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ANTIMICROBIAL USE AND SUSCEPTIBILITY PROFILE OF SELECTED ZOONOTIC PATHOGEN IN DAIRY FARM AND THEIR PUBLIC HEALTH IMPLICATION IN ADAMA AND MODJO, ETHIOPIA

MVSc Thesis

By

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BISHOFTU, ETHIOPIA

ANTIMICROBIAL USE AND SUSCEPTIBILITY PROFILE OF SELECTED ZOONOTIC PATHOGEN IN DAIRY FARM AND THEIR PUBLIC HEALTH IMPLICATION IN ADAMA AND MODJO, ETHIOPIA

A Thesis Submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Veterinary Science in Veterinary Public Health

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Approval Sheet

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Title**: Antimicrobial Use and Susceptibility Profile of Selected Zoonotic Pathogen in Dairy Farm and Their Public Health Implication in Adama and Modjo, Ethiopia**

As member of the Examining Board of the final MVSc open defense, we certify that we have read and evaluated the Thesis prepared by: Munera Ahmednur, titled: **Antimicrobial Use and Susceptibility Profile of Selected Zoonotic Pathogen in Dairy Farm and Their Public Health Implication in Adama and Modjo, Ethiopia** and recommended that it be accepted as fulfilling the thesis requirement for the degree of Master of Science in Veterinary Public Health.

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STATEMENT OF AUTHOR

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

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LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celsius
AAU-CVMA	Addis Ababa University College of Veterinary Medicine and Agriculture
AG	Aminoglycoside
AMR	Antimicrobial Resistance
AMU	Antimicrobial Use
BPW	Buffered Peptone Water
CDC	Centera for Diseas Contorol and prevetion
CLSI	Clinical Laboratory Standard Institute
E. coli	*Escherichia coli*
EHEC	Entero hemorrhagic E. coli
EMB	Eosin Methylene Blue
H_2S	Hydrogen Sulfide
HGT	Horizontal Gene Transfer
ISO	International Organizations for Standardization
KAP	Knowledge, Attitudes and Practices
Km `	Kilometer
KOH	Potassium Hydroxide
MDR	Multidrug Resistant
m-RNA	Messenger Ribonucleic Acid
OM	Outer Membrane
PBP	Penicillin Binding Proteins
r-RNA	Ribosomal Ribonucleic Acid

RVSB	Rappaport Vassiliadis Soy broth
S. aureus	*Staphylococcus aureus*
SFB	Selenite F Broth
S-S	Salmonella Shigella
t-RNA	Transfer RNA
TSA	Tryptic Soy Agar
TSI	Triple Sugar Iron Agar
VP	Vogue Proskauer
WHO	World Health Organation
XLD	Xylose Lysine Deoxycholate

ABSTRACT

A cross-sectional study was conducted from November 2018 to May 2019 in Adama and Modjo. The main objectives of the study were to isolate and identify selected zoonotic pathogens from dairy cattle, to assess practical usage of drugs and to determine the *in vitro* antimicrobial resistance profiles of the isolates. Structured questionnaire was designed and administered to dairy farm owners and dairy food consumers to assess their consumption behavior and antibiotics usage. A total of 607 samples consisting of fresh cow milk, fecal sample, nasal swab, pooled milkers' hand swabs, pooled floor swabs and tank milk samples were collected from 20 dairy farms. The samples were examined for the presence of selected zoonotic pathogens. Total of 396 samples were analyzed for *S. aureus* and 15.7% (62) were positive. Total of 396 samples were analyzed for *E. coli* and 7.6% (30) were positive. From 271of the total analyzed samples for *Salmonella* 4.8% (13) were positive. A total of 62 *S. aureus*, 30 *E. coli* and 13 *Salmonella* isolates were observed resistant to at least one or more antimicrobials tested. Penicillin, Methicillin and Sulphametoxazole trimethoprim were drugs to which a large proportion of isolated *S. aureus* were (100%to90%) highly resistant. From 30 tested *E. coli* show resistance to Tetracycline (83%) and Vancomycin (80%). The resistances of Salmonella were 69% to Nalidxic Acid and 54% to Vancomycin. Multiple drug resistance was detected in high (98.4%) for *S. aureus*, (56.7%) for *E. coli* and (53.9%) for *Salmonella*. High proportion of the current misuses of drugs and occurrence of resistance isolates to drugs in the dairy farms alerts concern for animal and public health as these drugs is used widely for treatment and prophylaxis in animals and humans.

Keywords: *Dairy farms*; *E. coli; Multidrug resistance; Salmonella; S. aureus. Ethiopia*

1. INTRODUCTION

The safety of dairy products and by products with respect to food borne disease is a great concern worldwide. Raw milk may contain pathogenic microorganisms and it may occasionally play a great role in the transmission of this pathogenic microorganisms to humans (Vahedi *et al.*, 2013).The consumption of raw milk and its derivatives is common in Ethiopia, which is not safe for consumers in a health point of view as it may lead to the transmission of various diseases. Even though milk from a healthy udder contains a few bacteria, it picks up many bacteria from the time it leaves the teat of the animal depending on the hygienic level exercised during milking. These microorganisms are indicators of both manner of handling milk from milking till consumption and the quality of the milk (Elmoslemany *et al.*, 2010; Hadrya *et al.*, 2012; Lunder and Brenne, 1996; Shunda *et al.*, 2013).

The milk and milk products have been a threat to the human, because it may contains pathogenic microorganisms. This pathogenic microorganism has been a threat living things. To counter these living threat agents, several measures, especially administration of antimicrobials, are employed globally. Antibiotics are natural, synthetic, or semi-synthetic substances which interfere that the growth of or kill microorganisms, specifically bacteria, and are used to treat or prevent infections in humans and animals. Antibiotics are now an "endangered species" facing extinction due to the worldwide emergence of antimicrobials resistance (AMR) and the void in the development of new therapeutic substances (O'Neill, 2015; WHO, 2016).Know a day a global analysis of antimicrobial usage revealed that, the worldwide consumption of antimicrobials in food animal production is estimated at ≥57,000t (1 t = 1,000 kg) and projected a 67% increase in total usage by 2030 to ≥95,000 t (Van Boeckel *et al.*, 2015).

Antimicrobial resistance (AMR) means the ability of a microorganism to survive and reproduce in the presence of antibiotic doses that were previously thought effective against them which has become an emerging problem globally. In present time, AMR both in human and veterinary medicine has reached alarming levels in most parts of the world and has been recognized as a significant emerging threat to global public health and food security (Chuanchuen *et al.*, 2014). Even though, global livestock production has been growing rapidly and has moved increasingly where antimicrobial use (AMU) is an integral part of production their misuse or over use may produce antimicrobial-resistant bacteria (Phu *et al.*, 2016).

Research over the last 40 years has been suggested that AMR in east Africa is associated with human-animal contact, high levels of antibiotic use in small production systems, lack of withdrawal for human consumption of meat and milk products from recently treated animals, and frequent or less prudent AMU, which is reported to be one major reason for failure of treatment of infectious diseases which brought the AMR (Erb *et al.,* 2007). In Ethiopia the control of drugs from the government authorities and information on the actual rational drug use pertaining to veterinary drug use is very limited and misuses of drugs are common among the various sectors including veterinary and public health (Bedada *et al.*, 2012).

In Ethiopia, milk production systems can be categorized into urban, peri-urban and rural, based on location, which around 97% of the annual milk production is accounted by the traditional milk processing system (Felleke, 2003).The traditional milk processing materials used are also similar among different areas which generally poor in quality of processing, includes; plastic container, Bottle gourd and clay pot (Duguma and Janssens, 2014; Wafula *et al.*, 2016). The fresh milk is sold unpasteurized to the public either directly from small producers, via informal markets or through dairy farmers cooperatives which has been a great challenge for milk quality control at all levels (Godefay and Molla, 2000).

Antibiotics have been used in the dairy industry for more than five decades to treat or prevent disease and to increase milk production or improve feed efficiency (Riediker *et al.,* 2004). As such, food products such as milk, cheese, yoghurt and other dairy products have been implicated as potential sources for the transmission of the pathogen to humans (Normanno *et al.,* 2007).

Furthermore, foods may contaminated with antibiotic resistant bacteria represent ideal vehicles for the transmission of antibiotic resistant strains (Angulo *et al.,* 2004; Phillips *et al.,* 2004). So, food is an important vehicle for the transfer of AMR factor to intestinal tract of consumer's very efficiently (Hageman *et al.,* 2006; Spanu *et al.,* 2012). This further transfer of AMR bacteria to humans via the food chain (Angulo *et al.,* 2004) and from livestock has been well documented, which indicates livestock and livestock product like milk and meat may serve as reservoirs for human infections (Normanno *et al.,* 2007).

Recently several antimicrobial-resistant food-borne pathogens have been emerged in the food-production chain, which can transmit to, and cause infections in, humans. *Escherichia coli, Salmonella and Staphylococcus aurous* resistance to antimicrobials is one of them and creating trouble to the healthcare system worldwide (Kashef *et al.,* 2010). Different studies also conducted in Ethiopia revealed fragmented substantial prevalence as well as antimicrobial susceptibility of *Salmonella*, *E. coli* and *S. aureus* in veterinary and public health setups (Alemayehu *et al.,* 2003; Beyene and Tesega, 2014; Ejeta *et al.,* 2004; Hailu *et al.,* 2015; Molla *et al.,* 2003, 2006; Zewdu and Cornelius, 2009). However, reports from coinciding study on apparently healthy animals at farm level, personnel and equipment used in the farms is limited especially in the current study area. Additionally, the risk of consumption of contaminated milk by these resistant bacteria and the selection of resistance in human due to on farm drug misuse or drug residue in milk is not well confirmed. So, this study aimed isolate and identify selected zoonotic pathogens from dairy cattle, to assess practical usage of drugs and to determine the *in vitro* antimicrobial resistance profiles.

Therefore, the objectives of the current study were:

- ✓ To isolate and determine antimicrobial susceptibility profile of *E. coli*, *S. aureus and Salmonella* from selected dairy farm.
- ✓ The possible health risks to consumers based on the presence of antibiotic resistance profiles of the isolates were also investigated.

2. LITRATURE REVIEW

2.1. The Emergence of Antimicrobial Resistance in Bacteria

Antimicrobial resistance was first described in 1940 in Bacillus coli (now known as *Escherichia coli*) by Abraham and Chain (1940), shortly before the start of the use of penicillin to treat infectious diseases in humans in the same year (Chain *et al.,* 1940) and not long after its discovery by Fleming (1929). Since most antimicrobials in clinical use are naturally produced by soil microorganisms, such microorganisms are the source of many resistance genes now found in clinically relevant bacteria, as was demonstrated more than 40 years ago (Benveniste and Davies, 1973). Further phylogenetic analysis has shed some light on the evolutionary origins of resistance, indicating that bacteria evolved AMR genes long before the antibiotic era (Baquero *et al.,* 2008; Finley *et al.,* 2013; Wellington *et al.,* 2013), and even developed defenses against synthetic compounds (D'Costa *et al.,* 2011). There is growing evidence that AMR is in fact an ancient and natural part of the genome of environmental bacteria (Bhullar *et al.,* 2012). However, it is important to realize that AMR was very rare in clinical isolates predating the introduction of antibiotics, as demonstrated in a retrospective analysis by Høiby *et al.* (2010), which provides strong evidence for the central role of AMU in the emergence and spread of AMR as a public health threat.

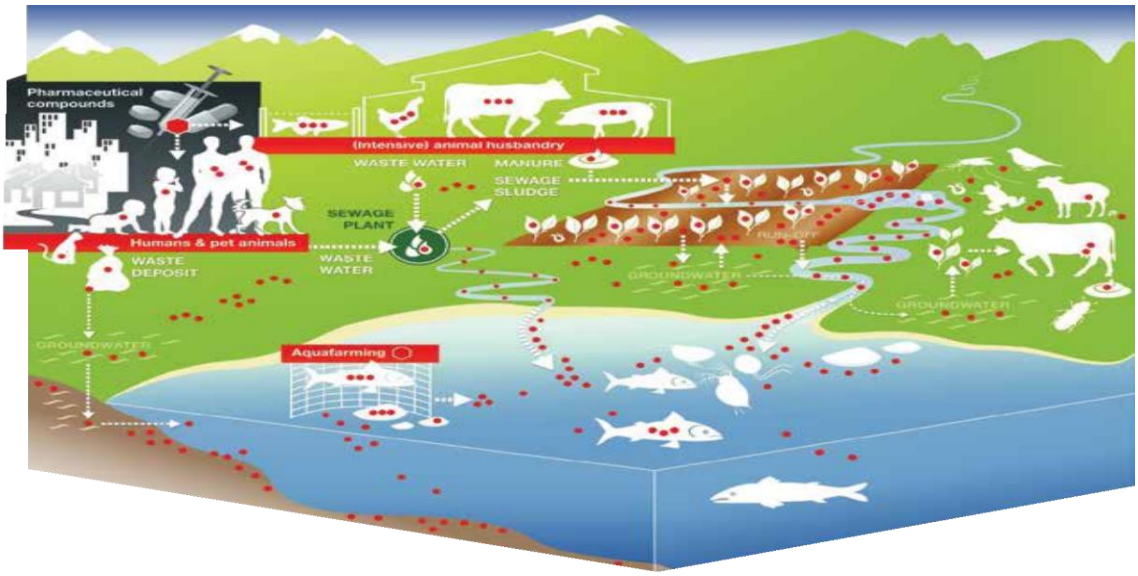

Figure 1: Antimicrobial usage in humans, animals and agriculture.

Resulting dispersion of antimicrobial residues in to aquatic and terrestrial environments (represented by red dots).

Source:(Van Boeckel *et al.*, 2015).

2.2. Antimicrobial Use and Resistance Development

Antimicrobial drug use is the single most important factor leading to resistance (CDC, 2013). Antimicrobial drugs are used in a variety of settings including hospitals, outpatient clinics, and long-term care facilities as well as animal-associated settings such as veterinary clinics, farms, and feedlots. They are used widely to prevent or treat disease in food animals. The major part of the usage is for prevention of disease where nearly all feed for growing animals is supplemented with antimicrobials in various doses, ranging from so-called "sub-therapeutic concentrations" to full therapeutic doses. Antimicrobial agents are also used in food processing plants to eliminate the growth of microorganisms that affect the quality and safety of the end products (Marshall and Levy, 2011).

Apart from decade of research and a considerable body of scientific evidence, the link between antimicrobial drug use on farms and antimicrobial resistant infections in humans remains contested by critics (Marshall and Levy, 2011). It is estimated that the volumes of antimicrobials used in food animals exceeds the use in humans worldwide, and nearly all the classes of antimicrobials that are used for humans are also being used in food animals (Aarestrup *et al.*, 2008). Excessive use and misuse of antimicrobials are widely recognized as two of the major drivers for acquired AMR, both directly and indirectly, due to the selection pressure imposed on human and animal microbiota (WHO, 2016), and on environmental bacteria (Martinez and Baquero, 2001).

Use of antimicrobials may unblock gene expression, resulting in the development of resistance genes in bacteria (Courvalin, 2008; Lambert, 2012) or promoting the occurrence of mutations (Martinez and Baquero, 2001). The resistant gene can be transferred from agricultural and food bacteria, via gene swapping, to bacteria that cause diseases in people. This issue is a worldwide public health concern because a resistant infection can spread from one person to many others. The persistence of antimicrobial residues in feed and animal waste contaminating soil and water also affects the aquatic and environmental microbiomes (You and Silbergeld, 2014). It has been estimated that 75 to 90 percent of antimicrobials used in livestock are excreted, mostly unmetabolized and the concentration of antimicrobial residues in these farm environments is likely to be high, which is a crucial risk factor for the emergence of AMR (Marshall and Levy, 2011).

Prolonged antimicrobial exposure has been associated with acquisition of multidrug resistance in enteric bacteria in both humans and animals (Levy and Marshall, 2004), and in aquaculture (Nonaka *et al.*, 2007). The use of combinations of antimicrobials may also result in the selection of multiple drug resistance (MDR) bacterial strains (Martinez and Baquero, 2001). Antimicrobials at low dosages (i.e. residual levels, sub-lethal or sub-therapeutic dosages) are also factors contributing to resistance as they promote genetic and phenotypic variability in exposed bacteria (Andersson and Hughes, 2014; You and Silbergeld, 2014).

Dairy markets in most sub-Saharan African countries are mostly supplied by small-scale farm enterprises. Sick dairy animals are often treated with antibiotics; failure to follow required withdrawal time/periods after these treatments is the most commonly cited reason for drug residues in marketed milk (Zwald *et al.,* 2004). They are also used prophylactically to prevent the occurrence and spread of infections in intensive production. The presence of antibiotics drug residues in milk above allowable limits is a serious food safety risk because it may lead undesirable effects which can be classified as toxicological, selection of resistant strains of bacteria and allergic reactions. A major public health concern is the increasing occurrence of strains of bacteria resistant to antibiotics (Kurwijila *et al.,* 2012). This phenomenon has resulted in the removal or restriction on the use of certain antibiotics and prolonged exposure to antibiotics as a result of consuming residues contained food is contributing to antibiotics resistance (Shitandi, 2004).

Milk can be contaminated with feed pathogens that glycerol from an organic boric acid and the alkalinity exhibit resistance to antibiotic and raw milk products have been implicated as mechanisms for transferring antibiotic resistant organism from farm environments to humans (Kalman *et al.,* 2000). Antibiotic residues may also impact the manufacturing process of milk products. The public health risk to antibiotics residues seems particularly high in developing countries, where there may be no local legislations regulating maximum tolerance limits for marketed products and recommended withdrawal times are violated (Kurwijila *et al.,* 2012; Shitandi, 2004).

2.3. Antimicrobial Resistance

2.3.1. Antimicrobial Agent and Mechanism of action

The term antibiotics encompass a wide range of chemical substances that are produced naturally, semi-synthetically, and synthetically, and are used to inhibit (bacteriostatic) bacterial growth or kill them (bactericidal) (Gillings, 2013; Martínez, 2012; Milić *et al.,* 2013). They are categorized based on their effects as either bacteriostatic or bactericidal, and on their series of efficacy, as narrow or broad-spectrum antibiotics. The most commonly used antibiotics in feed animals can be grouped into five major classes. These include the beta-lactams (ß-lactams) like Penicillins and

Cephalosporins, Tetracyclines like Oxytetracycline, Tetracycline and Chlortetracycline, Aminoglycosides like Streptomycin and Gentamicin, Macrolides like Erythromycin and Sulfonamides which also include Sulfamethazine (Mitchell *et al.*, 2013).

Antibiotics kill or inhibit the growth of microorganisms in several ways with variation from one antibiotic to the other. So, the drug can kill or inhibit the growth of microorganisms by inhibiting cell wall synthesis, inhibiting nucleic acid function, antagonizing the metabolic activity, damaging cell membrane, or inhibiting protein synthesis (Dubey and Maheshwari, 2005). The β-lactams drug inhibits cell wall synthesis. The primary targets of the β-lactam agents are the penicillin binding proteins (PBPs).It has been hypothesized that the β-lactam ring mimics the D-alanyl D-alanine portion of peptide chain that is normally bound by PBP. The PBP interacts with β-lactam ring and are not available for the synthesis of new peptidoglycan. The disruption of peptidoglycan layer leads to the lysis of bacterium (Džidić *et al.*, 2008).

Some antimicrobials inhibit protein biosynthesis by targeting the 30S or 50S sub unit of the bacterial ribosome (Johnston *et al.*, 2002; Vannuffel and Cocito, 1996). The aminoglycosides (AG's) drugs are one of them and are positively-charged molecules which attach to the outer membrane (OM) which is negatively charged leading to formation of large pores, and thus allow antibiotic penetration inside the bacterium. AG's works by interacting with the 16S r-RNA of the 30S sub unit then, they cause misreading and premature termination of translation of mRNA. The Tetracyclines, such as Tetracycline, Chlortetracycline, Doxycycline, or Minocycline, are also act upon the conserved sequences of the 16S r-RNA of the 30S ribosomal sub unit to prevent binding of t-RNA to the A site (Wise, 1999; Yoneyama and Katsumata, 2006).

Others drug like macrolides are affect the early stage of protein synthesis, namely translocation, by targeting the conserved sequences of the peptidyl transferase center of the 23S r-RNA of the 50S ribosomal subunit, which results in a premature detachment of incomplete peptide chains. The sulfonamides groups of drugs are work by inhibiting dihydropteroate synthase in a competitive manner with higher affinity for the enzyme than the natural substrate, p-amino benzoic acid then; agents such as trimethoprim work by acting at a later stage of folic acid synthesis and inhibit the enzyme dihydrofolate reductase (Yoneyama and Katsumata, 2006).

2.3.2. Mechanism of Resistance Development and Bacterial Resistance Transfer

Resistance is the ability of a micro-organism to withstand the effects of antibiotics. Antibiotic resistance may evolve via natural selection acting upon random mutation. It can also be engineered by applying an evolutionary stress factor on a population of bacteria. Resistance may either phenotypic or genotypic. There are two types of phenotypic resistance, the L-form bacteria which lack a cell wall but survive in an isosmolar environment despite continued exposure to antibiotics (Inglis, 2003) and the other type is called persisters because they represent a small fraction of the initial bacterial population that persists after the start of antimicrobial therapy (Adam *et al.*, 2008). Genotypic resistance is determined by resistance genes carried in the chromosome or plasmids and there is a possibility of genes being re-assorted leading to a unique recombinant. Recombination ensures more efficient adaptation to rapidly changing antibiotic environment (Jacob, 2007).

Sequences of genes that could recombine with both the chromosome and plasmids are known as transposons. The accumulation of resistance factors on a transposon may result in chromosomally coded antibiotic resistance which transferred first to a plasmid and then to other species. Finally, one of the most efficient mechanisms for accumulating antimicrobial resistance genes is represented by integrons, which are site-specific recombination systems capable of recruiting open reading frames in the form of mobile gene cassettes. Integrons provide an efficient and rather simple mechanism for the addition of new genes into bacterial chromosomes, along with the necessary machinery to ensure their expression; a robust strategy of genetic interchange and one of the main drivers of bacterial evolution (Inglis, 2003).

Resistance to antibiotics may also be either intrinsic or acquired. Intrinsic resistance is the innate ability of a bacterial species to resist activity of an antimicrobial agent through its inherent structural or functional characteristics, which allow tolerance of a particular drug or antimicrobial class (Abebe *et al.*, 2016). This can also be called "insensitivity" since it occurs in organisms that have never been susceptible to that drug. Such natural insensitivity can be due to: lack of affinity of the drug for the bacterial target, inaccessibility of the drug chromosomally encoded active exporters and innate production of enzymes that inactivate the drug (Ahmad *et al.*, 2009). Acquired resistance is applicable to those organisms that were previously susceptible to the antibiotics. This

form of resistance causes great concern because of its potential for reducing the range of previously useful antibiotics available. Acquired resistance can be further divided into horizontally acquired resistance and mutational acquired resistance (Ahmad *et al.,* 2009).

Horizontally acquired resistance refers to the situation where resistance emerges because of horizontal gene transfer (HGT). Three mechanisms have been identified for inter- and intra-transfer of genetic material and resistance genes; those include transformation, transduction and conjugation. Transformation involves direct transfer of free DNA originating, for example from lysed bacteria. Transformation may the simplest type of HGT, but only a handful of clinically relevant bacterial species are able to "naturally" incorporate naked DNA to develop resistance. In transduction transmission of genetic material from one bacterium to another is done by bacteriophage, which is common in gram positive bacteria (Hotopp, 2011).

Conjugation uses mobile genetic elements such as plasmids and transposons as a vehicles to share valuable genetic information, although direct transfer from chromosome to chromosome has also been well characterized (Manson *et al.,* 2010). A plasmid or other genetic material is transferred from the donor bacterium to the recipient via cytoplasmic bridge (pilus). Conjugation may occur between bacteria of the same species, within species of the same genera or between species of different families. Emergence of resistance in the clinical environment often involves conjugation, a very efficient method of gene transfer that involves cell-to-cell contact and is likely to occur at high rates in the gastrointestinal tract of human's under antibiotic treatment (Thomas, 2000).

Mutational acquired resistance occurs when the bacterial genome mutates to overcome the effect of an antibiotic and normally only involves the change of one or a few nucleotides. In many instances, mutational changes leading to resistance are costly to cell homeostasis and are only maintained if needed in the presence of the antibiotic. Resistance to antibiotics typically occurs by one or more of the following mechanisms. These mechanisms can be organized into five broad categories; decreased accumulation of the antimicrobial within the cell, either through diminished permeability and/or active efflux of the antimicrobial from the bacterial cell, enzymatic modification or degradation of the antimicrobial, acquisition of alternative metabolic pathways to

those inhibited by the antimicrobial, modification or protection of the antimicrobial target and overproduction of the target enzyme (van Hoek *et al.,* 2011).

Bacteria have developed mechanisms to prevent the antibiotic from reaching its intracellular or periplasmic target by decreasing the uptake of the antimicrobial molecule however; many of the antibiotics used in clinical practice have intracellular bacterial targets or, in case of gram negative bacteria, located in the cytoplasmic membrane (the inner membrane). Hydrophilic molecules such as β-lactams, Tetracyclines and some Fluoroquinolones are particularly affected by changes in permeability of the outer membrane since they often use water-filled diffusion channels known asporins to cross this barrier (Pages *et al.,* 2008).

Membrane proteins that export antibiotics from the cell and maintain their low-intracellular concentrations are called efflux pumps (Džidić *et al.,* 2008). At the same speed, where these antimicrobials are entering the cell, efflux mechanisms are pumping them out again, before they reach their target (Wise, 1999). These pumps are present in the cytoplasmic membrane, unlike porins which are present in OM. These systems may be substrate-specific or with broad substrate specificity, which are usually found in MDR bacteria (Poole, 2005). Antibiotics of all classes except polymyxin are susceptible to the activation of efflux systems (Lambert, 2002). Efflux pumps can be specific to antibiotics. Most of them are multidrug transporters that are capable to pump a wide range of unrelated antibiotics such as, macrolides and Tetracyclines (Džidić *et al.,* 2008).

The production of enzymes capable of introducing chemical changes to the antimicrobial molecule is a well-known mechanism of acquired antibiotic resistance in both gram-negative and gram-positive bacteria. The resulting effect is often related to steric hindrance that decreases the avidity of the drug for its target. One of the best examples of resistance via enzymatic modification of the drug is the presence of aminoglycoside modifying enzymes (AMEs) that covalently modify the hydroxyl or amino groups of the aminoglycoside molecule (Ramirez and Tolmasky, 2010; Wilson, 2014).

Natural variations or acquired changes in the target sites of antimicrobials that prevent drug binding are a common mechanism of resistance. Target site changes often result from spontaneous mutation of a bacterial gene on the chromosome. Since antibiotic interaction with target molecule is generally quite specific, minor alteration of the target molecule can have important effect on antibiotic binding and the most common mechanisms of antibiotic resistance of bacteria. These target changes may consist of; point mutations in the genes encoding the target site, enzymatic alterations of the binding site and/or replacement or bypass of the original target. Although some of the genetic determinants coding for proteins that mediate target protection have been found in the bacterial chromosome, most of the clinically relevant genes involved in this mechanism of resistance are carried by mutations in the genes encoding (Kapoor *et al.,* 2017). Mechanism of drug resistance was summarized in Figure 1.

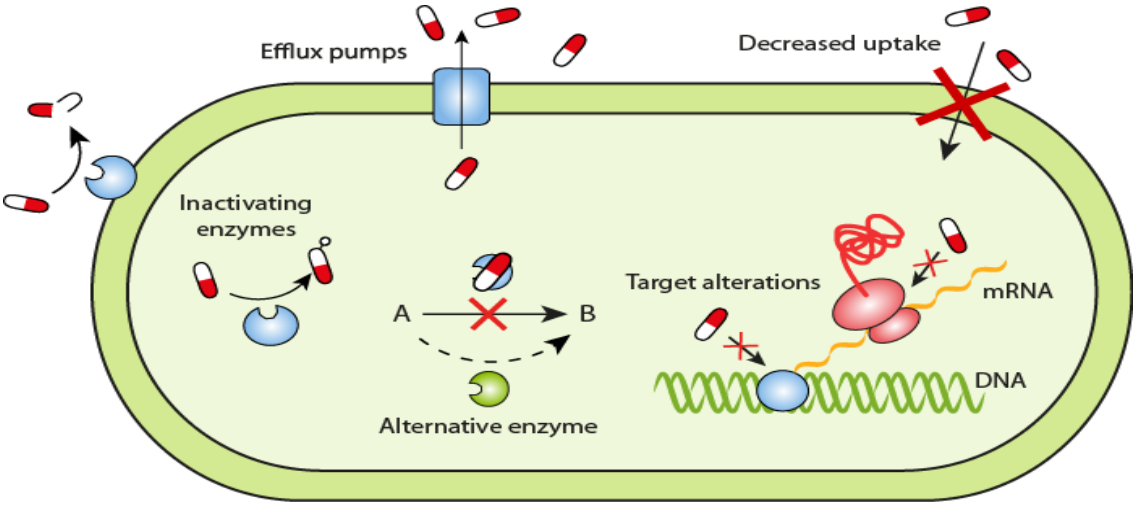

Figure 2: Mechanism of Drug Resistance

Source : (Martínez and Baquero, 2014).

Biofilm is a structured population of bacteria embedded in a matrix, which is composed by polysaccharides, protein and extracellular DNA. It has been shown than cells growing in biofilms are less susceptible to antibiotics than those growing planktonically (Singh *et al.,* 2010). The antibiotic resistance associated with biofilms depends on several causes, some due to the structure of the extracellular matrix, some other to the physiological state of biofilm-growing bacteria;

which is different to that of planktonic cells. Even inside the biofilm, bacteria show different metabolic states, because there is a gradient of nutrients and oxygen between the surface of the biofilm and its deeper region. The extracellular matrix may change the activity of the antibiotics by two different reasons; by diminishing the diffusion of the antibiotic or by sequestering it through its binding to the matrix. This is not a general trend, since in several occasions; slow diffusion of the antibiotic is not the most important element in the phenotypic resistance displayed by biofilms. Biofilm production occurs in many loci, including teeth plaque, water environments, medical catheters, trauma wounds, etc (Szczotka-Flynn *et al.,* 2010). As such, microorganisms that are found in biofilms are protected from the entry of multiple antimicrobial agents (Høiby *et al.,* 2010).

2.4. Mechanisms of spread of antimicrobial resistance between animals and humans

Both pathogenic and non-pathogenic resistant bacteria can be transmitted from livestock to humans via food consumption, or via direct contact with animals or their waste in the environment (Marshall and Levy, 2011). Fomites can also play an important role in the local and wider spread of resistant bacteria. In Denmark, farm-to-farm spread of multidrug-resistant Salmonella enterica serovar typhimurium DT204 has been closely studied, and shared farm equipment (e.g. machinery) was identified as an important route (Aarestrup and Schwarz, 2006).

Any mechanism that helps spread bacteria has the potential to transfer resistant bacteria. Resistance may also be conferred by the exchange of genetic elements between bacteria of the same or different strains or species, and such transfer can occur in any environment where resistant bacteria have the opportunity to mix with a susceptible bacterial population, such as in the human or animal gut, in slurry spread on agricultural soil, or in aquatic environments (Baquero *et al.,* 2008; Wooldridge, 2012). An example of a framework in which resistance genes could spread from poultry production to humans. If resistance develops in environmental bacteria, this can create an animal or human health problem when such bacteria contaminate water, food crops or animal feed, introducing the opportunity for bacterial mixing with commensal or pathogenic species in the animal or human gut (Aarestrup, 2006; Finley *et al.,* 2013; Marti *et al.,* 2013).

Many antimicrobial preparations used for livestock are given orally so that antimicrobial residues excreted in animal faeces have the potential to exert selection pressure on bacterial populations in soil or water (Wooldridge, 2012). However, evidence is scarce as to how important this mechanism is in transferring resistance (Hong *et al.,* 2010; Novo *et al.,* 2013), and different antimicrobials have different fates in the environment (Kemper, 2008). It must be considered that residues resulting from human treatment with antimicrobials or from pharmaceutical manufacturing can also exert selection pressure on environmental bacteria (Baquero *et al.,* 2008; Finley *et al.,* 2013; Novo *et al.,* 2013; Wellington *et al.,* 2013). Indeed, effluent from drug manufacturing has been found to contain extremely high concentrations of antimicrobial residues, as previously reported in countries with large pharmaceutical industries such as India (Larsson *et al.,* 2007; Sim *et al.,* 2011; Wellington *et al.,* 2013).

2.5. The Great Challenge of Antibiotics Resistance

The routine employment of antibiotics, for prevention and growth promotion purposes in livestock farming, selects for antibiotic resistance among commensal and pathogenic bacteria. Owing to the fact that most of these antibiotics are not fully metabolized but released into the environment as waste products, antibiotic resistance has an ecological impact, since these waste products still have the potential to influence the bacteria population and promote antibiotic resistance. Cogliani *et al.* (2011) pointed out that the low concentrations of these antibiotics in the environment bring about random and spontaneous mutagenesis. Therefore, the environment has been viewed as a plausible reservoir or pool of antibiotics and antibiotic-resistant bacteria, as well as their resistance genes. It is a situation of great concern to public health facilities worldwide, as bacteria have the capability to transfer resistance genes between strains of the same species and between different species (Marti *et al.,* 2013). This is, however, possible due to the fact that the antibiotic resistance genes are located on elements, including transposons, integrons, and plasmids, that can be immobilized (Chang *et al.,* 2015). The transmission of these resistance genes is termed horizontal gene transfer (HGT) or lateral gene transfer (LGT), and it does occur via transformation, conjugation, and transduction processes (Landecker, 2016). These processes are responsible for the increasing antibiotic resistance worldwide (because of gene transfers between different bacteria species). LGT has been implicated in the distribution of numerous antimicrobial-resistance determinants,

and as the cause of an epidemic in nosocomial and community infections, by conferring resistance to many classes of antimicrobials, which leads to multidrug resistance (Warnes *et al.,* 2012). Moreover, the employment of broad-spectrum antibiotics creates selective pressure on the bacterial flora, thus increasing the advent of multidrug-resistant bacteria which results in the production of new antibiotic-resistant bacteria with cycles of unpleasant treatments (Schjørring and Krogfelt, 2011).

2.6. Impact of Antibiotic Resistance and Ways of Prevention

Combating antimicrobial resistance has been recognized as a priority for global public health. In 2015, WHO issued a global action plan on antimicrobial resistance and recommend each country to formulate its own plan. If we want to preserve antibiotics as a valuable therapeutic tool, we must seriously address the crisis of antibiotic resistance. Treatment of resistant infections is associated with higher costs for second line drugs, additional investigations, and longer hospitalization (Coast *et al.,* 1996). Other indirect costs associated with AMR include productivity losses due to excess morbidity and mortality (Coast *et al.,* 1998; Kaier and Frank, 2010). There is a tested, effective approach to the problem of antibiotic resistance: simply phase out the use of antibiotics as routine animal feed additives and limit the damage which arises from antibiotic use. We know that agricultural antibiotic use increases the human carriage of resistant organisms and that phasing out this use results in a markedly decreased incidence of human carriage (Aarestrup *et al.*, 2001).

Some studies show that, agricultural antibiotic use may be more important than hospital antibiotic use in generating the asymptomatic carrier state. Clearly, discovery of new antimicrobials as well as finding strategies to expand the useful life of existing antibiotics is important to combat the ever-increasing antimicrobial resistance. Study the molecular basis of resistance development so that we can prevent and overcome antibiotic resistance by targeting resistance mechanisms, which will make the existing and novel antibiotics more effective and sustainable. Therefore, to mitigate antibiotic resistance and prevent transfer of resistance gen, we should cautiously use antibiotics from a One Health perspective(Smith *et al.,* 2005).

2.7. Prevalence and Resistance Profile of *Escherichia coli, Salmonella* and *Staphylococcus aureus*

2.7.1. Escherichia coli

Escherichia coli (E. coli) are gram-negative coli, rod-shaped and predominant among the facultative anaerobic bacteria in the gut of humans and animals. They are motile, and some especially those from extra intestinal infections, may produce a polysaccharide capsule. This microorganism was first described by Theodor Escherich in 1885. The majority of *E. coli* rods do not constitute a serious health hazard, but some serotypes can cause food poisoning and alimentary intoxications. These pathogenic *E. coli* can be categorized based on sero-groups, pathogenicity mechanisms, clinical symptoms, or virulence factors (Kaper *et al.,* 2004; Nataro and Kaper, 1998). The most dangerous among them are entero-hemorrhagic *E. coli* strains which produce Shiga toxins (Stxs) and cause hemorrhagic colitis (HC), hemolytic uremic syndrome and thrombotic thrombocytopenic purpura especially serotype O157:H7 (Picozzi *et al.,* 2005; Reuben *et al.,* 2013).

E. coli has been found in the intestines of healthy cattle, deer, goats, and sheep. However, cattle have been identified as a major reservoir and consumption of foods of bovine origin such as beef and dairy products have been associated with some of the largest food poisoning outbreaks in which this organism was identified as the etiologic agent (Bedasa *et al.,* 2018). It can get access to milk and dairy products and considered as a reliable indicator of contamination by manure, soil, and contaminated water which indicates poor sanitary practices during milking and further handling processes (Disassa *et al.,* 2017; Oliver *et al.,* 2009).

Numerous outbreaks associated with Enteropathogenic *E. coli* (EPEC) and Enterohemorrhagic *E. coli* (EHEC) in milk and other foods has been recently reported (Oliver *et al.,* 2009). In Ethiopia, few studies have been done on prevalence, distribution and associated virulent genes of *E. coli O175: H7* in humans, animals or in foods of animal origin (Hiko et al., 2008a). The study conducted on the prevalence and antimicrobial susceptibility pattern of *E. coli and E. coli o157:H7* isolated from traditionally marketed raw cow milk in and around Asosa town shows that out of 380 raw milk samples examined, 129 (33.9%) and 11 (2.9%) were contaminated with E. coli and E. coli O157:H7 respectively (Disassa *et al.,* 2017).

2.7.2. Salmonella

Salmonella is the second leading cause of food borne illness in most developed countries causing diarrhea, cramps, vomiting, and often fever. Salmonella is a diverse bacterial species comprising over 2600 serotypes. There are two species of Salmonella: Salmonella enterica and Salmonella bongori (Guibourdenche *et al.,* 2010). Serotyping differentiates the strains and *Salmonella enterica*is further classified into 6 subspecies (*Salmonella enterica* subspecies *enterica*, *S. enterica* Subspecies *salmae*, S. *enterica* Subspecies *arizonae*, *S. enterica* Subspecies *diarizonae*, *S. enterica* Subspecies *hautenae* and *S. enterica* Subspecies *indica*). Most of the Salmonella serotypes are part of *S. enterica* subspecies *enterica*, and over 99 % of human and animal infections are caused by serotypes under this subspecies (Uzzau *et al.,* 2000).

Members of the genus are gram negative, facultative anaerobic, oxidase-negative, rod-shaped bacteria and most of motile by peritrichous flagella except S. Pullorum and S. Gallinarum, which lack flagella and chemoorganotrophic, with ability to metabolize nutrients by the respiratory and fermentative pathways (D'Aoust and Maurer, 2007; Forshell and Wierup, 2006). *Salmonella* is transmitted to animals and humans through the fecal-oral route. Animals can become infected after ingestion of feed and water contaminated with Salmonella. Similarly, humans can become infected by foodborne transmission or after direct or indirect contact with infected animals (Hoelzer *et al.,* 2011), consumption of contaminated food products (Forshell and Wierup, 2006; Kusumaningrum *et al.,* 2003).

Occurrence of non-typhoidal *Salmonella* serotypes commonly infecting humans in dairy cattle, particularly, those stains resistant to antimicrobial agents commonly used in human medicine, are a serious threat to human health. Some multi-drug resistant (MDR) *Salmonella* outbreaks in humans have been linked to exposure to dairy farms or contaminated dairy products (Gupta *et al.,* 2003).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 (Zewdu and Cornelius, 2009).

The worst food poisoning incident due to Salmonella occurred in USA in 1985 and there was a cause of 16,289 human cases and 7 deaths as the result of recontamination of pasteurized milk with a potent strain of Salmonella typhimurium (White *et al.,* 2001).Salmonellosis is one of the most wide spread food-borne zoonoses in Ethiopia. Different studies conducted in Ethiopia revealed fragmented substantial prevalence as well as antimicrobial susceptibility of Salmonella in veterinary and public health setups (Alemayehu *et al.,* 2003; Beyene and Tesega, 2014; Ejeta *et al.,* 2004; Hailu *et al.,* 2015; Molla *et al.,* 2003, 2006; Zewdu and Cornelius, 2009).

2.7.3. Staphylococcus aureus

The genus Staphylococcus comprises of several species and subspecies (Kwok and Chow, 2003). The genus is broadly grouped into two, namely, coagulase-positive and coagulase-negative Staphylococcus(Becker *et al.,* 2014; Sasaki *et al.,* 2010). From those of coagulase-positive Staphylococci group, the three major pathogenic ones are *S. aureus, S. intermediu s*and *S. hyicus* (Devriese, 1990; Tanner et al., 2000). *S. aureus* is ubiquitous: detected in animals, cow sheds, farm and dairy workers, processing environments and ultimately the cheese (Jørgensen *et al.,* 2015; Lim *et al.,* 2017). Furthermore, *S. aureus* have evolved to cause mastitis in farm animals (Capurro et al., 1999) and can be transmitted into the dairy food chain, leading to contaminated dairy products, and possible staphylococcal food poisoning (SFP). It is one of the bacteria that easily grow and establish themselves as commensals on the skin and mucous membranes of warm-blooded animals (Jørgensen *et al.,* 2015; Sá *et al.,* 2004).

They are easily spread between animals and under certain conditions to humans by skin contact with excretions which contain Staphylococci spp., such as saliva, or aerosols released during sneezing and coughing. Moreover, it may be spread by animal products such as non-pasteurized milk (Werckenthin *et al.*, 2001). It also to cause a number of pathological conditions in humans that range from mild skin infections, bacteremia, systemic diseases, osteomyelitis to the more complicated toxic shock syndrome associated with the consumption of raw milk and milk products(Hageman *et al.*, 2006; Le Loir *et al.*, 2003; Lina *et al.*, 1999). Multiple-drug resistant (MDR) staphylococcal isolates such as methicillin resistant *S. aureus* (MRSA) have been isolated primarily from human, and also from animal samples (Silbergeld *et al.*, 2008). A study conducted by Schmidt *et al.* (2015)in the Kwa Zulu Natal province of South Africa have reported humans working with farm animals carry far more AMR Staphylococci than the farm animals they work with (Schmidt *et al.*, 2015).

3. MATERIAL AND METHODS

3.1. Study Area

The study was conducted in Adama and Modjo towns of East Shoa zone of Oromia regional state from November 2018 to May 2019. Adama town is situated on the high way from Addis Ababa city to Harar. Adama has an altitude of 1666 meter above sea level and located at 100 km east of Addis Ababa. The annual average minimum and maximum temperatures of Adama town are 18°C and 32°C(CSA, 2008). Modjo town is located at 66 km Southeast of Addis Ababa and lies at 8°35'N and 39°7'E at an altitude 1790 meters above sea level. The average annual rainfall, temperature, and mean relative humidity are: 776mm, 19.4 ºC and 59.9% respectively (Abera *et al.*, 2012; CSA, 2008).

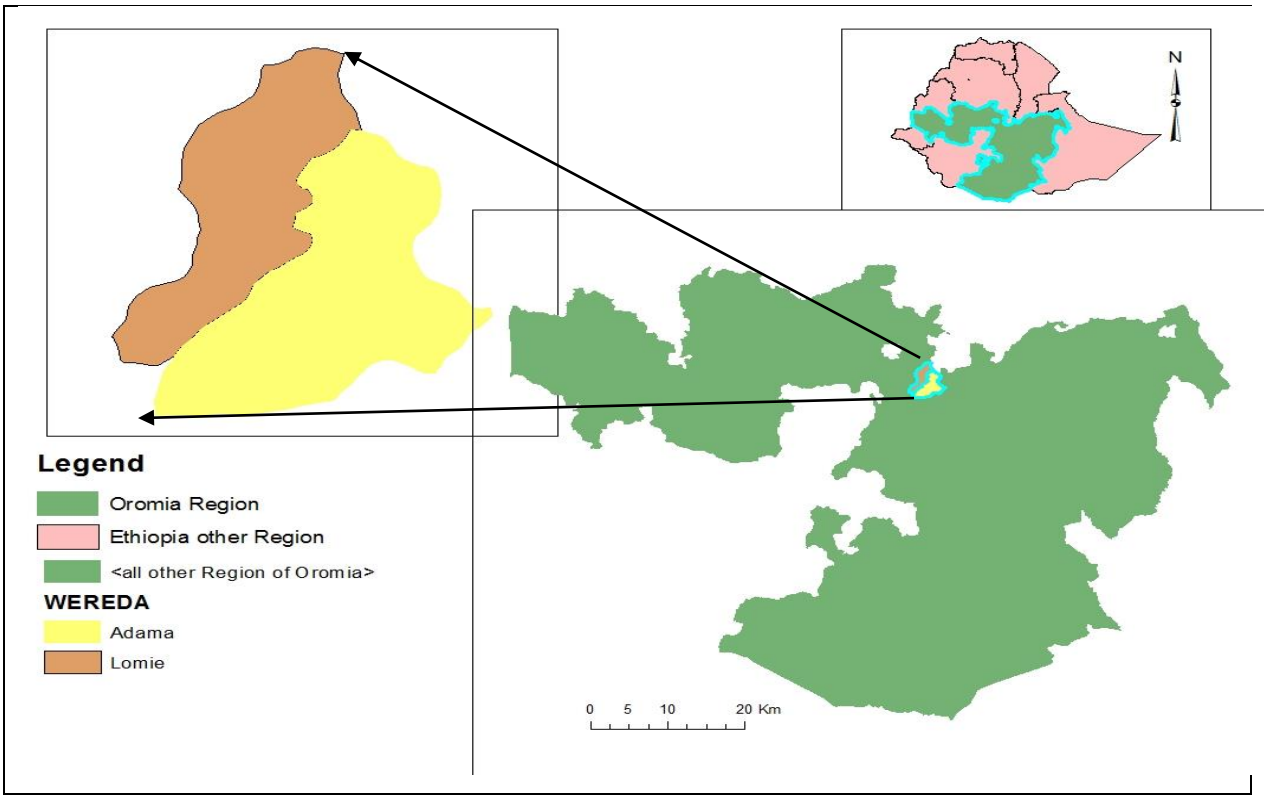

Figure 3: A map showing the study area in Oromia region, Ethiopia.

3.2. Study Population

The study populations were apparently healthy dairy cows, farm equipment used in the storage of milk, environmental sample from floor of the farms and personnel's (hand milkers) in dairy farms located in Modjo and Adama town.

3.3. Study Design

A cross sectional study was conducted to generate the desired data from November 2018 to May 2019.

3.4. Sample Size Determination and Sampling Technique

The sample size was calculated using the formula described by Thrusfield and Christley(2018) at 5% precision and 20% expected prevalence *E. coli* by Bedasa *etal.*(2018) in Bishoftu town which found near to selected town. Expected prevalence of *Salmonella* was 7.02% by Dabassa and Bacha(2011)and *S. aurous* were 16.1% by Beyene *et al.*(2017). Calculated sample size was 246, 100 and 208 for *E. coli*, *Salmonella* and *S. aureus* respectively however; the collected sample size was 211 because of unwillingness of farm owners. On the other hands the total sample size included in this study was 607 from different sample type.

20 dairy farms (proportional allocation of dairy farms from each selected towns) were included in this study. The sampling of the farm was employed purposively based on accessibility and willingness of the owners. From total selected farms founded in both towns, total animal of the farm was included except 2 farms which have large animal size. For those farms of large animal size proportional samples were selected randomly. The number and proportion of each sample from each location were shown in Table 1.

Table 1: Number of samples for each sample type with the isolate tested.

Sample type	Sample size	Proportion (%)	Tested for
Nasal swab	211	34.8	S.aureus
Feaces sample	211	34.8	*E. coli* & *Salmonella*
Udder milk	125	20.6	*S. aureus, E. coli* & *Salmonella*
Bulk milk	20	3.3	*S. aureus, E. coli* & *Salmonella*
Hand swab	20	3.3	*S. aureus, E. coli* & *Salmonella*
Floor swab	20	3.3	*S. aureus, E. coli* & *Salmonella*
Total	607	100	

3.5. Sample Collection and Transportation

Using the prepared sample registration chart (Appendix 1) all samples was documented. Samples from dairy cattle (feacal and nasal swab) and cattle derivative food (milk) were collected aseptically directly from randomly selected healthy dairy cattle in the farm. If diseased animals present on the day of sample collection, they were also sampled. Accordingly, approximately 10 ml of milk were collected aseptically from all teats and bulk milk in a sterile test tube. Nasal swabs of animals were collected directly from nose of animal. A pooled swab of milker hands and pooled swabs of floor (environment) were collected by using a sterile wooden cotton swab on the surface of material and insert in the 10ml test tube that contains sterile buffered peptone water (BPW) used as a pre-enrichment media for 24 hours at 37^{o}C.

The swabs were rotated and rubbed against the sampled surface several times. Faeces were collected directly from the rectum and put into 50 ml containing universal screwed caped bottle. Samples were properly coded based on collection date, sample source and sample type. Source of sample was classified as animal, personnel and equipment. All samples were transported in ice box to Addis Ababa University College of Veterinary Medicine and Agriculture (AAU-CVMA) microbiology laboratory for analysis. Samples were processed separately in pre-enrichment media and stored at +4 °C until processed for the presence of bacteria.

3.6. Isolation and identification

3.6.1. Staphylococcus aureus

The isolation and identification of *S. aureus* from different swabs and milk sample were performed at the AAU-CVMA by using techniques recommended by International Organizations for Standardization(ISO) (ISO, 2007). Initial culturing was made by streaking 50µl of each swab-BPW or milk sample on Tryptic Soy Agar (TSA) with a 5% sheep blood. Plates were incubated at 37^0C for 24 hours and yellow colonies formation with yellow zones after 24 hours of incubation at 37^0C on Mannitol Salt Agar (MSA) was appreciated. The presence of *S. aureus* colonies was evaluated using morphological aspects (round, smooth and white or yellow colonies) and hemolytic pattern (α, β, double hemolysis (α + β)) on the surface of blood agar plate and clotted when mixed with 0.5 ml of rabbit plasma.

To get a pure culture, presumed *S. aureus* colonies were further sub-cultured on nutrient agar plates and incubated at 37^0C for 24 hours. In addition to the above colony characteristics, final identification of *S. aureus* was conducted using Gram staining and series of biochemical tests such as coagulase test, catalase test, indole production, methyl red test, Voges-Proskauer reaction, urease production, citrate utilization and sugar fermentation. Gram-positive cocci in bunched, grapelike irregular clusters during Gram's stain will be seen. Bubble production was seen when a loop full of the pure colony mixed with a drop of 3% hydrogen peroxide on a clean glass slide within a few seconds. Then the bacterium that confirmed as *S. aureus* were sub-cultured onto nutrient agar and were incubated at 35 °C for 20 to 22 h for AM test.

3.6.2. E. coli

Detection of *E. coli* was carried out according to the protocol of (International Organization for Standardization (ISO), 2007) standard from feaces, milk and swabs samples. Approximately 1 ml of milk/ 1 g of feces (homogenized) were be suspended into 9 ml of modified Tryptone Soya Broth. Samples were vortexed and incubated overnight at 41 °C. After selective enrichment, 50µl of product were streaked onto MacConkey agar for primary isolation of *E. coli* and incubated

aerobically at 37°C for 24 hours. The plates were observed for the growth of *E. coli* (pink colony; lactose fermenter). A single, isolated colony were picked and sub-cultured on Eosin Methylene Blue (EMB) agar for formation of metallic sheen. Simultaneously another single colony with similar characteristics was stained with Gram's stain. The isolate was examined for stain and morphological characteristics using bright-field microscopy. KOH test is then employed to confirm the Gram's reaction (Quinn *et al.*, 2001).

Suspected colonies of *E. coli* (pinkish color appearance on MacConkey agar and metallic sheen on EMB were then sub-cultured onto blood agar to appreciate colony characteristics and then pure colonies taken from blood agar were inoculated on nutrient agar (non- selective media). Environmental samples were incubated overnight at 41 °C after suspended into modified tryptone soya broth (Oxoid) (at 1:9 ratios) and subjected to similar tests for bacteriological analysis as fecal and milk samples. Biochemical tests were performed to confirm the *E. coli* using catalase test, Indole Production test, Methyl red-Voges proskaur (MR-VP) test, and Simmon's Citrate test on tryptone broth, MR-VP medium and Simon citrate agar respectively (ISO, 2013). Then the bacterium that confirmed as *E. coli* were sub-cultured onto nutrient agar and were incubated at 35 °C for 20 to 22 h for antimicrobial susceptibility test.

3.6.3. *Salmonella*

The isolation and identification of *salmonella* from faeces, different pooled swabs, and bulk or whole milk sample were performed at the AAU-CVMA by using techniques ISO 5659, (2013). 5gm of fecal sample or 5ml of milk were pre-enriched with 45ml of BPW at a ratio of 1:9 and swabs taken from farm such as floor, hand and samples of bucket and tank milk were also pre-enriched with 10ml BPW and incubated for 24 hrs at 37°C. Then one ml of the pre-enriched culture was transferred to 10 ml of Selenite F Broth (SFB) tube and another 0.1 ml portion were transferred to 10 ml of Rappaport Vassiliadis Soy broth (RVSB) and incubated at 37°C for 24 hrs and 48 hrs, respectively.

Finally, one loop of broth culture from the incubated SFB and RVSB were inoculated and incubated on to Xylose Lysine Deoxycholate (XLD) at 37^0c for 48 hrs and Salmonella Shigella (S-S) agar at 37°C for 24hrs. Characteristic salmonella colonies were, having a slightly transparent zone of reddish color and a black center on XLD media and typical *Salmonella* colonies on S-S agar plate cause the color of the medium to be colorless or transparent colony with black center. When suspected colonies detected, sub cultivation of 4 *Salmonella* colonies from XLD and S-S agar on to a non-selective nutrient agar media plates for confirmation by using biochemical tests including Triple sugar iron agar (TSI), Indole test, urease test, Simon's citrate test, and MR-VP test. A typical biochemical reaction on TSI i.e. alkaline (red) slant, acidic (yellow) butt, H_2S and gas production, citrate utilization as a carbon source, Indole and urease negative, M-R positive, and V-P negative were appreciated. Biochemically conformed *Salmonella* were being inoculated in nutrient agar and incubated at 37^0c for 24hrs for antimicrobial susceptibility test (Schumann *et al.,* 2003).

3.7. Antimicrobial susceptibility testing

Antimicrobial susceptibility of isolates was tested according to Clinical Laboratory Standard Intuits (CLSI, 2013) using medically important selected drugs.

3.7.1. S. aureus

Identified *S. aureus* isolates were tested for their susceptibility to 11 different antimicrobials using the disk diffusion method with incubation at 37°C overnight. *S. aureus* isolates were cultured on agar plate and 1-3 colonies were picked to make suspension in sterile saline water. The turbidity of the suspension was adjusted to a 0.5 McFarland turbidity standard. A sterile swab was dipped into the standardized suspension of bacteria and excess fluid was expressed by pressing and rotating the swab firmly against the inside of the tube. The swab was streaked in three directions and continuously brushed over the Mueller Hinton Agar and allowed to stand for 3-5 minutes. The discs were placed onto the agar surface using sterile forceps and gently pressed with the point of a sterile forceps to ensure complete contact with the agar surface. The plates were incubated aerobically at 37°C for 18hr for all discs.

Inhibition zone diameters were measured and interpretations were done as described by the manual of the manufacturer and Clinical and Laboratory Standards Institute guidelines (CLSI). The isolates were classified as resistant, intermediate or susceptible to a particular antibiotic based on the cut-off value as indicated in Wayne (2011). Phenotypic multidrug resistant (MDR) were recorded for isolates that had resistance to three and more antimicrobials. Cefoxitin were used since CLSI recommends the cefoxitin-disk (30 µg) screen test as alternative methods of testing for methicillin resistance *S. aureus*.

3.7.2. E. coli and Salmonella

Antibiogram of *E. coli* and *Salmonella* isolates were evaluated against at least nine different antibiotics by using Kibry-Bauer disk diffusion method following Clinical and laboratory standards institute(CLSI), (2013). From each isolate, four biochemically confirmed well-isolated colonies grown on nutrient agar were transferred into tubes containing 5 ml of TSB (Oxoid, England). The broth culture was incubated at 37ºC 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 (Oxiod Basingstoke, England) with in a sterile safety cabinet. The plates were kept at room temperature for 15 minutes to allow drying. Antibiotic discs with known concentration of antimicrobials were placed and the plates were incubated for 24 hrs at 37ºC. Following incubation, the diameters of zone of inhibition were recorded to nearest millimeters for each disc used and then were classified as resistant, intermediate, and susceptible according to published interpretive chart of CLSI, (2013).Broth micro dilution assay were be done for determination microbial inhibition concentrations if evaluating incremental changes in the development of resistance is required.

3.8. Questionnaire Survey

Structured questionnaires were used to assess the knowledge, attitudes and practices (KAP) of selected study farms related to antimicrobial usage, residues in products, antimicrobial resistance containments and risk of milk consumption in the study area. All selected dairy farm owners were included in the study and data were collected using face-to-face interview and properly recorded on questionnaires format (APPENDIX-I). The second questionnaire was used to interview 18 randomly selected consumers on their dairy foods consumption habits and the use of antibiotics.

3.9. Data Management and Its Analysis

Data describing the presences of isolates in samples were classified filtered and coded using Microsoft Excel. The data was then exported to Stat 13 for appropriate statistical analysis. The prevalence of resistance isolates from all samples was determined by using descriptive statistics. Effects were reported as statistically significant if p-value is less than 0.05. Odds ratio and 95% confidence intervals was used to measure the strength of associations.

3.10. Ethical Considerations

The following study does not have any risk to the study subject, but the study was conducted by maintaining any ethical consideration set by the rule of the senate of the Addis Ababa University College of veterinary medicine and agriculture or responsible organization for the current study. Accordingly, the result obtained by the study was keeping the secret of the farm included under the study. The project PI obtained the Official ethical clearance certificate from the college Animal and Research Ethical Review Committee. The research team was ensuring to adhere to the code of practice recommended by the University's ethical review process with high reverence.

4. RESULTS

4.1. Isolation and Identification of *S. aureus, E. coli* and *Salmonella*

Out of 607 samples collected from 20dairy farms, 396 samples were analyzed for *S. aureus* and 62(15.7%) isolates were identified. The occurrence of *S. aureus* in the different sample types which includes nasal swabs, udder milk, pooled hand swabs of personal, milking utensils (bulk milk sample) and pooled floor swabs was indicated Table 2.

Table 2: Isolate of *S. aureus* derived from the different sample types.

Sample type	**No. of sample**	**Positive**	**Prevalence (%)**
Nasal swaps	211	35	16.7
Udder milk	125	19	15.2
Bulk milk	20	6	30
Pooled hand swabs	20	2	10
Pooled floor swabs	20	0	0
Over all	396	62	15.7

$X^2 = 7.4714$ P- value = 0.113

Out of 607 samples collected from 20dairy farms, 396 samples were analyzed for *E. coli* and 30(7.6%) isolates were identified. The occurrence of *E. coli* in the different sample types which includes feaces sample, udder milk, pooled hand swabs of personal, milking utensils (bulk milk sample) and pooled floor swabs, was indicated Table 3.

Table 3: Isolate of *E. coli* derived from the different sample types.

Sample type	**No. of sample**	**Positive**	**Prevalence %**
Feaces	211	17	8.1
Udder milk	125	12	9.6
Bulk milk	20	0	0
Pooled hand swabs	20	0	0
Pooled floor swabs	20	1	5
Over all	396	30	7.6

$X^2 = 4.3915$ P- value = 0.356

Out of 607 collected samples from 20dairy farms, 271 samples analyzed for *Salmonella* and 13(4.8%) isolates were identified. The occurrence of *Salmonella* in the different sample types which includes feaces sample, pooled hand swabs of personal, milking utensils (bulk milk sample) and pooled floor swabs, was indicated Table 4

Table 4: Isolate of *Salmonella* derived from the different sample types.

Sample type	**No. of sample**	**Positive**	**Prevalence %**
Feaces	211	10	4.7
Bulk milk	20	2	10
Pooled hand swabs	20	0	0
Pooled floor swabs	20	1	5
Over all	271	13	4.8

$X^2 = 2.1966$ P- value = 0.533

NOTE: P value - Level of significance Significant when P-value ≤ 0.05.

4.2 Antimicrobial Susceptibility Profiles of *S. aureus, E. coli and Salmonella* Species

The result of antimicrobial susceptibility test of *S. aureus, E. coli* and *Salmonella* isolates to the selected eleven (*S. aureus*) and nine (*E. coli* and *Salmonella*) different antimicrobial disks are indicated in Table 5.The number of *S. aureus* isolates tested for antimicrobial resistance was 62 all of isolates show resistant to all of selected drug except Gentamycin and Ciprofloxacin which show 93.6% and 61.3% susceptibility. Penicillin, Methicillin and Sulphametoxazole-trimethoprim were drugs to which a large proportion of *S. aureus* isolates were highly resistant. As it is indicated in Table5, most isolates (100%to90%) were resistant to these three drugs. *S. aureus* isolates were also resistant o Cefxetine (71%), Tetracycline (66%), Erythromycin (58%), Nitrofurantoin (41%), Streptomycin (40%), and Kanamycin (32%).

The number of *E. coli* isolates tested for antimicrobial resistance was 30 and they were found to be resistant to most antimicrobials except Gentamycin, Nitrofurantoin and Ciprofloxacin which show (90%), (70%) and (63.3%) susceptibility. From the total of 30 tested isolates 83% were resistant to Tetracycline, 80% to Vancomycin, 43% to Ceftriaxone, 23% Streptomycin, 20% Cefxetine and 17% to Nalidxic Acid.

The resistance level of 13 *Salmonella* isolates was 69% to Nalidxic Acid, 54% to Vancomycin, 46 % to Streptomycin,46%toTetracycline, 38% to Ciprofloxacin, and 20% to Cefxetine. Some others drugs like Gentamycin, Nitrofurantoin, and Ceftriaxone were show 85%, 69% and 61.5% susceptible.

Table 5: Antimicrobial susceptibility profiles of *S. aureus*, *E. coli* and *Salmonella*.

Antibiotics	**Species**	**Susceptible**		**Intermediate**		**Resistant**	
		No	%	No	%	No	%
Ceftriaxone	*S.aureus*	-	-	-	-	-	-
	E.coli	16	53.33	1	3.33	13	43.33
	Salmonella	8	61.5	4	30.8	1	7.8
Cefxetine	*S.aureus*	18	29	-	-	44	71
	E.coli	17	56.7	7	23.3	6	20
	Salmonella	5	38.5	5	38.5	3	23
Ciprofloxacin	*S.aureus*	38	61.3	17.34	27.4	7	11.3
	E.coli	19	63.3	10	33.3	1	3.3
	Salmonella	-	-	8	61.5	5	38.5
Methicillin	*S.aureus*	3	4.8	1	1.6	58	93.
Gentamycin	*S.aureus*	58	93.6	-	-	4	6.5
	E.coli	27	90	-	-	3	10
	Salmonella	11	85	1	7.8	1	7.2
Erythromycin	*S.aureus*	12	19.4	14	22.6	36	58
	E.coli	-	-	-	-	-	-
	Salmonella	-	-	-	-	-	-
NalidxicAcid	*S.aureus*	-	-	-	-	-	-
	E.coli	18	60	7	23.3	5	16.67
	Salmonella	3	23	1	7	9	70
Nitrofurantoin	*S.aureus*	28	45.16	8	12.9	26	41.94
	E.coli	21	70	6	20	3	10
	Salmonella	9	69.2	3	23	1	7.8
Penicillin	*S.aureus*	-	-	-	-	62	100
	E.coli	-	-	-	-	-	-
	Salmonella	-	-	-	-	-	-
Streptomycin	*S.aureus*	8	12.90	29	46.77	25	40.32
	E.coli	14	46.67	9	30	7	23.33
	Salmonella	6	46.15	1	7.69	6	46.15
Sulphametoxazole	*S.aureus*	4	6.45	1	1.61	57	91.94
-trimethoprim	*E.coli*	-	-	-	-	-	-
	Salmonella	-	-	-	-	-	-
Tetracycline	*S.aureus*	13	20.97	8	12.90	41	66.13
	E.coli	5	16.67	-	-	25	83.3-
	Salmonella	7	53.85	-	-	6	46.15
Vancomycin	*S.aureus*	-	-	-	-	-	-
	E.coli	5	16.67	1	3.3	24	80
	Salmonella	3	23.08	3	23.08	7	53.85
Kanamycin	*S.aureus*	30	48.39	12	19.35	20	32.26
MDR	*S.aureus*	1	1.61	-	-	61	98.39
	E.coli	13	43.33	-	-	17	56.67
	Salmonella	6	46.15	-	-	7	53.85

The present study revealed that 98.4% (58/62) show multidrug resistant (MDR) to *S. aureus* isolated from milk of lactating cows, from nasal swabs of animal, from bulk milk (milking utensils) and milkers hands. From all 62 tested isolates of *S. aureus*, one isolate was resistant for all selected drug expect Gentamycin. Two isolates of *S. aureus* were resistant to 9 drugs except Gentamycin and Ciprofloxacin. Eight isolate were resistant to 8 drugs and seventeen isolate were resistant to 7 drugs. On the contrary, from all 62 tested isolates of *S. aureus* 58% were susceptible to Gentamycin and 38% of them susceptible Ciprofloxacin.

From total of 30 tested isolates of *E. coli*, 17 (56.7%) were show MDR. Six isolate of *E. coli* was found to be resistant to Tetracycline, Vancomycin and Ceftraxon. On the other hand, most isolate were susceptible to Gentamycin, Ceftraxon and Nitrofurantion. From total of 13 tested isolates of *Salmonella*, 7(56.7%) were show MDR. From all tested isolates of *Salmonella* 11 were susceptible to Gentamycin and 9 Nitrofurantion, but all of five tested isolates were resistant for both Vancomycin and Tetracycline.

Upon comparison of the MDR between sample types; total tested *S. aureus* isolates derived from all four sources, floor, udder milk and bulk milk sample were show 100% MDR but, from tested nasal swabs 97% were show MDR. From total tested *E. coli* isolates derived from floor sample, milk samples and faeces; 1 isolate of floor sample were tested and it show resistant to three of the selected antimicrobials tested. From 12 tested *E. coli* isolated from milk samples were show 5(41.7%) MDR. On the other hand, all 17 *E. coli* isolated from feces were show 11(64.7%) MDR. From total tested *Salmonella* isolates derived from floor, bulk milk and faeces samples; 1 tested isolate of floor sample and 2 isolate of bulk milk sample were tested and show 100% MDR. On the other hand, 10 tested isolates of feces sample were show 4(40%) MDR.

Table 6: Multidrug Resistance pattern of *S. aureus*, *E. coli* and *Salmonella* isolates.

Bacteria	Antimicrobial resistance level	Number of isolates	Multidrug Resistance, Percent (%)
S. aureus	One	One	1.61
(n=62)	Three	Two	3.23
	Four	Seven	11.29
	Five	Ten	16.13
	Six	Fourteen	22.58
	Seven	Seventeen	27.42
	Eight	Eight	12.90
	Nein	Two	3.23
	Ten	One	1.61
	Total	Sixty two	100
E. coli	One	Three	10.00
(n=30)	Two	Ten	33.33
	Three	Eight	26.67
	Four	Six	20.00
	Five	Two	6.67
	Six	One	3.33
	Total	Thirty	100
Salmonella	One	Three	23.08
(n=13)	Two	Three	23.08
	Three	One	7.69
	Four	Five	38.46
	Seven	One	7.69
	Total	Thirteen	100

4.3. Questionnaire Survey

The first questioner study was conducted on 20 respondents of farm owners, consisting of 19 males and 1 female. The conducted survey showed that educational level of respondents was 8(40%) completed secondary school, 10(50%) was completed high school and 2(10%) was graduated from college. With regard to location of the farm all selected farms found in the urban location. The dairy cow managed 11(55%) intensively, 8(40%) semi intensively and 1(5%) ware extensive. All respondent use plastic can for milk collection; none the farm sells their milk to industries, 45% sell to small shops, 15% were sell to café and restaurants and 40% were sell to neighbors. Only 20% of farms test their milk, the other 80% of the farms were not taste their milk and all of the farms not pasteurized their milk.

Overall, 51.2% of the dairy herdsmen reported clinical mastitis and dystocia (29.9%). Lameness, left-displaced abomasum, and bloat were also reported. Among herdsmen, 50% reported that they call veterinarian to examined the animals before antimicrobial treatment, 20% reported that they buy medicine from veterinary pharmacy and administered the drugs by themselves, 10% were go to clinic and 20% were use traditional medicine. In addition, 45% of the farm owner says they administer full dose however, 55% of them stopped the administration of drugs before attaining the complete dose recommended by the clinician. The most commonly used antimicrobials on the dairy farms were 15% ox tetracycline, 40% pen strep, 25% penicillin, and 20% sulfa drug. The response to knowledge of withdrawal time of drugs they used, 80% of them know and 20% of them don't know about drug withdrawal time. None of the farm discards milk obtained from a cow in a withdrawal time, 55% the farm gives the milk to calf, 40% of them sell and 5% of them consume at home.75% of the respondent say yes when they asked if they know AMR and 25% does not know about AMR.

Question were also developed for the selected farm client consumer. Consumers interviewed in this study indicated that in how they consume the milk they obtain from these farms and from 18 respondents 11(61%) of say they boiled the milk before they consume however 3(16.7%) use milk for yoghurt or cheese and the other 4(22%) consumed in both ways. Among them, 53.7% explained their knowledge on the side-effects of raw/undercooked milk consumption.

5. DISCUSSIONS

Antibiotic use drives the evolution of antibiotic resistance (Albrich *et al.*, 2004). Cow's milk may be contaminated by AMR pathogen from different sources like animals itself, unclean milk containers, the milk handlers, airborne dust or droplets at the site of production and during processing that presents a health hazard (Getahun and Gebre-Selassie, 2003). Over the past years, the dissemination of antimicrobial resistance (AR) in bacteria, including *S. aureus, E. coli* and *salmonella* has increased and poses public health risks. This is best narrating by the multidrug resistant bacterial strains that cause infections which is difficult to treat. Preserving the effectiveness of existing antibiotics by minimizing the emergence and spread of multidrug resistant microorganisms which come pass from animal or animal product like milk and meat is important so that; we can maximize the existing antibiotics become effective (Koning *et al.*, 2003).

In the present study the prevalence of *S. aureus* from 396 samples was 15.7% (62). *S. aureus* was isolated from nearly all sample types with different proportion, except environmental sample (floor swabs) (0.0%). This finding was agreed with the study done by Abebe *et al.*(2014) who reported 15.5 % in Tigrai, Beyene *et al.*(2017) who reported 16.1% in Addis Ababa, Gizaw and Bishoftu (2014)who reported 17.2% in Addis Ababa, Osman(2010), who reported 17.2% in Egypt. The occurrence of *S. aureus* in the present study was higher than the report from Debre Zeit which indicated 5% in cottage cheese and 10 % in raw milk (Addis *et al.*, 2011). Lower prevalence of 6.6% and 10.8% were also reported in India (Kumar and Prasad, 2010) and Brazil (Fagundes et al., 2010), respectively. Conversely, a higher prevalence of 40.6% and 42.1 %, 40%, 74.5%, and 100% has been reported in Southern (Daka and Yihdego, 2012) and central Ethiopia (Abera e*t al.*, 2010), in Morocco (Bendahou *et al.*, 2008), India (Sarkar *et al.*, 2014), and South Africa (Ateba *et al.*, 2010), respectively.

The prevalence of *E. coli* from 396 samples were 7.6% (30/396); of which 8.1%(17/211), 9.6%(12/125), 0% (0/20), 0% (0/20) and 5% (1/20) were positive from faeces , udder milk, bulk milk, pooled hand swab, and pooled floor swab respectively. It was not possible to compare the overall prevalence obtained from these five samples types because studies made on *E. coli* isolated

from faeces, bulk milk, pooled hand swab, and pooled floor swab was not clearly studied and published in Ethiopia or elsewhere in the world. The current finding was agreed with the studies by Lye *et al.* (2013) and Addo *et al,* (2011) who reported 8.75% and 11.2% from Malaysia and Ghana, respectively. This finding was also lower when we compare with Abebe *et al.* (2014) finding that showed 23.7% from Tigray, 26% by Farhan *et al.* (2014) from Ethiopia and report of 23.3% by Elbagory *et al.* (2015)from Egypt. In addition, the prevalence is far lower when compared to the report of (44%) by Shunda *et al.* (2013)from Mekelle towon Ethiopia, report of 33.9% by Disassa *et al.* (2017) from Ethiopia, report of 69% by Fadaei (2014) from Iran, report of 63% by Ali and Abdelgadir (2011) from Khartoum and report of 90.67% Lubote *et al.* (2014) from Tanzania.

In the present study the prevalence of 4.8% (13/271) of *Salmonella*, of which 4.7% (10/211), 10% (2/20), 0% (0/20) and 5% (1/20) were positive from faeces, bulk milk, pooled hand swab, and pooled floor swab respectively. It was also not possible to compare the overall prevalence obtained from these four sample types because studies made on *Salmonella* isolated from faeces, bulk milk, pooled hand swab, and pooled floor swab was not clearly studied in Ethiopia and elsewhere. Overall prevalence of 4.8 % *Salmonella* from 271 samples of the present study is not in agree with the finding of Abunna *et al*. (2017)who reported 12.1% in udder milk from Modjo town, Ethiopia, 20% reported by Tadesse and Dabassa (2012) from Kersa district of Ethiopia. There is also a report of lower prevalence 3.08% by Addis *et al.* (2011) from central Ethiopia and by Eguale *et al.* (2016) 2.3% *Salmonella* from central Ethiopia.

The above finding difference in the present study from previous studies might be attributed from differences in environmental conditions of study site, sample size or due to differences in management and hygienic practices. In this study from the sample type's higher number of *Salmonella* was isolated from faecal (4.7%) samples and prevalence of *E. coli* in faecal (8.1%) is also high next to milk (9.6%). The prevalence of fecal shedding indicates the magnitude of environmental contamination possible. The result of 5% for both *Salmonella* and *E. coli* which isolated from floor swabs shows some level of floor contamination. Thus fecal collected from these cows could contaminate the milk, farm equipment's, and personnel handlers. Improper hygienic

practices and collecting milk in plastic container were also observed during questioner survey in current study. This may pose risk to the farm personnel and the community at large for infection.

The antimicrobial susceptibility tests carried out in current study indicated that 100% of *S. aureus* were resistant to Penicillin followed by 93% to Methicillin and 91% Sulphametoxazole trimethoprim. Resistance to penicillin (100%) was more frequently observed than other drugs. This result is comparable with the study by Ateba *et al*. (2010) and Gandhale *et al.* (2017) who reported the resistance of 100% and 91.5% of penicillin, respectively. In contrary this result higher than the study by Massawe *et al.* (2019), and Al-Thani and Al-Ali (2012), who reported the resistance of 55.5% and 60% of penicillin, respectively. Resistance to erythromycin (58%) observed in this study is agreed with report by (Kenar *et al.,* 2017), who found resistance of 61.5% in Turkey. But disagreed with the report by Mohanta and Mazumder (2015) and Massawe *et al*. (2019), who found resistance among 20.5% and 29% of isolates, respectively. On the other hand, isolates showed moderately low resistance to Ciprofloxacin and sensitivity to Gentamicin.

The present study showed that the majority of *E. coli* isolates were resistant to Tetracycline with 83.3%, and Vancomycin with 80%. Resistance to Tetracycline was more frequently observed than other drugs. This result was agreed with the study by (Bekele *et al.,* 2014; Shecho *et al.,* 2017). But in Dire Dawa, Ahmed and Van Velkinburgh (2014) reported that *E. coli* was susceptible to tetracycline, which is contrary to the results of the present study. *E. coli* isolates were sensitive to Streptomycin, this result disagree with the study by Hiko *et al.* (2008b) and Bekele *et al.* (2014) from Ethiopia and Magwira *et al.* (2005) from Botswana revealed that the resistance of *E. coli* mainly to streptomycin. Meanwhile, isolates were sensitive to Gentamicin, Ciprofloxacin and Nitrofurantoin.

All of the 13 isolates of *Salmonella* from animal and farm were tested against 10 commonly used antibiotics. Resistance rates of isolated *Salmonella* was, 70% to Nalidxic Acid, 54% to Vancomycin, 46 % to Streptomycin and 46.15% to Tetracycline. In the current study Nalidxic Acid showed a least efficacy against *Salmonella* isolates. In addition, the resistance to Nalidxic Acid (70%) is agree with the prevalence of 80% reported from Ethiopia (Tadesse and Dabassa, 2012). But slightly disagreed with the prevalence of 89-92% reported from Kenya (Lakshmi *et al.,*

2006). The result for Streptomycin resistance (46 %) of *isolated Salmonella* in this study was higher than 13.3% and 25%, which was reported by Addis *et al.* (2011) and Tadesse and Dabassa (2012), respectively. The effectiveness of Gentamycin in to isolated *Salmonella* in this study 85% is which agreed to the result reported by 73.3% and 75% Addis *et al.* (2011) and Tadesse and Dabassa (2012), respectively. But lower than Tesfaw *et al.* (2013), who reported 100%.

The frequency of MAR to three or more antibiotics was observed 98.39% in all most in all isolates of *S. aureus*. The present study has demonstrated the presence of high level of multi drug resistance of isolated *S. aureus* were 98.39% (61/62), especially among commonly used drugs like penicillin and tetracycline. The current study was agree with Tafa *et al.* (2015), Taddesse *et al.* (2014) and Abera *et al.* (2010),who reported 87.6%, 90% and 94.4% of *S. aureus* resistance to multiple drugs, respectively. But the results of this study are higher with that reported in Brazil (dos Santos *et al.*, 2013) and Ethiopia (Asrat *et al.,* 2013) where 64.4 and 45.1% of *S. aureus* isolates, respectively, resisted three or more antibiotics. According to this study, penicillin, Methicillin, Erythromycin, and tetracycline were the most frequently observed pattern.

Multidrug resistance analysis of *E. coli* showed that 56.67% of tested isolates were resistant to different combinations of tested antibiotics. This is in disagreement with the report of Bedasa et al. (2018), who showed 92.5% of isolates were multidrug resistant. Moreover, various authors (Atnafie *et al.,* 2017; Bekele *et al.,* 2014; Iweriebor *et al.,* 2015) from the country and abroad reported multidrug resistance patterns. In the present study, 53.85% of *Salmonella* isolates were resistant to at least three or more types of antimicrobials (MDR) and compared with the work of Tesfaw *et al.* (2013) who reported 50% , but lessen than the study of Dabassa and Bacha, (2011) who report 83.3% also Tadesse and Dabassa (2012), who reported 70% for multiple antimicrobial resistance.

This difference of AMR in the present study for all isolated bacteria was might be due to small sample sizes for the data, nature of drug, presence of different strain of the bacteria, development of resistant gene, their low frequency usage of some drugs (Gentamycin) for prevention and control of disease in food animals in the study area. This is a concern because the resistant strain can be transmitted to human by consumption of milk and its products. Furthermore, the consumption of

food carrying antibiotic-resistant bacteria can directly or indirectly result in the acquisition of antibiotic-resistant infections (Marshall and Levy, 2011).

Highest resistance levels observed in the present study might be due to none judicious use of antibiotics in dairy farm level. The result of MDR reflects the uses of those antibiotics in the study area were common, and it shows that *S. aureus*, *E. coli* and *Salmonella* has been exposed to these drugs. From questioner survey common drug used in dairy farms shows 15% Oxtetracycline, 40% Penstrep, 25% Penicillin, and 20% Sulfa drug which all most all tested isolates of bacteria develop resistance to these drugs. It was noticed that these drugs are widely available from agro-vet distributors and can be purchased easily without any prescription from an authorized facility. Among herdsmen, 20% reported that they buy medicine from veterinary pharmacy these result showed us farm owners buy drugs without prescriptions at some level. Dairy animal owners as well as consumers involved in the study clearly indicated their use of available antibiotics without prescription and appropriate clinical examination/medical consultation.

Evidence (Akoachere *et al.,* 2009; Mahami *et al.,* 2011) indicates that the global rise of antimicrobial resistance is mainly due to in discriminate use of drug for treatment of both human and animal diseases. Some of the irrational practices in antibiotics usage in the study areas showed self-administration of drugs without proper clinical examination and cessation of drug usage before complete dose, as indicated 55% of farm respondents don't administer full dose. There was also poor knowledge of withdrawal time of drugs they used 20% of them don't know about drug withdrawal time and none of the farm discards milk obtained from a cow in a withdrawal time. Another possible reason for the observed pattern is antimicrobial-resistant bacteria in raw milk may be able to colonize the gut if consumed by humans, thus making infections difficult to treat the availability and price of these drugs. From the consumer consumption habit, we learned that people can consume raw/undercooked milk products. This aggravates the situation when geared with the irrational use of drugs as investigated in this study. Although different antibiotic classes of drugs are used in animal health management and in human medicine, the selection of resistance to one drug class may lead to cross-resistance to another (Haran *et al*., 2012).

6. CONCLUSION AND RECOMMENDATIONS

In the present study isolation of *S. aureus, E. coli* and *Salmonella* at dairy farms level showed that dairy cattle and their environment are important sources of milk contamination. This indicates poor hygiene which is great concern to the consumer's health. This study clearly indicates that *E. coli* and *Salmonella* isolates shaded from feces can contaminate the milk, the farm equipment's and personnel and result in bad hygienic standards of the farms. Detection of high proportion of multiple antimicrobial resistant isolates (98.4%) for *S. aureus,* (56.7%) *E. coli* and (53.9%) for *Salmonella* in the dairy farms alerts concern for animal and public health as these drugs is used widely for treatment and prophylaxis of various bacterial infections in animals and humans. It also reveals evidences of the irrational use of antibiotics in the dairy production and raw milk consumer habit. The main reasons for the occurrence of a high number of resistant strains in this study are the use of sub-therapeutic level of antibiotics and/or short treatment. Finally, due to the high resistance levels detected in the present study, it is believed that it is necessary to set up permanent resistance surveillance programs in the country.

Based on the above remarks, the following recommendations need to be considered:

- ✓ To ensure the quality of raw milk, everyone engaged in milk and dairy production chain should be trained for hygienic practices.
- ✓ In order to protect consumers from zoonotic AMR, food safety management programmed should be implemented and highly considered.
- ✓ Public awareness should be given on the proper use, handling, and storage of antibiotics should be prioritized for livestock farmers and other drug users.

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8. APPENDICES

APPENDIX 1: Sample collection and laboratory activities work sheet for laboratory analysis

Data collection					**Laboratory analysis**				
No	Date	Species	Sample type	Sample ID	Pre-enrichment	Enrichment and	Colony Char.	Biochemical test	Antimicrobial Susceptibility Test

APPENDIX 2: Primary Tests

All the media used in this study were prepared according to manufacturer's instructions. Details of the preparations and handling of different types of media used is hereby shown:

1.1. **Nutrient Agar (NA**)

The medium was prepared according to the manufacturer's (AccumixR 500g) instructions whereby 23 g of the powdered medium was suspended into 1 litre of distilled water, mixed well and left on the bench to stand until the mixture is uniform. Then the mixed solution was heated with gentle agitation and boiled until completely dissolved. The medium solution was sterilized in the autoclave at 121°C for 15 minutes then allowed to cool to 45oC and poured onto sterile Petri dishes. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility and to dry the condensed vapour on the plate cover.

1.2. Tryptone Soya Broth (Enrichment media)

The medium was prepared according to manufacturer's (oxiod CM0129) instructions whereby 30 gm of the powdered medium was dissolved in 1 litre of distilled water. Tryptone Soya Broth is used in food bacteriology as the basal medium to which variety selective agents are added for selective enrichment of *Staphylococcus aureus* and *Escherichia coli* O157. The medium was prepared according to the manufacturer's instructions whereby

1.3. Buffered Peptone Water (BPW)

The medium was prepared according to manufacturer's (72993(BM)) instructions whereby 20 gm of the powdered medium was dissolved in 1 litre of distilled water. The culture medium was mixed well and each 10 ml were dispensed into capped test tubes. Then, the test tubes were sterilized by autoclaving at 121°C for 15 minutes and cooled to 25°C before use.

1.4. MacConkey Agar

The medium was prepared according to the manufacturer's (76875(MM011) instructions whereby 52 gm of the powdered medium was suspended into 1 litre of distilled water. The medium was boiled to dissolve completely followed by sterilization by autoclaving at 121°C for 15 minutes and cooled to below 45°C and poured onto sterile Petri dishes. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility and to dry the condensed vapour on the plate cover

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1.5. Mannitol Salt Agar

Suspend 111.02 grams in 1000 ml purified/ distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes or as per validated cycle. Cool to 45-50°C. Mix well and pour into sterile Petru plates.

Note: This product contains 7.5% Sodium chloride as one of its ingredients. On repeated exposure to air and absorption moisture sodium chloride has tendency to form lumps, therefore we strongly recommend storage in tightly closed containers in dry place away from bright light.

1.6. Eosin Methylene Blue (EMB) Agar

The medium was prepared according to the manufacturer's M317-500g instructions whereby 35.96 grams of EMB Agar in 1000 mls of distilled water. Heat to dissolve the medium completely. Sterilize by autoclaving at 15 lbs. pressure (121 °C) for 15 minutes. Avoid overheating. Cool to 50 °C and dispense onto sterile Petri dishes. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility and to dry the condensed vapour on the plate cover

1.7. Rappaport-Vassiliadis (RV) M880-500gEnrichment Broth media

The medium was prepared according to the manufacturer's instructions by weighing 30 g of the powdered medium added into 1 litre of distilled water and heated gently until completely dissolved. Then, 10 ml of the medium were dispensed into capped test tubes followed by autoclaving at 1210C for 15 minutes and cooling to about 25°C prior to use.

1.8. Selenite Broth Base oxiod CM0395 (Enrichment broth media)

Suspend 19 g of the dehydrated powder in 1 litre of purified water (if twin pack is available for use; dissolve 4g of sodium biselenite in 1 litre of distilled water and then add 19g of Selenite Broth Base- check manufacturer's instruction labeled/available in the pack). Sterilize in a boiling water bath, or in free flowing steam, for 10 minutes. Avoid overheating (as it is detrimental). Do not autoclave. Distribute in sterile test tubes. (Note: Discard the prepared medium if large amount of red precipitate is seen at the bottom of the tube/bottle). Incubate in ambient air at 35-37°C for up to 24 hours (Coliforms may overgrow the pathogens if incubated for longer than 24 hours). After incubation, subculture the broth into selective and differential enteric culture medium on XLD.

1.9. Xylose Lysine Deoxycholate (XLD) Agar (53556 (XM 011)

XLD Agar is a selective differential medium for the isolation of Gram-negative enteric pathogens from fecal specimens and other clinical material. It is especially suitable for the isolation of Shigella and *Salmonella* species. Microbiological testing of foods, water and dairy products. Suspend 56.68 grams of dehydrated medium in 1000 ml purified or distilled water. Heat with frequent agitation until the medium boils. Note: DO NOT AUTOCLAVE. Transfer immediately to a water bath at 50°C. After cooling, pour into sterile Petri plates. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility and to dry the condensed vapour on the plate cover. Note: It is advisable not to prepare large volumes, which will require prolonged heating and may produce precipitate.

1.10. Standard Plate Count (SPC)

For total plate count, appropriate a decimal dilution was selected that would give the expected total number of colonies on a plate. The standard plate count (SPC) agar was cooled to 45°C for 15 minute after autoclaved and before pouring to petri-dish. One ml from each sample of raw milk were transferred to 9 ml sterile saline water (0.1%) and thoroughly mixed to give 1:10 dilution 'first dilution and serial dilution of 10-6 and mixed thoroughly. Serial dilutions were made by transferring 1ml of the previous dilution in 9ml of 0.1% sterile saline water. Aerobic Bacterial Count was made by incubating surface plated duplicate decimal dilutions of milk samples on Plate Count Agar (Oxid) plates. Colonies were counted after the culture media is incubated at 37 C for 24hours. Total number of colonies on plates 30 to 300 per plates was selected and colonies were counted.

1.11. Triple Sugar-Iron Agar

Triple Sugar Iron Aga is recommended for identification of gram-negative enteric bacilli on the basis of dextrose, lactose and sucrose fermentation and hydrogen sulphide production in accordance with Indian Pharmacopoeia. Suspend 64.63 grams (the equivalent weight of dehydrated medium per litre) in 1000 ml purified/ distilled water. Heat to boiling to dissolve the medium completely. Mix well and distribute into test tubes and Sterilize by maintaining at 10lbs pressure (115°C) for 30 minutes or as per validated cycle.

PROCEDURE

Specimen Collection: This product is not intended for primary isolation of patient specimens. It should be used only with cultures of isolated organism. This product is used in conjunction with other biochemical tests to identify cultures of isolated organism. Method of Use: Allow the TSI Agar to warm to room temperature before use. Using one isolated, pure colony inoculates, stab, and streak the specimen on the agar as soon as possible after collection. Incubate tubes aerobically at 35-37ºC. For 18-24 hours

1.12. Simmons Citrate Agar

Simmons Citrate Agar is recommended for differentiation the members of Enterobactericeae on the basis of citrate utilization from clinical and non-clinical samples. Suspend 24.28 grams in 1000 ml distilled water. Heat, to boiling, to dissolve the medium completely. Mix well and distribute in tubes or flasks. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

PROCEDURE

Specimen Collection: Consult listed references for information on specimen collection. (2-4, 6, 7) Infectious material should be submitted directly to the laboratory without delay and protected from excessive heat and cold. If there is to be a delay in processing, the specimen should be inoculated onto an appropriate transport medium and refrigerated until inoculation. Method of use: This medium is not intended to be used for primary isolation. Using a light inoculum, inoculate medium with growth from a pure culture. Inoculate the slant by streaking the surface in a serpentine manner. Replace caps loosely on the tube(s). Incubate aerobically for 24-96 hours at 35 degrees C. Examine daily for growth and color change.

1.13. SIM Medium

SIM Medium is recommended for determination of hydrogen sulphide production, indole formation and motility of enteric bacilli. Suspend 36.23 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense in tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Allow the tubes to cool in an upright position.

PROCEDURE

The medium should be dispensed in tubes or bottles and when cool, inoculated once with a pure culture, by inserting a straight wire to about one third of the depth of the medium. If papers are used for the detection of indole, then these are wedged between the cotton wool plug or cap, and side of the container. The inoculated medium is incubated at 35°C for 18 hours or longer, if necessary, and examined for motility, hydrogen sulphide production and finally indole production from tryptophan.

Kovac's Indole Reagent: Lightly inoculate Tryptone Broth or Peptone Broth with the test organism. Incubate 24-48 hours at 37ºC. Add 4-5 drops of Kovacs Reagent to the tube, shake gently. Formation of a red coloured ring indicates positive indole test.

1.14. **MR-VP Medium (Glucose Phosphate Broth**)

MR-VP Medium (Glucose Phosphate Broth) is recommended for studying Methyl Red and Voges-Proskauer tests to differentiation amongst coli-aerogenes group. Add 17g to 1 litre of distilled water. Mix well, distribute into final containers and sterilize by autoclaving at 121°C for 15 minutes.

PROCEDURE

- Prior to inoculation, allow medium to equilibrate to room temperature.
- Using organisms taken from an 18-24 hour pure culture, lightly inoculate the medium.
- Incubate aerobically at 35ºC for 24 hours.
- Following 24 hours of incubation, aliquot 1ml of the broth to a clean test tube.
- Incubate the remaining broth for an additional 24 hours.

VOGES-PROSKAUER (VP) TEST

- ✓ To the aliquot (step 4 above), add 0.6ml of 5% alpha-naphthol. Next add 0.2ml of 40% KOH.
- ✓ Gently shake the tube to expose the medium to atmospheric oxygen.
- ✓ Allow the tube to remain undisturbed for 10-15 minutes.
- ✓ Observe the medium for a pink-red color development. The test may be read for up to, but not beyond, one hour following addition of the reagents.

Note: If test reactions are negative (no red color produced) or questionable, the test can be repeated using the incubated broth (without reagents) from step 5 above. Incubation and repeat testing can be performed for up to 5 days.

METHYL RED (MR) TEST

- ✓ Following 48 hours of incubation (step 5 above), aliquot 2.5ml of the broth to a clean test tube.
- ✓ Add five drops of Methyl Red reagent (Cat. no. Z117).
- ✓ Observe the medium for the immediate development of a red color.

Appendix 3: Biochemical Tests

Commonly used Biochemical tests for the identification of *Staphylococcus aureus* and their results are as follows,

Gram's staining procedures

1. Make a thin bacterial colony smear and allow it to dry on the air
2. Fix the dried smear by passing through the Bunsen flame two to three times taking care not to overheat the smear
3. Flood the fixed smear with Gram's crystal violet (primary stain).Let stand for 60 seconds.
4. Pour off the stain and gently wash with tape water.
5. Flood with Gram's iodine (mordant) solution. Allow it to remain for 60 seconds.
6. Pour off the iodine solution and gently wash with tape water.
7. Decolorize with Gram's decolorizer solution (95% acetone alcohol) for 15-20 seconds until the blue dye no longer flows from the smear and gently wash the smear with tape water.
8. Counter stain with Gram's safranin solution or carbolfuschin (counterstain) for 60 seconds.
9. Wash off the red safranin solution with water. Blot with bibulous paper to remove the excess water. Alternatively, the slide may shake to remove most of the water and air-dried.
10. Examine the finished slide under a microscope (oil immersion objective).

11. Interpretation: Bluish purple colour indicate Gram positive and pinkish colour indicate Gram negative bacteria

Catalase test procedure

1. Pickacolonyfroman18-24hourscultureandplaceitona clean glass slide.
2. Put one drop of 3% H_2O_2 over the organism on the slide.
3. Observe for immediate bubbling (gas liberation) and record the result.
4. Interpretation: A positive result is the rapid evolution of O_2 as evidenced by bubbling and a negative result is no bubbles or only a few scattered bubbles.

Procedure for oxidation and fermentation test

1. Prepare O-F base medium and when it is cooled at 50^{o}c, add 10ml of sterile glucose in to 100ml of O-F base, for a final concentration of 10% glucose and dispense in to a sterilized tube.
2. Heat two tubes of medium in boiling water for 10 minutes to drive off the oxygen, cool and inoculate by inserting a straight wire vertically
3. Incubate one tube aerobically and the second tube anaerobically or seal the surface with a layer of sterile liquid paraffin oil to create anaerobic condition
4. Incubate both tubes at 37°C for 24-48 hours or more, up to 7dayswith the caps loose. Longer incubation may be required for slowly growing species.
5. Examine tubes daily for color change.

Interpretation:

Fermentation: Yellow colour in both tubes with or without gas.

Oxidation: Yellow colour only in tube without oil.

No oxidation/fermentation: No change in the colour of the tubes. The carbohydrates have not been fermented or oxidized.

Coagulase test procedure

1. Three test tubes are taken and labeled “test”, “negative control” and “positive control”.

2. Each tube is filled with 0.5ml of 1in10 diluted rabbit plasma. To the tube labelled test, 0.1mlofover night broth culture of test bacteria is added.

3. To the tube labelled positive control l, 0.1 ml of overnight broth culture of known *S. aureus* is added and to the tube labelled negative control, only 0.1ml of sterile broth is added.

4. All the tubes are mixed gently, incubated at 37^{O}C and observed up to four hours. If the test remains negative until four hours at 37^{O}C, the tube is kept at room temperature for overnight incubation.

5. Avoid shaking or agitating the tube during reading. Doubtful or false negative results may occur due to break down of the clot.

Result: Positive result is indicated from a loose clot suspended to a solid clot that is immovable, which remains in place even after inverting the tube. No degree of clotting is observed in negative result.

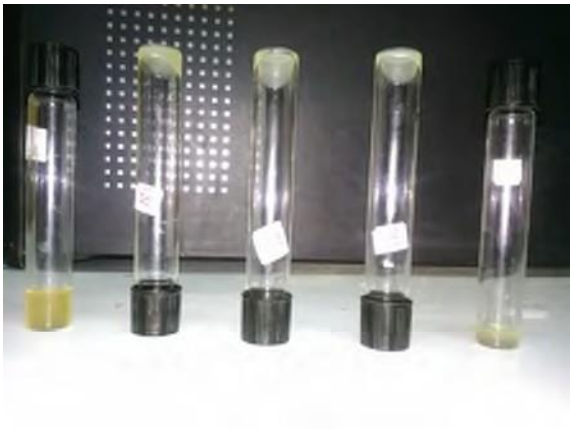

Figure 1: Tube coagulase test.

The second, third and fourth test tubes produced heavy clot and stayed there in the test tube while the test tube stood up side down which represents coagulase positive *Staphylococcus* species. But the first and fifth test tube not produced clotting. They are categorized in to CNS.

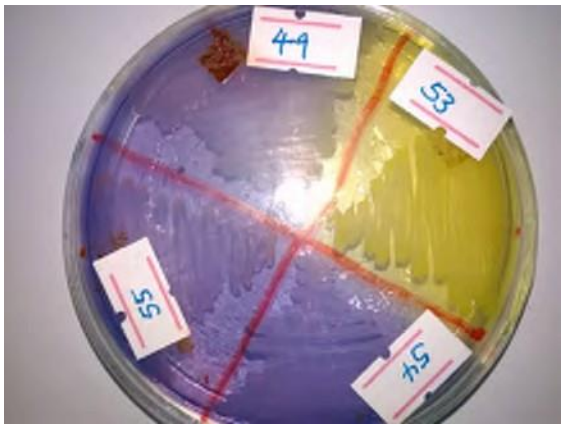

Figure 2: *Staphylococcus* Species Colonies grown on purple agar plate

These colonies changed the color of the medium in to yellow. Colonies changed purple color in to yellow was *S. aureus.*

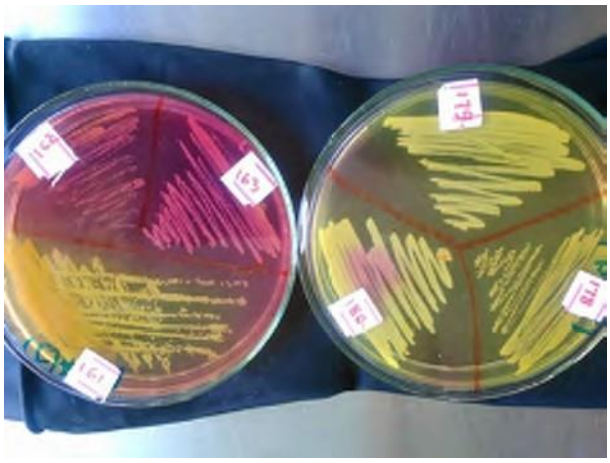

Figure 3: *Staphylococcus* species Colonies grown on Mannitol Salt Agar plate.

The yellow colonies fermented the Mannitol and the red ones are non-ferment.

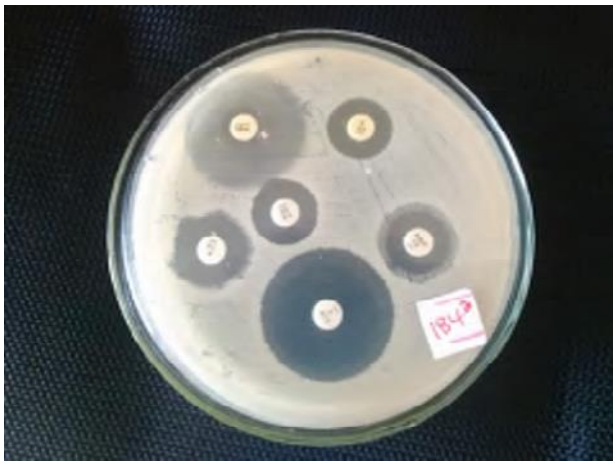

Figure 4: Plate indicated antibiotic diffusion methods

Commonly used Biochemical tests for the identification of *E. coli* and their results are as follows

- ✓ Indole Positive (+ve)
- ✓ MR (Methyl Red) Positive (+ve)
- ✓ VP (Voges Proskauer)Negative (-ve)
- ✓ Simmons Citrate Negative (-ve)
- ✓ TSIA (Triple Sugar Iron Agar) Acid/Acid, Gas +ve

Commonly used Biochemical tests for the identification of *Salmonella* and their results are as follows,

- ➢ Indole-negative. (Tryptophan broth)
- ➢ Triple Sugar Iron Agar-TSI Test: A/A, H2S
- ➢ Voges-Proskauer (VP)-negative (media; MR/VP Broth).
- ➢ Methyl-Red Test: Positive
- ➢ Citrate Test-positive (media: Simmon's Citrate Agar).

APPENDIX 4: Susceptibility Tests

Antimicrobial susceptibility tests (ASTs) basically measures the ability of an antibiotic or other antimicrobial agent to inhibit the invitro microbial growth. This ability may be estimated by either the dilution method or the diffusion method.

Procedure

- ✓ Select a pure culture plate of one of the organisms to be tested.
- ✓ Aseptically emulsify a colony from the plate in the sterile saline solution. Mix it thoroughly to ensure that no solid material from the colony is visible in the saline solution.
- ✓ Repeat until the turbidity of the saline solution visually match that of the standard turbidity.
- ✓ Take a sterile swab and dip it into the broth culture of organism.
- ✓ Gently squeeze the swab against the inside of the tube in order to remove excess fluid in the swab.
- ✓ Take a sterile Mueller-Hinton agar (MHA) plate or a nutrient agar (NA) plate.
- ✓ Use the swab with the test organism to streak a MHA plate or a NA plate for a lawn of growth.
- ✓ After the streaking is complete, allow the plate to dry for 5 minutes.
- ✓ Antibiotic discs can be placed on the surface of the agar using sterilized forceps.
- ✓ Gently press the discs onto the surface of the agar using flame sterilized forceps or inoculation loop.
- ✓ Carefully invert the inoculated plates and incubate for 24 hours at 37° C.
- ✓ After incubation, use a metric ruler to measure the diameter of the zone of inhibition for each antibiotic used.
- ✓ Compare the measurement obtained from the individual antibiotics with the standard table to determine the sensitivity zone.
- ✓ Compare the measurement obtained from the individual antibiotics to the standard table to determine whether the tested bacterial species is sensitive or resistant to the tested antibiotic.

McFarland turbidity standards are prepared by mixing various volumes of 1% sulfuric acid and 1% barium chloride to obtain solutions with specific optical densities. 0.5 McFarland turbidity

standard provides an optical density comparable to the density of a bacterial suspension 1.5x 10^8 colony forming units (CFU/ml). 0.5 McFarland standard is commercially available.

APPENDIX 5: Zone Diameter (mm) and Microbial Inhibition Concentration

Table 3: *S. aureus*

Antimicrobial	**Concentration (µg/disc)**	**Susceptible (mm)**	**Resistant (mm)**	**Intermediate (mm)**
Methicillin (Met)	10	≥17	≤13	14-16
Erythromycin (ERY)	15	≥18	≤ 12	13-17
Cefoxitin (Cxt)	30	≥22	≤21	-
Ciprofloxacin (CIP)	5	≥21	≤15	16-20
Gentamicin (GM)	10	≥15	≤12	13-14
Penicillin (P)	10	≥28	≤29	-
Nitrofurantoin (Nit)	30	≥18	≤13	14-18
Streptomycin (S)	10	≥15	≤11	12-14
Tetracycline (TE)	10	≥15	≤11	12-14
Kanamycin (K)	30	≥18	≤13	14-17
Sulfametoxazole-trimethoprim(ST)	5	≥16	≤10	11–15

Table 4: Enterobacteriaceae

Antimicrobial	Concentration (µg/disc)	Susceptible (mm)	Resistant (mm)	Intermediate (mm)
Vancomycin	30	≥17	≤13	14-16
Ceftriaxone (C)	30	≥18	≤ 12	13-17
Ciprofloxacin (CIP)	5	≥21	≤15	16-20
Gentamicin (GEN)	10	≥15	≤12	13-14
Nalidixic Acid (NA)	30	≥18	≤13	14-18
Streptomycin (S)	10	≥15	≤11	12-14
Tetracycline (TE)	30	≥15	≤11	12-14
Kanamycin (K)	30	≥18	≤13	14-17
Trimethoprim(TR)	5	≥16	≤10	11–15

APPENDIX 6: Questionnaire Format

Assessment of Knowledge, Attitude and Practices (KAP) of animal owners on antimicrobials use, residue and resistance and hygiene management and good dairy farm practices (Interview Questionnaire intended for dairy cattle farm owners at farm level)

Date: _____

Questionnaire code: ……

A) General Information/ Socio demographic characteristic of animal owners/respondents

1. Respondent's name: - …… /Farm name: …… Address (Tell): …… District: ……Kebele/FA……
2. Gender: a) Male b) Female
3. Age (in years): ……
4. Education: a) not able to read and write b) Read and write or Primary school (grade 1-8) c) High school (grade 9-12) d) Vocational/college diploma e) University degree
5. How many years is your dairy farming experience (in years)? ……
6. How many employees (full-/part time or paid and not paid) including family members work in the farm? ……..
7. What breed of animal do you have?
   a) Pure exotic: _____L/kg b) zebu: _____L/kg
   c) Cross bred: _____L/kg

B) Housing design, Feeding and Watering of the Farm animals

8. What is the production system of the farm? (Observation):
   a) Intensive b) semi-intensive and small holder c) extensive
9. What is the housing design of the dairy farm? Observation:
   a) Indoor b) Outdoor c) Semi-indoor
10. What do you feed your animals?

a) Industrial mixed b) homemade c) both d) others (specify)……

11. What type of barn do you own?

a) Housed b) fenced c) no barn

12. How frequent do you clean your cow's house/barn?

a) daily b) two times a week c) three times a week d)once a week e)do not clean

13. What are the sources of water?

a) pipeline b) water well c) lake water d) other (specify) ……

14. How many milking cows: total (……), only local breeds (#……), cross-breed ……. % (# ……) and pure exotic breeds (…….) currently available in the farm?

C) Hygienic practices during milking

15. How often do you clean (wash) your dairy animals? …… (per month); the floor…… (per month)?

16. How many times (frequency of milking) do you milk your cow per day (per 24 hrs)? ……

17. What do you use to milk a cow?

a) hand milking b) machine milking c) both

18. If the answer is (a) do you wash your hands before milking?

a) Yes…… b)No……

19. Do milkers use glove during milking? a) yes b) no

20. What material do you use to collect milk?

a) plastic can b) metallic can c) other (specify) ……

21. What do you use to clean milking instruments?

a) Clean water b) water with detergents c) other (specify)……

22. Do you wash your cow's udder before milking? a) Yes….. b) No……..

23. If yes, when do you wash it?

a) Cleaned before milking only b) Cleaned after milking only c) Cleaned before and after milking

24. If you wash the udder what materials do you use for drying?

a) Collective towel b) Individual towel c) Just with hands d) others (specify)

25. What is the source of the water used for washing the hand, udder and milk utensils?

a) Piped/ tap…… b) River/ stream c) Hand dug well d) Other (specify)……

26. Who is milking the cows?

a) owner/operator b) family member(s) of animal owner c) hired worker(s) d) others (specify)…

27. Is a milkier works at other farms as well? a) yes b) no

D) Milk chain and quality assessment

28. Where do you sell the milk/products?

a) Industries b) small shops c) neighbors d) other (specify) ……

29. Do you cool the milk before sale? a) Yes____ b) No____

30. If yes how? a) Refrigerator b)Traditional cooling system c)If others _____

31. Do you transport your products to market places? a) Yes b) No

32. If YES, what is the means of transportation?

a) on foot b) horse cart c) on animal back d) Public transport e) Private car

f) other means (specify)_____

33. Do the workers at collecting centers test the quality of milk before adding to the pool?

a) Yes b) No

34. If YES, indicate method of quality test and criteria use

a) Alcohol test b) Density test c) Clot on boiling test d) lacto scan e) Other (Specify)

35. Has your milk been rejected by the cooperative? a) Yes…….. b) No……..…

36. If yes, why was it rejected? a) Low fat b) Abnormal color c) Failed Alcohol test

d) Low Density e) Abnormal smell f) Dirt g) Other (Specify)

37. If you consume the milk at home in what form of do you use?

a) Fresh whole milk……..... b) Ergo (naturally fermented).

c) Butter…….…….. d) Arera (defatted sour milk) ..

e) Nitir kibe (Ghee) ……..... f) Ayib (cottage cheese). g) Aguat (whey) ……...

E) Farm animal health

38. Other than dairy animals, which species, type and how many animals do you keep?

a) Cattle (……) and type……..…? b) Chicken (……) and type……?

c) Sheep (……) d) goat (……) e) Others (specify) ……31.

39. What common dairy cow diseases/symptoms you have experience/know or aware

40. What did you do when encounter with such animals' illness? More than one response is possible.

a) Call for private animal HCP/Vet b) go to government animal health clinic

c) go to private vet clinic d) go to a nearby veterinary pharmacy and buy medicines

e) go to any shop/open market and buy medicines f) use traditional medicines in own area

g) slaughter the animal h) other (specify) ……

41. Do you have health/medicines use records of each animal? a) yes b) no

F) Waste disposal system

42. Do you have a proper waste disposal /management/ system in the farm?

a) Yes b) no

43. For what purposes do you use the animal faeces/dung?

a) Urea source for vegetables b) for biogas c) for fuel d) others (specify)……

44. Which method of manure-storage or treatment used in your farm?

a) Under-floor pit b) fabricated manure storage tanks c) dry manure storage d) others (specify)…….

45. Where do you dispose the manure? …… ……

G) Antimicrobials/Antibiotics Use, Residues and Resistance

46. Do you know/familiar with what antimicrobials mean?

a) yes b) no (check by e.g. and displaying the drugs) c) don't know

47. List three most common AMs that you know? a) ……

b) …… c) …… d) Do not remember

48. Do you know/ familiar with what antibiotics mean?

a) Yes b) No (check by e.g. and displaying the drugs) c) don't know

49. Have you ever used (self-prescribed) AMs for your animals in the last one month?

a) Yes b) No--→move to 48

50. If yes to Q48, for which animals do you use antimicrobials/antibiotics?

a) Calves b) heifers c) milking cows d) pregnant/dry cows e) others (specify) ……

51. If yes to Q49, why do you use antimicrobials/antibiotics?

a) Treat individual sick animal b) after parturition c) dry cow treatment

d) prevent a group of animals from being sick (prophylactic/meta phloxes)

e) others (specify) ……

52. If yes to Q64, how did you get/select the medicines used?

a) Prescribed by animal healthcare provider b) self-selected

c) Recommended or given by neighbors d) Others (specify)……

53. Who is administering the AMs?

a) Veterinarian/ animal health professionals b) animal owners (non-vet)

c) farm supervisor (non-vet) d) other (specify……

54. Which AMs/Abs is commonly used in your farm?

a) Ox tetracycline b) Pen strep c) Penicillin d) sulfa drugs e) others (specify)……

55. Do you know any kind of antibiotics or AMs that are not allowed to use in livestock production?

a) Yes (specify)…… …… b) No c) Others (specify)……

56. Were any of the animals have had any adverse medicines events/reactions during administration of the medicines (such as hives, collapsing, abortion, decrease in milk production, fever, lethargy, respiratory distress, lumps or swelling in the injection area, medicines do not work as expected etc)?

a) Yes b) No

57. How frequent do you use antimicrobials/antibiotic over 1-month period?

a) once b) 2-5 times c) more than 5 times d) Specify (……..)

58. What are the sources of antimicrobials you used? Multiple responses are possible.

a) veterinary pharmacies/drug shops b) human pharmacies c) open market/ any shop

d) veterinary clinics e) traditional practitioners f) community animal health worker

g) others (specify)……

59. Have you ever self-prescribed (administered) AMs for your neighbors' sick animals?

a) Yes b) No

60. Who advised you or provided information to administer antimicrobials/antibiotics? Multiple responses are possible.

a) Veterinarian or animal health worker b) veterinary pharmacy

c) Human pharmacy professional in pharmacy d) pharmaceutical or feed distributer

e) Traditional medicine practitioner f) other farmer g) own experiences

61. Do you administer the full dose and course of the antimicrobials as recommended by the product labels? a) Yes b) No
62. If no to Q76, why not? Multiple responses are possible.

a) Have no sufficient money b) believed that it is enough

c) Advised by others d) others (specify) ……

63. Do you ever stop giving antimicrobials before you were supposed to?

a) Yes b) No

64. If yes to Q78, why did you stop giving antimicrobials?

a) Because the antimicrobial does not work b) believed that the animal has improved or cured

c) To save for later use d) others (specify) ……

65. Do you share antimicrobials of one animal to another animal? a) Yes b) No
66. Do you reserve antimicrobials for later use other than the current use? a) Yes b) No
67. If the antimicrobials in your hands or those for some reason bought are expired, what do you do with them?

a) Will use them when needed b) will not use them

c) throw away d) return back to where you bought

APPENDIX 7: Checklist for General Information of Dairy Farms

1) Housing systems for cattle
2) Feeding and feed sources
3) Major crops
4) Breeds commonly used
5) Water sources
6) Types of dairy production commonly produced
7) Animal healthcare and health record
8) Milking and handling systems
9) Milk hygiene and material used for milking

10) Manure management
11) Average production level?
12) Milk chain
13) Knowledge of AM
14) Type of AM commonly used
15) Who prescribe AM
16) Dose of prescriptions
17) Knowledge of withdrawal time
18) Management of milk during withdrawals time or drug residue
19) Knowledge of AMR

APPENDIX 8: Some Pictures Taken During Study Time