

Isolation and Multiple Drug Resistance Patterns of *Salmonella* Isolates from Selected Dairy Farms in Hawassa Town, Ethiopia

Keywords: Dairy farms; Isolation; Hawassa; Multiple drug; Antimicrobial resistance; *Salmonella*

Abstract

A cross-sectional study was conducted from November 2017 to May 2018 on selected dairy farms in Hawassa town to isolate and assess the in-vitro antibiotic biogram of *Salmonella* from lactating dairy cows, personnel's and equipments at farms. A total of 216 samples were collected from selected dairy farms. All samples were processed bacteriologically following standard procedures outlined by ISO 6579: 2002. The overall prevalence of *Salmonella* was 12.9% (28/216) of the total samples. Out of total, 64.3% (18/28), 10.7% (3/28) and 25% (7/28) were from lactating cows, personnel's, and equipments, respectively. Based on antimicrobial susceptibility testing, all isolates were resistant at least to one or more antimicrobials tested. Accordingly, 96.4% (27/28), 82.1% (23/28) and 75.0% (21/28) isolates showed resistance for oxytetracycline, kanamycin, and nalidixic acid, respectively. Out of all the resistant isolates, 96.4% (27/28) showed multiple antibiotic resistance (resistance to two or more antibiotics) patterns. Multiple antimicrobials resistance was observed in 66.7% (18/27), 7.4% (2/27) and 25.9% (7/27) from lactating cows, personnel's, and equipments, respectively. Thus, awareness creation to the public regarding the public health importance of multiple drug-resistant *Salmonella* species and the consumption of unpasteurized milk and milk products is important.

Introduction

Milk has been described as a nearly perfect food since it contains the vital nutrients essential to the body, but it is also considered as a good medium for many microorganisms [1]. Raw untreated milk is still used by a large number of farm families and workers. In the raw milk value chain, milk producers, vendors and shop outlets can influence the prevalence of harmful pathogens in milk through poor animal husbandry, adulteration, washing equipment, udder and hands with unsafe water, storing and transportation in unhygienic condition and abuse of storage temperature [2]. Especially, the safety of dairy products with respect to foodborne diseases is a major global issue especially in the developing countries where production of milk and milk products takes place under poor hygienic, sanitary and Agricultural practices [3]. Milk contamination by zoonotic pathogenesis often natural but can also occur through handling milk in unhygienic conditions [1,4].

Food-borne bacterial diseases are a serious challenge to public health in developed and developing countries. There are more than 250 different food-borne diseases and most of these diseases are infections, caused by a variety of bacteria, viruses, parasites, and poisonings caused by harmful toxins or chemicals like poisonous mushrooms [5,6]. There different bacteria that cause foodborne diseases such as *Salmonella*, *Campylobacter*, *Listeria*, pathogenic *Escherichia coli*, *Yersinia*, *Shigella*, and *Enterobacter*. *Salmonella* is



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one of the most important bacterial species that infect a wide variety of hosts including humans and numerous farm animals; such as pigs, cattle, horses, and chickens [7].

Salmonella is comprised of different species and more than 2,600 different serovars of *Salmonella* have been characterized based on the surface 'O' antigen, which is a part of the variable long chain of lipopolysaccharide on the bacterial outer membrane [8]. Out of these 2,600 serovars, nearly 1500 belong to the *Salmonella* subspecies enterica. Serovars of the enterica subspecies can be divided into three groups depending upon their ability to infect a wide variety of hosts. The first group includes serovars that have a broad host range also called unrestricted serovars as these infect nearly all animals. This group includes serovars like *Salmonella typhimurium* and *Salmonella enteritidis*. Nevertheless, these serovars are of high importance with respect to their epidemiology as these have developed mechanisms to invade different hosts without any greater resistance. Thus, these serovars pose a greater zoonotic potential than their other counterparts [9].

The second group includes serovars that cause highly severe systemic infection in their preferred host and are usually excreted without any clinical symptoms when they accidentally infect hosts others than their most adapted or preferred. Serovars such as Dublin, Choleraesuis fall into this category, as these prove to only cause systemic infection in cattle and pigs respectively [10]; however, these upon infection into other hosts like rodents and humans are usually excreted making these hosts as 'carriers'. Serovars of this group are referred to as the 'Host-adapted Serovars'. The third group comprises of serovars which are restricted very strictly with one very specific host only; these serovars are called 'host restricted serovars'. They exclusively cause systemic infection, which often proves to be fatal within their host. Serovars such as Typhi, Gallinarum, Abortus equi, ectecra belong to this group [11].

Salmonella is transmitted to animals and humans through consumption of contaminated food products (milk, eggs, and meats), direct contact with infected animals, through contaminated equipments such as stainless steel, hanging material, bucket, where milk is collected and stored, are a key mechanism for pathogens to contaminate food products [12]. In livestock, clinical signs typically appear 6-24 hr after exposure and include profuse diarrhea, fever,

dehydration, in appetite, foul-smelling feces, and mucus or blood in feces [13]. Disease manifestations in people include diarrhea, fever, abdominal cramps and septicemia in severe cases, appearing 12-72 hr after ingestion. *Salmonella* can also be carried subclinically by both humans and animals [14,15].

The prevalence of salmonella infection varies across regions, however, the diseases caused by *S. enteric* serovars are especially prevalent in developing areas, such as Southeast Asia, Africa and South America that leads to an estimated 20 million cases of humans and 200,000 deaths each year. Challenges such as antibiotic-resistant *Salmonella* strains also pose a significant threat to deliver reliable therapies [16]. In Ethiopia, as in other developing countries, it is difficult to evaluate the burden of Salmonellosis because of the limited scope of studies and the lack of coordinated epidemiological surveillance systems. In addition, under-reporting of cases and the presence of other diseases considered to be of high priority may have overshadowed the problem of Salmonellosis. Continuous surveillance of the prevalent *Salmonella* serovars and assessing their antimicrobial resistance pattern is essential to control the spread of the pathogen [17].

Antibiotic resistance in *Salmonella* is a rising problem over the past decades. Improper use of antibiotics in both human and veterinary medication has caused bacteria to develop resistance against therapeutic antibiotics [18,19]. Using antimicrobial agents for cattle has been implicated as a source of human infection with Antimicrobial-Resistant (AMR) *Salmonella* through direct contact with livestock and consumption of raw milk, meat and contaminated material [20]. Antimicrobial-resistant *Salmonella* are increasing due to the use of antimicrobial agents in food animals at subtherapeutic level or prophylactic doses that may promote growth and markedly increase the human health risks associated with consumption of contaminated milk and meat products through mutation, acquisition of resistance encoding genes and irrational use of antimicrobials in food animals [21-23].

Accordingly, there are limited studies regarding the assessment of the pathogens isolated from apparently healthy animals at farm level, personnels' and different types of equipment. Thus, the screening of milk and other dairy products against pathogenic organisms will play a vital role in curtailing human infection. Studying the prevalence and antimicrobial resistance of *Salmonella* from cattle and in contact with humans in dairy farms is the most important to design methods of minimizing the possible transmission of *Salmonella* between humans and cattle. Moreover, it is important in combating the emergence of antibiotic-resistant strains of *Salmonella* [11]. Hence, this study was conducted to isolate, identify and assess the multiple drug resistance pattern of *Salmonella* isolates from selected dairy farms in Hawassa town.

Materials and Methods

Study area

The current study was conducted from November 2017 to May 2018 in selected dairy cattle farms of Hawassa towns. It is located 275 km south of Addis Ababa. Hawassa is situated at an altitude of 1750 m above sea level and according to an estimate, it lies between 6°83' to 7°17' N and 38°24' to 38°72' E. Hawassa receives an average annual

rainfall of 955 mm with mean annual temperature of 20 °C and the city has a total area of about 50 km² divided into eight sub-cities and 32 kebeles (kebeles are the smallest administrative unit below the sub-city/woreda level) [24].

Study population

The study animals were apparently healthy dairy cows that were located in Hawassa town. The study includes dairy cattle kept under different (extensive, intensive and semi-intensive) management systems as well as farm personnels and equipments. There are different types of farms including small, medium and large scale having dairy cattle ranging from five to twenty two. Besides, the farms were selected purposively based on the availability of lactating cows and the willingness of the owners.

Study design and Sampling technique

A cross-sectional study was carried out from November 2017 to May 2018 to isolate, identify and assess the multi-drug resistance pattern of the *salmonella* isolates from selected dairy farms. The farms are selected purposively based on the availability and accessibility of study animals. Accordingly, a total of 216 samples were collected from selected dairy cattle farms in the study area.

Sample collection, handling and transportation

Samples were collected aseptically from apparently dairy cows (milk and feces), hands of personnel working in the farms and from equipment (container and buckets). Then the all samples were collected after getting proper consent from the personnel and Hawassa university to perform the research activity. Fecal samples were collected directly from the rectum and put into 50 ml containing a universal screwed capped bottle and 10 ml of milk was collected aseptically from all teats in a sterile test tube after aseptically preparing the teats thorough scrubbing with a cotton moistened with 70% denatured alcohol and the first 3-4 streams of milk were discarded. All types of swab samples (milkers' hand, container and buckets) were collected before the commencement of the milking process using a sterile wooden cotton swab and were put into a sterile test tube containing 10 ml buffered peptone water used as transport media. All sample types were properly labeled with permanent marker. Then, the samples were immediately transported using an icebox to the Microbiology Laboratory of Hawassa University for further bacteriological examination.

Isolation and identification of salmonella

The isolation and identification of *Salmonella* was performed at the Microbiology laboratory of Hawassa University by using techniques recommended by International Organizations for Standardization (ISO-6579, 2002) [25]. The detection of *salmonella* was performed based on the following four successive stages: Firstly, All samples were pre-enriched in non-selective liquid media and processed separately. Then, 1 gm of fecal sample and 1 ml of milk was pre-enriched with 9 ml of Buffered Peptone Water (BPW) and incubated for 24 hrs at 37 °C. Secondly, all samples were transferred to selective media such as Tetrathionate Broth and Rappaport Vassiliadis *Salmonella* Enrichment Broth. A 1 ml of pre-enriched sample was transferred aseptically into a tube containing 10 ml of Tetrathionate Broth and incubated at 37±1 °C for 24±3 hours. Another 0.1 ml of the culture

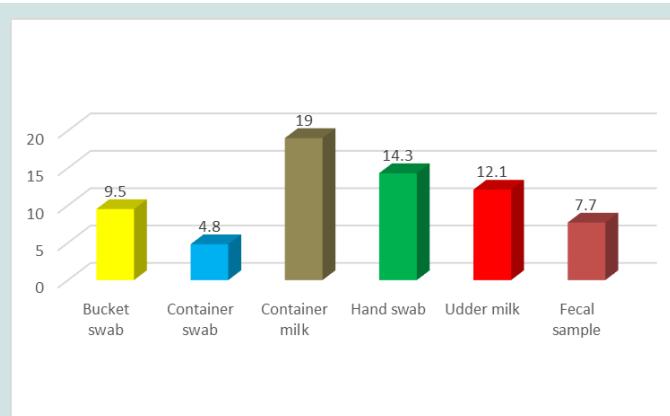


Figure 1: Proportion of *Salmonella* isolates from different samples types.

Table 1: Antibiotic Susceptibility Profiles of *Salmonella* isolates in dairy farms.

Antibiotics disc	Antibiotic Susceptibility Patterns		
	Sensitive (%)	Intermediate (%)	Resistance (%)
Amoxicillin	10 (35.7)	11 (39.3)	7 (25.0)
Cefoxitin	25 (89.3)	0 (0.00)	3 (10.7)
Chloramphenicol	14 (50.0)	9 (32.1)	5 (17.9)
Ciprofloxacin	27 (96.4)	0 (0.00)	1 (3.6)
Gentamycin	28 (100.0)	0 (0.00)	0 (0.00)
Kanamycin	2 (7.1)	3 (10.7)	23 (82.1)
Nalidixic acid	0 (0.00)	7 (25.0)	21 (75.0)
Oxytetracycline	0 (0.00)	1 (3.6)	27 (96.4)
Streptomycin	16 (57.1)	9 (32.1)	3 (10.7)
Trimethoprim-sulphamethaxazole	22 (78.6)	3 (10.7)	3 (10.7)

obtained in pre-enrichment broth was transferred aseptically into a tube containing 10 ml Rappaport Vassiliadis *Salmonella* Enrichment Broth (Harmonized) and incubated at 41.5 ± 1 °C for 24 ± 3 hrs.

Thirdly, Plating out and identification of the samples were conducted using Xylose lysine Desoxycholate (XLD) agar and *Salmonella-Shigella* (SS) agar plates. A loopful of inoculum from cultures of the selective enrichment media were streaked on to XLD and SS agar plates and incubated at 37 °C for 24 hrs. Then, all colonies that grow on the XLD medium, produces hydrogen sulfide (H_2S) and colorless colonies with black center on SS medium were streaked onto Nutrient Agar and incubated at 37 °C for 24 hrs for further confirmation through serious of biochemical tests. Finally, All suspected colonies were subjected to a series of different biochemical tests using the procedure of (ISO 6579, 2002; to confirm salmonella [25]. Triple Sugar Iron Agar (TSIA), Urease, Citrate, Indole, Methyl red and Voges Proskouer (VP) tests were performed on all suspected isolates to confirm the salmonella. All presumptive salmonella Isolate were cultured on Nutrient Agar for further antimicrobial susceptibility testing.

Antimicrobial susceptibility test

The antibiotic susceptibility tests of the *Salmonella* isolates were performed according to the Clinical and Laboratory Standards Institute (CLSI) method using Kirby-Bauer disk diffusion test on Muller-Hinton Agar (HIMEDIA, India) [26]. Pure colonies on nutrient agar were taken with a wire loop and transferred to a tube

containing 5 ml of Saline water and emulsified. The broth culture was incubated at 37 °C for 4 hrs until it achieved the 0.5 McFarland turbidity standards. Sterile cotton swab was dipped into the suspension and the bacteria were swabbed uniformly over the surface of Muller-Hinton agar plate within a sterile safety cabinet. The plates were held at room temperature for 15 minutes to allow drying. Antibiotic discs with a known concentration of antimicrobials were placed and the plates were incubated for 24 hrs at 37 °C.

Amoxicillin (AML) (25 µg), Cefoxitin (FOX) (30 µg), Chloramphenicol (C) (30 µg), Gentamycin (CN) (10 µg), Streptomycin (S) (10 µg), Kanamycin (K) (30 µg), Nalidixic acid (NA) (30 µg), Ciprofloxacin (CIP) (5 µg), Oxytetracycline (OT) (30 µg) and Trimethoprim-sulphamethoxazole (SXT) (25 µg), were selected based on availability and their current use in human and veterinary medicine. All the antibiotics were from Oxitod Hampshire, England, and the expiry date was properly checked before application. Zone of inhibition of individual antibiotic agent was interpreted in to susceptible, intermediate, and resistance categories by referring recommended clinical and laboratory standards institute [26].

Data analysis

Data collected from field and laboratory investigations were recorded, and coded using Microsoft Excel 2013 program and analyzed using STATA version 13.0. Descriptive statistics were used to figure out the proportions of *Salmonella* isolate. Moreover, the antibiotic efficacy of each drug was determined by comparing the

Table 2: Multiple antibiotic resistance MAR profile of *Salmonella* isolates.

MAR pattern	Number of isolates	Multiple drug resistance patterns	Number of isolates (%)
Two	3	OT, NA,	5(18.5)
	2	OT, K	
Three	7	OT, K, NA	8(29.6)
	1	OT, K	
Four	2	NA, OT, K	7(25.9)
	1	OT, CIP, C	
	1	OT, AML, NA	
	1	NA, OT, SXT, K	
	1	OT, AML, SXT, K	
Five	1	NA, S, OT, K	4 (14.8)
	1	NA, C, OT, K	
	2	OT, AML, K, NA	
Six	1	FOX, C, OT, K, NA	1 (3.7)
Seven	1	FOX, S, AML, K, NA	1 (3.7)
Eight	1	OT, AML, SXT, K, NA, C	1 (3.7)
		S, C, OT, AML, K, NA, FOX	1 (3.7)

zone of inhibition with the standard one.

Results

Frequency of *Salmonella* isolates

In this study, out of 216, the overall prevalence of *Salmonella* was 12.9% (28/216). From the overall proportion, 64.3% (18/28), 10.7% (3/28) and 25% (7/28) were isolated from the milk and feces of dairy cows, personnel and equipments, respectively. 19% of *Salmonella* were isolated from plastic container milk. A higher proportion of *Salmonella* was isolated from milk samples (12.1%) than fecal samples (7.7 %) (Figure 1).

Antimicrobial susceptibility of *Salmonella* isolates

In the present study, out of 28 isolates, 27 isolates showed multiple drug resistance. Accordingly, all isolates were susceptible to ciprofloxacin, cefoxitin and trimethoprim-sulphamethoxazole with proportion of 96.4%, 89.3%, and 78.6%, respectively. However, all isolates were 96.4%, 82.1% and 75.0% resistant to oxytetracycline, kanamycin and nalidixic acid, respectively. On the other hand, all isolates were 100% sensitive to gentamycin (Table 1).

Multiple drug resistance patterns of *Salmonella* isolates

Multiple drug resistance (isolates that were resistant for two or more antibiotics) were detected in 96.4% (27/28) of the *Salmonella* isolates. Out of these, 66.7% (18/27), 7.4% (2/27) and 25.9% (7/27) isolates were from lactating cows, personals', and equipments, respectively. The higher multi-drug resistance pattern was observed in K, NA, OT, with the proportion of 25.9% followed by K, NA, OT, AML with the proportion of 7.4%. Besides, 11.1% of the resistant isolates were resistant to six and more antibiotics (Table 2).

Discussion

In this study, out of 216 samples collected from selected dairy farms in Hawassa town, the overall proportion of *Salmonella* isolated from dairy cows, personals' and equipment were 12.9%. This was higher than the reports of where 7.2% were found in slaughtered small ruminants and environment in Modjo export abattoir [27], 7.1% from apparently healthy slaughtered cattle in Debre Zeit and the study on cheese and milk in Debre Zeit (2.1%) as well as dairy product

in Addis Ababa (1.6%) [21,28-30]. However, the current finding was comparable with 10.5% from apparently healthy dairy cows in Modjo [31], 10.76% from lactating cows and in contact humans in dairy farms of Addis Ababa and (11.5%) among chicken table eggs at Kombolcha [18], Ethiopia [32]. The present result was lower than the findings of who reported 20% in raw milk from the Korsa district and Ejeta et al., 2004 who reported 14.7% from minced beef, mutton and pork samples among supermarkets in Addis Ababa [33].

In this study, the prevalence of *Salmonella* from milk and feces of apparently healthy lactating dairy cows was 64.3%. This was higher than who reported 7.1% from apparently healthy slaughtered cattle [28]. This variation could be due to the test procedures and techniques used since pre-enrichment steps using buffered peptone water was employed in this study and source of sample. Similarly, the report of [34]; from England (0.2% and 4%), from Northern Thailand (3%) and from Cameroon (27%) are much lower than the current study [35,36]. The current result was higher than the prevalence recorded in Iran 4% and in USA 7.3% and in Nigeria (15%) and 10.9% reported in Namibia on bovine and ovine bone-and-meat meal and blood meal samples [19,37,38]. This may be attributed to the variation in agroecological location of the cattle, housing conditions, feeding habits, and types of feed provided for the cattle.

According to the current investigation, *Salmonella* was isolated from the fecal samples of apparently healthy lactating dairy cows with a rate of 7.7%. This finding was higher than the report of from Egypt where prevalence in on fecal shedding of *Salmonella* among dairy cattle was 1.56 [39]. However, this result was lower than from the United States (9.7%) [40], from central Texas [41], USA where *Salmonella* shedding rate from fecal samples of dairy calf was 70%. This huge difference might be in the report from Texas, all isolates were one serotype (*S. kinshasa*) and this serotype might have specific host requirement.

In the present study, *Salmonella* was isolated from milkers' hand swab with a rate of 14.3%. This was higher than the report of (8.9%) from small ruminants slaughtered in Modjo export abattoir [27]. However, it was lower than the work of Beyene et al., 2016 (28.6%) from pooled milkers' hand swab of personals' working in Asella Municipal abattoir.

The variation in the prevalence of *Salmonella* isolation between the present-day study and the previous studies at different areas of the country could be associated with different risk factors that contribute to the occurrence of *Salmonella*. These are host-related risk factors that include age, breed, the physiological state of the animals, feeding strategies, vaccination status [29]. Environment-related risk factors are often related to hygienic and management practice, stocking density, type and amounts of feed, accessible water supplies, infection load in the environment, usage of contaminated utensil, housing type, ventilation, flooded grassing areas, movement of animals, calving environment, and production facilities in different areas are also plays a role for *Salmonella* occurrence [12]. Additionally, epidemiological patterns of *Salmonella* differ greatly between geographical areas depending on climate, population density, land use, farming practice, food harvesting and processing technologies and consumer habits [42].

The current study revealed that 96.4% of the isolates were resistant for two or more antibiotics which was comparable with the finding of [31]. However, it was higher than the previous studies conducted in Ethiopia and elsewhere in the world [21,28,43-46]. This difference may be due to the increasing rate of inappropriate utilization of antibiotics in the dairy farms which favors selection pressure that increased the advantage of maintaining resistance genes in bacteria [47,48].

The result of the current research indicated *Salmonella* isolates were resistant to Oxytetracycline, kanamycin, and nalidixic acid with a resistance rate of 96.4%, 82.1%, and 75% respectively. Similarly, reported that the isolates of *Salmonella* from food items and personnel from Addis Ababa were resistant to the commonly used antibiotics including streptomycin [21], and oxytetracycline. However, resistance rates to oxytetracycline are very high compared to results documented in America reported 95.6% and 87.8% sensitivity levels [37], respectively and Iran reported 42.58% sensitivity for both antibiotics [19]. In this study, 96.4% of the isolates showed resistance to two or more antibiotics which is lower than a report from Addis Ababa, Ethiopia (83%) [18].

According to the study, *Salmonella* isolates were susceptible to gentamycin and ciprofloxacin with the rate of susceptibility 100% and 96.4% respectively. This was in agreement with the reports of where *Salmonella* isolated from apparently healthy slaughtered sheep in Turkey showed 100% sensitivity to these antibiotics [36], with and with the report of in Iran where ciprofloxacin was 100% effective [19,30]. However, it was higher than who reported 73.3% and 83.3% [11], who reported 75% and 95% for ciprofloxacin and gentamycin, respectively [33]. This variation might be due to small sample sizes for the data, nature of the drug, presence of different strains of the bacteria, development of resistant gene, their low-frequency usage for prevention and control of disease in food animals in the study area.

The present study revealed that *Salmonella* isolates were resistant to tetracycline and ampicillin with a rate of 96.4% and 39%, respectively which disagrees with the report of in Egypt reported that each of the ampicillin and tetracycline was 85.7% effective against *Salmonella* species isolated in dairy cattle [39]. In addition, in the present study trimethoprim-sulphamethoxazole was an effective drug (78.6%) against salmonella isolates that disagrees with the report by

who reported 100% resistance to trimethoprim-sulphamethoxazole [39]. A higher activities of gentamycin (100%) observed in the current study disagree with a study in Texas, USA, reported 85% and this difference might be due to availability and overuse of the drug in the farm of the current study [41]. In the current study, ciprofloxacin was 96.4% effective against all isolates which was in line with a report in Sudan where ciprofloxacin was 100% effective to all human and cattle *Salmonella* isolates [49]. The result for streptomycin resistance in this study (10.7%) was lower than 13.3% and 25%, which was reported by and [18,33], respectively. Amoxicillin resistance in this study (25%) was higher than 16.7% reported by [30]. The resistance of chloramphenicol in this study 17.9% is consistent with 16.7% reported by and [18,30], and lower than 25% reported by [33].

According to the antimicrobial susceptibility testing, all of the isolates showed multiple drug resistance to at least one or more drugs tested were observed which was in line with the report of [30,33,50]. Moreover, 96.4% of the isolates showed multiple drug resistance for two or more types of antimicrobials. This was higher as compared to the report of who reported 70% and 30% [33], who report 83.3% and 16.3% [50], and who reported 50% and 50% for multiple and single antimicrobial resistance, respectively [30].

In general, antimicrobial use is a key driver of resistance development, which is either overuse for minor infectious, misuse due to lack of access to appropriate treatment and underuse due to inadequate dosing, poor adherence or substandard antimicrobial and lack of financial support to complete treatment course. The present study indicated the importance of cattle products (milk), personnel working in the farms and materials/equipment used as a potential source of *Salmonella* infection.

Conclusion and Recommendations

In the present study, the isolation of 12.9% *Salmonella* at dairy farms level showed that dairy cattle and their environment are important sources of milk contamination with the organism, and consumption of raw milk and other unpasteurized dairy products can lead to infection with zoonotic *Salmonellosis*. The presence of a high proportion of multiple antimicrobial-resistant isolates (96.4%) in the dairy farms to antimicrobials that are commonly used in the veterinary and public health set up in this study further signifies the public health importance of *Salmonella* in addition to treatment failure. In this study, all the isolated *Salmonella* revealed resistance at least to one of the antibiotics tested. In general, awareness creation to the public about the public health importance of foodborne diseases and the consumption of unpasteurized milk and milk products is important. Gentamycin and Ciprofloxacin should still be used as a choice to treat *Salmonellosis*. Further, the molecular characterization of the isolates with emphasis on resistant strains is important to identify mechanisms of antibiotic resistance.

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