subsequently, he worked as team leader in Ethiopian Agricultural Authority since March 1, 2018 G.C. for five consecutive years. In October 2021 G.C., he joined Faculty of Veterinary Medicine, School of Graduate Studies, Hawassa University to pursue a Master of Science (MSc) degree in Veterinary Microbiology.