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Isolation, Identification and Antimicrobial Resistance Profiles of *Salmonella* from Dairy Farms in Adama and Modjo Towns, Central Ethiopia

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ABSTRACT

A cross-sectional study was carried out from February 2019 to May 2019 in Adama and Modjo aiming at isolating *Salmonella* from dairy cattle farms and determining the antimicrobial susceptibility testing of the isolates. A total of 117 samples from dairy farms: faces, bulk tank milk, personnel hand swab and contaminated floor samples were collected and screened for the presence of *Salmonella*. Ten (8.5%) of the samples tested were found to be positive for *Salmonella*. Of 89 faces, 10 bulk tank milk, 9 personnel hand swab and 9 contaminated floor samples, no positive was found in the milker's hand swab samples from both Adama and Modjo areas and the isolation frequencies of *Salmonella* were 8.98%, 10%, and 11.1% in faces, bulk tank milk and floor sample, respectively. The antibiogram testing revealed differential multi-drug resistance among *Salmonella* isolates in lactating cow and cows environment samples. Most the isolates were resistant to methicillin, streptomycin, and nalidixic acid whereas sensitivity was recorded for gentamicin. In conclusion, the relatively high resistance among the bacteria present in dairy farms could pose public health and therapeutic problems to consumers as potential vehicles of resistant *Salmonella* food borne infections.

Keywords: Adama, *Salmonella*, Antimicrobials, Dairy farms, Isolation, Modjo, and Multidrug resistance.

INTRODUCTION

Food-borne diseases are a public health problem in developed and developing countries. More than 250 different food-borne diseases have been described. Most of these diseases are infections caused by a variety of bacteria, viruses and parasites. *Salmonella* has been one of the most commonly reported causes of food-borne pathogens from distant and recent times (Pui *et al.*, 2011; Hoffmann *et al.*, 2012). According to a recent study (Kirk *et al.*, 2015) commissioned by the WHO on the global disease burden of food-borne diseases in humans, food-borne illnesses from diarrheal and invasive non-typhoidal *Salmonella enterica*, resulted in the largest disease burden, highlighting the significant public health importance of *Salmonella* infections and the urgency for control, particularly in low-and middle-income countries where most burden of diseases and occurrence of mortality cases are reported. *Salmonella* species belong to Gram-negative, rod shaped, facultative intracellular bacteria that successfully infects a wide variety of hosts. *Salmonella* is comprised of two species, *Salmonella bongori* and *S. enterica* (Guibourdenche *et al.*, 2010).

Out of these 2,700 serovars, nearly 1500 belong to the *S. enterica* subsp. *enterica*. Serovars of the *enterica* sub species can be divided into three groups depending upon their ability to infect a wide variety of hosts: Serovars which have a broad host range also called as unrestricted serovars as these infect nearly all animals and pose a greater zoonotic potential than their other counterparts, Serovars which accidentally infect hosts other than their most adapted or preferred (McCuddin *et al.,* 2006; Shahen *et al.,* 2019), and serovars which are restricted to one specific host only. *Salmonella* transmits to humans can occur through several routes. these are consumption of contaminated food products (milk, eggs, and meats), direct contact with animals and their environment, cross-contamination through direct contact of foods to contaminated surfaces such as stainless steel, hanging material, knife, bucket where milk are collected are a key mechanism for pathogens to contaminate food products (Kusumaningrum *et al.*, 2003; Uddin *et al.*, 2017).

Excretion of *Salmonella* with faces can contaminate water, soil, other animals and feed. Although *Salmonella* primarily intestinal bacteria, due to its ubiquitous nature common in the environment and commonly found in farm effluents human sewage and in any materials subject to faecal contamination as a result it leads the contamination of milk and meat products to originate either from infected live animals or from cross-contamination while during processing. Despite the controls that have already been put into place, *Salmonella* infection arising from contaminated food continues to be an immense problem with millions of cases occurring annually throughout the world. In addition to the misery caused, financial loss is enormous (Hendriksen, 2003). An increasing proportion of *Salmonella* isolates is resistant to commonly used antibiotics in both developing and developed countries (Threlfall, 2002), and this increase is seen in both veterinary and public health sectors (Kemal, 2014; Van Boeckel *et al.*, 2015).

Using antimicrobial agents for cattle have 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 materials (Alexander *et al*., 2009; Sharif *et al*., 2019). Antimicrobial resistant *Salmonella* are increasing due to the use of antimicrobial agents in food animals at sub-therapeutic level or prophylactic doses for growth promotion and markedly increase the human health risks associated with consumption of contaminated milk and meat products (Zewdu and Cornelius, 2009; Md *et al.,* 2006), through mutation, acquisition of resistance encoding genes (Fluit, 2005) and irrational use of antimicrobials in food animals (Fluit, 2005; Beyene and Tesega, 2014).

In Ethiopia, as in other developing countries, it is difficult to evaluate the burden of food-borne diseases, because of the limited scope of studies and lack of coordinated epidemiological surveillance systems. In addition, under reporting of cases and the presence of other diseases considered to be of higher priority may have overshadowed the problem of food-borne diseases including *Salmonellosis*. Therefore, the aims of the current study were: To isolate and identify *Salmonella* from dairy cows' feces, personnel hand, equipment and contaminated floor sample and to evaluate the antibiogram pattern of the isolates to commonly used antibiotic agents from selected dairy farms in Modjo and Adama towns.

MATERIALS AND METHODS

Study Area – The study was conducted in Modjo and Adama towns from February 2019 to June 2019. Modjo is the administrative center of Lome district, located in the East Shewa Zone of the Oromia Region, Ethiopia. It is located at 66 Km Southeast of Addis Ababa and lies at latitude 8°35'N and longitude 39°7'E at an altitude of 1790 meters above sea level. The area gain rainfall twice a year those known as long and short rainy season. The main rainy season extends from June to September. The average annual rainfall, temperature, and mean relative humidity are: 776 mm, 19.4ºC and 59.9% respectively (CSA, 2005).Adama town, East Shoa, Ethiopia, which is located about 100 km south east of Addis Ababa at an altitude of 1650 meter above sea level. Its annual temperature ranges from

13.9°C - 29°C. The mean annual rainfall of the area is 1024 mm. The livestock population of the area in 2004/2002 estimated to be 70,622 cattle, 36,142 sheep, 42,968 goats and 2,193 equines (CSA, 2005).

Study Population - The study population is lactating dairy cows in Modjo and Adama towns and the study animals were apparently healthy dairy cows in small- and large-scale dairy farms located in and around Modjo and Adama towns. The farms were selected by using simple random sampling strategies based on data obtained from both areas livestock and fishery resource development. In this study, the majority of farms found in the study areas were small scale having herd sizes not more than six cows. All of the available lactating cows present in each farm were sampled. According to personal observation, the hygienic status of the cows and their environment was more or less good even though some animals were reared under poor hygienic condition plus in a manner mixed with other activities of the households. Farm equipment used in the milking and storage of milk and personnel's (milkers') were also part of the study.

Study Design - A cross-sectional study was carried out to isolate, identify and detect antimicrobial susceptibility profile of the *Salmonella* from dairy farms. Sampling days were randomly assigned and each farm was visited only once during the study period. Types of sample collected include feces, bulk tank milk, milkers' hand swab, contamination of floor.

Sample Collection and Transportation - Samples from dairy cows (faces), hand swab of personnel working in the farms (milker's), from equipment and from contaminated environment (contaminated floor sample) were aseptically collected from the selected dairy farms. Samples from dairy cows were collected from apparently health lactating cows. Fresh fecal samples were collected directly from the rectum of healthy lactating dairy cows using disposable gloves into sterile plastic bags. Pooled milkers' hand swab is collected before the beginning of milking process by using a sterile cotton swab. Floor samples were collected from bedding of the animal house and from the environment and equipment which the cows feed using a sterile a sterile cotton swab and Samples were properly coded based on collection date, sample source and sample type. Source of sample was classified as animal, personnel and equipment. Types of samples collected in quantity were faces (89), pooled milkers' hand swab (9), bulk tank milk (10) and floor sample (9). Then samples were immediately transported under cold condition (ice box) to the Microbiology Laboratory of College of Veterinary Medicine and Agriculture, Addis Ababa University, Bishoft. Upon arrival, samples were processed separately by pre-enriching in pre-enrichment media or were stored overnight in a refrigerator at +4°C until examined the next day. Then further processes were followed after samples were incubated for 24 hrs.

Isolation and Identification of *Salmonella*

Pre-enrichment and selective enrichment - The feces samples were pre-enriched in appropriate amount of buffered peptone water in (1:9) ratio and incubated at 37°C for 24 hrs. Rappaport-Vassiliadis medium (RV) broth and Selenite (SB) broth were used for selective enrichment of the samples. About 0.5 and ml of the pre-enriched sample was transferred into a tube containing 10 ml of Rappaport-Vassiliadis medium (RV broth) and incubated at 42°C for 24 hours. Another 1.0 ml of the pre-enriched broth was transferred into a tube containing 10ml of Selenite broth and incubated at 37°C for 24 hours.

Plating out and identification – Xylose lysine deso-xycholate (XLD) agar plate was used for plating out and identification. A loop full of inoculums from each RV and Selenite broth cultures were plated onto XLD plate and incubated at 37°C for 24 hours. After incubation, the plate was examined for the presence of typical and suspect colonies. Typical colonies of *Salmonella* grown on XLD-agar have a black center and a lightly transparent zone of reddish color due to the color change of the media while H_2S negative variants grown on XLD agar are pink with a darker pink center. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening. Five typical or suspected colonies were selected from the

selective plating media, streaked onto the surface of pre-dried nutrient agar plates and incubated at 37°C for 24hrs. All suspected *Salmonella* colonies were picked from the nutrient agar and inoculated into the following biochemical tubes for identification: triple sugar iron (OXOID CM0277, England) agar, Simmon's citrate agar (HIMEDIA M099, India), Indole tests (Becton Dickinson, USA), Methyl red-Voges-Proskauer (HIMEDIA M070, India) broth and then incubated for 24 to 48 hrs at 37°C

Biochemical Confirmation of *Salmonella* Isolates - All suspected *Salmonella* isolates were subjected to the following biochemical tests for confirmation: Triple Sugar Iron (TSI) test, Indole test, Citrate utilization test, Methyl red test, and Vogues Proskauer (VP) test. Colonies producing red slant (alkaline), with yellow butt (acid) on TSIA with blackening due to hydrogen sulphide (H_2S) production and e (gas production) in butt, negative for Indole test, positive for Methyl red test (red broth culture), positive for citrate utilization (deep blue slant), and negative for VP test were considered to be *Salmonella* positive (Quinn *et al.*, 2004). Presumptive *Salmonella* isolates that were found fulfilled the *Salmonella* characteristics on all biochemical tests indicated above were transferred and cultured on Nutrient Agar (NA) for antimicrobial sensitivity and motility tests.

Antimicrobial Susceptibility Testing - The antimicrobial susceptibility testing of the isolates was performed with Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute of U.S.A (CLSI, USA) and Kirby Bauer Disk Diffusion Susceptibility Test Protocol on Muller Hinton agar medium. From each biochemically confirmed isolate, loop full of well grown colonies on nutrient agar were transferred with sterile loop into sterile tubes containing 5ml of normal saline solution (0.85% NaCl). The inoculated colonies mixed well with saline solution by vortex until smooth suspension was formed. Saline solution (if suspension more turbid) or colonies (if suspension less turgid) were added to the suspension until it achieved to the 0.5 McFarland turbidity standards. Then sterile cotton swab was dipped into the suspension and the bacteria were swabbed uniformly over the entire surface of Muller Hilton Agar plate. The plates were being held at room temperature for 3 minutes in bio-safety cabinet to allow drying. Ten antimicrobial disks with known concentration of antimicrobial were placed on the Muller Hinton Agar plate; nine of them in circular pattern and one at the center and the plates were incubated for 22 hrs at 37ºC.

Each isolate was tested for a series of eleven antimicrobials: Tetracycline (TE) (30µg), Cefoxitin (FOX) (30µg), Cefuroxime (30µg), Ciprofloxacin (CIP) (5µg), Gentamycin (CN) (10µg), Nitro-furantoine (NIT) (300µg), Nalidixic acid (NA) (30µg), Streptomycin (S) (10µg), Erythromycine (ERY) (15) and Methicillin (ME) (5). The diameters of clear zone of inhibition produced by diffused antimicrobial on lawn inoculated bacterial colonies were measured to the nearest mm using caliper. All eleven zone of inhibition against eleven antimicrobial agents for each isolate were recorded and compared with standards and interpreted as resistant, intermediate, or susceptible according to published interpretive chart (CLSI, 2013).

Data Management and Analysis - The raw data generated from the study were arranged, organized, coded and entered to Excel spread sheet (Microsoft® office excel 2007). Then the data was analyzed using SPSS version 20 through descriptive analysis with chi-square statistics. The results of analyses were mostly described in proportion. Proportion were estimated as the numbers of samples detected positive to *Salmonella* from the total sample tested as well as the numbers of antimicrobial resistant isolate to the detected positive isolate.

RESULTS

Growth on Solid Media - On nutrient agar *Salmonell*a colonies were moderately large (2-4 mm), circular with smooth surface and grayish- white in color after 24 hours at 37ºC (**Fig 1**). Growth on deoxycholate agar showed slight opaque dome-shaped colonies measured (2-4 mm) with central black spots (indicated production of hydrogen sulfide) surrounded by a zone of clearance after 48

hours at 37ºC (**Fig 2**). On triple sugar iron agar *Salmonella* colonies produced hydrogen sulfide which was indicated by black discoloration, gas production causes bubbles in the agar, and pH change was indicated by production of red color in the slant (**Fig 3**).

Proportion of Bacteria Isolation - *Salmonella* was isolated from 10/117 (8.5%) of the total samples. Out of the 10 *Salmonella* isolates, 8 (80%), 1 (10%), (and 1 (10%) were from feces, bulk tank milk, and floor sample, respectively (**Table 1**). From a total of 89 cow's feces examined, 8.98% (8/89), 10 bulk tank milk samples, 10% (1/10), and out of 9 contaminated floor sample, 11.1% (1/9) were confirmed positive for *Salmonella* from both cities, Adama and Modjo.

Fig 1: Growth of *Salmonella* on Nutrient Agar.

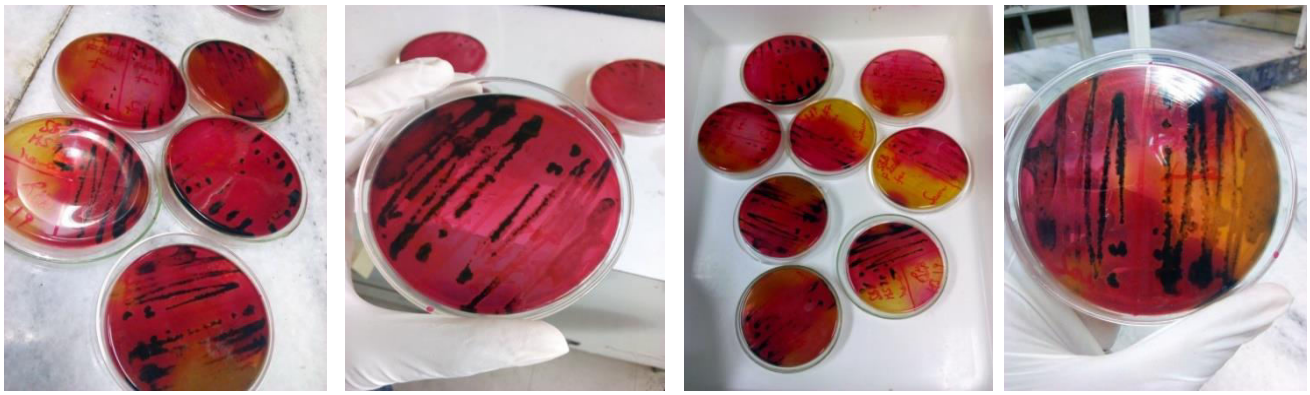

Fig 2: Growth of *Salmonella* on Xylose Lysine Desoxychocolate.

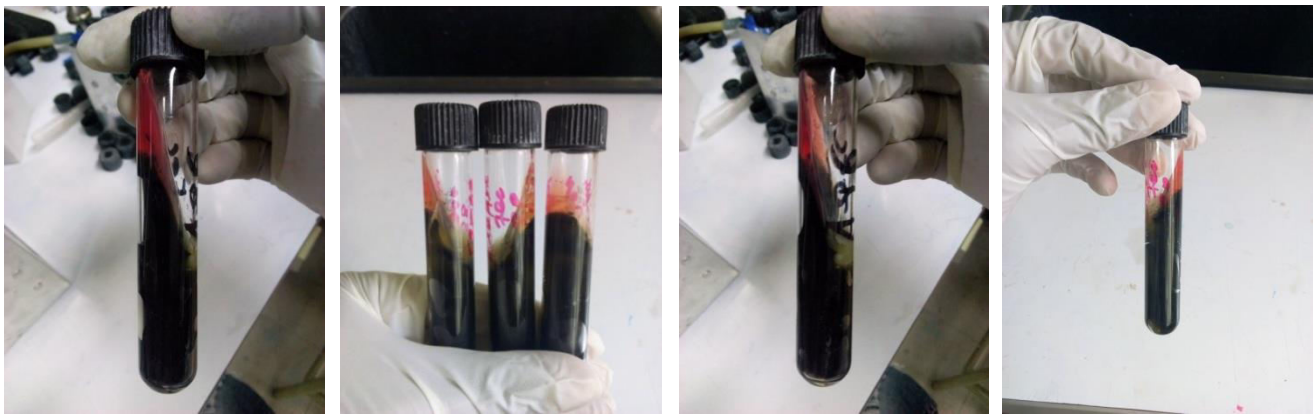

Fig 3: Growth of *Salmonella* on Triple Sugar Iron Agar (TSI).

Table 1: Frequency of *Salmonella* isolated from different sample types in the study area.

Site	Sample type	Sample collected	No. of positives	Percentage of positives (%)
Adama	Bulk tank milk	6	1	(16.7)
	Feces	46	7	(15.2)
	Floor sample	5	1	(20)
	Hand swab	5	-	-
	Sub total	62	8	12.9
Modjo	Bulk tank milk	4	-	-
	Feces	43	1	(2.3)
	Floor sample	4	-	-
	Hand swab	4	-	-
	Sub total	55	1	1.8
	Grand total	**117**	**10**	**8.5**

There is no statistically significant difference between isolates derived from different sample types of the studied dairy farms ($\chi 2$=0.966, p=0.809). From a total of 10 (8.5%) isolates, nine (90%) were from Adama and 1 (10%) was from Modjo. There is statistically significant difference between isolates derived from Adama and Modjo of studied sites ($\chi 2$=6.012, p=0.014) (**Table 1**).

Antibiotic Susceptibility Testing - All the 10 isolates were tested against ten commonly used antimicrobials (**Table 2**). All isolates were resistant at least to one or more antimicrobials. Nine of the 10 isolates were resistant to two or more antimicrobials. The antibiotic susceptibility profiles of the isolates showed that the isolates were 60% resistant to all of the antibiotics which are streptomycin, methicillin and nalidixic acid. Gentamycin was the most effective antibiotic. Ninety percent (9/10) of the isolates were found to be susceptible to gentamycin (**Fig 4**).

Multi-Drug Resistance Frequency Distribution - Among 10 resistant isolates, 8 (80%) were resistant to two or more antimicrobials (multidrug resistance (MDR)). The large proportion of multi-drug resistant isolates 6 (60%) were resistant to four to seven different antimicrobials while the other two resistant isolates were resistant to a single antimicrobial. 2 (6.67%), 5 (16.67%), 4 (13.33%), and 7 (23.33%) were tetra-resistant, quadra-resistant, hexa-resistant, hepta-resistant, respectively with 11 different resistance patterns. Among 8 MDR isolates, 6 (75%) from feces, 1 (12.5%) bulk tank milk, and 1 (12.5%) floor sample isolates (**Table 3**).

Sensitivity to antimicrobial agents - Sensitivity test to the ten *Salmonella* isolates against 10 antibacterial agents was carried out. 60% of *Salmonella* isolates were found resistant to streptomycin, methicillin and nalidixic acid, and 90% of the isolates sensitive to gentamycin (**Fig 5**).

DISCUSSION

Salmonellosis is the most common food borne disease in both developing and developed countries, although incidence rates vary according to the country (Stevens *et al*., 2006). The fecal wastes from infected animals and humans are important sources of bacterial contamination of the environment and the food chain (Ponce *et al*., 2008).

Salmonella infection in dairy cattle persists to be a major problem worldwide. Considerable economic losses were manifested through mortality and poor growth of infected animals as well as the risk of transmission to humans either through food chain or direct animal contact (Alam *et al*., 2017). Hence, detection of animals contacting humans and equipment are essential to control *Salmonella* on farm and its spread to the public (Rotimi *et al*., 2008).

Table 2: Antibiotic susceptibility profiles of *Salmonella* isolates in dairy farms.

Types of antimicrobials	No. of isolates		
	Resistant (%)	Intermediate (%)	Susceptible (%)
Ciprofloxacin 5 µg	10	30	60
Cefoxitin 30 µg	20	0	80
Gentamycin 10 µg	10	0	90
Nalidixic acid 30 µg	60	10	30
Methicillin 5 µg	60	0	40
Cefuroxime 30µg	10	20	70
Erythromycin 15 µg	50	30	20
Tetracycline 10 µg	50	10	40
Nitrofurantoin 300 µg	10	20	70
Streptomycin 10 µg	60	00	40

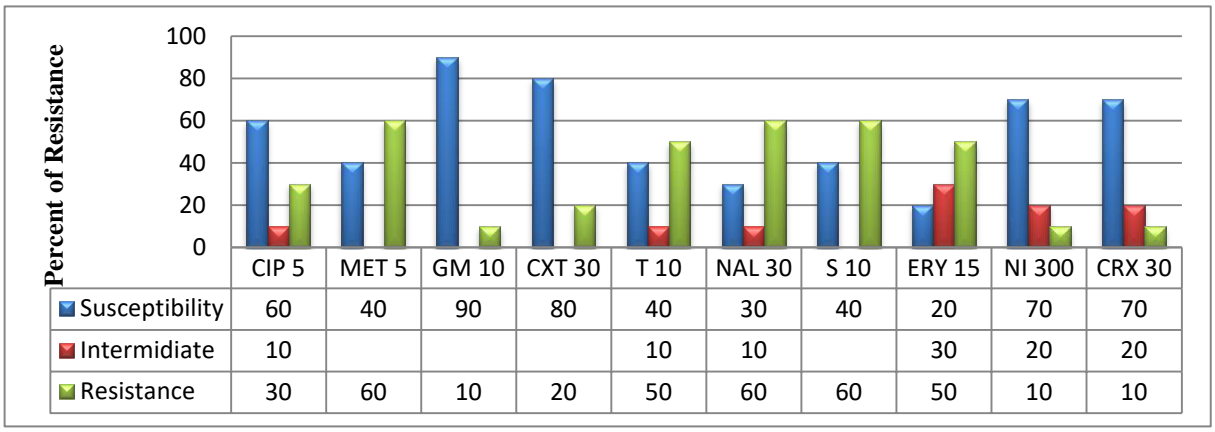

Fig 4: Antimicrobial susceptibility profiles of *Salmonella* isolates.

Table 3: Multiple antimicrobial resistances of the isolated *Salmonella.*

Number of antimicrobial resistances	Antimicrobial resistance patterns (number of isolates)	Number of isolates (%)
Three	2	2(20)
Four	3	3(30)
Six	2	2(20)
Seven	1	1(10)

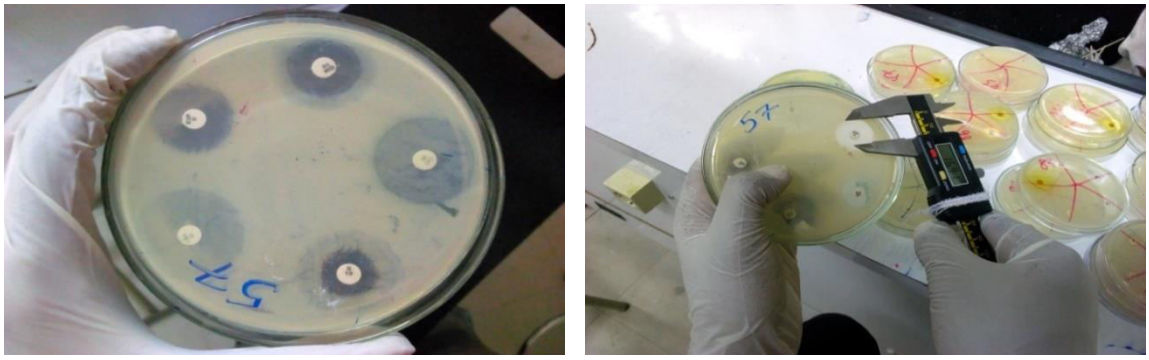

Fig 5: Antimicrobial Susceptibility of *Salmonella* to Different Antibiotics.

The present study was conducted to investigate *Salmonella* from cow's feces, equipment, personnel's hand swab and the contamination of cattle's environment in dairy farms from Adama and Modjo. In this study, the prevalence of *Salmonella* was (8.5%) is lower than (20%) in raw milk from Kersa district; Ethiopia (Tadesse and Anbessa, 2012), (12.5%) This was relatively comparable with studies conducted in other parts of Ethiopia such as Harar (11.5%) (Ayalu *et al*., 2011), Adama (8.6%), Modjo (10.5%) (Fufa *et al*., 2017), and Butajira (10.5%) (Demissie *et al*., 2017), but it was higher than another study conducted in Adama (5.7%), (Beyene *et al*., 2016) (6.5%).The variation among the above-mentioned findings might be largely due to substandard environmental and personal hygiene, ignorance of health promotion, and methodological difference (sample size, study design, study period, and diagnostic techniques).

Fecal prevalence of *Salmonella* among lactating dairy cattle in the current study was 8.98% (8/89) which is higher than the fecal *Salmonella* isolation rate of 7.7% in lactating cows and in contact humans in dairy farms of Addis Ababa (Zelalem *et al*., 2011) and (Fufa *et al*., 2017). However, it is lower than the fecal *Salmonella* isolation rate of 9.7% in United States (Callaway *et al*., 2005). The current study also revealed 10% (1/10) of *Salmonella* isolates from bulk tank milk which is lower than that reported by 19% (4/21) (Fufa *et al*., 2017) in and around Modjo, but higher than the work of (Teklu and Negussie, 2011) (8.9%) in slaughtered small ruminants and environment in Modjo export abattoir. The difference in amount and relative occurrence of *Salmonella* isolate between the present and previous studies at different areas of the Ethiopia could be attributed to difference in 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 (Liza, 2003). Environment related risk factors such as hygienic and management practice, stocking density, type and amounts of feed, accessible water supplies, usage of contaminated utensils, housing type, ventilation, and movement of animals, calving environment, and production facilities in different areas also play role for *Salmonella* occurrence (Karin *et al*., 2011).

Resistance for two or more of antimicrobials (90%) which was observed in this study was higher than other studies conducted in Ethiopia (Molla *et al*., 2006; Zelalem *et al.*, 2011), but lower than (96.4%) (Fufa Abunna *et al*., 2017).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 (Mathew *et al*., 2007).

Zewdu and Cornelius (2009) reported that the isolates of *Salmonella* from food items and personnel from Addis Ababa were resistant to the commonly used antibiotics including streptomycin, ampicillin, and tetracycline. The result of the current research also indicated resistance of *Salmonella* isolates to commonly used antimicrobials including strepto-mycin, methicillin and nalidixic acid with resistance rate of 60% to all of the antibiotics mentioned above (Rahman *et al*., 2019). Among all isolated *Salmonella*, in the current study, 60% of them were resistant to streptomycin.

This finding is lower than other reports (Beshatu Ferede, 2014) (81.8%), (Zelalem, 2011) (66.7%), but higher than other reports (10.7%), (Beyene *et al*., 2016) (19.4%). This finding is higher than the previous report from Addis Ababa (Zelalem *et al.*, 2011) which reported 26.7% resistance to tetracycline. In the current study gentamycin showed a good antimicrobial activity against 90% of the tested isolates and one intermediate isolate against this drug was detected. This result is higher than the reports by (Zelalem *et al*., 2011) from Addis Ababa who reported a resistance rate of 73.3%, but lower than the report by (Fufa *et al.,* 2017).

Among 10 resistant isolates, 8 (80%) were resistant to two or more antimicrobials (resistant to two or more antimicrobials). This finding is in line with previous report by (Zelalem *et al*., 2011) from Addis Ababa on lactating dairy cows and in contact humans in dairy farms in which 83.3% of the isolates were resistant to greater than one antimicrobial agent (s) and all isolated from dairy farm related samples were resistant to at least one antimicrobial agent (Happy *et al*., 2018).

CONCLUSION AND RECOMMENDATIONS

This cross-sectional study showed that *Salmonella* was isolated (8.5%) from dairy farms in Adama (12.9%) and Modjo (1.8%) with variable isolation rate. This result is significantly high to be a potential source of food borne Salmonellosis in humans. High proportion (80%) of *Salmonella* isolates were resistant to two or more of the antimicrobials that are commonly used in the veterinary and public health set up. This may pose difficulties in the treatment of human clinical cases and other bacterial diseases. Based on the above conclusion the following recommendations are forwarded: Hygiene status of the dairy farms should be improved to minimize cross contamination of *Salmonella* from milking containers and cattle's environment, Since *Salmonella* is resistant to most common drugs, attention should be taken in selecting antimicrobials in treating *Salmonella* infection both in animals and human being based on antimicrobial susceptibility test, Further study ought to be conducted to identify the source of contamination, and Molecular characterization of the isolates with emphasis on resistant strains is also necessary to identify mechanisms of antibiotic resistance.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest about the publication of the article.

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