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Identification and Antibiogram of Staphylococcus Aureus and Molecular Detection of Methicillin Resistant Staphylococcus Aureus from Beef Line in Bahir Dar and Debre Markos Municipal Abattoirs, Northwest Ethiopia

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SCHOOL OF ANIMAL SCIENCE AND VETERINARY MEDICINE

MASTER OF SCIENCE IN VETERINARY MICROBIOLOGY

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MSc. Thesis

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Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science (MSc) in Veterinary Microbiology

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THESIS APPROVAL SHEET

As member of the Board of Examiners of the Master of Sciences (M.Sc.) thesis open defense examination, we have read and evaluated this thesis prepared by **Samuel Abie Wondie entitled "Identification and Antibiogram of *Staphylococcus aureus* and Molecular Detection of Methicillin Resistant *Staphylococcus aureus* from Beef line in Bahir Dar and Debre Markos Municipal Abattoirs, Northwest Ethiopia."** We hereby certify that, the thesis was accepted in partial fulfillment of the requirements for the award of Degree of Masters of Science in **Veterinary Microbiology.**

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DECLARATION

This is to certify that this thesis entitled "**Identification and Antibiogram of *Staphylococcus aureus* and Molecular Detection of Methicillin Resistant *Staphylococcus aureus* From Beef line in Bahir Dar and Debre Markos Municipal Abattoirs, Northwest Ethiopia**", submitted in partial fulfillment of the requirements for the award of Degree of **Master of Science in Veterinary Microbiology** to the post graduate Program of the College of Agriculture and Environmental Sciences, Bahir Dar University by **Samuel Abie Wondie (ID BDU1206715PR)** is an authentic work carried out by him under our guidance. The matter embodied in this project work has not been submitted earlier for award of any degree or diploma to the best of our knowledge and belief.

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LIST OF ABBREVIATIONS

CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
FBD	Food-borne diseases
MDR	Multi-drug Resistant
MRSA	Methicillin resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar
PCR	Polymerase Chain Reaction
PPE	Personal Protective Equipment
SE	Staphylococcal Enterotoxins
SFD	Staphylococcal Food-Borne Disease
SPSS	Statistical Package for Social Science
TAE	Tris Acetate EDTA
TSS	Toxic Shock Syndrome
WHO	World health organization

ABSTRACT

Staphylococcus aureus (S. aureus) is one of the most common zoonotic bacteria, which causes diseases and responsible for the development of resistance against various antibiotic agents. In Ethiopia, data about the pattern of S. aureus and its Methicillin resistant strain is limited. Therefore, the main aim of the current study was to provide the basic data on the detection of S. aureus, its Methicillin resistant strain and antibiogram assessment in Bahir Dar and Debre Markos municipal abattoirs. A cross-sectional study was conducted from January 2021 to April 2022. One hundred fifty swab samples were purposively collected from beef carcasses, knives, splitting axes, cutting tables, hooks, walls of the abattoir houses and personnel hands and cloths. Isolation and identification of S. aureus was performed according to ISO6888-2 and antibiogram assessment was conducted for ten selected antibiotic agents by the disk diffusion method based on Clinical and Laboratory Standards Institute guidelines. Conventional polymerase chain reaction was applied for the detection of mecA gene. S. aureus was detected as 25.3% (38/150) of the samples, out of which, 27.1%, 23.1%, and 26.9% from beef carcass, abattoir environment and abattoir workers, respectively. About 22.7% of S. aureus was isolated from Bahir Dar municipal abattoir, while 28% was from Debre Markos municipal abattoir. The highest proportion of S. aureus was detected from hands and hooks samples (35.7%), the lowest in the splitting axes (11.1%). Furthermore, the isolates were detected from knives, tables, walls and workers' cloths with the proportion of 26.7%, 23.1%, 14.3% and 16.7%, *respectively. All isolates were completely susceptible to Gentamicin; but 100% resistant were recorded to Penicillin and Methicillin. Around 84.2% of S. aureus isolates showed multi-drug resistance. Furthermore, the mecA gene was detected from five isolates (33.3%) of the 15 S. aureus isolates. The contamination of beef carcass, abattoir environment and abattoir workers with S. aureus may have significant risks on the public health and economic aspects in the study areas. Therefore, to minimize the risk of this pathogen, prevention and control strategies such as using most sensitive drugs, creating good abattoir hygiene, equipment and abattoir workers' sanitation and good carcass handling were recommended.*

Key words; *Abattoir, Bahir Dar, Beef, Carcass, Debre Markos, mecA, S. aureus*

Chapter 1. INTRODUCTION

1.1. Background and Justification

Food borne diseases (FBD) are defined as diseases of infectious or toxic nature which are caused by the consumption of contaminated food or water (Kadariya *et al*., 2014). There are around 250 different foodborne diseases in the world. Among them, two thirds are caused by bacterial agents (Loir *et al*., 2003)**.** Food-borne pathogens are microorganisms, such as bacteria, viruses, fungi and a group of parasites (Xihong *et al*., 2014). Bacterial pathogens, including *Staphylococcus aureus* (*S. aureus*), *Escherichia coli (E.coli), Listeria monocytogenes (L. monocytogenes), Salmonella* species and *Campylobacter* species are the most common causes of food-borne diseases and even death in the world (Xihong *et al*., 2014).

Staphylococcus aureus is a Gram-positive, non-spore forming and biochemically, catalase and coagulase positive pathogen that belongs to the family *Staphylococcaceae* (Sadiq *et al*., 2020). It can be transmitted from the contaminated foods of animal origin meat, (i.e. beef, mutton and pork), dairy products and eggs to the healthy individuals (Ash, 2008; Kadariya *et al*., 2014). This organism can be confirmed based on gram staining, hemolysis of blood agar, biochemical tests and growth on mannitol salt agar as well as based on the molecular detection techniques (ISO, 2005; Quinn *et al*., 2004).

Some strains of Staphylococcus have acquired different genes making them resistant to multiple drugs. The antimicrobial resistance strains of *S. aureus* have the quality of persistent availability in the environment and the possible contamination of water and food (Normanno *et al*., 2007). In the early 1960s, the first isolates of Methicillin Resistant *Staphylococcus aureus* (MRSA) were detected in the United Kingdom (Enright *et al*., 2002). The worldwide spread of MRSA strain has become also a serious challenge for human infection control and antibiotic therapy (Pantosti, 2012).

Staphylococcus aureus is a commensal of the skin and hair of the animal from which the meat was obtained (Voss *et al*., 2005) and nostrils and mucous membranes of humans and animals that can cause opportunistic infections following trauma of the skin and mucous membranes

(Lozano *et al*., 2011). *S. aureus* is present in a variety of locations on the slaughterhouse environment that can play significant roles in the dissemination of pathogens from different slaughterhouse zones to the finished meat products (Lavilla *et al*., 2014).

When *S. aureus* gets the opportunity to grow in foods, it can produce different types of enterotoxins preformed in food (Doyle *et al*., 2012; Kadariya *et al*., 2014) that are responsible for the cause of food poisoning and gastrointestinal disease (Luca *et al*., 2006). The implications of food borne diseases are great including health and economic losses (Loir *et al*., 2003**).** The World Health Organization (WHO) estimated that, in developed countries up to 30% of the population suffers from food borne diseases, whereas in developing countries up to 2 million deaths in each year (WHO, 2007).

Even though the information is limited, some researchers conducted various proportions on the detection of *S. aureus* and its antibiotic profile from municipal abattoirs setting. For instance, high isolation rate of *S. aureus,* (56.04%) from abattoir settings and 71.4% in polled knives swab were detected by Million Weldeselassie *et al*. **(**2020) and Fufa Abunna *et al*. (2016), respectively. As well as the low level of *S. aureus* isolates (9.4%) was conducted by Feben *et al.* (2018) in Ethiopia. Abdi Hassan and his cofounders have been also detected as overall of 36.4% of *S. aureus* isolates, of which, 34.3% in beef carcass, 48.6% in abattoir environmental samples (Abdi Hassan *et al*., 2018) and also 53.0% in meat swab, 42.9% in slaughter line swab and 42.9% in polled abattoir hand swab (Fufa Abunna *et al*., 2016). According to the finding of Feben Adugna *et al*. (2018) the isolated *S. aureus* were 100% resistant for Methicillin and 95% of the isolates against tetracycline. The resistance profile of *S. aureus* isolates to penicillin G were 95.3%, 93.8% and 95.5%, which was reported by Takele Beyene *et al*. (2017), Million Weldeselassie *et al*. (2020), Fufa Abunna *et al*. (2016) in Ethiopia, respectively. MRSA has been isolated in livestock meat (pork, beef and chicken), which poses a threat that can potentially lead to the spread of MRSA to consumers through the food chain (De Neeling *et al*., 2007; Wulf and Voss, 2008). The report of Okorie-Kanu *et al*. (2020) indicated that, the overall confirmation of MRSA based on the detection of the *mecA* gene was 0.9%.

1.2. Statement of the Problem

The potential sources of external microbial contamination of meat has pointed to many sources including poor sanitation, dirt, unclean equipment and water, intestinal contents, skin and superficial wounds, hands or clothing of personnel, the physical facilities themselves and numerous other unhygienic factors, mainly along with slaughtering chains during skinning, evisceration, storage and distribution at slaughterhouses (Nouichi and Hamdi, 2009). These factors all lead to contamination of the meat, bacterial multiplication and possible toxin production. In some cases where, abattoir workers' remuneration is linked to the number of head of cattle slaughtered in the abattoir, workers tend to increase all slaughter line speed (Dillard, 2008). The very high speed implies inadequate time for slaughter operations to perform their work that exposed to increased chances of spillage during evisceration and unsterilized equipment lead to increased risk of beef carcasses contamination.

Among the bacteria predominantly involved in meat contamination, *S. aureus* is the major cause of food intoxication (Behling *et al*., 2010). The magnitude of beef carcass, abattoirs and abattoir environment contamination may vary from place to place based on over control of slaughtering activities. However, there is scarce data in Ethiopia, including Bahir Dar and Debre Markos municipal abattoirs, regarding to the detection of *S. aureus* and its Methicillin resistant strain. Thus, there is a need to generate more data from abattoir settings, by taking samples from abattoir environment, personnel and from contaminated regions of the beef carcass.

Staphylococcus aureus is resistant to many groups of antimicrobial agents, and its resistance has been reported in both veterinary and human health sectors (Hanson *et al*., 2011). Prevalence of antimicrobial resistance increased during the recent decades (Van *et al*., 2007). As parts of Ethiopia, in the study areas, raw meat consumption which may result in food-borne diseases and intoxication, including Staphylococcal infection (Setegn Eshetie *et al*., 2016) is a common habit and the full value chain of meat supply from abattoirs distribution to final consumers are not properly handled to ensure the microbial quality and safety.

1.3. Objectives

1.3.1. General objective

The general objective of this study was to identify and assess Antibiogram profile of *S. aureus* and molecular detection of MRSA from beef line in Bahir Dar and Debre Markos municipal abattoirs.

1.3.2. Specific objectives

- To isolate and identify *S. aureus* from the beef line of two municipal abattoirs in Amhara Region, Ethiopia.
- To assess antibiogram profile of *S. aureus* from the beef line of two municipal abattoirs in Amhara Region, Ethiopia.
- To characterize Methicillin resistant *S. aureus* strain from the beef line of two municipal abattoirs in Amhara Region, Ethiopia.

1.4. Research Questions

- Is there *S. aureus* along the beef line of Bahir Dar and Debre Markos municipal abattoirs?
- How is the antibiogram profile of *S. aureus* against antibiotic of choice?
- Is there any Methicillin resistant *S. aureus* strain along the beef line of Bahir Dar and Debre Markos municipal abattoirs?

Chapter 2. LITERATURE REVIEW

2.1. General Characteristics of *Staphylococcus aureus*

The term staphylococci was derived from two Greek words *staphyle* which means "bunch of grapes" and *coccus* that means "spherical bacteria" whereas *aureus* is a Latin word which stands for "gold". This was given to the bacteria because of yellow to yellowish white colonial appearance on enriched medium (Freeman-cook, 2006). It is non-motile**,** facultative anaerobe that occurs as an irregularly clusters resembling bunch of grapes and sometimes singly or in pairs when observed under light microscope by Gram staining (Licitra, 2013) and 0.8-1 µm in diameter, colonies are smooth raised yellow to golden yellow color (Plata *et al.*, 2009; Pal, 2007).

The bacteria can grow on a variety range of temperature from 5 to 45ºC and PH 4-10 (Ercolini *et al*., 2006). Foods with a pH around 7 are ideal for bacterial growth, such as most animal food products including meat, fish, poultry, eggs and milk. The organism is resistant to drying and may grow and produce enterotoxins in foods at low water activity (Ash, 2008). Even though, cooking foods destroys the bacteria, the toxin produced is heat stable and proteolytic enzyme resistant enterotoxins that resist heating at 100°C for 30-70 min. The organism is often hemolytic in blood agar containing 5% sheep or horse blood due to production of hemolysins (Dinges *et al*., 2000). It is salt tolerant, which is able to grow in mannitol-salt agar medium containing 7.5% sodium chloride (Brown *et al.*, 2005).

Staphylococcus aureus multiplies by binary division, and under suitable conditions of environment and temperature, the multiplication occurs every 15-30 minutes. Thus, one cell could become over 2 million in 7 hours and 7000 million cells after 12 hours continuous growth (Jay, 2000).

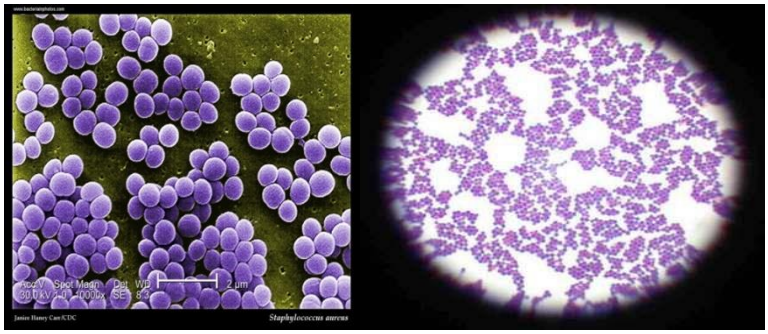

Figure 1. Shape of *S. aureus* under light microscope (CDC, 2022).

2.2. Nomenclature of Staphylococcus

The genus Staphylococcus is under the family of *Staphylococcaceae*. Currently, 53 species of *staphylococci* and 28 subspecies are recognized in the genus Staphylococcus that is collectively referred to as staphylococci, most of which are found only in lower mammals (PHE, 2020). Kingdom Bacteria, Phylum *Firmicutes*, Class Bacilli, Order *Bacillales*, Family *staphylococcaceae* and Genus Staphylococcus are the scientific classification of *Staphylococcus aureus*. The pathogenic *staphylococci* are *S. aureus, S. intermedius and S. hyicus* (Morrison, 2008). There are currently 2 subspecies of *S. aureus*; these are *S. aureus subspecies aureus* and *S. aureus subspecies anaerobius* (PHE, 2020).

2.3. Epidemiology of *Staphylococcus aureus*

Staphylococcus aureus and its drug-resistant strains, MRSA are ubiquitous and found on the skin glands of humans, other mammals, soil, water and air (den Heijer *et al*., 2013; Taylor and Unakal, 2022). They are sometimes found in the blood, mouth, intestinal genitourinary and upper respiratory tracts of the hosts.

The bacteria is a very hardy organism and can survive on dry surfaces over a long period; it is resistant to desiccation and can survive high level of salt concentration a basis for selection on growth media from other bacteria (Ash, 2008). Staphylococcal related diseases may be associated with overcrowding, problems with hygiene, sanitation, housing conditions, food and water quality, particularly in developing countries (Heath, 2006). Studies in few parts of Ethiopia show the detection level of *S. aureus* in food of animal origin (Table 1).

Table 1. Status of *S. aureus* from animal origin food in Ethiopia

Source of Sample	Prevalence (%)	Source
Abattoir source	13.2	Takele Beyene *et al*., 2017
Beef carcass swab	11.7	
Cutting table swab	15	Feben Adugna *et al*., 2018
Knife swab	22.5	
Abattoirs' cloth swab	40	Abdi Hassan *et al*., 2018
Abattoir workers' hand swab	60	
Hook swab	60	
Axe swab	50	

2.4. Reservoirs and Transmission

Staphylococcus aureus is transmitted from the contaminated animal-source foodstuff by direct contact and or with infected people and ingestion of contaminated animal origin food (Shimelis Argaw and Mekonnen Addis, 2015).The species of *Staphylococcus* mainly, Methicillin resistant *S. aureus* can be transmitted from person to person, as well as from animals to humans and vice-versa. *S. aureus* has the potential to contaminate animal products and may enter the food chain during processing, preparation, wrapping, mincing, and storage (Wang *et al*., 2017).

Various food items can be contaminated by staphylococcal enterotoxins, especially moist food containing starch and protein (Wu *et al*., 2016). Several food materials including beef, pork, mutton and poultry are common vehicles that are frequently implicated in Staphylococcal food poisoning (Wang *et al*., 2017. Raw meat is a good medium for *S. aureus* survival and spread of drug-resistant *S. aureus* in the community (Melaku Tefera *et al*., 2019). Additionally, abattoir workers, butcher men and any meat handlers and processers carrying *S. aureus* on their bodies or gloves, nasal secretions, sneezing, coughing can also contaminate food (Wang *et al*., 2017).

The nasal passage of humans and the skin and hair of warm-blooded animals and food origin are the primary habitat of *S. aureus.* Mainly, humans are the major reservoir host for this

organism (Guven *et al*., 2010). In carrier state, the organism found in the anterior nares and can remain without causing infections for weeks or months. People who carry *S. aureus* can contaminate the food with their contaminated hands. If food is contaminated with *S. aureus*, the bacteria can multiply in the food and produce toxins that can make illness (Shimelis Argaw and Mekonnen Addis, 2015).

2.5. Pathogenesis

The process of *S. aureus* infections involves colonization, local infection, systemic dissemination and/or sepsis, metastatic infections and toxinosis (Moormeier and Bayles, 2017). The colonization proceeds to infection under certain predisposing factors i.e. immune suppression, surgeries, chronic metabolic diseases and use of invasive medical devices . Localized skin abscess develop when the organism is inoculated into the skin from a site of carriage. After entering into the blood, the organism can spread systemically to different organs and causes sepsis. Specific syndromes can occur without a blood stream infection due to extra cellular toxins of *S. aureus*. *S. aureus* is one of the most important pathogenic members of the genus Staphylococci and a leading cause of nosocomial, community and livestock associated infection (Bloemendaal *et al.*, 2010). The pathogenicity nature of *S. aureus* is because of the bacterial structures and extracellular products, such as toxins, which could cause staphylococcal related diseases which are transmitted by food (Kadariya *et al*., 2014).

2.6. Virulence Factors of *Staphylococcus aureus*

Staphylococcus aureus possess battery of virulence factors. These virulence factors enable the organism to become successful as pathogen that causes huge of human and animal infections. Virulence factors of the organism help in attachment to host cells, breaking down the host immune shield, tissue invasion, causing sepsis and elicit toxin-mediated syndromes. This is the basis for persistent staphylococcal infections without strong host immune response (Kima *et al*., 2016). *S. aureus* virulence factors include hemolysins, leukocidins, proteases, enterotoxins, exfoliative toxins, and immune-modulatory factors (Foster, 2005). The expression of these factors is totally regulated during growth of the organism. The *agr* system, known as the quorum-sensing system, is known to play a central role in the regulation of

virulence factors (AgrAC is a two-component system (TCS) that consists of a histidine kinase and a response regulator. The Agr system regulates the expression of the gene coding for small RNA, known as RNA III, which is localized divergently at the *agr* operon and regulates the expression of many virulence factors, such as hemolysins, leukocidins, and protein A (Novick and Geisinger 2008). The virulence factors of *S. aureus coa*, and *spa* genes are directly linked to pathogenesis and the magnitude of staphylococcal infection, respectively. Both virulent genes are highly polymorphic and can provide critical information on strain variations (Sadiq *et al*., 2020**).**

2.7. Staphylococcal Enterotoxins and Food Poisoning

Staphylococcal enterotoxins (SEs) are in the family of more than 20 different types enterotoxins. Among these serological type enterotoxin, includes, staphylococcal enterotoxin A-E (SEA, SEB, SEC, SED, and SEE) and other entities as toxic shock syndrome toxin1 (TSST-1) and exfoliative toxins A and B. TSST-1 is the toxin responsible for toxic shock syndrome (TSS) and is only caused by strains carrying the TSST-1 gene (Behling *et al*., 2010). Staphylococcal enterotoxins are stable and highly resistant to heat and environmental conditions such as freezing and drying and also for proteolytic enzymes, like pepsin or trypsin and for low pH value (Argudin *et al*., 2010). The most common SEs are SEA and SEB. Of these, SEA is most frequently involved in food poisoning. SEB is not only involved in food poisoning but identified as a potential biological weapon of war and terrorism (Pinchuk *et al*., 2010).

FBD are intoxication, infection and toxico-infections (Dhama *et. al.,* 2013). Staphylococcal food borne disease (SFD) is one of the most common FBD in the world that are resulted after releasing of enterotoxins into the food and toxic shock syndrome by release of super antigens into the bloodstream. Heat stability is one of the most important properties of SEs with regard to food safety (Behling *et al*., 2010). However, it is believed that SEs directly affects intestinal epithelium and vagus nerve causing stimulation of the emetic center. The onset of FBD caused by *S. aureus* is rapid following ingestion of contaminated food (usually 3– 5 hours). This is because of the production of one or more toxins by the bacteria during growth at permissive temperatures (Loir *et al*., 2003).

2.8. Clinical signs and Diseases Caused by *Staphylococcus aureus*

The onset of symptoms in staphylococcal food poisoning is usually rapid, typically within 1–6hr, and is influenced by individual susceptibility to the toxin, amount of contaminated food consumed, amount of toxin in the food ingested, and general health status (Ash, 2008). Localized skin abscess can further spread and results in various clinical manifestations of localized infections such as carbuncle, cellulitis, and impetigo bullosa or wound infection. Serious infections may result in, pneumonia, meningitis, mastitis, renal carbuncle, septic arthritis and epidural abscess, urinary tract infections and deep-seated infections such as osteomyelitis and endocarditis most of which are minor and not life-threatening(Shaw *et al*., 2004).

Extra cellular toxins of *S. aureus* can cause toxic shock syndrome, scalded skin syndrome and food borne gastroenteritis (Ferry *et al*., 2005). The most common symptoms are also nausea, vomiting, abdominal cramping, diarrhea, sweating, headache, prostration, and sometimes a fall in body temperature (Behling *et al*., 2010).

2.9. Diagnosis

For localized infections, the clinical appearance is generally sufficient without the need for analysis of microbiological cultures. However, in patients with systemic infections, more extensive microbiological testing and their susceptibility to different antibiotics is indicated in order to provide appropriate treatment, and to initiate relevant control measures (Schofer *et al*., 2011). For diagnosis, the former steps are isolation and identification of the bacteria from appropriate specimens. Bacteriological culture, serological testing and molecular techniques are the crucial methods for confirmatory diagnosis of staphylococcal infections (Quinn *et al*., 2004).

Mannitol Salt Agar is a type of media that act as selective and differential medium for the isolation and identification of *S. aureus* collected from suspected cases. It contains peptones and beef extract, which supply nitrogen, vitamins, minerals and amino acids essential for growth. The 7.5% salt concentration of the medium is used to partial or complete inhibition of bacterial organisms other than staphylococci and used to supplies essential electrolytes for

transport and osmotic balance. On MSA, pathogenic *S. aureus* ferments mannitol, thereby changing the color of the medium from red to yellow. The reason for this color change is that *S. aureus* have the ability to ferment the mannitol, producing an acid, which changes the phenol red indicator color from red to yellow. This growth differentiates mannitol fermenter *S. aureus* from non-mannitol fermenter Staphylococci species which forms colonies with red zones. For identification of *S. aureus*, the specimen was inoculated the MSA and incubate from 24-48 hours at 37°C (Jorgensen and Turnidge, 2015).

Catalase test is important to distinguish staphylococci which are catalase-positive from streptococci (catalase-negative). Organisms such as staphylococci which produce catalase enzyme break down the hydrogen peroxide and results O_2 production which produces bubbles in the reagent drop indicating a positive test. Organisms that have not the cytochrome system also lack the catalase enzyme and could not break down hydrogen peroxide, into O_2 and water and are catalase negative (Quinn *et al*., 2004).

Coagulase test is used to distinguish between coagulase-positive *S. aureus* and coagulase negative, other pathogenic Staphylococcus such as *S. epidermidis* and *S. saprophyticus* (Morrison, 2008). *S. aureus* isolates produce free and bound coagulase. Bound coagulase is a cell wall associated protein that can be detected in slide coagulase test. After incubation at 37^oC for 24 hours, the tube test used to detect free coagulase usually within 4 hours. Fresh or reconstituted commercial freeze-dried rabbit plasma is the reagent used which contains fibrinogen that is converted to fibrin by the staphylococcal coagulase enzyme (Harley and Prescott, 2002).

Antibiotic susceptibility testing is important to confirm susceptibility to chosen empirical antimicrobial agents, or to detect resistance in every bacterial isolates. The disk diffusion method is simple and has been well-standardized. The test is performed following inoculation of the bacteria on the surface of a large Mueller-Hinton agar plate. The Antibiotic disks are commercially-prepared, having fixed concentration and paper like antibiotic disks that can be placed on the inoculated agar surface. Plates are incubated for 16–24 hours at 35°C. The zones of growth inhibition around each of the antibiotic disks are related to the susceptibility pattern of the isolate and to the diffusion rate of the drug in to the agar medium. The

diameters of inhibition zones of each drug can be interpreted based on the Clinical and Laboratory Standards Institute (CLSI) as indicated in table 2 (FDA, 2003). The results of disk diffusion test are "qualitative," in the category of susceptibility (susceptible, intermediate or resistant) with reference of 0.5 McFarland standards on Muller Hinton agar plats (CLSI, 2021).

Table 2. Types and diameter of inhibition zone of antibiotics for *S. aureus*

Antibiotic Agents	**Disk Content**	**Zone of Diameter Interpretive Criteria (mm)**		
		Susceptible	Intermediate	Resistant
Penicillin	10IU	≥29	___	≤28
Ampicillin	10µg	≥36	27-35	≤26
Gentamicin	10µg	≥15	13-14	≤12
Erythromycin	15µg	≥23	14-22	≤13
Ceftazidime	30µg	≥21	16-20	≤15
Norfloxacin	10µg	≥17	13-16	≤12
Doxycycline Hydrochloride	30µg	≥19	15-18	≤14
Clindamycin	2µg	≥21	15-20	≤14
Methicillin	5µg	≥23	17-22	≤16
Co-Trimoxazole Sulfamethoxazole	25µg	≥16	11-16	≤10

Molecular diagnostic techniques of *S.aureus* include targeting on *nuc, mecA, femA, coa, spa, 16S rRNA, Sa442* and other genes (He *et al*., 2010). Detection of *S. aureus* genes associated with antibiotic resistance including *mecA, aacA-aphD, tetM, ermA, ermC, vatA, vatB, vatC* facilitates the appropriate detection and control of pathogens. The molecular confirmation of *S. aureus* and MRSA are conducted via Polymerase Chain Reaction (PCR), which is used for detection of *16S rRNA* gene that is specific for bacterial identification, *nuc* gene that confirms *S. aureus* presence, *mecA* gene that codes for penicillin binding protein 2a *(*PBP2a*)* and is used for the detection of MRSA and for two virulence factors (*spa* gene and *coa* gene) of highly pathogenic *S. aureus (*Sadiq *et al.*, 2020*).*

Polymerase chain reaction has been also used to detect Staphylococcus enterotoxins' by amplifying their corresponding genes, but couldn't indicate whether enterotoxins are produced (Wu *et al*., 2016). The whole genomic sequences of *S. aureus* strains yields the great opportunity to carry out detailed investigation of the molecular mechanisms, virulence and pathogenesis in the microbial genome levels. Comparative analyses of *S. aureus* sequences can identify coding and conserved non-coding regions, including regulatory elements and species-marker (Liu *et al*., 2007).

2.10. Treatment

Antimicrobial agents such as Penicillin, Erythromycin, Neomycine, Vancomycin, Streptomcin, Trimethoprim, Norfloxacin and Tetracycline (Pal, 2007) are widely used for prophylaxis and treatment of *S. aureus* infections in human and animal. However widespread use of antibiotic drugs in hospitals and veterinary clinics, especially in food-producing animals develops selective pressure for *S. aureus* to become resistant to antibiotics (Threlfall *et al*., 2000).

Staphylococcus aureus develops resistance to antimicrobials by different mechanisms. These mechanisms include limiting uptake of the drug, modification of the drug target, enzymatic inactivation of the drug, and active efflux of the drug (Smith *et al*., 2002). Most strains of *S. aureus* contain plasmids that encode β-lactamase, the enzyme that degrades penicillin. Some strains of *S. aureus* are resistant to Methicillin which is commonly known as MRSA, which is the major cause of nosocomial/community acquired infection in human (Otter and French, 2008).

2.10.1 Emerging of Methicillin-resistant *Staphylococcus aureus*

For the first time, Methicillin was introduced into clinics in 1961; however, in less than a year, resistance of MRSA was reported and MRSA outbreaks were mentioned in different parts of the world mainly from the European countries. The notable feature of these reports is that, the incidences were from hospitals and thus MRSA emerged as a hospital-borne pathogen (Klevens *et al*., 2007).

Methicillin-resistant *S. aureus* are strain of *S. aureus* that carry a *mecA* gene, which codes for additional penicillin-binding protein, PBP-2a. The beta-lactam antibiotics exert their antibacterial activity by inactivation of penicillin-binding proteins (PBPs), which are essential enzymes for bacterial cell wall synthesis and catalyzes the production of the peptidoglycan in the bacterial cell wall. However, the antibiotics have a low affinity towards PBP2a, thus this enzyme evades from inactivation and carry out the role of essential PBPs resulting in cell wall synthesis and survival of bacteria even in availability of beta-lactam antibiotics. Therefore, due to the presence of *mecA* gene, MRSA are resistant to nearly all beta-lactam antibiotics (Fuda *et al*., 2004).The *mecA* gene is a DNA segment, which is a part of mobile genetic element that is non-native to *S. aureus* and it is inserted in a large block of exogenous DNA, known as the staphylococcal cassette chromosome *mec* (SCC*mec*) (Katayama *et al.,* 2000). The gene also contains genetic structures such as Tn554, pUB110 and pT181 (Wielders *et al*., 2002; Enright *et al*., 2002).

The *mec A* gene *S. aureus* strains that are phenotypically resistant to Methicillin, but they do not harbor the *mecA* gene (Velasco *et al*., 2019). The phenotypic Methicillin resistance has been associated with variations of the *mecA* gene, such as the $mecA_{LGA251}$ renamed as *mecC* (Stegger *et al*., 2012; Garcia-Alvarez *et al.,* 2011), the *mecB* gene (Tsubakishita *et al*., 2010), and others that are not as well-known (Velasco *et al*., 2015). The *mecC* gene is located on the staphylococcal cassette chromosome *mec* type XI (*SSCmec* XI) and exhibits 70% sequence homology with the *mecA* gene (Ito *et al*., 2012; Garcia-Alvarez *et al.,* 2011). In addition to these, MRSA lacking *mec* genes may have uncommon phenotypes, such as the β-lactamase hyper production, which partially hydrolyzes the β-lactam ring and different nucleotide mutations in PBP genes (Nadarajah *et al*., 2006).

2.11. Prevention and Control

Eliminating *S. aureus* from the environment is impossible, due to its ubiquitous nature. The way of prevention of staphylococcal infections or intoxication requires strategies to interrupt different modes of transmissions (Shimelis Argaw and Mekonnen Addis, 2015). Cooking food thoroughly, maintaining critical points, preventing contamination and cross-contamination are effective ways to prevent staphylococcal infections. Public awareness regarding safe meat-handling and other public health interventions could be a cornerstone in

preventing the outbreak (Kadariya *et al*., 2014). To prevent food-poisoning outbreaks, it is necessary to keep foods either refrigerated (-10°C) or hot (45°C) to prevent proliferation of the organism to such numbers (10^5 cells/g) necessary for detectable toxin formation (Bennett and Monday, 2003).

Controlling program of *S. aureus* and MRSA include improvements in personal hygiene practices among health professionals and food handlers, decontamination of equipment, surfaces, clothing, judicious use of antibiotics, proper cooking and storage of food, and screening programs (Shimelis Argaw and Mekonnen Addis, 2015). Microbiological guidelines such as Hazard Analysis and Critical Control Points (HACCP), Good Manufacturing Practice and good hygienic practices developed by the WHO and the United States Food and Drug Administration should be implemented strictly to prevent and control *S. aureus* contamination (Kadariya *et al*., 2014)**.**

2.12. Public Health and Economic Importance

Staphylococcus aureus is the major pathogen of public health concern throughout the world. MRSA has become a pathogen of increasing importance in nosocomial infection, the community, and also in the livestock. Staphylococcal food poisoning is an intoxication which is caused by the ingestion of contaminated food with pre-formed staphylococcal enterotoxins (Argudin *et al*., 2010). WHO and the US Centers for Disease Control and Prevention (CDC) report every year a large number of people affected by foodborne illnesses (Luca *et al.*, 2006) The presence of antibiotic-resistant strains has become an emerging zoonotic issue of public health concern (Hachemi *et al*., 2019).

In addition to illnesses and deaths, *S. aureus* infections have great economic impact due to treatment costs and hospitalization. It may also causes organ or carcass condemnation at abattoir level (Halasa *et al*., 2009) and there may be losses from export rejection of different food items as well as trade rejections. This disadvantage mainly arises from food product processors and exporters failed to meet the importing country hygiene and processing quality standards (Smith *et al*., 2002)**.**

Chapter 3. MATERIALS AND METHODS

3.1. Study Area Setting

The study was conducted in municipal abattoirs of Bahir Dar and Debre Markos, Amhara Region, Northwest, Ethiopia (Figure 2). Bahir Dar is the capital city of Amhara regional state that is found in the North-Western part of Ethiopia. It is found 565 kilo meters far from Addis Ababa, the capital of Ethiopia, and is located between 11° 25' 19" to 11° 57' 7"N latitude and 37° 14' 35" to 37° 29' 7"E Longitude. It is at1500-2600 m.a.s.l altitude on the South of Lake Tana where Abay River starts. Its annual average rainfall ranges from 1200 to 1600 mm and the temperature is 38°c (BARD, 2018). Debre Markos is the capital city of East Gojjam Administrative Zone, which is located in the North-West of Addis Ababa at a distance of 300 kilo meters and South- East of Bahir Dar at a distance of 265 kilo meters. The geographical location of Debre Markos is located between 10°17′00″ to 10°21′30″ N latitudes and 37°42′00″ to 37°45′30″ E longitudes and its elevation ranges in altitude from 2350 -2500 m.a.s.l. The city has 1380 mm average annual rainfall and minimum and maximum temperatures of 15^{o}c and 22^{o}c respectively (DMTA, 2011).

Cattle slaughtered from the two municipal abattoirs originated from different districts in and around the study areas. Based on the schedule of slaughtering practice of both abattoirs, the average daily slaughtered cattle were 13 and 8 in Bahir Dar and Debre Markos municipal abattoirs, respectively during the study period. There were also 4 and 1 Veterinary professional/s and on average 13 and 10 abattoir workers at Bahir Dar and Debre Markos abattoirs, respectively. Veterinarians performed anti-mortem and post-mortem inspections, and the remaining activities were carried out by abattoir workers. There was no clear division/sectioning of the slaughtering process into stunning, bleeding, skinning, evisceration, or cutting delivery in both municipality abattoirs.

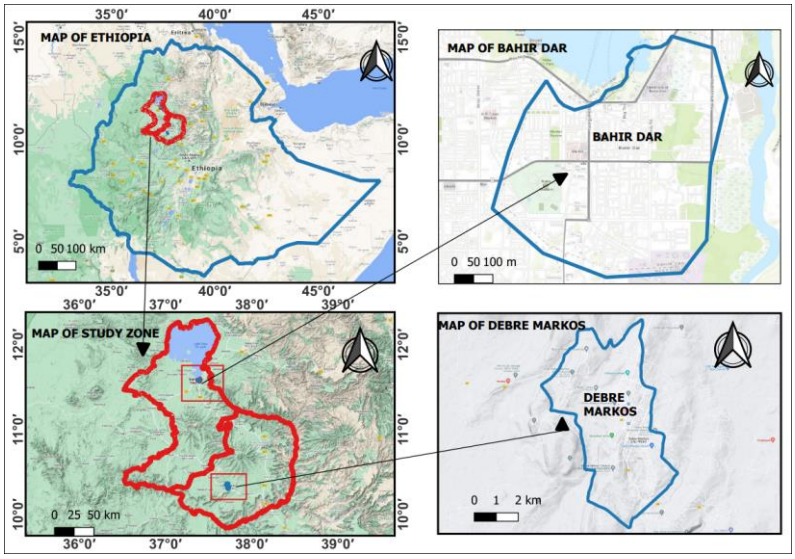

Figure 3. Map of the study areas

3.2. Study Design

A cross-sectional study was carried out from January 2021 to April 2022 for identification, antibiogram assessment and molecular detection of *S. aureus* from beef line in Bahir Dar and Debre Markos municipal abattoirs.

3.3. Study population

The study population includes beef carcass, abattoir equipment (i.e. knife, splitting axe, cutting table and hook) and wall of slaughterhouses as well as hand and cloth of abattoir workers that were found in the abattoirs of the study areas.

3.4. Sampling Method and Sample Size Determination

Non-probability sampling method was used to select the two municipal abattoirs (Bahir Dar and Debre Markos) from the list of municipal abattoirs in Amhara region.

Purposively, 150 swab samples (Beef carcass =59, Knife=15, Cutting table=13 Hook=14, Wall=14, Splitting axe=9, Hand=14 and Cloth=12) were collected from the two municipal abattoirs. In the other way, 65, 59 and 26 swab samples were collected from abattoir environment, carcass and abattoir workers, respectively. Convenience sampling was used to take swab samples from the study population in the two abattoirs. The number of samples to

be included in each abattoir was determined by proportional allocation based on, the similarity of number and working habit of abattoir workers, the same geographical location of abattoirs and comparable daily slaughtering capacity of cattle as well the abattoirs applied the same slaughtering activities and waste disposal systems.

3.4. Sample Collection and Transportation

Swab samples were collected from beef carcass, abattoir workers, and abattoir environment by using the method described in ISO6888-2 (ISO, 2005). Beside the beef carcass, the abdomen (flank), thorax (lateral), crutch and breast (lateral) regions which may have the highest contamination were chosen to collect swab samples. From each site the single swab sample was collected and recorded, separately. The selected beef carcasses were swabbed by placing sterile template (10 × 10 cm) on specific sites of a beef carcass. A sterile cotton tipped swab (2 × 3 cm) was fitted with shaft and first soaked in an approximately 10 ml of buffered peptone water (Oxoid Ltd., Hampshire, England) were rubbed horizontally and then vertically several times on the beef carcasses (Tizeta Bekele *et al*., 2014).

Swab samples were also collected from knife, splitting axe, hook and cutting table, wall of slaughter houses as well as from hand and cloth of abattoir workers during active slaughtering activities/operations. On completion of the rubbing process, the shaft was broken by pressing it against the inner wall of the test tube and was disposed leaving the cotton swab in the test tube. Finally, all samples were collected under aseptic conditions and then were transported with ice box in to Bahir Dar Animal Health Investigation and Diagnostic Laboratory for isolation, identification and antibiogram assessment of *S. aureus*.

3.5. Isolation and Identification of *Staphylococcus aureus*

The collected samples were inoculated on blood agar base enriched with 5% sheep blood and incubated aerobically at 37°C for 24 hours. The plates were then examined for gross colony morphology and hemolytic characteristics within 24 hours. Colonies that showed hemolytic character were selected and sub cultured on nutrient agar and incubated aerobically at 37°C for 24 hours to get pure colonies (Mesele Abera *et al*., 2010). The pure colony was then inoculated on the mannitol salt agar and incubated for 24 hours at 37°C. Based on hemolytic

characteristics on blood agar, Gram's stain reaction, fermentation of mannitol salt agar, catalase activity and coagulase test, *S. aureus* was identified. Following the phenotypic study of *S. aureus*, isolates were stored in a nutrient broth with an addition of 20% glycerol to prevent any frost shock due to crystal formation in bacterial cells and was stored at -20^{o}c, and then the molecular detection was conducted further.

3.6. Antibiogram Assessment of *Staphylococcus aureus*

Antibiogram assessment of *S. aureus* isolates was performed for selected antibiotic disks by using the disk diffusion method based on CLSI (CLSI, 2018; CLSI 2021). Ten antibiotic disks including: Ampicillin (AMP 10µg), Methicillin (Met 5µg), Penicillin G (P-10IU), Doxycycline Hydrochloride (DO-30µg), Erythromycin (E-15µg), Gentamycin (CN-10µg), Clindamycin (CD-2µg), Co-Trimoxazole (COT-25µg), Ceftazidime (CAZ-30µg) and Norfloxacin (NX-10µg) were used. Well isolated colonies of *S. aureus* were selected and the suspension was made in nutrient broth with 5 ml sterile and transparent tubes. The turbidity of the suspension was adjusted by comparison with a 0.5ml McFarland turbidity standard. The sterile swab was dipped into the standardized suspension of bacteria and then excess fluid was minimized by pressing and rotating the swab against the inside of the sterile tube above the fluid level. The swab was streaked in three directions and continuously brushed over the Mueller Hinton agar and inoculated plates were allowed to stand for 5 minutes. The disks 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 and the plates were incubated aerobically at 37°C for 24 hours (CLSI, 2021). The inhibition zones, where bacterial growth was absent was measured across the center of the disks to the nearest mm using a ruler and reported as the diameter of the individual disk. Based on this, the isolates were defined as resistant, intermediate and susceptible according to the guide lines CLSI (CLSI, 2018; CLSI, 2021).

3.7. Molecular Detection of Methicillin Resistant *Staphylococcus aureus*

The genomic detection of Methicillin resistant *S. aureus* was conducted via PCR, which was typically used for detection of *mecA* gene that codes for penicillin binding protein 2a (PBP2a). From a total isolates of *S. aureus*, due to resource limitation 39.5%, (15/38) were selected for

molecular detection of MRSA. The selection criteria among sample types was number of positive samples in each sample type and based on proportional allocation, 7/16 (43.8%), 5/15(33.3%), 3/7(42.9%) samples from beef carcass, abattoir environments and abattoir workers were selected, respectively.

3.7.1. DNA extraction

Genomic DNA extraction from staphylococcal cells was performed using DNeasy Blood and Tissue Kits (QIAGEN, Germany extraction kit). *S. aureus* isolates were transferred into 1.5-ml Eppendorf tubes. Bacteria were lysed by adding AL buffer and after proceeding steps, the suspension was carefully transferred to DNeasy Mini spin column within 2ml collection tubes. After addition of AW1 and AW2 washing buffers AE buffer was added in to new labeled eppendorf tube. Finally, the extracted DNA was eluted and stored at 4°C till PCR amplification. The crude extract thus obtained was used for PCR as the DNA template.

3.7.2. Polymerase chain reaction for detection of *mecA* gene

The extracted genomic DNA of *S. aureus* isolates were amplified by conventional PCR for the presence of *mecA* gene using forward primer "*mecA*-F (5'-AAA ATC GAT GGT AAA GGT TGG C-3')" and reverse primer "*mecA*-R (5'-AGT TCT GCA GTA CCG GAT TTG C-3')". PCR reaction mixture (22µl) was prepared using 3µl of nuclease-free water, 10µl of IQ super mix and 2µl of each primer. 5µl of DNA template was added to each PCR reaction tubes containing master mix components. Amplification was carried out in a applied bio-system 2720 PCR thermocycler (Singapor) with thermal cycling conditions of an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec and extension at 72°C for 30 sec, and with final extension at 72°C for 7 min.

3.7.3. Agarose gel electrophoresis

The 2% of agarose gel was prepared. Four microliter of loading dye containing gel red was mixed with PCR products and then 10µl of this mixture was poured in to the gel wells. Ten microliter of DNA ladder was added in to wells at both sides of the agarose gel.

Electrophoresis was conducted in a horizontal electrophoresis tanker containing 1X TAE buffer for 60 min at 120 V.

3.8. **Ethical Consideration**

The study protocol was reviewed and approved by institutional review board of Bahir Dar University, College of Agriculture And Environmental Science, School of Animal Science and Veterinary Medicine. A letter of support was obtained from College of Agriculture and Environmental Science and permission at the office level was requested from the concerned higher officials Bahir Dar and Debre Markos cities administration municipal abattoirs. Abattoir workers' permission was obtained to take the swab sample from their hands and clothes, after briefly explaining objectives and relevance of the study.

3.9. Data Management and Analysis

Data were coded and entered in to Microsoft Excel 2010 spread sheet. STATA version 16 was used to compute descriptive statistics. Pearson's chi-square (χ^2) and/or fisher's exact test were used to assess the difference in the isolation of *S. aureus* by study area and sources of samples. A p-value < 0.05 was considered indicative of a statistical significance.

Chapter 4. RESULTS

4.1. Isolation and Identification of *Staphylococcus aureus*

From the total 150 swab samples, 38 (25.3%) were positive for *S. aureus* using MSA, gram stain and biochemical tests (Figure 3). Among these 17(22.7%) were isolated in Bahir Dar and 21 (28%) in Debre Markos municipal abattoirs (Table 3).

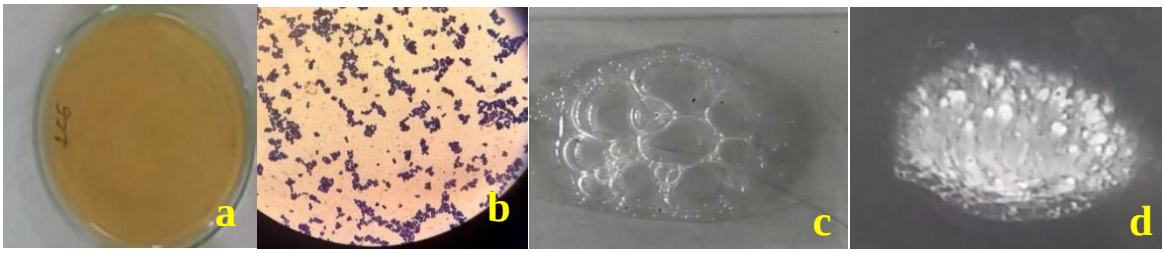

Figure 4. Identification of *S. aureus* (a) MSA, (b) Gram's staining, (c) Catalase test, (d) Coagulase test

The 27.1% (16/59) of *S. aureus* were isolated on the beef carcass, 23.1% (15/65) were from the abattoir environment and 7/26 (26.9%) were from abattoir workers' swab samples (Table 3).

Table 3. Proportion of *S. aureus* isolated from Bahir Dar and Debre Markos abattoirs

Sample source	Sample area	Total sample	*S. aureus* sub-total (%)	Total (%)
Beef carcass	Bahir Dar	29	7(24.1)	16 (27.1)
	Debre Markos	30	9(30)	
Abattoir	Bahir Dar	33	8(24.2)	15 (23.1)
environment	Debre Markos	32	7(21.9)	
Abattoir worker	Bahir Dar	13	2(15.4)	7 (26.9)
	Debre Markos	13	5(38.5)	
Sub-total	Bahir Dar	75	17(22.7)	38(25.3)
	Debre Markos	75	21(28)	
Total		**150**	**38(25.3)**	**38(25.3)**

From 59 swab samples that were collected from beef carcass, 16(27.1%) were positive for *S. aureus*. Among these, *S. aureus* was isolated from abdomen, thorax, crutch and breast regions of the beef carcass (Figure 4).

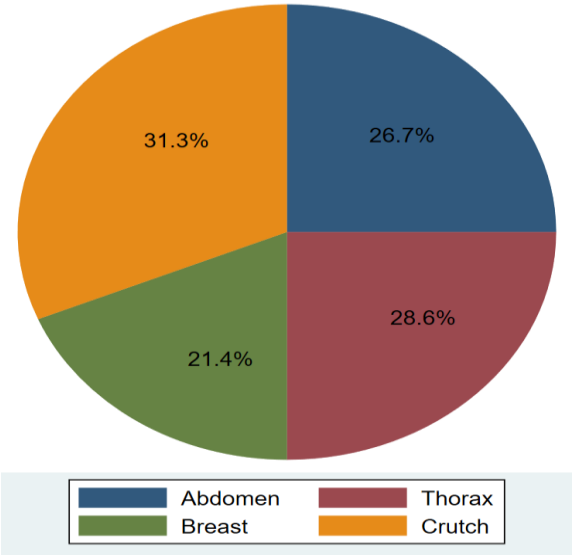

Figure 5. *S. aureus* isolates from the beef carcass

Among 65 abattoir environmental samples, 15 (23.1%) of *S. aureus* were isolated. The frequency of isolation of *S. aureus* varied between sample types and ranged from 11.1% to 35.7%. From abattoir environmental samples, the highest proportion of *S. aureus* was isolated in hook swab samples, 35.7%, however the lowest proportion was observed in splitting axe (11.1%) (Figure 5).

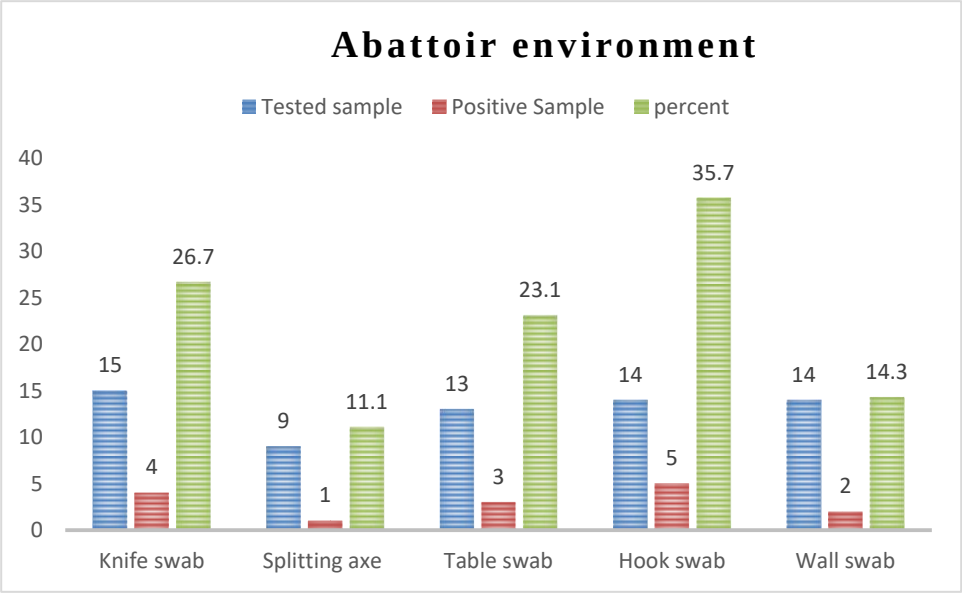

Figure 6. *S. aureus* isolates from abattoir environment

Staphylococcus aureus was assessed from 26 swab samples and 7/26 (26.9%) of *S. aureus* were isolated. Relatively, higher proportion was found in abattoir workers' hand swab, 35.7% than that of isolates from cloth swab samples, 16.7% (Figure 6).

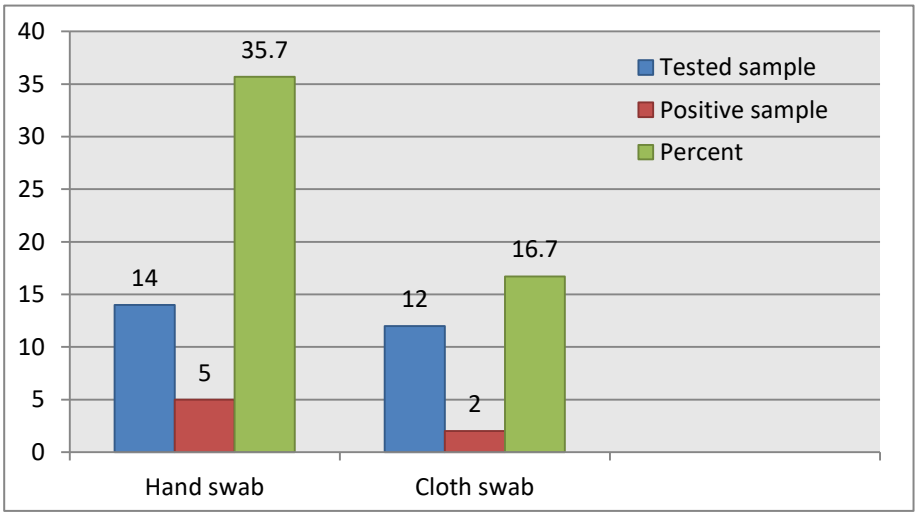

Figure 7. *S. aureus* isolates from abattoir workers

Chi square and/or fisher's exact test indicated that there was no significance difference (P > 0.05) on positive isolates of *S. aureus* in all type of samples between Bahir Dar and Debre Markos Municipal abattoirs (Table 4).

Table 4. Chi square and/or fisher's exact test result

Sample source	**Site of abattoir**	**Positive**	**Negative**	χ^2	**P- value**
Beef carcass	Bahir Dar	7	22	0.256	0.613
	Debre Markos	9	21		
Abattoir	Bahir Dar	8	25	0.051	0.821
Environment	Debre Markos	7	25		
Abattoir	Bahir Dar	2	11		0.378
workers	Debre Markos	5	8		
Overall	Bahir Dar	17	58	0.564	0.453
	Debre Markos	21	54		

50% of cells were less than or equal to 5, so that fisher's exact test p-value were taken

4.2. Antibiogram Assessment

The antibiogram assessment was performed on 38 *S. aureus* isolates. All isolates were 100% susceptible to Gentamycin and 86.8% to Norfloxacin. On the contrary, they were found to be totally resistant to Penicillin and Methicillin. Resistance to Penicillin-G and Methicillin (100%) was the highest widespread among the isolates in the current study; followed by resistance to Doxycycline Hydrochloride 63.2%, Clindamycin 23 (60.5%) and Ampicillin 22 (57.9%). Generally, from ten antibiotics assessed, Gentamycin was the most effective drug for all 38 (100%) isolates, followed by Norfloxacin 33 (86.8%) and Co-Trimoxazole 24 (63.2%)**.** The comparison of antibiogram profiles among various sample types shown in (Table 5).

Table 5. Antibiogram assessments of *Staphylococcus aureus*

Antibiotic Disks	**Susceptible n (%)**	**Intermediate n (%)**	**Resistant n (%)**
Ampicillin (AMP-10µg**)**	13 (34.2)	3 (7.9)	22 (57.9)
Erythromycin (E-15µg)	11 (28.9)	23 (60.5)	4 (10.5)
Gentamicin (CN-10µg)	38 (100)	0 (0.0)	0 (0.0)
Penicillin-G (P-10IU)	0 (0)	0 (0.0)	38 (100)
Co-Trimoxazole Sulfamethoxazole-(COT-25µg)	24 (63.2)	4 (10.5)	10 (26.3)
Doxycycline Hydrochloride (DO-30µg)	9 (23.7)	5 (13.2)	24 (63.2)
Clindamycin (CD-2µg)	15 (39.5)	0 (0)	23 (60.5)
Ceftazidime (CAZ-30µg)	13 (34.2)	11(28.9)	14(36.8)
Norfloxacin (NX-10µg)	33 (86.8)	0 (0)	5 (13.2)
Methicillin (Met-5µg)	0(0)	0(0)	38(100)

The susceptibility profile of isolates varied among ten different antibiotic drugs (Figure 8).

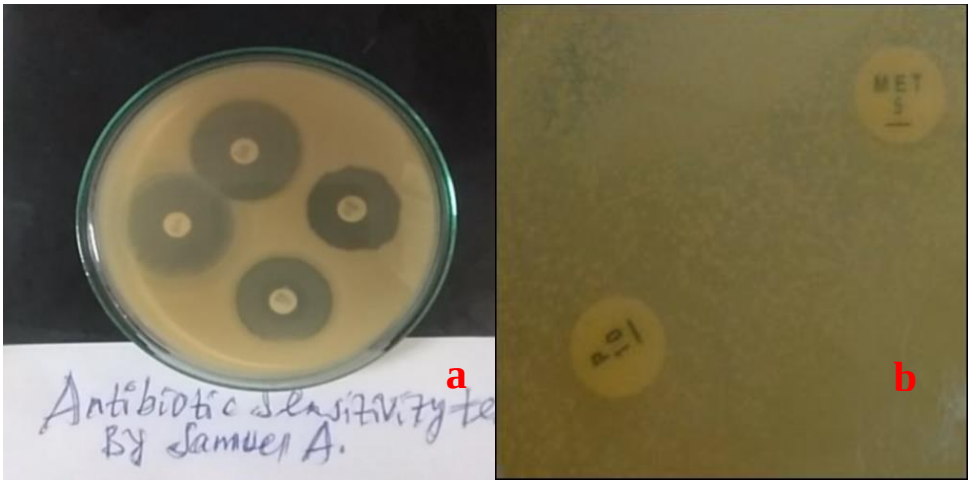

Figure 9. Antibiotic susceptibility profiles of *S. aureus* (a) clear zone of inhibition (b) no zone of inhibition.

4.2.1. Multidrug resistance profile of *Staphylococcus aureus*

In the current study, most of the *S. aureus* (n = 32, 84.2%), isolates exhibited Multidrug Resistance (MDR) as each isolate at least resistant to ≥3 drugs simultaneously. Eleven isolates were resistant to five types of antibiotics and seven isolates were resistant to four antibiotics, similarly eight *S. aureus* isolates were also resistant to six different antibiotics (Figure 8 & 9). Among 38 isolates the one that was detected in the beef carcass swab sample showed a high resistance against eight different types of antibiotics and seven drugs scored resistant profile against two isolates that were identified from hook and workers' hand swab sample.

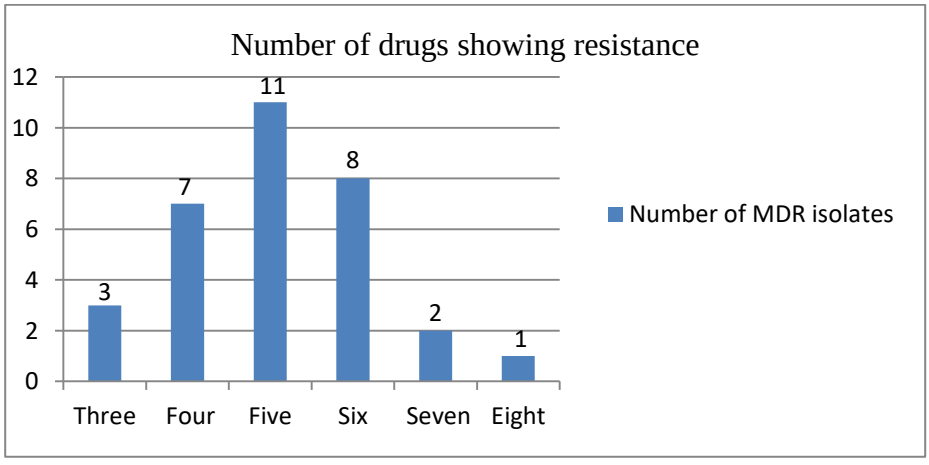

Figure 10. Multiple drug-resistance profile of *S. aureus* isolates

The presence of MDR of *S. aureus* isolates indicates the possible significant risk of the resistant strain along the studied beef line.

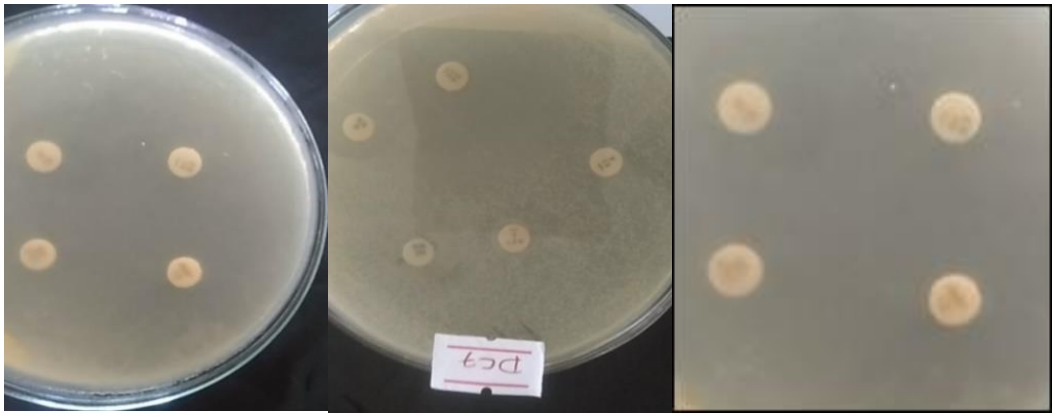

Figure 11. Resistance profile of *S. aureus* for multiple drugs

4.3. Molecular Detection of Methicillin Resistant *Staphylococcus aureus*

After the gel electrophoresis was completed, PCR amplicon with 533 bp band size for *mecA* gene was visualized under Transilluminator UV-light (France) as shown (Figure 10). The molecular detection of *mecA* gene among *S. aureus* isolates was 5/15, (33.3%). Out of 15 *S. aureus* isolates selected, only five isolates were positive for *mecA* gene. The highest proportion of *mecA* gene was detected in abattoir workers' swab samples (2/3, 66.7%) followed by beef carcass (3/7, 42.9). Among the two municipal abattoirs, the higher proportion of MRSA was characterized in Debre Markos 3(60%) than Bahir Dar municipal abattoirs 2(20%) (Table 6).

Table 7. Molecular detection of *mecA* gene

Sample source	Sample area	Total sample	*mecA* gene (%)	Total (%)
Beef carcass	Bahir Dar	4	1(25)	3 (42.9)
	Debre Markos	3	2(66.7)	
Abattoir	Bahir Dar	4	0(0)	**0 (0)**
environment	Debre Markos	1	0(0)	
Abattoir worker	Bahir Dar	2	1(50)	2(66.7)
	Debre Markos	1	1(100)	
Sub-total	Bahir Dar	10	2(20)	5(33.3)
	Debre Markos	5	3(60)	
Total		**15**	**5(33.3)**	**5(33.3)**

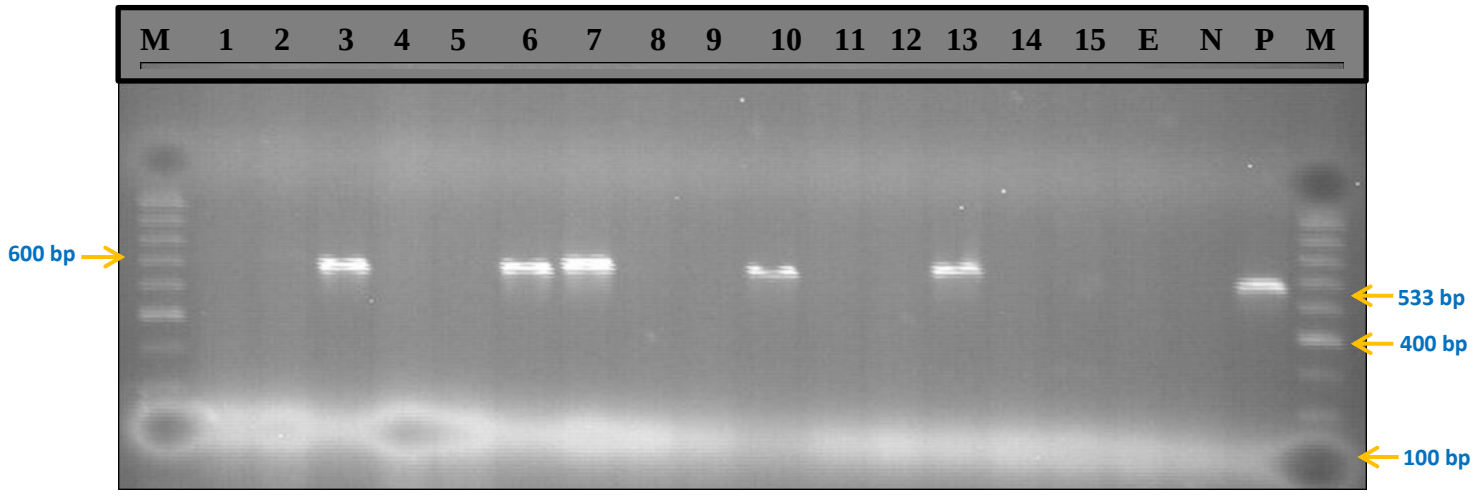

Figure 12. Detection of *mecA* gene from *S. aureus* isolates Agarose (2%) gel electrophoresis image of the *mecA* gene (533bp). Lane M is a 100-bp DNA ladder, lanes 1 to 15 are test samples/templates, lane E is an extraction control, lane N is a negative control and lane P is a Positive control.

Chapter 5. DISCUSSION

The overall proportion of *S. aureus* in the current study was 25.3%, in which the higher proportion was isolated in Debre Markos (28%) than Bahir Dar (22.7%) municipal abattoirs. The current finding was lower than 41.27%, 49.09% and 36.4% which were reported by Jirata Shiferaw and Seid Yimmam (2020) in Bishoftu, Million Weldeselassie *et al*. (2020) in Mekelle and Abdi Hassen *et al*. (2020) in Asella, Ethiopia municipal abattoirs respectively. However, it was higher than 13.2% and 9.4% finding of Takele Beyene *et al*. (2017) and Feben Adugna *et al*., (2018) from Addis Ababa municipal abattoir, Ethiopia. Those variations might be due to difference in degrees of environmental hygiene, accessibility of water and sterilizer, sampling sources/origin, sample size, sampling time and sampling at different slaughter process stages can affect the results.

Staphylococcus aureus, isolated from the beef carcass (27.1%) was more or less can correlate with the result of Abdi Hassan *et al. (*2018) who reported 34.3% in beef samples in Ethiopia and Pekana and Green (2018) who announced 20.4% in South Africa. The current finding was lower than the findings of Sadiq *et al*. (2020) who reported as 75% in Pakistan, 57.5% by Ebrahim and his cofounders (Ebrahim *et al*., 2013) in Iran from beef samples. However, it was higher than 11.7% and 5.6% by Takele Beyene *et al.* (2017) in Addis Ababa and Matewos Kebede (2020) in Bishoftu, Ethiopia, respectively.

Among 15 swab samples taken from the knife, 4(26.7%) were identified as *S. aureus* which was relatively consistent with 33.3% in Addis Ababa, Ethiopia (Takele Beyene *et al*., 2017) and 22.5% from knife swabs in Pakistan (Sadiq *et al*., 2020). The present result was lower than the findings of Abdi Hassan *et.al. (*2018) who reported as 50% from knife swab samples in Asella, Ethiopia and 55% in Pakistan(Sadiq *et al*., 2020), but it was higher than the null report of Matewos Kebede (2020) from Bishoftu, Ethiopia. The isolates of *S. aureus* in the hook swab samples (35.7%) were agreed with 33.3% isolated in hook swab samples (Takele Beyene *et al*., 2017). The result was higher than 15% **(**Feben Adugna *et.al*. 2018), but lower than the result of 60% that was reported by Abdi Hassan *et al*. (2018)**.** In the current study 23.1% of *S. aureus* was identified from table swab samples, which was greater than 1.6%, conducted by Odetokun *et al*. (2018) in Nigeria. Among

samples swabbed from splitting axe, 11.1% was isolated as *S. aureus,* which was less than 50%, reported by Abdi Hassen *et al. (*2020). *S. aureus* was identified as 14.3% in the wall of municipal abattoirs at the time of active slaughtering activities which was lower than 1.8%, Odetokun *et al*. (2018) in Nigeria. The difference might be due to variation of food safety tool application, sampling time i.e. sampling before and after washing and sterilization of equipment as well as before and after contacting with carcass and personnel can affect the results.

The proportion of *S. aureus* isolates from workers' hand swab, 35.7% were lower than 60.0% from the same source of sample (Abdi Hassen *et al.,* 2020) in Asella Municipal abattoir, but higher than 16% (Mulat Dagnew *et al.,* 2013) from finger nail content of cafeteria food handlers in university of Gondar, 0% from workers' hand swab (Takele Beyene *et al., 2017*) in Addis Ababa and 9.1% (Matewos Kebede, 2020) in Bishoftu, Ethiopia. The current result was similar with 42.9% in Asella (Fufa Abunna *et al*., 2016). Isolates of *S. aureus* in abattoir workers' cloth, 16.7% was lower than 40% that was isolated from Asella, Ethiopia (Abdi Hassen *et al.,* 2020). The variation of results on abattoir workers might be depend on the difference in an individual hygiene, PPE usage and level contact with animate and inanimate objects.

Comparing the proportion of *S. aureus* in knife, table, axe, hook, abattoir workers' hand and cloth, wall of abattoir houses and beef swabs in abattoirs was that, the highest proportion was seen in workers' hand and hook swabs. This might be attributed to the effects of meat contamination at different points of slaughtering process during handling of the hook with contaminated hands. Next to the hook and workers' hand swabs, high number of *S. aureus* was isolated from beef carcass swab than any other swab samples. This could be attributed to the highest sample size of the beef carcass samples than other sources of samples and the contaminated personnel at abattoir might have also contributed to beef carcass contamination.

The results of antibiogram assessment showed that *S. aureus* isolates were 100% susceptible to Gentamicin which was completely aligned with the work of Matewos Kebede (2020) in Bishoftu and Takele Beyene *et al*. (2017) in Addis Ababa, but it was

disagreed with the records of Million Weldeselassie *et al.* (2020) in Mekelle and Abebe Mekuria *et al*. (2013) in Addis Ababa who reported 50% and 41.2%, respectively. The susceptibility profile of all isolates against Norfloxacin, 86.8% was similar with 95.5% (Senait and Moorty, 2016) in Debre-Zeit. These levels of sensitivity might be due to least frequently used of these drugs in and around the study areas. In contrary, all isolates were found to be completely resistant (100%) to Penicillin G and Methicillin. The resistance level of isolates to Penicillin G was similar with the finding of Jirata Shiferaw and Seid Yimmam, (2020) in Bishoftu, Ethiopia and Sergelidis *et al*. (2015) in Greece who reported as 100% resistance. However the result was higher than the findings reported by Sophia (2011), 74.2% in USA and Birhanu Abera *et al.* (2013), 51.7% in Asella, Ethiopia. The complete resistance of *S. aureus* isolates against Methicillin was totally aligned with the work of Feben Adugna *et al*. (2018) in Addis Ababa, Ethiopia and Sadiq *et al*. (2020) in Pakistan. The resistance pattern of *S. aureus* isolates, 57.9% to ampicillin was similar with the work of Hiroi *et al*. (2012), (59%), whereas it was lower than the report of Haimanot Tassew *et al.* (2010) who reported as 85%.

The development of antibiotic resistance variation might be as a result of repeated therapeutic use and/or inappropriate usage of the drugs and the existence of different strains of the bacteria in different parts of the Country. The development of MDR of the isolates, 84.2% was comparable with 72.7% (Fufa Abunna *et al*., 2016) in and around Asella, Ethiopia. However, it was slightly higher than 59.3% (Sergelidis *et al.*, 2015) and 51.7% (Velasco *et al*., 2019). This was indicated that *S. aureus* strains have developed multidrug resistance worldwide, including the current study areas with broad diversity in proportional rate in different regions.

Molecular detection of MRSA, 33.3% was relatively consistent with 22.6% *mecA* gene detection from pig and chicken carcass (Okorie-Kanu *et al*., 2020), but higher than 1.1% (Odetokun *et al*., 2018) from slaughterhouses in Nigeria. The current finding was relatively lower than 63% in beef swab (Sadiq, *et al.*, 2020) in Pakistan. MRSA was mostly detected in abattoir workers than other source of samples which was similar with the report of Odetokun *et al*. (2018). Out of fifteen selected *S. aureus* isolates, *mecA* gene was detected only from five isolates. However, based on antibiogram assessment, all isolates were

phenotypic resistance to Methicillin. The reason might be, such isolates could contain other types of resistant genes, i.e. *mecC, mecB, mecD* and other genes rather than *mecA* gene. Similarly, Velasco and coworkers indicated that, *S. aureus* strains that were phenotypically resistant to Methicillin, but they did not harbor the *mecA* gene (Velasco *et al*., 2019). The similar result was reported as *S. aureus* isolates were phenotypic resistant to Methicillin, but lack of *mecA* or *mecC* genes (Ba *et al*., 2014) and also Ozdemir and Keyvan (2016) reported that, from 114 *S. aureus* isolates none of the isolates contained *mecA* gene but some of them were identified as resistant to a group of antibiotics, including Methicillin. Additionally, the *mecC* gene was mentioned by Stegger *et al*. (2012; Garcia-Alvarez *et al.,* 2011), the *mecB* gene by Tsubakishita *et al*. (2010) and others that were not as well-known (Velasco *et al*., 2015) and also Schwendener *et al*. (2017) reported a new *mec* gene called *mecD*. The higher proportion of MRSA was detected in Debre Markos (60%) than Bahir Dar (20%) municipal abattoirs. The difference might be due to probability of sample size variation and/or the wide spread of Methicillin resistance strains in and around Debre Markos city.

Chapter 6. CONCLUSION AND RECOMMENDATIONS

Staphylococcus aureus is one of the most important pathogen globally, having imposed heavy burdens on the animal and human healthcare systems. The present findings show the contamination of the beef carcass that might be regarding to the presence of poor personnel and environmental hygiene, absence of separated areas in slaughter houses, such as stunning, bleeding, skinning, evisceration and cutting rooms and lack of safety rules in both municipal abattoirs. The detection of *S. aureus* and its Methicillin resistant strain play an important role for understanding the pathogen and developing the treatment and prevention protocols. All *S. aureus* isolates were 100% susceptible to Gentamicin, however 100% resistant to penicillin G and Methicillin, antibiotics. Most of *S. aureus* isolates showed MDR which indicates the wide distribution of MDR S. *aureus* stains among the beef line.

Based on the above remarks, the following recommendations are forwarded:

- Good beef carcass handling techniques, good hygiene and sanitation, availability of water for frequent washing, complete dressing of PPE should be practiced in municipal abattoirs.
- Awareness should be created for Abattoir workers about the impact of *S. aureus* and MRSA on the health and economical aspects.
- Infections caused by *S. aureus* should be treated using most sensitive antibiotics, i.e. Gentamycin, Norfloxacin and Co-Trimoxazole group of drugs, however Penicillin and Methicillin should not be a treatment choice in the study areas.
- Conducting widened and detailed research regarding the epidemiology, antibiogram assessment and molecular detection of *S. aureus* strains could help.

Chapter 7. REFERENCES

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Chapter 8. APPENDICES

Appendix 1. Laboratory record format to identify *S. aureus* and MRSA detection

S/no	**Sample source and ID**	**Isolation and cultural characteristics of *S. aureus***				**Biochemical test results**		**Molecular detection of MRSA**
		Blood agar	Mannitol salt agar	Nutrient agar	Gram stain	Catalase test	Coagulase test	*mecA* detection

Appendix 2. Procedures for biochemical tests

1. Catalase Test

- Take a pure bacterial colony and place on a clean microscopic slide.
- Add a drop of 3% H_2O_2 then, mix through by inoculating needle.
- Immediately, observe vigorous bubble formation, which are visible to the naked eye. Take the test colonies for catalase test from media without blood since erythrocytes possess catalase activities (Quinn *et al*., 2004).

Interpretation:

Bubbling indicates a positive (+) test for *S. aureus* and no bubbling indicate a negative (-) test.

2. Coagulase Test

- Place a drop of physiological saline on two clean slides
- Add and emulsify an isolated colony on each drops by sterile platinum wire loop
- Drop reconstituted rabbit plasma to the suspension and mix gently.
- Look for clumping of the organisms within 10 seconds. No plasma is added to the second suspension that could be act as control.

Interpretation:

The development of clumping is considered as coagulase positive test and no clumping formation considered as negative for *S. aureus* (Kompanikova *et al*., 2016).

Appendix 3. Procedures for DNA extraction

1. Take a pure colony of bacteria from overnight culture and place in a test tube containing 5ml of broth and incubate at 37^{o}C overnight.

2. After completely mixing the sample, suspend 200µl in to 1.5ml Eppendorf tube.

3. Add 200µl of AL buffer in the sample and mix by pulse-vortexing for 15sec.

4. Add 20µl QIAGEN protease kinase in to 1.5ml Eppendorf tube

5. Incubate at 56°C for 10min.

6. Briefly centrifuge 1.5ml Eppendorf tube to remove drops from inside the lid.

7. Add 200µl of ethanol into 1.5ml microfuge tube and mix again by pulse-vortexing and centrifuge.

8. Carefully transfer the mixture to-DNeasy *Mini* spin column within 2ml collection tube and centrifuge at 12000rpm for 1min then discarded collection tube which contain the filtrate.

9. Carefully open the DNeasy *Mini* spin column and add 500µl AW1 buffer in to the suspended extract, without wetting the rim. Close the cap and centrifuge at 12000rpm for 1min. Place DNeasy *Mini* spin column in a clean 2ml collection tube and then discarded collection tubes which contain the filtrate.

10. Add 500µl AW2 buffer and centrifuge at 12000rpm for 1min. Finally placed DNeasy *Mini* spin column in a clean 1.5ml microfuge tube and discard the tubes which contain the filtrate.

11. Add 200µl AE buffer and incubate at room temperature for 5min, then centrifuge at 12000rpm for 1min.Then store the extracted DNA at 4°C till PCR amplification.

Appendix 4. Antibigram assessment recording sheet

Sample ID	Type and content of antibiotic agents	Zone diameter break points to the nearest whole mm

Appendix 5. List of some photos captured during laboratory works

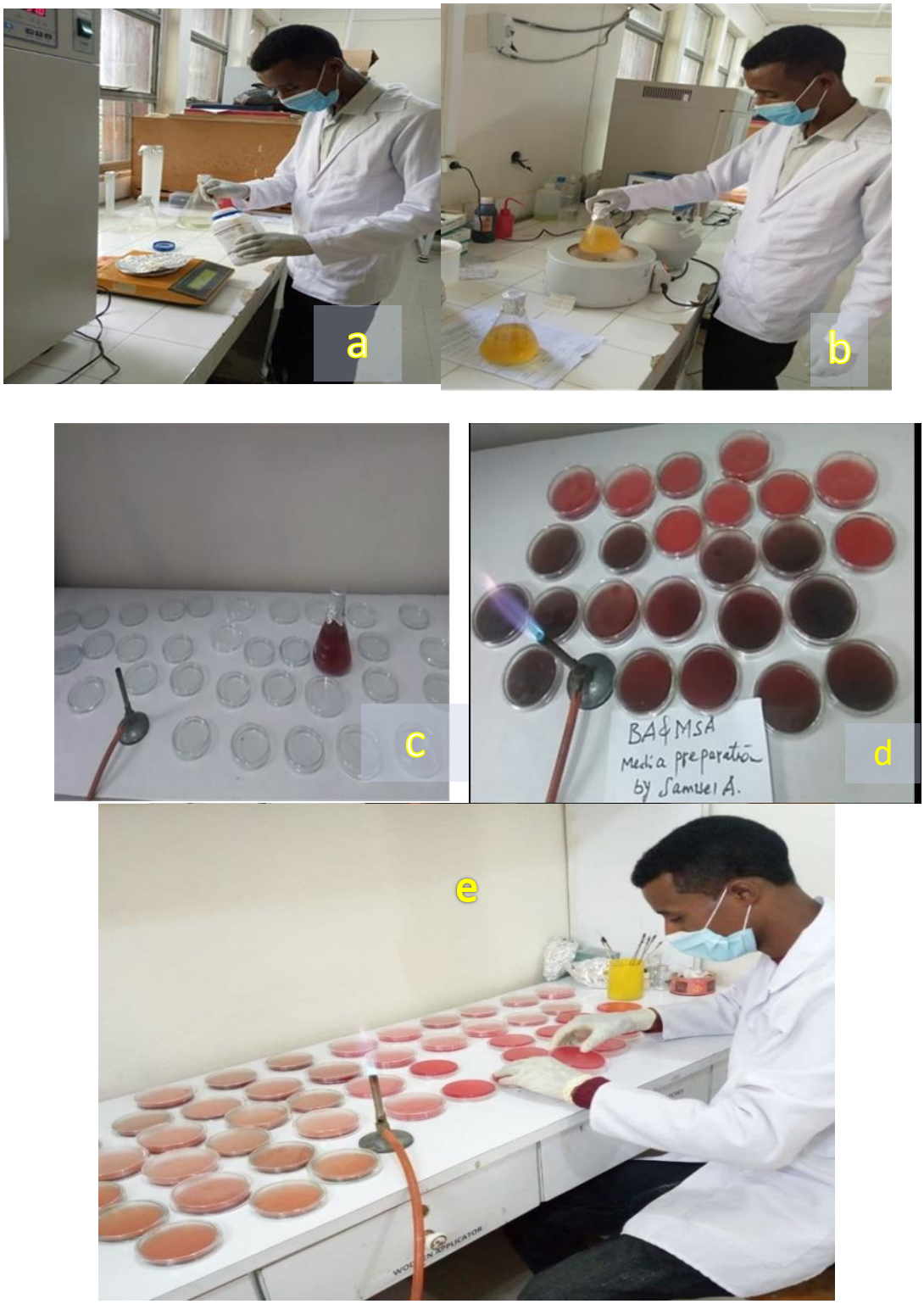

Figure. Media preparation a) Measuring the agar b) Boiling for complete mixing c) Dispensing d) Prepared Blood and MS agar e) Inverting solidified media

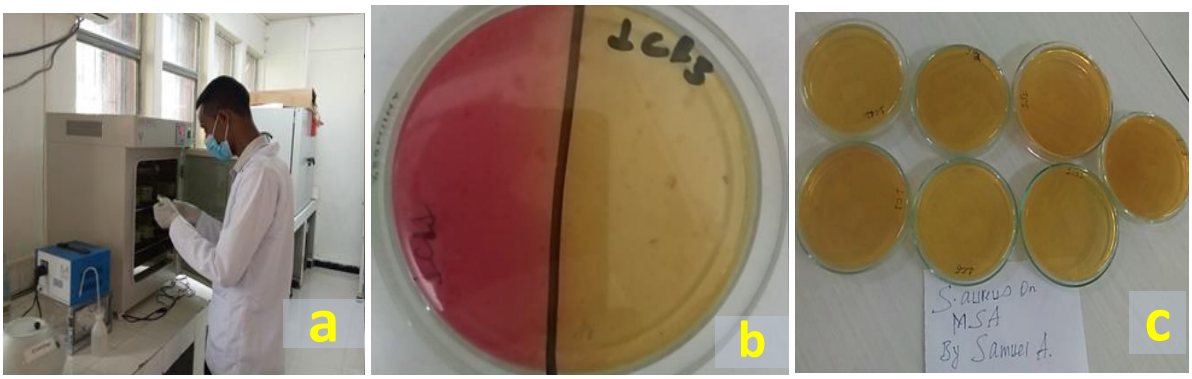

Figure. a) Incubating the cultured Medias b&c) MSA fermentation *by S. aureus*

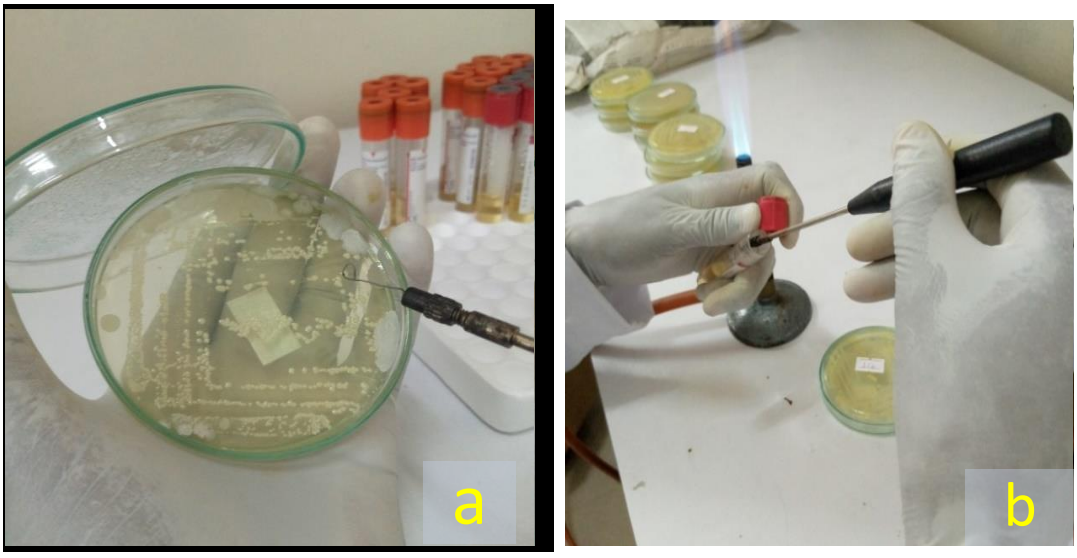

Figure. a) Pure colony of *S. aureus* on NA b) Inoculating in to NB

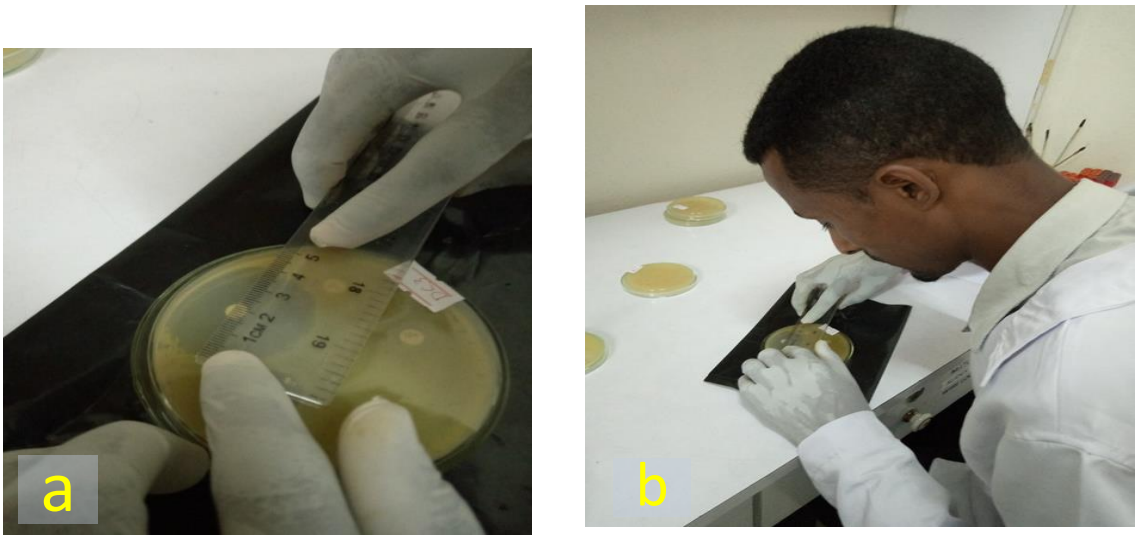

Figure. a &b Measuring diameters of antibiotic inhibition zones

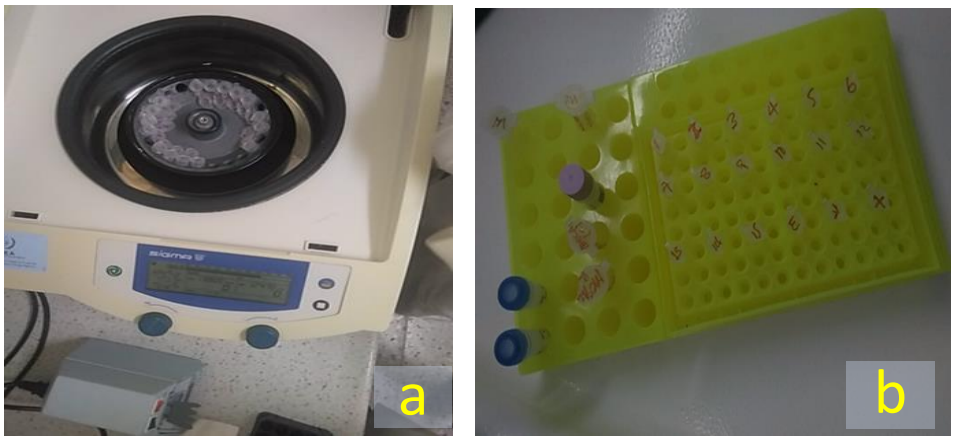

Figure. a) Centrifugation for DNA extraction b) Master mix preparation

Figure . a) Adjusting PCR conditions b) Inserted PCR tubes in to PCR machine

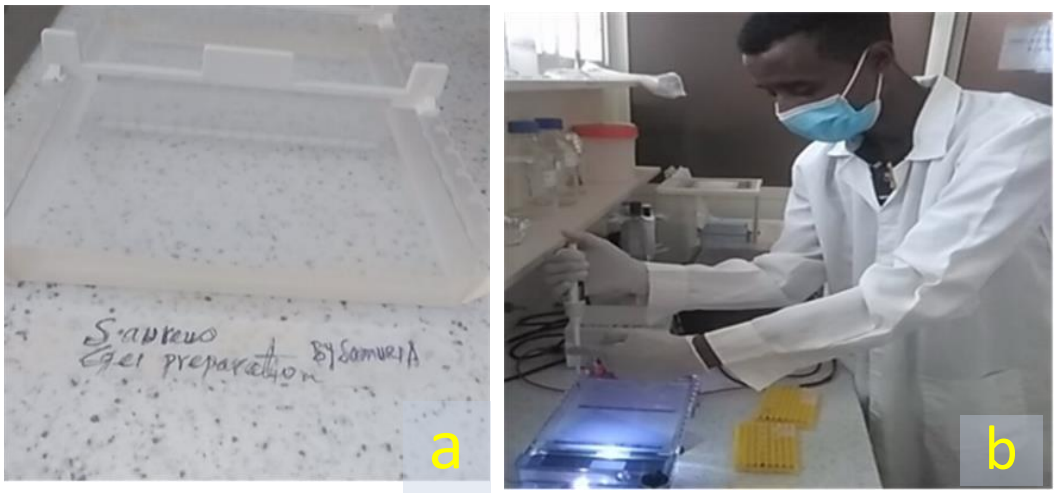

Figure. a) Gel preparation b) Loading in to gel wells