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Isolation, Identification and Antimicrobial Susceptibility Profiles of Staphylococcus Isolates from Dairy Farms in and Around Modjo Town, Ethiopia

Haimanot Belete Wodaje, Takele Beyene

[1.] Assosa University, College of Agriculture and Natural Resource, Department of Veterinary Science, Western Ethiopia, P.O.BOX 18, Email Address: haimanotbelete0266@gmail.com
[2.] Addis Ababa University, College of Veterinary Medicine and Agriculture, East Shoa, Ethiopia

Abstract: A cross sectional study was conducted between December 2015 to April 2016 in and around Modjo town, central Ethiopia aiming at isolation and identification *Staphylococci* from dairy cattle farms and establishing their antimicrobials resistance (AMR) profile for the isolates. Among a total of 212 samples collected from 26 small holder and semi intensive dairy farms, 167 of them were raw bovine milk and a total of 45 swab samples. Occurrence, characterization, and antimicrobial susceptibility profile of *Staphylococcus* isolated from the study area were investigated. The isolation results showed 82 (38.8%) positive for *Staphylococci*. Of the total isolates, 81.7% (67/82) were from bovine raw milk at the level of the farm and collection center, whereas 18.3% (15/82) were from milk container and milkers hand swab. *S. aureus* was the most frequently isolated species among different samples accounting for 32 (15.1%) followed by; *S. intermedius* 22 (10.4%), Coagulase positive staphylococcus (CPS) 18 (8.5%) and *S. hyicus* 10 (4.7%). Furthermore, the antimicrobial susceptibility test of *Staphylococcus* species (n=82) revealed AMR to at least one of the tested antimicrobials, was seen in 98.8% of the isolates. The comparative efficacies of the tested antimicrobials indicated Ciprofloxacin and Gentamycin were the most effective with susceptibility of 92.7% and 87.8% respectively. Conversely, a large proportion of the isolates were resistant to Penicillin G (98.8%), Cloxacillin (64.6%), and Amoxicillin (79.3%). Multidrug resistance (MDR) was detected in 92.7% (n=76/82) of the isolates (96.9% *S. aureus*, 77.3% *S. intermedius*, 10% *S. hyicus*, 38.9% CNS). In general, the isolates showed the development of MDR to commonly used antimicrobial agents in the study area. Thus, implementing strict hygienic control measures in dairy farms is vital in order to guarantee the quality of milk and milk products. The occurrence of MDR *Staphylococcus* particularly Coagulase positive *Staphylococcus* (CPS) should be under consideration during selection of antimicrobials for the treatment of dairy cattle.

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1. Introduction

Staphylococci are a genus of worldwide distributed bacteria correlated to several infections of different sites in human and animals. The genus *Staphylococcus* is in the bacterial family *Staphylococcaceae*. They are Gram-positive cocci bacteria that occur in clusters resembling unmoving grapelike structure due to its perpendicular division planes where cells remains attached to one another following each successive division (Kloos and Bannerman, 1995). They can be divided into two groups, CPS and CNS, according to production of coagulase enzyme, which is capable of coagulating blood plasma (Cunha and Calsolari, 2008). Its importance is not only because of its distribution and pathogenicity but especially due to its ability to overcome antimicrobial effects (Souza *et al*., 2012). The species of the *Staphylococci* genus are ubiquitously disseminated in the environment, with a number of species inhabiting specific ecological niches. They are found living naturally on the skin and mucous membranes of warm-blooded animals and humans, which generally imply a commensal or symbiotic relationship with their host (Heikens, *et al*., 2005).

Infections due to *Staphylococci* are of major importance to veterinary and human medicine (Zadoks, 2002). In animals, it is commonly associated with mastitis leading to contamination of milk and dairy products. In the last few decades, *Staphylococcal* food poisoning has been reported as third cause of food-borne illnesses in the world. Among the foods implicated in *Staphylococcal* food poisoning, milk, dairy products and particularly handled foods, play a

vital role since enterotoxiginic strains of *Staphylococcus* species have been commonly isolated in them (Ateba *et al*., 2010). Long-term surveys suggest that the significance of *Staphylococcus* in the dairy industry has remained unchanged (Swinkels *et al*., 2005).

The cattle derivative foods that are most often involved in *Staphylococcal* food poisoning have been documented by a number of workers in developed countries. In the United Kingdom, for example, 8% of the *Staphylococcal* food poisonings were due to milk products (Wieneke *et al*., 1993), in France, among the Staphylococcal food poisonings milk products and especially cheeses were responsible for 32% of the cases and meats for 22% (Haeghebaert *et al*., 2002).

Milk and milk-based food products are highly susceptible to microbial attack because of their rich composition, which provides a favorable medium for growth of a host of spoilage agents (De Buyser, 2001). Unpasteurized milk may become contaminated with enterotoxiginic CPS species, either through contact with the cow's udder during milking or by cross contamination during processing (Ramesh *et al.,* 2002). Symptoms such as nausea, vomiting, abdominal cramps and diarrhea usually appear 1–6 hours after ingestion of the enterotoxins produced in contaminated milk (Asao *et al*., 2003). Consequently, enumeration and identification of *Staphylococci* in dairy products is a priority in developing public health measures to reduce food-borne disease outbreaks (Ercolini *et al*., 2004).

Antimicrobials are regarded as either bactericidal or bacteriostatic, depending on their mode of action. Antimicrobial agents may be categorized by mechanism of action: inhibition of cell wall synthesis, protein synthesis, nucleic acids, synthesis of important metabolites, and plasma membrane damage (Tortora *et al*, 2010). However, different microbial species, and strains within the same species, may vary in susceptibility to antimicrobials.

The increasing handling of antimicrobials has led to a worldwide problem in the development of AMR among bacterial populations during recent decades (Shryock and Richwine, 2010). The emergence of AMR among humans has prompted concerns about the public health implications of antibiotic use in agriculture. Some bacteria have developed resistances to all of the different classes of antibiotics discovered up to date, and the most frequent type of resistance is acquired and transmitted horizontally via mobile genetic elements such as plasmids, transposons and integrons (Tiemersma *et al.,* 2004). The improper antibacterial treatment and over use of antibiotics for agricultural purposes have contributed to the increased incidence of AMR in farm animals (Shryock and Richwine, 2010). This AMR has been documented by a number of workers in different areas. For example, 20–30% for Denmark and Norway, 85% in Ireland (De Oliveira *et al*., 2000) and Brazil (Costa *et al.,* 2000)

Therefore, the uncontrolled application of antimicrobials in the environment is leading to a constant increase in the rate of AMR among community-acquired *Staphylococci* (Harakeh *et al*., 2006). *Staphylococcus* species can rapidly acquire resistance to a broad range of antimicrobials, thereby posing a major concern in the treatment of *Staphylococcal* infections (Bozdogan *et al.,* 2004). Studying AMR in humans and animals is important for detecting changing patterns of resistance, implementing control measures on the use of antimicrobial agents and preventing the spread of MDR strains of bacteria (Van Duijkeren *et al*., 2003).

Despite of the above mentioned situation, there is little published information about the importance of risk factors of *Staphylococcus* pathogens in food-borne diseases and its risks of AMR development in cattle, food chains and humans particularly in study area. In addition a systematically organized coordinated study to generate information should be conducted to explore the magnitude of the problem and to develop an effective strategy to reduce the outbreak of food-borne illnesses and resistance burden in the community.

Therefore this study was designed to:

- ❖ Isolate and identify *Staphylococcus* and *Staphylococcus* species from dairy cattle farms.
- ❖ Evaluate antimicrobial susceptibility patterns of *Staphylococci* species.

2. Materials And Methods

2.1. Study Area

The study was conducted in the privately owned dairy farms found in and around Modjo town from December 2015 to April 2016. Modjo is a town in central Ethiopia, located in the East Shoa zone of Oromia Regional State at a distance of 70kms South East of Addis Ababa. The latitude and longitude of the town is 8°N and 39°E, respectively, with an altitude of between 1788 and 1825 meters above sea level. The average minimum and maximum temperature is 18°C and 28°C, respectively and has an experience of bimodal rain fall pattern in which the main rainy season occurs between June and September and Short rainy season from March to May. The average annual rain fall is of 800mm (ILRI, 2005).

2.2. Study Design and Study Population

A cross-sectional study was conducted from December 2015 to April 2016 to isolate and identify *Staphylococcus* species from selected dairy farms of Modjo town, Eastern Shoa., Ethiopia. The study animals were all lactating dairy cows found in

randomly selected cows' population from conveniently selected dairy farms in Modjo town, Eastern Ethiopia. The study animals for this study represent cattle and cattle derived milk foods. The breeds include Holiestin Frisian (HF), cross and indigenous breed lactating dairy cows managed under intensive and semi intensive system. Moreover, dairy personnel and milking utensil (milking bucket and milk storage in farm) were also included.

2.3. Sampling Method

Using simple random sampling technique, depending on herd size twenty six with 21 semi intensive and 5 intensive dairy farms and 3 collection centers in the study area were incorporated in the study. The sampling frame from the study site indicates most of the farms were small holder dairy farms having an average of 5 cows from each respective herd/farm were included in the study population. The study involved total of 212 samples from dairy farms of which 152 udder milk, 15 tank milk of the selected dairy farms and collection center, 15 milking bucket swab, 15 tank swab, 15 milkers' hand swab.

2.4. Study Methodology

2.4.1. Sample type

Raw milk samples were collected from two critical control points (directly from the cows' udder and, from container at farm level) together with milkers, bucket and container swab at farm level and from collection center (tank milk and tank swab) that were considered to be associated with the hazard, where a measurement can be conducted and when control measures can be taken in order to reduce the hazard to an acceptable level. The swab samples were collected before milk sampling by using sterile swabs.

2.4.2. Sampling procedure, transportation and storage

Strict aseptic procedure was followed when collecting milk samples in order to prevent contamination with microorganisms present on the skin udder and teats, on the hands of samplers and on the barn environment. Teat ends were cleaned and disinfected with ethanol (70%) before sampling. Strict foremilk (first jets) were discharged to reduce the number of contamination of teat canal (Quinn *et al.,* 1999). Sterile universal bottle with tight fitting cups were used. The universal bottle was labeled with permanent marker before sampling. To reduce contamination of teat ends during sample collection, the near teats were sampled first and then followed by the far ones (Quinn *et al.,* 1999).

Briefly, milk in the bulk containers was agitated before collection, and samples taken from the top of the bulk tank using a sanitized dipper. Identification of samples were made by date of collection and collection centers (MCC) of the milk. All samples were kept in an icebox containing ice packs and taken immediately to the microbiology laboratory of Addis Ababa University, college of Veterinary Medicine and Agriculture, Bishoftu for microbiological analysis. Upon arrival, the samples were stored overnight in a refrigerator at 4° C until examined the next day (Fanta, 2010).

2.5. Laboratory Examination

Final isolation and identification of *Staphylococci* organisms and species was done based on culture characteristics, Gram staining, series of biochemical tests for example catalase test, coagulase test and sugar oxidation fermentation as described by Quinn *et al*., (2002).

2.5.1. Culturing procedure and colony appearance

A loop of milk sample was streaked on 5% sheep blood agar (BAP) and the plates were incubated aerobically at 37°C and examined after 24 hours of incubation for growth. The bacteriological media used was prepared according to the manufacturer's recommendations. The plates were examined for the presence of *Staphylococcus* colonies. Isolates supposed to belong to *Staphylococcus* species on the basis of their morphological aspects (round, smooth and white or yellow colonies) and haemolytic pattern on the surface of BAP were collected. The representative colonies were sub cultured on nutrient agar plate (NAP) and incubated at 37 °C. The plates were preserved and maintained for characterizing the isolates. Final identification of the organisms and species was done based on gram staining, catalase test, oxidative-fermentative test, coagulase test, growth on mannitol salt agar and on purple agar base were performed and *Staphylococcus* species were isolated for further test. Pure cultures of a single colony type from the NAP were inoculated into nutrient slants and incubated at 37°C for 24-48 hours under aerobic culture conditions. The pure isolates in the nutrient slant were preserved and maintained at 4°C for further need (Quinn *et al*., 2002).

2.5.2. Isolation and identification of Staphylococcus species

Gram's staining*:* All suspected cultures of *Staphylococci* species were subjected to Gram's stain and observed under a light microscope for Gram's reaction, size, shape and cell arrangements. The Gram stained smears from typical colonies that showed Gram-positive cocci occurring in bunched, grapelike irregular clusters were taken as presumptive *Staphylococcus* species (Quinn *et al.,* 2002).

Catalase test*:* The culture to be tested for catalase test was picked up by bacteriological loop (nichrom wire loop) from the agar slant and mixed with a drop of 3% hydrogen peroxide on a clean slide. If the organism is positive, bubbles of oxygen is liberated

within a few seconds. Those positive cocci were considered as *Staphylococci* (Quinn *et al.,* 1999).

Oxidation-Fermentation *(O-F) test:* The convectional O-F medium is most suitable for non-fastidious Gram negative bacteria. The modification of the O-F medium which is applied for the identification of *Staphylococcus* and *Micrococcus* was used. Bacteria that are metabolized glucose under either aerobic or anaerobic are facultative anaerobes which are the characteristics of *Staphylococcus* (Quinn *et al.,* 2002).

Mannitol salt agar (MSA) and mannitol fermentation test*:* The colonies that were identified by Gram-staining reaction, O-F glucose, oxidase and catalase test as *Staphylococcus* were streaked on MSA plates and incubated at 37°C and examined after 24-48 hours for growth and change in the color of the medium. The presence of growth and change of pH in the media (red to yellow color) were regarded as confirmative identification of *Staphylococci*. Phenol red pH indicator detected the acidic metabolic product of mannitol. Fermentation of mannitol by *S. aureus* causes yellow discoloration of the medium. Colonies that develop weak or delayed yellow color after 24 hours of incubation were taken as *S. intermedius* and colonies that failed to produce any change on the medium were considered as *S. hyicus* and CNS (Quinn *et al*., 2002).

Coagulase test*:* The tube coagulase test was performed in sterile tubes by adding 0.5 ml of selected isolates of *Staphylococcus* grown on nutrient broth (NB) at 37°C for 24 hours to 0.5 ml of fresh rabbit plasma. After mixing by gentle rotation, the tubes were incubated at 37°C along with a negative control tube containing a mixture of 0.5 ml of sterile NB and 0.5 ml of rabbit plasma. Clotting was evaluated at 30 minutes intervals for the first 4 hours of the test and then after 24 hours incubation. The reaction was considered positive, if any degree of clotting from a loose clot to a solid clot that is immovable when the tube is inverted (tilted) was visible within the tube and no degree of clotting would be taken as negative (Quinn *et al*., 2002).

Purple agar base*:* Purple agar base (PAB) with the addition of 1 % maltose was used to differentiate the pathogenic *Staphylococci,* particularly the coagulase-positive isolates. The suspected culture was inoculated on PAB media plate with 1% of maltose and incubated at 37°C for 24-48 hours. The identification was based on the fact that *S. aureus* rapidly ferment maltose and the acid metabolic products cause the pH indicator (bromocresol purple) to change the medium and colonies to yellow *S. intermedius* gives a weak or delayed reaction and *S. hyicus* did not ferment maltose but attacks the peptone in the medium producing an alkaline reaction (a deeper purple) around the colonies (Quinn *et al*., 2002).

2.5.3. In vitro antimicrobial susceptibility test

The antimicrobial resistance patterns of the isolates (n=82) identified to species level of CPS and CNS species were tested against 14 selected antibiotics using the Kirby-Bauer disk diffusion technique on Mueller Hinton Agar (Quinn *et al*., 1999; NCCLS, 1999), using fresh nutrient broth culture and antibiotic discs as described in the standard manual.

The disk diffusion method was used to determine the susceptibility of all selected isolates to fourteen different antimicrobial drugs (Oxoid, Hampshire, England) were used: Amoxicillin (AML-25µg), Cefoxitin (FOX-30µg), Chloramphenicol (C-30µg), Ciprofloxacin (CIP-5µg), Cloxacillin (OB-5µg), Erythromycin (E-15µg), Gentamycin (CN-10µg), Kanamycin (K-30µg), Nalidixic acid (NA-30µg), Nitrofurantoin (F-50µg), Penicillin G (P-10U), Streptomycin (S-10µg), Sulphamethoxazole Trimethoprim (SXT-25µg), and Vancomycin (VA-30µg).

Well isolated colonies of the same morphological type were selected from a non-selective agar plate and suspension was made in sterile saline. The turbidity of the suspension was adjusted by comparison with 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 inoculated plates were 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 and the plates were incubated aerobically at 37°C for 16hour-18hour for all disc except for Vancomycin 24hour (CLSI, 2007; CLSI, 2012; Oxoid, Hampshire, England).

Inhibition zone diameters were measured and values obtained from the Clinical laboratory standard institute (CLSI, 2007; CLSI, 2012; CLSI, 2013) and manual of the manufacturer (Oxoid) were also used to interpret the results obtained. The isolates were then classified as resistant, intermediate or susceptible to a particular antibiotic based on the cut-off value as indicated on CLSI. Intermediate results were considered resistant (Huber *et al*., 2011).

2.6. Data Management and Analysis

Collected data was coded and entered to Microsoft Excel spreadsheet and checked for accuracy. After validation, it was transferred and processed using computer software statistical package for social science (SPSS) version 20 for analysis. Pearson's chi-square tests were used when appropriate

to analyze the proportions of categorical data. Odds ratio and 95% CI were computed and the results were considered significant at $p<0.05$.

3. Results

3.1. Bacterial Isolation and Identification Results

Out of the 212 samples 38.8% (82/212) resulted contaminated with *Staphylococcus* species. After isolation and identification of *Staphylococcus* species 32 (15.1%), 22 (10.4%), 10 (4.7%) and 18 (8.5%) were *S. aureus, S. intermedius, S. hyicus* and coagulase negative staphylococci (CNS) respectively, from the total sample (212). The result indicated that *S. aureus* was the most prevalent *Staphylococcal* species followed by *S. intermedius*, CNS and *S. hyicus* (Table 1).

The frequency of isolation of *Staphylococcus* varied between different sample types. The prevalence of staphylococci were 36.8% (56/152), 73.3% (11/15), 33.3% (5/15), 46.7% (7/15) and 20% (3/15) from udder milk, tank milk, hand swab, bucket swab and tank swab respectively. The results showed that relatively higher isolation of *Staphylococcus* from tanks milk (73.3%) than other swabs. The results of biochemical characterization of these isolates showed that *S. aureus* was the most frequently isolated species among different sample types accounting for 32 (15.1%) the population studied (Table.1).

Table 1: Number and percentage of staphylococcus species with sample type taken in and around Modjo town, dairy farms

Sample type	*S. aureus*	*S. intermedius*	*S. hyicus*	CNS	Total
UM (n=152)	22 (14.5)	15 (9.9)	7 (4.6)	12 (7.9)	56 (36.8)
TM (n=15)	4 (26.7)	1 (6.7)	3(20)	3 (20)	11 (73.3)
HS (n=15)	3 (20)	1(6.7)	0 (0)	1 (6.7)	5 (33.3)
BS (n=15)	1 (6.7)	4 (26.7)	0 (0)	2 (13.3)	7 (46.7)
TS (n=15)	2 (13.3)	1 (6.7)	0 (0)	0 (0)	3 (20)
Total (n=212)	**32 (15.1)**	**22 (10.4)**	**10 (4.7)**	**18 (8.5)**	**82 (38.7)**
X^2 (p=value)	2.8 (0.60)	5 (0.28)	10 (0.04)	4.5 (0.34)	24.6 (0.07)

Key: UM=udder milk, TM=tanker milk, HS=hand swab, BS=bucket swab, TS=tanker swab, CNS=coagulase negative *Staphylococcus*

3.2. Antimicrobial Susceptibility Test Results

Antimicrobial susceptibility test was conducted for all 82 isolates against 14 antimicrobial agents. The result indicated Ciprofloxacin and Gentamycin were the most effective antimicrobials with susceptibility percentage of 92.7and 87.8 respectively. Conversely 98.8 % of the *Staphylococcus* species were found to be resistance to penicillin G. The resistance profile of Cloxacillin and Amoxicillin were 64.6% and 79.3% respectively (Table 2).

Table 2: Antimicrobial susceptibility of *Staphylococcus* isolates (n=82).

List of drugs drug	code	Susceptible n (%)	Intermediate n (%)	Resistant n (%)
Penicillin G	P-10U	1 (1.2)	_	81 (98.8)
Erythromycin	E-15µg	16 (19.5)	46 (56.1)	20 (24.4)
Chloramphenicol	C-30µg	50 (61)	20 (24.4)	12 (14.6)
SM-TMP	SXT-25µg	70 (85)	5 (6.1)	7 (8.5)
Nitrofurantoin	F-50µg	28 (34.1)	14 (17.1)	40 (4.9)
Cloxacillin	OB-5µg	5 (6.1)	7 (8.5)	53 (64.6)
Amoxicillin	AML-25µg	17 (20.7)	_	65 (79.3)
Vancomycin	VA-30µg	61 (74.4)	_	20 (24.4)
Nalidixic acid	NA-30µg	22 (26.8)	35 (42.7)	25 (30.5)
Kanamycin	K-30µg	60 (73.2)	19 (23.2)	3 (3.7)
Ciprofloxacin	CIP-5µg	76 (92.7)	5 (6.1)	1 (1.2)
Streptomycin	S-10µg	53 (64.6)	17 (20.7)	12 (14.6)
Gentamycin	CN-10µg	72 (87.8)	7 (8.5)	3 (3.7)
Cefoxitin	FOX-30µg	6 (7.3)	_	39 (47.6)

Key: SM-TMP=Sulphamethoxazole Trimethoprim

As the result showed *Staphylococcus* species have variable susceptibility pattern towards antimicrobials. *S. aureus* is highly susceptible to both Ciprofloxacin and Gentamycin with percentage of 96.9 and 87.5 respectively, and highly resistant to Penicillin G (100%). *S. intermedius* also showed better susceptibility towards Ciprofloxacin (86.4%) and Vancomycin, Sulphamethoxazole Trimethoprim and Gentamycin with a percentage of 81.8. Similarly *S. hyicus* showed greater susceptibility (80%) towards Ciprofloxacin, Gentamycin and Sulphamethoxazole.

Trimethoprim but it was highly resistant to penicillin G and Cefoxitin with percentage of (100%). Moreover, CNS has susceptibility of Ciprofloxacin and Gentamycin with 100% for both; but it was resistant to Penicillin G (94.4%) and Cefoxitin (90.9%) (Table 3).

Table 3: Antimicrobial susceptibility patterns of each *Staphylococcus* species

Drug code	Susceptibility pattern of *Staphylococcus* species							
	S. aureus		***S. intermidius***		***S. hyicus***		***CNS***	
	S n (%)	R n (%)	S n (%)	R n (%)	S n (%)	R n (%)	S n (%)	R n (%)
P	0 (0)	32(100)	0(0)	22(100)	0(0)	10(100)	1(5.6)	17(94.4)
E	6(18.8)	11(34.4)	4(18.2)	5(22.7)	0(0.0)	3 (30)	6 (33.3)	1(5.6)
C	15(46.9)	4(12.5)	15(68.2)	5(22.7)	7(70)	1(10)	13(72.2)	2(11.1)
SXT	27(84.4)	4(12.5)	18(81.8)	2(9.1)	8(80.0)	0(0)	17(94.4)	1(5.6)
F	9(28.1)	14(43.8)	5(22.7)	15(68.2)	3(30)	7(70)	11(61.1)	4(22.2)
OB	4(12.5)	24(77.4)	0(0.0)	11(84.6)	0(0.0)	6(85.7)	1(7.1)	12(85.7)
AML	4(12.5)	28(87.5)	3(13.6)	19(86.4)	2(20.0)	8(80)	8(44.4)	10(55.6)
VA	23(74.2)	8(25.8)	18(81.8)	4(18.2)	6(60)	4(40)	14(77.8)	4(22.2)
NA	3(9.4)	13(40.6)	9(40.9)	7(38.9)	3(30)	0(0)	7(38.9)	5(27.8)
K	22(68.8)	3(9.4)	16(72.7)	0(0)	8(80.0)	0(0.0)	14(77.8)	0(0.0)
CIP	31(96.9)	0(0.0)	19(86.4)	1(4.5)	8(80)	0(0.0)	18(100.0)	0(0.0)
S	17(53.1)	5(15.6)	17(77.3)	2(9.1)	5(50.0)	4(40)	14(77.8)	1(5.6)
CN	28(87.5)	3(9.4)	18(81.8)	0(0.0)	8(80.0)	0(0.0)	18(100.0)	0(0.0)
FOX-	5(16.7)	25(83.3)	0(0.0)	3(100)	0(0.0)	1(100)	1(9.1)	10(90.9)

Key: S=Susceptible; R= Resistance; n= number of isolates; %= percentage of the isolates; CNS= Coagulase negative *Staphylococcus*

Almost 92.7% (76/82) of the isolates were observed with MDR to majority of the antimicrobial agents tested. About 98.9% of *S. aureus* showed MDR to all tested antimicrobials. However, very small proportions of *S. aureus* were resistance to Ciprofloxacin, Gentamycin and Kanamycin. Penicillin G, Cloxacillin, Cefoxitin and Amoxicillin were the drugs to which a large proportion of the isolates were resistant. The result also showed that 98.8% and 95.1% of MDR were developed in *S. hyicus* and CNS respectively (Table 4).

Table 4: Multidrug resistance profile of *Staphylococci* species with number of their drug resistance and frequency

No. of resisted drugs	**Staphylococci species**				**Total**
	S. aureus n (%)	S. intermedius n (%)	S. hyicus n %	CNS n %	n %
0	0 (%)	0 (0)	0 (0)	1 (5.6)	1 (1.2)
1	0 (0)	0 (0)	0 (0)	1 (5.6)	1 (1.2)
2	1 (3.1)	1(4.5)	1 (10)	1 (5.6)	3 (3.6)
3	2 (6.3)	7 (31.8)	3 (30)	6 (33.3)	18 (22.0)
4	6 (18.8)	4 (18.2)	2 (20)	3 (16.7)	15 (18.3)
5	9 (28.1)	5 (2.3)	2 (20)	3 (16.7)	19 (23.2)
6	6 (18.8)	4 (18.2)	1 (10)	1 (5.6)	12 (14.6)
7	5 (15.6)	0 (0)	1 (10)	2 (20)	8 (9.8)
8	2 (6.3)	1 (4.5)	0 (0)	0 (0)	3 (3.6)
9	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
10	1 (3.1)	0 (0)	0 (0)	0 (0)	1 (1.2)
Total	**32 (100)**	**22 (100)**	**10 (100)**	**18 (100)**	**82 (100)**
MDR	31 (96.9)	21 (95.5)	9 (90)	15 (83.3)	76 (92.7)

Key: MDR= Multidrug resistance (isolates showed resistance to ≥3 tested antimicrobials)

Antimicrobials of veterinary and human health relevance were considered in this study has demonstrated the existence of alarming levels of resistance of *Staphylococcus* to commonly used antimicrobial agents in the study area (farms).

4. Discussion

The surveillance of food for microbial contamination is vital for the protection of public health and consumer interest. Production of safe food also has important economic implications in an increasing competitive global market. *Staphylococcus* organisms can gain access to row milk by direct excretion from udders having clinical and subclinical *Staphylococcal* mastitis or by contamination from food handlers and pose public health risk to consumers (Yilma *et. al*., 2007). Different investigators have reported that *Staphylococcus* species isolated from the dairy products of bovine are able to produce high levels of *Staphylococcal* enterotoxins. Smith (2007) reported that 54% of bovine mastitic milk isolates to be enterotoxiginic, and Salandra *et. al*., (2008) reported that 55.9% enterotoxin producing *Staphylococcus* isolates from dairy products in Italy.

In this study, the presence of *Staphylococci* was detected in 82 out of 212 analyzed samples. The detection was made in pooled udder milk, tank milk from farm and collection center, pooled bucket swab, farm tank swab, and hand swab of milker's. The overall prevalence of *Staphylococcus* in dairy farms was 38.7%. This is in line with the findings of Mekonen *et. al*., (2011) who reported 39.5% in Adaa-Liben district dairy and dairy product producer and Mekonnen and Tesfaye, (2010) who reported 35.8% in dairy farm in Adama. But it was higher than Abunna *et. al*., and Zeryehun *et. al*., (2013), who reported 21% and 28.7% in Addis Ababa, respectively.

Coagulase positive *Staphylococci* (CPS*)* isolates 30.2% (64/212) dominated in prevalence than the coagulase negative *Staphylococci* (CNS) with prevalence of 8.5% (18/212). This is similar with the finding of Siraj (2012) who report in Ambo that CNS is the second most prevalent pathogens next to CPS. However, it is contrary to the findings of Bendahou *et. al.,* (2008) who reported CNS (54 %) is the predominantly prevailing isolates. The differences in prevalence reports of *Staphylococcus* species in the present study and other reports could be attributable to difference in sample type, differences in the origin of the samples or by geographical differences, differences in study methods and materials employed by the investigators.

The products specific prevalence of *Staphylococcus* were found to be 36.8%, 73.3%, 33.3%, 46.7% and 20% from udders milk, farm tanks milk, milkers hand swab, buckets swab and farm tanks swab, respectively. This finding comparable with finding of Fikru, (2014) who reported 47.1%, 58%, 38% 34%, and 38% from udders milk, farm tanks milk, farm tanks swab, buckets swab, and hand swab of milkers samples, and Mekonnen *et al.,* (2011) who reported 33% *Staphylococcus* prevalence in tank milk, respectively, in Debre Zeit. A high prevalence of *Staphylococcus* was recorded in farm raw tank milk than in farm tanks swab and buckets swab. This finding factors that could be hypothesized to be causes of contamination of milk in this study include insufficient pre-milking udder preparation, insufficient cleaning of milkers' hands, milking buckets and storage containers. Most of small holder farms used milk tanks which were made up of plastics in the current study area. According to Soomro *et al*., (2003) plastic containers scratch easily and provide hiding places for bacteria during cleaning and sanitization and plastic containers are poor conductor of heat and hence will hinder effective sanitization by heat.

From the total sample *staphylococcus* species isolated among different sample types *S. aureus* accounting for 15.1% (32/212) the population studied. The other *Staphylococci* isolates were distributed as, *S. hyicus* 4.7% (10/212); *S. intermedius* 10.4% (22/212); CNS 8.5% (18/212). *S. aureus* was the most frequently species which was comparable to Fikru, (2014) who reported 17.2% from dairy farm.

The prevalence and degree of antimicrobial resistance in veterinary medicine are increasing worldwide (Werckenthin *et al*., 2001). The dissemination of MDR *Staphylococci* is presenting a challenge to both human and animal health professionals. Therefore the epidemiological and clinical importance of *Staphylococcal* species is not only because of its distribution and pathogenicity but especially due to its ability to overcome antimicrobial effects. So there is a need for continued vigilance and systematic study to enlarge the understanding of its dynamic. Considering the spread of methicillin resistant *S. aureus* strains it is necessary to determine risk factors for animal infections, especially for household animals that live in strict contact to human, the relationship between animal and human carriage, and the genetic relationship of animal and human strains (Miliane *et al*., 2012).

The current study of antimicrobial susceptibility testing showed among, 82 *Staphylococci* species tested, 98.8% (n=81) isolates showed AMR patterns to at least one of the antimicrobials tested. This study revealed that milk, and container and material used in the farm and at the milk collection center may be contaminated with MDR *Staphylococcus* species.

Staphylococcus species isolated from human (hand swab) also showed MDR to different drugs. The high frequency of resistance observed with Penicillin G (98.8%), Amoxicillin (79.3%), Cloxacillin (64.6%), and Cefoxitin (47.6%). This might indicate transfer of resistant strain among environment, livestock and human (Martil *et. al.,* 2004); though, these antimicrobials are not used in veterinary practice in the study area. However, very small proportions of *S. aureus* were resistant to Gentamycin (9.4%) and Kanamycin (9.4%) but no resistance was observed to Ciprofloxacin (0.0%). The resistance status of *S. aureus* to Sulphamethoxazole-Trimethoprim is in line with the reports of 15.7% to 19% in South Africa by Ateba, (2010).

Generally, this antimicrobial susceptibility study showed that overall 92.7% *Staphylococcus* species isolated from dairy farm samples (milk of dairy cows, container swab and milker hand swab, and tank swab of collection center) are MDR, means the isolates were resistant to more than two antimicrobials. *S. aureus* developed higher degree of MDR 31/32 (96.9%) which was slightly higher than Nihal *et al.,* (2011) and Chao *et al.,* (2007) who reported the rate of multi-drug resistant of *S. aureus* (80%) and (79%) respectively. Moreover, the present investigation showed *S. aureus* strains have develop MDR worldwide with broad diversity in prevalence rate in different regions (Normanno *et al*., 2007).

5. Conclusion And Recommendations

Infections due to *Staphylococci* are of major importance to veterinary and human medicine. In this investigation, CPS isolates dominated in occurrence than the CNS. Among the CPS, *S. aureus,* the important causes of milk borne illness associated with the consumption of raw milk and milk products, was the most frequently isolated species. This study highlighted the high incidence of AMR *Staphylococci* species isolated from dairy farms. According to the findings from this study the hand swabs of human, dairy cow, container used for milking might be a major source of contamination of animal milk with bacteria, *Staphylococcus* and their contamination were indicators for poor hygienic practice. Most *Staphylococci* species isolates showed MDR to various antimicrobial agents. Indiscriminate use of antimicrobial agents might account, at least in part, for such a high resistance.

Based on the findings of the present study the following recommendations are made:

- Awareness creation for dairy cow owners and milkers, especially to those of small holder dairy farms, regarding the importance of adequate udder preparation, use of clean dairy equipment and utensils, hygienic milking technique, and milkers hands should be practiced.
- Multiple drug resistant *Staphylococci* species *(S. aureus*) have a wide distribution in milk and therefore care should be taken in to account during dairy products processing to destroy the microorganisms and thereby to avoid the risk of human infection.
- The occurrence of MDR *Staphylococcus* particularly CPS should be under consideration during selection of antimicrobials for the treatment of bacterial infection in dairy cattle.
- Veterinarian and medical researchers in collaboration should make further investigation on public health significance of MDR associated with milk at regional and national level.

Finally, further studies should be conducted to investigate the impact of the high prevalence of multi-drug resistant *Staphylococci* on human and animal health in Ethiopia.

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7. Annexes

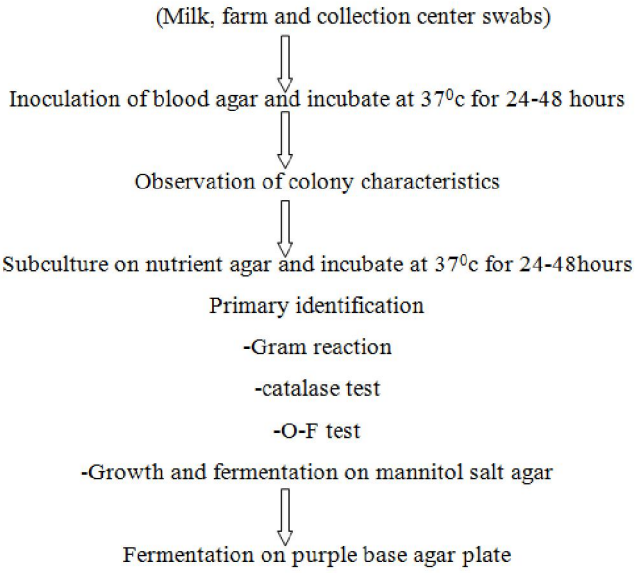
Annex 1. Sample collection sheet for bacteriological analysis

Serial Number	Date of collection	Type of sample	Source	Sample code	Number of samples

Annex 2. Record sheet for laboratory isolation and identification of staphylococcus

Serial number	Type of sample	Sample code	Colony characteristics on BPA	Haemolysis	Gram stain	Catalase test	Coagulase reaction	Growth on (MSA)	Growth on (MSA)	O-F test	Maltose fermentation (PAB)	Staphylococcus

Annex 3. A flow chart showing procedures followed in the isolation and identification of staphylococcus (ISO 6888-3: 2003):



Annex: 4. Differential tests used for identification of Staphylococcus species and their reactions (Quinn *et al*., 1999)

Serial number	Staphylococcus species	Haemolysis	Pigment production	Coagulase test	Fermentation of sugar	
					MSA	PAB
1	*S. aureus*	+	+	+	+	+
2	*S. intermidius*	+	-	+	±	±
3	*S. hyicus*	-	-	+	-	-
4	*CNS*	-	-	-	-	-

+ = 90% or more strains are positive, ± = 90% or more strains are weakly positive, - =90% or more strains are negative.

Annex 5: Zone of diameter interpretive standards for *Staphylococci* species

Antimicrobial agent	Disc code	Potency	Zone diameter nearest S I R		
Amoxicillin	AML	25µg	≥20		≤19
Cefoxitin	FOX	30µg	≥22*	-	≤21*
			≥25**		≥24**
Chloramphenicol	C	30µg	≥18	13-17	≤12
Ciprofloxacin	CIP	5µg	≥21	16-20	≤15
Cloxacillin	OB	5µg	13**	11-12	10**
Erythromycin	E	15µg	18*	14-22	17*
			≥23		≤13
Gentamycin	CN	10µg	≥15	13-14	≤12
Kanamycin	K	30µg	≥18	14-17	≤13
Nalidixic acid	NA	30µg	≥19	14-18	≤13
Nitrofurantoin	F	50µg	≥17	15-16	≤14
Penicillin G	P	10UNITS	≥29	-	≤28
Streptomycin	S	10µg	≥15	12-14	≤11
SM-TMP	SXT	25µg	≥16	11-15	≤10
Erythromycin	E	15µg	≥23	14-22	≤13
Vancomycin	VA	30µg	>15		≤15***

Key: **For *S. aureus* and *S. lugdunensis*. *For CNS except *S. lugdunensis*. *** All Staphylococci for which Vancomycin 14mm or less should be tested by reference MIC method (CLIS, 2007). S=susceptible, I=intermediate, R=resistance, SM-TMP= Sulphamethoxazole Trimethoprim

Annex 6. Media used for isolation and identification of *Staphylococcus*

(A) Blood agar base (BBL®, Becton Dickinson, USA)

Composition (g/l):

Heart muscle, infusion from (solids)	2.0
Pancreatic digest of casein	13.0
Yeast extract	5.0
Sodium chloride	5.0
Agar	15.0

Final PH 7.3 ± 0.2 at 25°c

Preparation: suspend 40 gram of the powder in 1 liter of distilled water and mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclaved at 121^0c for 15 minutes. Cool the base to 45-50 ^{0}c and add 5% of sterile sheep blood.

(B) Nutrient agar (Oxoid, Hampshire, England)

Composition (g/l):

”Lab-lemco” powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0

Final PH 7.4 ± 0.2 at 25°c

Preparation: suspend 28 gram in 1 liter of distilled water. Bring to boil to dissolve completely. Sterilize by autoclaving at 121^0c for 15 minutes.

(C) Mannitol salt agar (Difco, Detroit, USA)

Composition (g/l):

Protease peptone	No.310.0

Becton-beef extract	1.0
D-mannitol	10.0
Sodium chloride	75.0
Bacto agar	15.0
Phenol red	0.025

Final PH 7.5 ± 0.2 at 25°c

Preparation: suspend 111gram in 1 liter of distilled water and heat to boiling to dissolve completely. Sterilized in the autoclave for 15 minutes at 15 pounds pressure (121^0c) Cool to 45-50^0c and dispense into Petridishes.

(D) O-F basal medium (Merck, Darmstadt, Germany)

Composition (g/l):

Peptone from casein	2.0
Yeast extract	1.0
Sodium chloride	5.0
Dipotassium hydrogen phosphate	2.0
Bromothymol blue 0.08; agar-agar	2.5

Final PH 7.5 ± 0.2 at 25°c

Preparation: suspend 11 gram in 1 liter of distilled water by heating in a boiling water bath or in a current steam, autoclave at 121^0c for 15 minutes; at approximately 50^0c mix in 100ml/liter of filter sterilized 10% solution of D (+) glucose, lactose, sucrose, or other carbohydrates; dispense into tubes to give a depth approximately 5cm.

(E) Purple agar base (HIMEDIA, INDIA)

Composition (g/l):

Peptone, special	10.0
Beef extract	1.0
Sodium chloride	5.0
Bromo cresol purple	0.02
Agar	15.0

Final PH 6.8 ± 0.2 at 25°c

Preparation: suspend 31.02 grams in 1000 ml distilled water. Add 5 - 10 grams of the carbohydrate to be tested. Heat boiled to dissolve the medium completely. Dispense in tubes as desired and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Alternatively sterilize the basal medium prepared using 900 ml distilled water and add 100 ml separately sterilized 5 - 10% solution of the desired carbohydrate to it.

(F) Mueller-Hinton II agar (BBL®, Becton Dickinson, USA)

Composition (g/l):

Beef extract	2g/l
Acid hydrolase's of casein	17.5g/l
Starch	1.5g/l
Agar	17g/l

Final PH 7.5 ± 0.2 at 25°c

Preparation: Suspend 38 gram of the powder in 1 liter of purified water and mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121^0c for 15 minutes then and dispense to Petridishes.

(G) Buffered Peptone water (CONDA):

Composition (g/l):

Casein pancreatic digests	10g/l
Sodium chloride	5g/l
Disodium phosphates	3.5g/l
Monopotassium	1.5g/l

Final PH 7.3 ± 0.2 at 25°c

Preparation: Suspend 20g of the medium in one liter of distilled water. Mix well and dissolve by heating with frequent agitation. Boil for one minute until complete dissolution. Dispense in appropriate containers and sterilize in autoclave at 121^{O}C for 12 minutes.

(H) Tryptone soya broth (Oxoid, England)
Typical formula (g/l):

Pancreatic digest of casein	17.0
Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Di-basic potassium phosphate	3.5
Glucose	2.5

Final PH 7.3 + 0.2 at 25°c

Instructions for use: Dissolve 30 gram in liter of distilled water and distribute in to final container. Sterilize by autoclaving at 1210C for 15 minutes.

Annex 7. Primary identification tests

Gram's stain (Carter, 1999)

Procedure:

- Make a thin smear or film
- Allow the film to dry in air
- Fix the film by passing through the Bunsen burner flame several times
- Flood the slide with crystal violet for 30-60 seconds
- Pour of the stain and wash the remaining stain with iodine solution
- Wash of the iodine and shake the excess water from the slide
- Decolorized with acetone alcohol
- Counter stain with safranin for 30-60 seconds and wash with water

1. Catalase test (Quinn *et a*l., 1999)

Procedure:

- A loopful of the bacterial colony is taken from the nutrient agar medium.
- The bacterial cells are placed on a clean microscopic slide and a drop of 3%H_{2O2} is added.
- An effervescence of oxygen gas, within a few seconds, indicates a positive reaction.

2. O-F test (Quinn *et al*., 1999)

Procedure:

- Prepare O-F base medium and when the O-F has cooled to 50^{0}c, add 20 ml of sterile glucose solution into 200ml of O-F base, of a final concentration of 1% glucose and dispense into tubes.
- Two tubes of the O-F medium are heated in a beaker of boiling water immediately before use to derive off any dissolved oxygen and the tubes are then cooled rapidly under cold running water.
- Both tubes are stab inoculated with bacterium and a layer of sterile paraffin oil is layered on top of one of the tube (sealed tube) to a depth of about 1cm and the tubes are incubated at 37^{0}c and examined in 24 hours and then daily for 14 days.

Annex 8: Secondary identification tests (Quinn *et al*., 1999)

1. Slide coagulase test

Procedure:

- A loop full of the staphylococcal culture is emulsified in a drop of water on a microscope slide.
- A loop full of rabbit plasma is added and mixed well with the bacterial suspension.
- The slide is gently rocked and a positive reaction is indicated by clumping within one or two minutes.

2. Tube coagulase test:

Procedure:

0.5ml of rabbit plasma is placed in small (7mm) test tube. 0.5ml of an overnight broth culture of *staphylococcus* or a heavy suspension made from isolated culture in sterile water is added. The tube is rotated gently to mix the contents and then incubated at 37^{0}C, preferably in water bath. A positive test with clotting of the plasma occurs in 2 to 4 hours. However, many weak coagulase positive strains will coagulate the plasma only after overnight incubation (Quinn *et al*., 1999).

Annex 9: Procedure for the disk diffusion method

Inoculation of distinct colony in to 5ml nutrient broth incubated at 35-370c for about 8 hours. Then the turbidity is compared with 0.5 McFarland Standard. This standard is prepared by adding 0.5ml of 1 % (11.75g/liter) Bacl2 2H20 to 99.5ml of 1% (0.36N) H2SO4. Then a sterile cotton swab on a wooden applicator stick is used to transfer the diluted bacterial suspension to a plate; excess fluid must be squeezed out by rotating the swab against the sides of the tube. The plate is seeded uniformly by rubbing the swab against the entire agar surface in three different planes roughly 60 degrees to each others'.

Within 15 minutes (time used to dry the inoculums) after the plates are inoculated, antibiotic

impregnated discs are applied to the surface of the inoculated plates by hand using a sterile forceps. All discs gently pressed down on to the agar with forceps to ensure complete contact with the agar surface. The disc should no closer than 1.5 cm to the edge of the plate and they should rest 24 mm apart from each other. The large Petridishes accommodate 6 discs in outer ring and three in the center, where as no more than 5 should be placed in small plates (10cm plates). Incubate the plates inverted aerobically for 24 hours at 350c but not 370c.

Zone of inhibition is measured in millimeters using a transparent ruler on the under surface of the Petri dish. For measuring purpose, the end is taken as complete inhibition of growth as determined by naked eye. The result is interpreted according to the table presented below.

Annex 10: Picture gallery of some laboratory *Staphylococci* tests

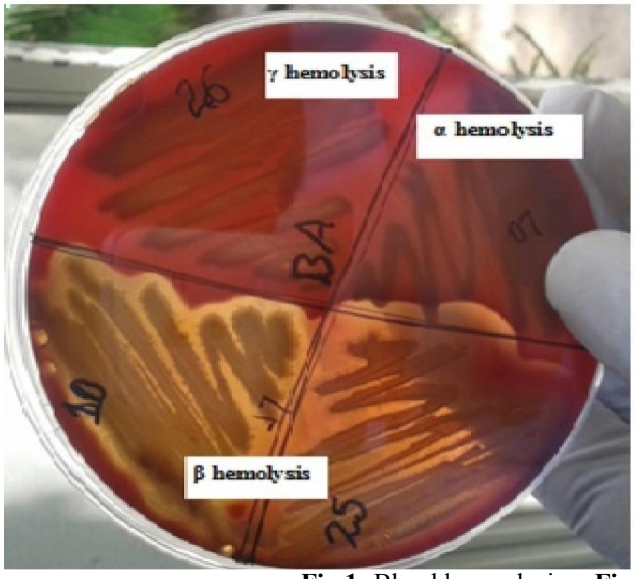


Fig.1: Blood haemolysis

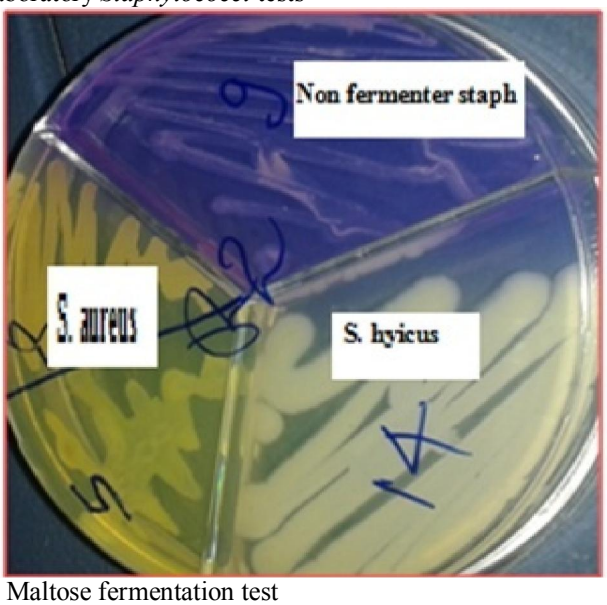


Fig. 2: Maltose fermentation test

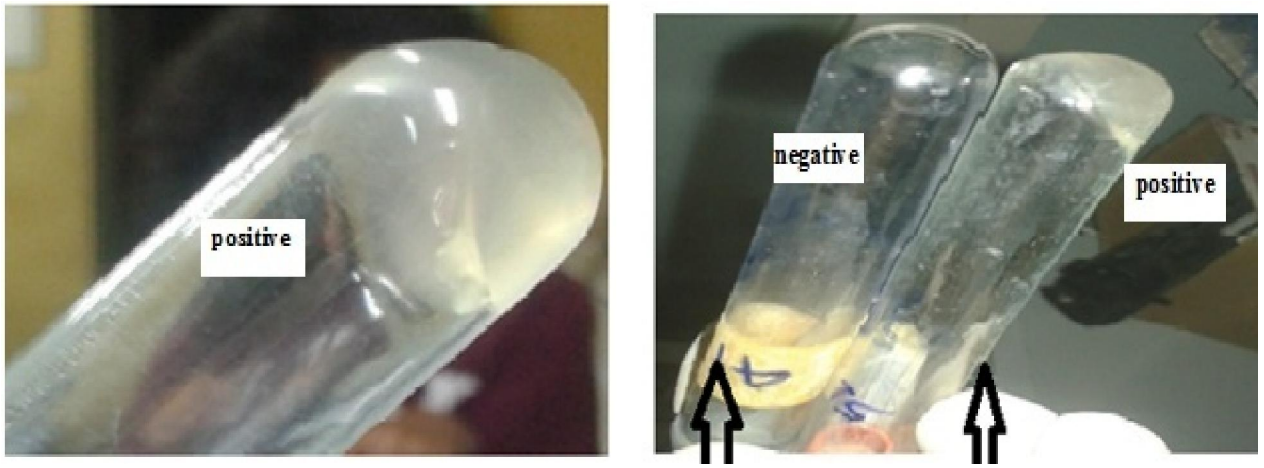


Fig. 3: Coagulase test of staphylococcus to rabbit plasma

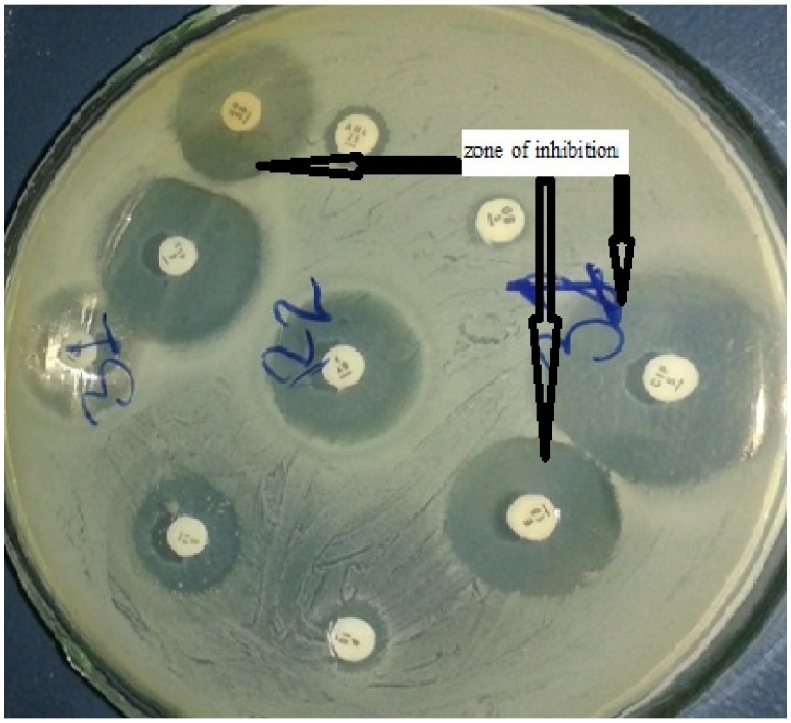


Fig. 4: Antibiotic susceptibility test

2/25/2021