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Original Article

Isolation and Antimicrobial Susceptibility Test of Non-typhoidal Salmonella from Raw Bovine Milk and Assessments of Hygienic Practices in Gursum District, Eastern Hararghe, Ethiopia

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Abstract

Introduction: Non-typhoidal *Salmonella* (NTS) is a significant human and animal pathogen worldwide. Most human infections are foodborne, but direct or indirect contact with animals also contributes annually. Epidemiological data on *Salmonella* are crucial to establish effective control measures.

Methods: A cross-sectional study was conducted from July 2022 to January 2023 in Gursum District, Eastern Hararghe Zone, Ethiopia, to isolate NTS from raw bovine milk and assess associated sanitary practices. A total of 480 samples were collected from bucket swabs, milk handlers' hands, and milk. Following pre-enrichment in buffered peptone water, samples were transferred to selenite cysteine and Rappaport-Vassilidis broths before plating on xylose lysine deoxycholate agar. Biochemical and antimicrobial susceptibility tests were performed.

Results: Among 56 *Salmonella* isolates recovered, 22(13.7%) were from milk, 20(12.5%) from bucket swabs, and 14(8.8%) from hand swabs. Abadir and Funyan Bira kebeles recorded the highest isolation rates (20%) from bucket swabs. There was no statistically significant variation in *Salmonella* isolation across kebeles. The majority (95%) of respondents were female, and 83% were illiterate. While 20% reported milking cows while ill, only 14% routinely washed udders before milking, though 85% cleaned their hands and 90% cleaned milking equipment. All isolates were resistant to at least two antibiotics, with resistance rates of 91.07% to tetracycline, 82.14% to ampicillin, and 73.21% to penicillin. However, all isolates were sensitive to chloramphenicol (100%), nalidixic acid (91.07%), and ciprofloxacin (91.07%).

Conclusion: The highest *Salmonella* isolation rates were found in milk and milk-contact surfaces at markets, indicating unhygienic handling practices from production to market. The widespread antibiotic resistance among isolates highlights the challenge of treating Salmonellosis and underscores the need to limit the misuse of antibiotics.

Keywords: Antimicrobial, Bovine milk, Hygienic practices, Isolation, Non-typhoidal *Salmonella*

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Introduction

Food-borne diseases are infectious or toxic disorders induced by the intake of contaminated foods infected with bacteria and/or their toxins, parasites, viruses, or chemicals (1). It is a global problem, with roughly 600 million cases of illness and 420 000 deaths caused by foodborne microorganisms each year. When compared to high-income countries, the issue was frequently more severe and less reported in low- and middle-income countries (2). Numerous bacteria cause foodborne illness in humans. Salmonellosis is one of the major zoonotic diseases all over the world, with annual estimates of 22 million cases and 200 000 deaths due to typhoid fever and 93.8 million cases of gastroenteritis and 155 000 deaths due to non-typhoidal *Salmonella* (NTS) (3).

Salmonella is a significant cause of foodborne human salmonellosis globally (4). NTS can be transmitted to humans by the ingestion of animal-derived goods such as bovine milk, eggs, and poultry meat, as well as direct contact with animals or their habitats (5). More than 2500 *Salmonella enterica* serovars have been identified, many of which can cause human illnesses. However, in human infections, NTS serovars, particularly Enteritidis and Typhimurium, are the most commonly isolated serotypes (6). NTS salmonellosis can produce severe invasive disease with complicated extra-intestinal infections, bacteremia, and meningitis in young children, the elderly, and individuals with compromised immune systems (7). These infections can be hazardous, especially in individuals with impaired immune systems.

Nontyphoidal Salmonella is a leading source of foodborne illness, causing diarrhea, bacteremia, and focal suppurative lesions. The majority of infections linked with humans and other mammals are caused by *S. enterica* subsp. *enterica*,

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S. enteritidis, and *S. typhimurium* (8). Human disorders caused by milk-borne bacteria range from gastrointestinal disturbances such as diarrhea and vomiting to generalized and even life-threatening foodborne illnesses. They are not only crucial for public health but also the economy (9). Because it has a high nutritional value and acts as a suitable medium for the growth of numerous spoilage or pathogenic microorganisms, it can cause product deterioration as well as infections and intoxications in consumers. Milk and milk derivatives play an essential role in feeding Ethiopia's rural and urban populations. It is manufactured daily, sold for cash, or easily processed. In milkshed areas, it is a cash crop, allowing families to purchase other commodities and significantly contributing to household food security (10).

Due to the lack of refrigeration facilities at the farm and household levels in poor countries of tropical regions with high ambient temperatures, raw milk is quickly contaminated with spoiled and harmful bacteria during milking, storage, transportation, and processing (11). Contaminated milk can harbor a variety of pathogenic and non-pathogenic microorganisms. As a result, milk could be a source of dangerous pathogens to consumers, leading to serious health problems. Bacteria such as Salmonella, *Escherichia coli*, *Klebsiella*, and *Enterobacter* have been found in milk products in Ethiopia (12).

The WHO estimates the distribution of non-typhoidal salmonellosis in Africa, allowing for the calculation of the Global Burden of Foodborne Disease in Africa. This report anticipated 59 000 global deaths owing to NTS in 2010, including 32 000 deaths in Africa and 22 000 deaths due to invasive illness, predominantly in children (13). Nonetheless, data to understand NTS epidemiology exist in the majority of Sub-Saharan African nations. Approximately 100 000 human cases are reported in the European Union (EU) every year. According to the European Food Safety Authority (EFSA), the overall economic cost of human Salmonellosis could be as high as EUR 3 billion per year (14). Due to the limited scope of studies and the lack of coordinated epidemiological monitoring systems in Ethiopia, estimating the burden of foodborne diseases is challenging. In Ethiopia, the prevalence of foodborne Salmonella infections has risen considerably in recent years (15).

Globally, the occurrence of antibiotic-resistant strains of Salmonella has increased in recent decades due to the widespread use of antibiotics for treatment and prophylaxis in animals (16). Animal health care in developing nations has been suboptimal due to an increased inclination for animal owners to carry pharmaceuticals in their homes and engage inexperienced persons, such as farmers and animal attendants, to treat animals, resulting in multidrug resistance (17). Antimicrobials are licensed in human medicine for the treatment and prevention of illnesses (18). According to WHO, more than half of all drugs in poor countries are incorrectly prescribed, delivered, or sold, and half of all patients fail to take them correctly. This, in conjunction with the use of antibiotics in animals, has led to the selection of antibiotic-resistant bacteria, which pollute animal food items and the environment (19). Although a few studies in Ethiopia have shown the incidence of Salmonella and antimicrobial susceptibility in humans, animals, and food of animal origin, further research is needed on the subject (20).

Statement of the Problem

NTS is a significant cause of foodborne infections and outbreaks worldwide, especially in developing countries. Raw bovine milk is a potential source of disease transmission to humans, as it can become contaminated by fecal matter or environmental sources during milking, handling, and/or storage. The consumption of raw or inadequately pasteurized milk and its products poses a significant public health risk, especially for children, pregnant women, and individuals with compromised immune systems. Furthermore, the emergence and spread of antimicrobial resistance among these isolates limit treatment options and increase the morbidity and mortality of the infection.

Raw bovine milk and its products are frequently consumed in Ethiopia, particularly in rural regions. However, there is little information available in the country about the prevalence, molecular characterization, and antibiotic susceptibility of Nontyphoidal Salmonella isolates from raw bovine milk. Furthermore, the sanitary practices of milk producers and consumers regarding bacterial contamination are not well-established. As a result, the purpose of this study is to isolate and identify bacteria from raw bovine milk samples collected in the Gursum area, Eastern Hararghe, Ethiopia, and to establish their antimicrobial susceptibility patterns. Additionally, the study will evaluate the hygienic procedures of milk producers and consumers in the study area and identify risk factors associated with NTS contamination. The findings of this study will provide valuable information for the prevention and control of Nontyphoidal Salmonella infections and the rational use of antimicrobials in the study area and beyond.

Objectives

General Objective

To assess the occurrence of NTS in raw bovine milk, milk handling and milk holding equipment, antimicrobial resistance, and the status of milk hygiene practices in the Gursum district of the Eastern Hararghe Zone, Ethiopia.

Specific Objectives

- To isolate and identify NTS from bovine milk and milk-contacting surfaces in the Gursum district of East Hararghe zone, Ethiopia.

- To assess the antimicrobial susceptibility profile of the NTS isolates.
- To assess cow milk hygiene practices concerning milk-borne pathogens.

Material and Methods

Description of the Study Area

The research was carried out in the Gursum district of Oromia Regional State, Ethiopia, from July 2022 to January 2023. Gursum district was one of the 20 districts in the East Hararghe zone of Oromia Regional State. The district's capital is Funyan Bira Town, which is 600 km east of Addis Ababa. The district is located between 9° 7" and 9° 32" North latitudes and 42° 17" and 42° 38" East longitudes. The district's overall land area was estimated to be 71 573 hectares, and it was home to approximately 168 476 people. The district's elevation spans from 1200 to 2950 m above sea level, with an annual rainfall of 650 to 750 mm (Figure 1). The district's mean yearly maximum and minimum temperatures were 25 °C and 18 °C, respectively (21).

The Gursum district shared boundaries with the Somali Regional State to the east, Harari Regional State to the west, Jarso District to the north, and Babble District to the south. The district has a population of roughly 168 476 people. The Gursum district's agroecology was divided into three categories: highland (5%), midland (45%), and lowland (50%). The two main cattle production systems were mixed-farm and extended production systems. The major crops farmed in the Gursum are sorghum, maize, and groundnuts, and the principal animal feeds include crop leftovers, grass, and some modified forages. Gursum district has 110 864 cattle, 32 786 sheep, 73 331 goats, 14 566 donkeys, 8735 camels, 32 horses, 52,881 poultry flocks, 5,192 traditional beehives, two transitional beehives, and two contemporary beehives (Gursum Agricultural Office) (22).

Study Design

From July 2022 to January 2023, a cross-sectional study design, supported by a structured questionnaire, was conducted in the Gursum district to isolate and identify NTS spp. from raw bovine milk and evaluate the antibiotic susceptibility profiles of the isolates in the study area.

Study Population

The study population consisted of apparently healthy lactating dairy cows selected from the Gursum district's kebeles of Ibsa, Abadir, Haro Bate, and Funyan Bira. In contrast, the target population consisted of all healthy lactating dairy cows reared in the study area. Lactating cows receiving antibiotics were omitted from the study. We also removed milk samples from homes where the animal owners refused to participate in the study. Data on hygienic handling methods of raw cow milk were also acquired through face-to-face interviews with nursing cow owners' family members.

Sample Size Determination

The desired sample size for the current study was

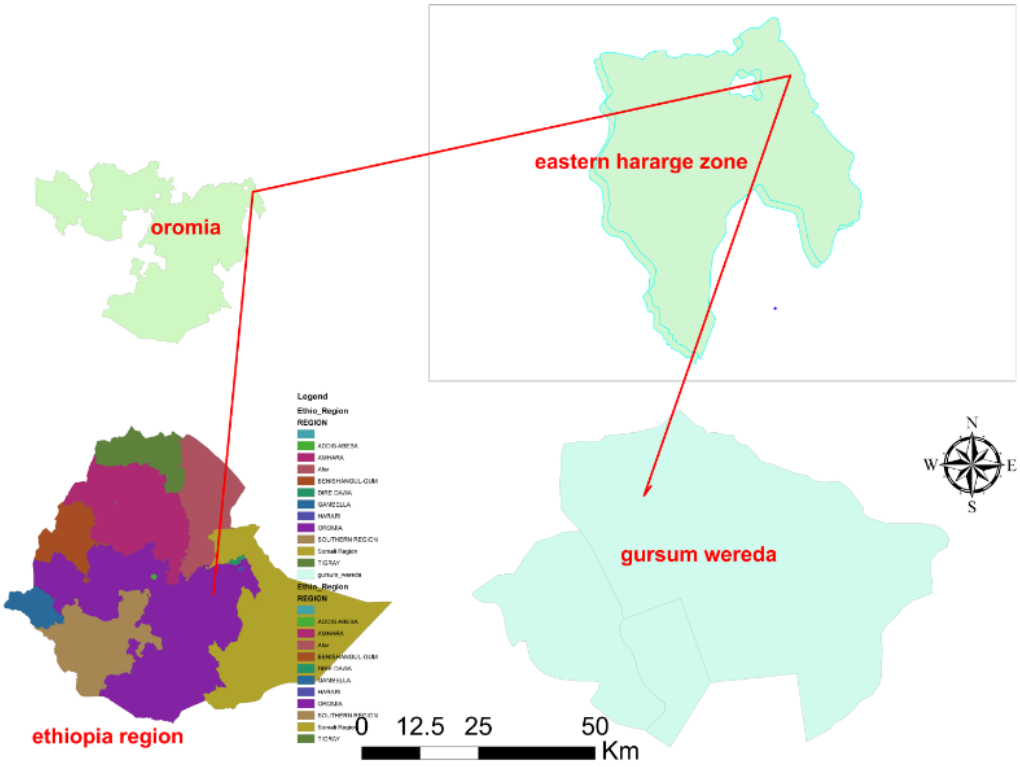

Figure 1. The map representing the study area from which study samples were taken (Source: ArcGIS Pro, 2023, developed offline)

calculated based on a 3.3% previous prevalence of Salmonella spp. in raw bovine milk, as reported by Reta et al (23) from Jigjiga. Hence, using a 5% desired level of precision at a 95% confidence level, therequired sample size was determined according to the formula described by Thrusfield (24) as follows:

$$n = \frac{Z^2 \times P_{exp}\left(1 - P_{exp}\right)}{d^2}$$

$$n = \frac{1.96^2 \times 0.033(1 - 0.033)}{0.05^2}$$

$$= \frac{3.8416 \times 0.033(1 - 0.033)}{0.0025} = 49.$$

Where, N = required sample size, Z = Confidence interval (95%), P_{exp} = Expected prevalence (3.3%), d = desired absolute precision (5%).

As a result, the projected prevalence is set at 50%. Consequently, the minimum required sample size (milk sample) was 49, although 160 raw cows' milk samples were obtained during the investigation to enhance precision. In addition to raw milk samples, swab samples from milkers' hands and a bucket of home milk were obtained for NTS isolation. Milk from the market equipment, a hand swab of the market, and swab samples from the container had all been collected. The interview process was approximated based on formal survey studies for milk safety practice assessment in the household and market (Funyan Bira) sites of sample collection, and the sample size was calculated using Ashram (25).

$$N = 0.25/SE^2 = 0.25/0.0025 = 100$$

Where N = Sample size, SE (Standard Error) at 5% precision and 95% confidence level.

Sampling Strategy and Sample Type

To collect raw cow milk, a multi-stage sample (District > Kebele > Household) using two stages of sampling technique (Kebele > household) was used. The Gursum district was chosen on purpose due to the ease of access to milk samples. According to the Gursum district agricultural office, Ibsa, Abadir, Haro Bate, and Funyan Bira kebeles were chosen from among the district's 39 kebeles for their potential in cow milk production. Lactating cow homes were located using district data and the assistance of Development Agent (DA) workers. Finally, an equal number of homes were carefully recruited from each Kebele using a systematic random sampling technique. Simultaneously, swab samples were obtained from milkers' hands and a pail of household milk, and family members of the nursing cows' owners were interviewed in the household. As a result, each Kebele received 40 raw cow's milk samples. Hand and bucket swabs were obtained from the same family and market (Table 1), and whenever there was more than one nursing cow in the household, a pooled milk sample was collected. Data on milk hygiene procedures were also collected using observational checklists created specifically for this study.

Table 1. Distribution of collected sample types from four Kebeles of the study area

Sample Type	Study Areas				Total
	Haro Bate	Ibsa	Abadir	Funyan Bira	
Milk	40	40	40	40	160
Bucket swab	40	40	40	40	160
Hand swab	40	40	40	40	160
Total	120	120	120	120	480

Sample Collections and Transportation

Once the households of nursing cow owners in the Kebeles were located, the households recruited for the study were chosen systematically. At the home level, approximately 25 mL of milk was collected from a bucket of individual nursing cow milk in the household, as well as a hand swab from milkers. Milk samples were obtained from buckets from all identified and visited households, and whenever more than one nursing cow was present in the household, a pooled milk sample was taken. Milkers' hand swabs and bucket swabs were collected from each cow milking individual in the specified household and milk container. Swab samples for bucket and hand swabs were collected before milking by rotating and rubbing a sterile hardwood cotton swab against the sampled area numerous times horizontally and vertically. After swabbing, the swabs were moistened in a sterile test tube containing 10 mL of buffered peptone water (BPW), a pre-enrichment medium.

All samples were processed for Salmonella isolation and antibiotic susceptibility testing. Meanwhile, volunteers involved in milk production (lactating cow owners) were asked about milk cleanliness and safety standards. Each sample was identified with a permanent marker and placed in a sterile plastic container before being placed in an ice box with an ice pack. The sampling site (a specific kebele), the sample type (such as individual cow milk from a family), or vendor at the marketing site, and the date of sample collection are all identified by the labeling code. Finally, the samples were transported in an ice box filled with ice. All samples were transported to the Veterinary Microbiology Laboratory of Haramaya University in an ice box with ice packs on the same day of sample collection and analyzed upon arrival or within 24 hours of sampling at the microbiology laboratory of the College of Veterinary Medicine, Haramaya University.

Isolation of Non-typhoidal Salmonella

NTS organisms identified from samples were isolated using the ISO 6579, 2002 standard microbiology of food

for the detection of NTS. According to the guidelines, four processes are necessary to achieve the desired results. Non-selective pre-enrichment, selective enrichment, plating on selective media, and biochemical confirmation are all phases in the isolation.

Non-selective Pre-enrichment

During the investigation, 25 mL of milk sample was aseptically measured, combined with 225 mL of buffered peptone water, i.e., homogenized, and incubated for 18 to 24 hrs at 37 °C ± 1 °C (ISO 6579, 2002) to proliferate and regenerate injured cells. Primary enrichment improves NTS recovery (ISO 6579, 2002).

Selective Enrichment Media

Following the non-selective pre-enrichment stage, 100 L of pre-enriched material was transferred and mixed aseptically into a tube containing 10 ml of Rappaport Vasiliadis (RV) broth. The cells were then injected and cultured at 41.5 0.5 °C for 18 hours to promote *Salmonella* growth.

Plating on Selective Media

A loop of RV broth inoculum was transferred aseptically and streaked onto the surface of Xylose lysine deoxycholate (XLD) agar plates prepared according to the manufacturer's instructions. The plates were incubated at 37 °C degrees for 18 to 24 hours. The plates were checked for the presence of *Salmonella* colonies. The plates were checked after incubation for the existence of usual and suspicious colonies. Due to the color change of the media, typical colonies of *Salmonella* produced on XLD-agar have a black center and a weakly translucent zone of reddish color (ISO 6579, 2002), whereas H2S negative variations developed on XLD agar are pink with a deeper pink center. Gram staining was done on *Salmonella* suspected colonies, and only gram-negative rod results were evaluated for the following procedures. A single colony was subcultured on nutrient agar from the probable *Salmonella* colonies for additional biochemical confirmation tests

Biochemical test of Non-typhoidal Salmonella isolates

Salmonella colonies isolated from non-selective (nutrient) agar and verified by gram staining were biochemically identified utilizing indole, Methyl red, Voges-Proskauer, urease, citrate utilization, triple sugar iron (TSI), and hydrogen sulfide synthesis tests (Figures 2 and 3). Colonies found to be urease negative (no color change) and producing alkaline slant (pink) and acidic butt (yellow) on TSI with or without H2S production (blackening for *Salmonella* and without H2S for *Shigella*) are then tested for IMViC. Only colonies displaying the *Salmonella* unique IMViC pattern are regarded as biochemically confirmed *Salmonella* isolates. Finally, a loop of pure colonies will be plated on TSA and incubated at 37 °C for 24 hours to create a suspension for the antimicrobial susceptibility profile test.

Antimicrobial Susceptibility Test

The isolates were tested for antibiotic resistance using the disc diffusion method, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2020) and CLSI (Clinical Laboratory Standards Institute, 2020). Single colonies were transferred from nutrient agar plates into tubes containing 5 mL of distilled water (Oxoid, England). The broth culture was incubated for 4 hours at 37 °C until it met the 0.5 McFarland turbidity criteria. To remove surplus inocula, a sterile cotton swab was dipped into the solution, turned several times, and firmly pressed on the inside wall of the tube above the level before being swabbed uniformly across the surface of the Muller Hinton agar (MHA) plate (Oxoid, England). The plates were allowed to dry for 30 minutes at room temperature. Antibiotic discs were placed correctly on the swabbed MHA plate, at least 15 mm apart from the plate's edge, to avoid inhibition zones from overlapping. The plates were then incubated for 24 hours at 37 °C.

The isolates' resistance to the following antibiotics was examined. Each Salmonella isolate was screened for eleven common antimicrobials. Ampicillin (AMP) 10 µg, gentamicin (CN) 10 µg, kanamycin (K) 30 µg, erythromycin (E) 5 µg, tetracycline (TE) 30 µg, ciprofloxacin (CIP) 5 µg, nalidixic acid (NA) 30 µg, chloramphenicol (C) 30 µg, vancomycin (VA) 30 µg, ceftriaxone (CRO) and penicillin (PG) 10 µg were placed at least 15 mm apart from the edge of the plates to prevent overlapping of the inhibition zones. The plates were incubated for 24 hours at 37 °C. After incubation, the diameters of the inhibitory zones were compared to the diameters of the control organism. According to the CLSI (2020) interpretation standards, it is classified as resistant, moderate, or susceptible (Figures 4 and 5).

Data Management and Analysis

The raw data were imported into an Excel spreadsheet and analyzed using SPSS version 26. Salmonella occurrence was indicated in terms of frequency and percentages in the descriptive statistics. Comparisons between each risk factor were also examined using the chi-square test and confirmed. Calculating chi-square values yielded statistically significant connections between variables at a 95% confidence interval and a 5% level of significance. A *P* value of less than 0.05 was judged statistically significant.

Results

Prevalence of Isolated Non-typhoidal Salmonella

Overall, 56 out of 480 (11.7%) total samples from raw bovine milk, bucket swabs, and manual milkers tested positive for NTS in the study area. In the study area,

NTS were isolated from caw milk (20.5%), bucket swab (22.5%), and milkers' hand swab (14.8%), respectively (Table 2). When compared to Ibsa 2 (5%) and Haro Bate 4 (10%) kebeles, Abadir and Funyan Bira had higher NTS isolation rates from bucket swabs, with equal values of 8% (20%). The study found no significant difference in NTS positivity between cow milk, bucket swabs, and hand swabs collected from the four kebeles in the study area ($P > 0.05$).

Antimicrobial Susceptibility of Non-Typhoidal Salmonella

All 56 NTS isolates were tested against eleven commonly used antimicrobials. The isolates were found to be sensitive to at least two antimicrobials tested. The antibiotic sensitivity profiles of the isolates showed that they were susceptible to chloramphenicol (100%), nalidixic acid (91.07%), ciprofloxacin (91.07%), ceftriaxone (82.14%), gentamycin, kanamycin, and vancomycin (73.21%), and erythromycin (62.5%), respectively. Erythromycin (37.5%), vancomycin, gentamycin, and kanamycin showed similar results at 26.79%, respectively (Figures 2-5). The high antimicrobial resistance of the isolates was recorded to tetracycline (91.07%), Ampicillin (82.14%), and penicillin (73.23%).

Socio-Demographic Characteristics of Respondents

Out of 100 total respondents in the research area, 95% were female and 5% were male. The age groups were 15-30, with 53% being under 30-50 (38%), and above 51 (9%). In terms of marital status, single (6%), married (83%), and divorced (5%), while illiterate (83%), primary (16%), and secondary (1%) (Table 3).

Hygienic Practices-Related Questionnaires

The research area's animal housing system consisted of 96% outside and 4% at home with family. The milking environment was classified as 74% under the roof, 21% in the shade, and 5% in the open air. According to the varieties of milk used for human consumption, more than half (62%) ingest raw milk, 26% boiled milk, and 12% fermented milk. The caw milking frequency per day is 87% twice per day and 13% once per day. At the farmer level, 38% of milk is produced for consumption, 26% for sale, and 36% for both home use and sales. During the study period, the most common milk products utilized were 56% butter, 15% fermented milk, and 29% cheese. Our study area's water sources for animal drinking were 67% pond, 30% river, and 3% tap water. The majority of milk storage containers (79%) were stainless steel, 17% plastic, and 4% traditional buckets. The milking equipment was

Figure 2. Indol test in Microbiology laboratory

Table 2. Sample type and sample site-wise prevalence of NTS

Type of Sample	Sample Site	No. of Sample	No. of Positive (%)	Total No. of Samples	Total No. of Positive (%)	*P* Value
Milk	Ibsa	40	4(10.0)	160	20(12.5)	0.822
	Abadir	40	6(15.0)			
	Haro Bate	40	4(10.0)			
	Funyan Bira	40	6(15.0)			
Bucket swab	Ibsa	40	2(5.0)	160	22(13.8)	0.128
	Abadir	40	8(20.0)			
	Haro Bate	40	4(10.0)			
	Funyan Bira	40	8(20.0)			
Hand swab	Ibsa	40	2(5.0)	160	14(8.8)	0.328
	Abadir	40	2(5.0)			
	Haro Bate	40	6(15.0)			
	Funyan Bira	40	4(10.0)			
	Total			480	56(11.7)	

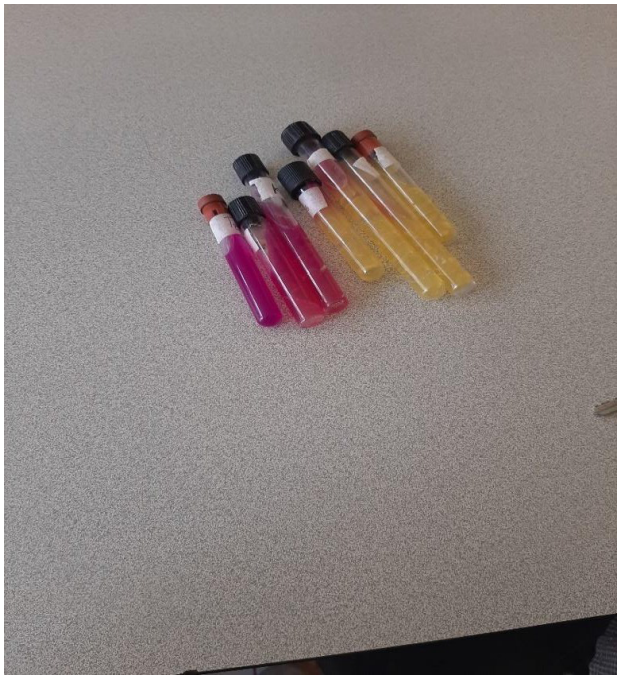

Figure 3. Urease Test in the Microbiology laboratory

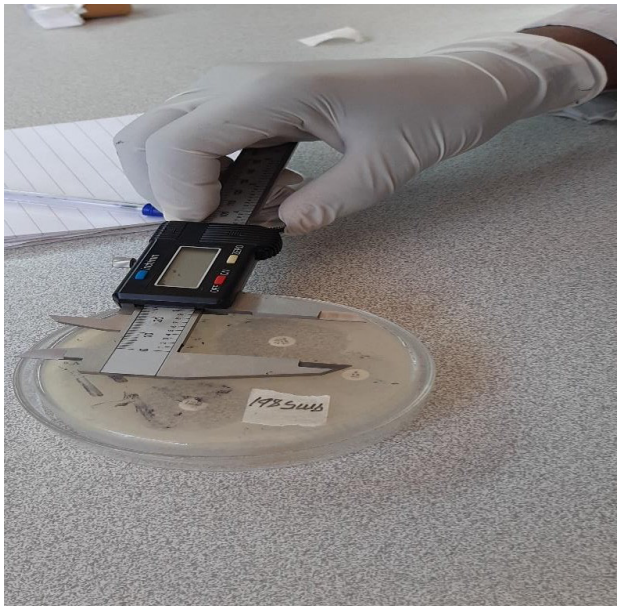

Figure 5. Measurement of Inhibition Zone by Digital Caliper

cleaned using several conventional methods, such as bakarkate (38%), hot water (37%), and both bakarkate and hot water (25%). Traditional uses of different trees as a smoker to container as a milk preservative were Berryessa 49% and Nadhalo 51% (Table 4).

The Knowledge, Attitude, and Practices of Milk Handlers about Nontyphoidal Salmonella

The KAPs of milk handlers have been assessed to determine whether the milk produced in the study area was handled under hygienic conditions. Almost all (100%) milk handlers know that lactating cows should not be milked while ill and should be treated when they

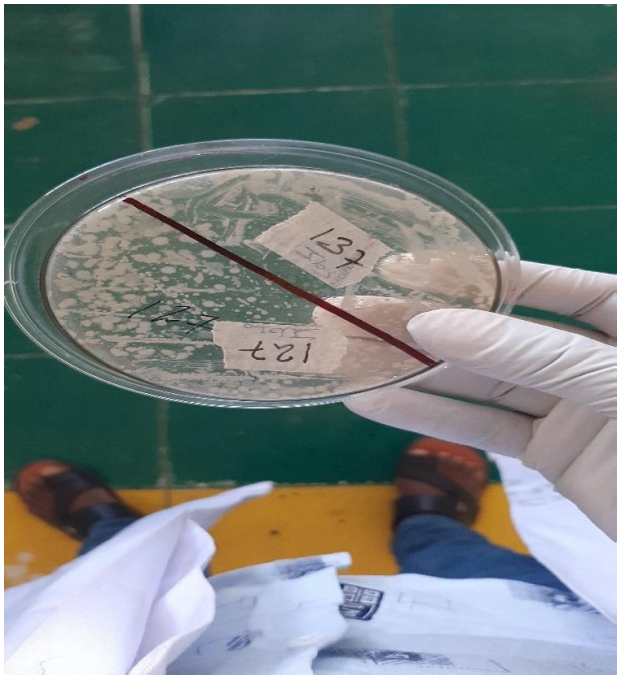

Figure 4. Colony of *Salmonella* on Nutrient Agar in the Microbiology Laboratory

Table 3. Demographic characteristics of milk handlers among respondents (n = 100)

Demographic characteristic	Category	Frequency	Percent
Sex	Female	95	95.0
	Male	5	5.0
Age	15-30	53	53.0
	30-50	38	38.0
	Above 51	9	9.0
Educational level	Illiterate	83	83.0
	Primary	16	16.0
	Secondary	1	1.0
Marital status	Single	6	6.0
	Married	83	83.0
	Divorced	5	5.0

are sick. To minimize microbial contamination of milk, 86% of milkers do not wash the udder before milking, while 14% do. Almost all milkers wash their hands before milking (85%), and 90% also clean the milking equipment (Table 5).

Discussion

Nontyphoidal *Salmonella*, as a major zoonotic pathogen, not only causes disease and death, but also causes a variety of socioeconomic damages. *Salmonella* infections in dairy cattle continue to be a significant problem worldwide. Furthermore, the risk of virus transfer to humans via the food chain poses a substantial burden in both developing and developed countries (26,27). *Salmonella* was one of the most common causes of foodborne sickness in the world (28).

Table 4. Milk hygiene practices for NTS

Hygiene-Related Question	Frequency	Percent
Animal housing system		
Home with family	4	4.0
Out side	96	96.0
Environment of milking		
Open air	5	5.0
Under the roof	74	74.0
Under the shade	21	21.0
Types of milk used for home consumption		
Raw	62	62.0
Boiled	26	26.0
Fermented	12	12.0
Milking frequency per day		
Once	13	13.0
Twice	87	87.0
Purpose of milk production		
Consumption	38	38.0
Sale	26	26.0
Both	36	36.0
Commonly milk product		
Butter	56	56.0
Fermented	15	15.0
Cheese	29	29.0
Water source for equipment cleaning		
Pond	67	67.0
River	30	30.0
Tap water	3	3.0
Types of smokers		
Nadhalo	51	51.0
Berryessa	49	49.0
Types of containers for milking		
Stainless	96	96.0
Traditional (okole)	4	4.0

Table 5. A questionnaire related to hygienic practices

Question	Yes	No
Milking while the animal was sick	20 (20%)	80 (80%)
Treating a cow while it is sick	100 (100%)	0 (0)
Wash the udder before milking	14 (14%)	86 (86%)
Wash your hands before and after milking	85 (85%)	15 (15 %)
Cleaning the equipment before Milking	90 (90%)	10 (10%)

Isolation of Non-Typhoidal Salmonella

In this investigation, the overall prevalence of NTS from cattle milk, buckets, and the hands of milkers was 11.7%. The findings agreed with prior studies (27). Salmonella isolation from milk, hand swabs, and milking equipment had a rate of 10.5% near Modjo town. However, the results were lower (20%) than those of (29) in Kersa district raw cow milk. Differences in outcomes were caused by worker hygiene, equipment cleaning methods, milking environment, and farming system types.

The prevalence of Salmonella in cow milk samples in this investigation was 12.5%, which corresponded with a previous result from Gondar town of 12.8% (30,31). Another report, lower than our study, was 5% from bucket milk, 3% from England (32), and 4.4% from the Asella dairy farm (33). Various investigations in various nations reported very low isolations of Salmonella from milk of 3.3% Reta et al (23), 4% of Shahabi et al (34), 2.17% Junaidu et al (35), and 1.43% Lailler et al (36). On the other hand, reports from Iran (17.0%) by Hossein et al (37) and Egypt (29.0%) by Omar et al (38) are significantly higher than the current study.

The current investigation also discovered a high prevalence of NTS isolates (8.8%) in milker's hand swabs. Fufa et al (27) reported a finding almost identical to a study from dairy farm workers in the Modjo town area, with a prevalence of 3 (10.7%). However, this is lower than a recent study from Meki, Shewa, which found a 19% prevalence (39), and 13.63% among Addis Ababa dairy farm workers. The prevalence of Salmonella from hand swabs was 5.26%, according to the lower data. In the research area, NTS was isolated from milking buckets at a rate of 13.8%. The findings were similar to those reported by Fufa et al (27), who discovered that the frequency of NTS from bucket swabs in dairy farms was 9.5%. However, the study results were 5% lower than those reported in previous work from Addis Ababa (40).

Antimicrobial Resistance of Nontyphoidal Salmonella

Since the introduction of antimicrobial medications, microbes have developed antimicrobial resistance. The failure of treatment regimens, the prophylactic use of antimicrobials, the use of antimicrobials as growth promoters, and the frequent use of antimicrobials in human practice are all known to induce bacterial resistance. Antimicrobial resistance has been identified as a significant therapeutic issue in both veterinary and human medicine. In this work, the antimicrobial resistance of NTS isolated from cow milk was evaluated using the disk diffusion method against eleven antimicrobial agents (41). Overall, antimicrobial resistance was found to be moderately low (73.21%). Antibiotic sensitivity patterns may be used to investigate multidrug-resistant (MDR) bacteria in cow milk, which may pose difficulties in humans if they become infected with MDR bacteria from cow milk. The study's findings highlight the most frequent bacterial infections circulating in cow milk, as well as their broad spectrum of resistance to numerous antibiotics commonly used for therapeutic purposes.

NTS isolates were resistant to all antibiotics tested in

this investigation. Tetracycline (91.07%) and ampicillin (82.14%) showed the highest resistance, followed by penicillin (73.21%). Only 73.21% of the isolates tested positive for penicillin resistance. The high resistance to these medications in gram-negative bacteria could be attributed to the transfer of resistance genes from gram-positive bacteria, notably β-lactamase genes (42). The current investigation contradicted the findings of previous researchers (43), who screened 56 isolates of NTS isolated from cow milk for different antibiotics and discovered low-level penicillin resistance. Meanwhile, they found the highest level of tetracycline antibiotic resistance.

Because these medications are extensively employed in the treatment of human patients and veterinary practice, the development of antimicrobial resistance by bacteria to these drugs constitutes a significant concern in both human and animal care. In Ethiopia, antimicrobial resistance in NTS isolates from animal and human sources has been observed (44). Consequently, controlling illness poses a significant challenge for safeguarding the population's consumption of cow milk. To mitigate the risk of disease transmission through disease management techniques, such as washing cow milk equipment, while also enhancing hygiene.

Salmonella isolates were shown to be resistant to tetracycline, ampicillin, and penicillin at rates of 91.07%, 82.14%, and 73.21%, respectively, in this investigation. This conclusion is consistent with the earlier findings of 96.4% and 39.3% reported by Fufa et al (27) from milk, hand swab, and bucket samples. Diriba et al (31) showed a lower antimicrobial resistance pattern of 67.8% for tetracycline. According to Hailu et al (45) of Alexandria, Egypt, 85.7% of *Salmonella* isolated from dairy calves were responsive to ampicillin and tetracycline. This finding contradicts the current study, which found that 96.4% and 39% of the isolates were resistant to tetracycline and ampicillin, respectively (46-51). The study rate was much higher than the published rate (91.07% and 82.14%). Resistance levels to ampicillin and tetracycline are relatively high when compared to the 4.4% and 12.2% resistance rates reported in America by Blau et al (46,52-55).

This study, on the other hand, revealed that all Salmonella identified were totally and or very susceptible to chloramphenicol (100%), nalidixic acid and ciprofloxacin (91.07%), and ceftriaxone (82.14%), with vancomycin, gentamycin, and kanamycin (73.21%) having the same sensitivity rate. This result, in conjunction with the findings of Diriba et al (47,56), demonstrated that Salmonella isolates were 100%, 80.65%, and 70.9% sensitive to ceftriaxone, nalidixic acid, and ciprofloxacin, respectively.

The study found that 70.9% of Salmonella species isolates were sensitive to ciprofloxacin, which is similar to the findings of Teshome and Anbessa (57) and Reta et al, who reported 83.3%, 75%, and 65.7% susceptibility to ciprofloxacin, respectively. Gentamycin was shown to be 73.21% effective in the study (23). However, several researchers disagreed with the result of 100% sensitivity to gentamicin, with gentamicin sensitivity observed at 45.2% (58). Antimicrobial-resistant NTS in raw milk may be able to colonize the intestines of milk consumers, making the treatment of diseases caused by NTS more complex. Evidence suggests that the global growth in antimicrobial resistance is mainly due to the indiscriminate use of drugs in veterinary and public health treatments (59,60).

The Knowledge, Attitude, and Hygienic Practices of Milk Handlers

Almost all (96%) nursing cows were housed in a barned housing arrangement where they were intermingled with diverse species, which favored contamination of the udder and teat, increasing the isolation of *Salmonella* from milk and milk-contacting surfaces. 62% of milk producers utilized raw milk without any temperature treatment, making it a potential source of contamination for both spoilage and dangerous bacteria. The majority of milking (74%) was done in the open air, when the wind, sun, and other variables led to milk contamination. The findings agreed with those of Hailu (50), who reported that food catering areas, including milk preparation and selling areas, should be sheltered from the sun, dust, wind, road traffic, garbage, and waste, as such regions undoubtedly expose food (like milk) to contamination from microorganisms.

When it is unattainable to keep food preparation and selling areas clean or protect them from contaminant agents (such as dust, wind, road traffic, flies, and other contaminant agents), the displayed food, including milk, and its handling equipment should be adequately covered or protected from contamination (61,62). The movement of spoilage and harmful bacteria as a result of dust, debris, and winds may trigger a foodborne outbreak. In terms of equipment cleaning procedures used in the study region, the findings revealed that the majority of milk producers (27%) cleaned their milk handling equipment with warm water, detergent, and *Lantana camara* leaves. Nonetheless, while the majority of milkers (85%) wash their hands and equipment (90%) before and after milking, just a few of them utilize warm water and soap to clean their hands and equipment. Instead, they use cool water without detergent. The study by Worku et al found that the majority (69.7%) of respondents wash their milk handling equipment with cold water and detergents. Washing hands with cold water without detergent results in insufficient germ removal and is a significant source of microbial contamination of milk (52).

Milk handling equipment is washed once a day across the supply chain, followed by drying (for 5-10 minutes) and fumigation with smoke from burning stems of particular species of plants, such as Bir'eensa (*Terminalia brownie*),

Nadhello, or Mi'eessaa (*Euclea schemperi*), used as a decontamination method to increase flavor and preserve milk from contamination. The findings were consistent with those of Getaneh and Girma (54,63). According to the study, smoking milk containers in the preparation of homemade yogurt increased microbiological quality and taste when compared to non-smoked containers. Another study conducted in Kenya by Wanjala et al revealed the usefulness of smoke containers in limiting microbial growth and so enhancing the keeping quality of camel milk. The primary goal of sanitizing is to destroy leftover bacteria on these surfaces soon before milking. Inadequate or incorrect cleaning and sanitizing allowed germs to linger on the equipment's surfaces, grow, and reproduce, resulting in an increased number of various bacteria (55,64).

According to personal observations, the majority of people did not adequately cover their milk handling equipment from flies, dust, and other filth. This could have been a source of milk contamination from both spoilage and harmful microbes. In contrast, the majority of milk producers (79%) used aluminum cans; other researchers have claimed that aluminum or stainless steel is favored to reduce contamination (65). Stainless steel equipment is preferred for handling milk because it is readily cleaned to remove dust and bacteria from the container.

Through the supply chain, the bulk of the pastoral community's milk producers (67%) used pond water, (34%) river water, and (3%) tap water. The findings are consistent with the findings of Bereda and his colleagues, who reported that the majority (64.4%) of respondents were milk producers in the Ezha district of Ethiopia. Water from non-tap sources is used by the majority of milk collectors and carriers who bring raw milk to Jigjiga (60%), Harar (60%), and Dire Dawa (66.7%) (66). The source of water influences the bacteriological quality of milk (67). The water used for milk hygiene activities must be drinkable. This finding suggests that the water used to clean equipment may be a source of spoilage and harmful microorganisms (68).

Conclusion

The investigation involved acquiring milk, hand, and bucket swab samples for the isolation of Nontyphoidal Salmonella, with an overall incidence of 11.6%. The contamination was attributed to unsanitary handling and improper disposal. Individuals who regularly consume raw milk are particularly vulnerable to health problems. As a result, antimicrobial susceptibility testing on Nontyphoidal Salmonella isolates revealed high concentrations of multidrug resistance, potentially affecting health and indicating incorrect drug usage in the study area.

Recommendations

Therefore, based on the above conclusion, the following recommendations are forwarded:

- ✓ Unpasteurized milk, a type of dairy product, is not safe to consume due to potential contamination with harmful bacteria. Hence, proper hygiene is crucial.
- ✓ Cook food thoroughly and keep it hot until served, using a food thermometer to ensure meat, poultry, and eggs reach a safe internal temperature to kill germs.
- ✓ To avoid cross-contamination, refrigerate or freeze perishable foods within 2 hours, separate raw and cooked foods, use different cutting boards, plates, and knives, and wash them well after each use.
- ✓ Further research is needed to assess risks, evaluate interventions, and investigate mechanisms of multidrug-resistant Nontyphoidal Salmonella, an emerging public health threat affecting raw milk safety and quality.

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Competing Interests

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