Prevalence, Associated Risk Factors and Antimicrobial Susceptibility of
*Staphylococcus aureus* Isolated from Bovine Mastitic Milk in
and Around Asella Town, Ethiopia

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**Abstract:** A cross sectional study was carried out from November 2016 to April 2017 in and around Asella town to determine the prevalence, associated risk factors and antimicrobial susceptibility of *Staphylococcus aureus* in Bovine mastitic milk. A total of 384 lactating dairy cows were screened for mastitis based on clinical examinations and California Mastitis Test (CMT). Out of 230 lactating crossbred cows with either clinical or subclinical mastitis examined for the involvement of *Staphylococcus aureus*. *Staphylococcus aureus* was isolated at a rate of 47.2% and 42.9% of the subclinical and clinical cases, respectively. The overall prevalence of *Staphylococcus aureus* scored in this study was 46.5%. Among the risk factors considered, Age, parity and lactation stage were found significantly associated with the occurrence of *S. aureus* in mastitic milk (p<0.05). The current study revealed that *S. aureus* has avery low susceptibility to penicillinG (0%), followed by tetracycline (14.2%). However, these randomly selected isolates were found to be susceptible (100%) to Kanamycin. Proper prevention and regular antimicrobial sensitivity testing help to select effective antibiotics and ultimately reduce the development of resistance towards commonly used antibiotics.

**Key words:** Antimicrobial · Asella town · Bovine · Mastitis

**INTRODUCTION**

Ethiopia is a resourceful country bestowed with the largest livestock resource in the Africa continent [1] and the cattle population is estimated to be 53.4 million with the potential to export substantial numbers of live animals and their products [2]. Cows represent the largest population of cattle of the country. However, the annual consumption of milk in Ethiopian is low as compared to the average milk consumption of developed and developing countries. Local milk production does not satisfy the country's milk requirement due to low input husbandry practice and wide spread livestock diseases [3]. In the present day, there is a national drive to alleviate the existing food deficit by devising different agricultural strategies including improvements of the productivity of livestock sector by controlling some of the major infectious diseases, has received little attention in the country, especially, mastitis, the common problem of dairies that is known by an inflammation of the mammary gland is the leading one, that can contribute to reduce, milk production [4]. It is primarily resulting from an invasion of mammary tissues by pathogenic microorganisms through the teat canal resulting in physical, chemical and pathological changes in glandular tissues and milk [5, 6].

Mastitis is one of the most common diseases of dairy cattle throughout the world causing huge economic losses [7], it may be caused by either infectious or noninfectious agents. Infectious mastitis results from bacterial, mycotic or algal pathogens. Non-infectious mastitis is the result of injury, chilling, bruising or rough or improper milking. But it is almost due to the effect of infection by bacteria or mycotic pathogens. Pathogens causing mastitis in cattle are divided into major pathogens (those that cause clinical mastitis) and minor pathogens (those that normally cause subclinical mastitis and less frequently clinical mastitis) [8]. Clinical mastitis refers to the condition where the cow’s immune system responds with enough intensity to indicate signs of inflammation that is physically observable such as swelling, discoloration and pain [6], whereas subclinical mastitis...
refers to inflammation of the mammary gland in the absence of visible gross lesions in the udder or its secretion with the presence of pathogenic microorganisms and usually high number of somatic cells in the milk [9].

Among several etiological agents, *S. aureus* is a major mastitis-causing pathogen that also poses food safety and antimicrobial resistance threats [7]. It is a versatile pathogen of humans and animals that causes a wide variety of the diseases [10] and is an important cause of clinical mastitis in dairy cows causing a huge economic loss worldwide [11]. Reports from Ethiopia also indicate that, *S. aureus* is the most predominant cause of mastitis in dairy cows [12] it can express a wide array of potential virulence factors, including surface proteins that promote adherence to damaged tissue and/or exotoxins and enzymes that can cause variety of infections in skin and soft tissues, including intramammary mastitis [13]. Some evidence suggests that biofilm formation can be a virulence factor associated with *S. aureus* mastitis [14].

The cure rate after antimicrobial treatment of clinical *S. aureus* mastitis is very variable due to both cow and bacterial factors such as parity of the cow, chronicity of the infection and bacterial genotype [11]. To approach appropriate treatment and control measures, it is important to perform antibiotic susceptibility test on relevant and most frequently used antimicrobials. Currently, in Ethiopia, the information on prevalence and distribution of *S. aureus* and the sensitivity to commonly used antimicrobials for treatment of *S. aureus* mastitis is scarce [4]. Therefore, the objective of this study was to determine the prevalence, associated risk factors and antimicrobial susceptibility of *S. aureus* in bovine mastitic milk.

**MATERIALS AND METHODS**

**Study Area:** The study was conducted in and around Asella town, located in Oromia regional state, South Eastern Ethiopia. Asella town, the capital of Ars zone, is located at about 175 km Southeast of Addis Ababa at 6° 59’ to 8° 49’ N latitudes and 38° 41’ to 40° 44’ E longitudes with an altitude of the area ranges from 2500 to 3000metre above sea level and the temperature, between, 10-25°c. The rain fall is 1147mm on average and agricultural production system of the area is of mixed crop and livestock production [15]. It is one of the highly populated areas in Ethiopia with estimated population of bovine-82,190; ovine-51,292; caprine-8,11479; poultry-5,62915 and equine-22,055 [16]. Dairy farming using improved cross breeds is a common practice and farming system exists in the study area were intensive, semi-intensive and extensive.

**Study Population:** The study population was lactating dairy cattle of cross breeds, introduced by the artificial insemination program that was found in the study area and kept under intensive and semi-intensive husbandry practice.

**Study Design:** A cross sectional study was undertaken from November 2016 to April 2017 in and around Asella town to determine the prevalence, associated risk factors and antimicrobial susceptibility of *Staphylococcus aureus* in bovine mastitis milk in the study area. During the study period questioners, California mastitis test (CMT) and culturing of milk for *S.aureus* isolation and identification was carried out during the study period.

**Sampling Methods and Determination of Sample Size:**

Simple random sampling was carried out and the sample size of the study was determined based on sample size determination method as described by Thrusfield [17] with a 95% confidence interval and 5% desired precision. Since there was no similar research conducted in the area, expected prevalence of 50% was assumed. The value of 50% was assumed and this was corresponding to a required minimum sample size of 384 of lactating cows.

\[
N = \frac{(1.96)^2 \times P_{exp} \times (1 - P_{exp})}{d^2}
\]

Where,

- \( n \) = required sample size
- \( P_{exp} \) = expected prevalence
- \( d^2 \) = desired absolute precision

**Study Methodology**

**California Mastitis Test (CMT):** Each selected lactating cow was screened for mastitis based on clinical examinations and California Mastitis Test (CMT).Clinical examination of the udder was based on the method previously indicated [6]. The clinical findings considered include abnormalities of the secretion, abnormalities of the udder and teat and systemic reaction. The California Mastitis Test was performed according to previously established method [18]. In brief about 2ml of milk from each quarter of the udder was placed in each of four shallow cups in the CMT paddle and an equal amount of the reagent was added. A gentle circular motion was
Sample Collection and Transportation: The milk sample was taken from mastitic (CMT) positive cows not treated early with either intra mammary or systematic antimicrobials agents and collected according to earlier protocol [18]. To explain: quarters were washed with tap water and dried. The teat ends were then cleaned with cotton soaked with 70% ethyl alcohol and 8-10 ml milk was collected aseptically into a sterile screw-capped, pre-labeled test tube; by holding it slantly, so that, the pathogens that going to be recovered come from mammary gland; after discarding the first three streams of milk. Finally, milk samples were held in an ice box for transportation to Asella regional laboratory for bacteriological examination to isolate S. aureus. The samples were immediately cultured or stored at 4 °C for a maximum of 24 hr. until cultured on standard bacteriological media. Bacterial isolation and identification were carried out based on standard bacteriological techniques previously established [18]. In addition, data on potential risk factors including age, parity, lactation stage, housing and husbandry system were collected from an interview of owners and observation.

Bacteriological Milk Sample Examination and Isolation of S. aureus: During the course of the study, a total of 230 milk samples were collected from 35 clinical and 195 sub-clinical mastitis CMT positive cows. For bacterial isolation, milk samples were cultured according to the procedures described by Quinn et al. [19]. A loop full of milk sample collected from each infected quarters was cultured on blood agar base enriched with 7% sheep blood. The inoculated plates were then incubated aerobically at 37°C for 24 to 48h. Identification of bacteria on primary culture was identified as staphylococci according to the following procedure: The plate was examined for growth, colony morphology features such as circular, golden yellow and white in color and B-hemolysis on blood agar within 24-48 hours. Gram staining characteristics of the pure colonies such as random clustering were recorded. Suspected colonies of S. aureus were then selected and further sub-cultured on mannitol salt agar and incubated aerobically at 37°C for 24-48 hours. S. aureus colonies which produced a yellow pigment on the medium were subjected to slide catalase and coagulase [18] and oxidation tests. Finally, Staphylococcus aureus was determined and identified as Gram-positive cocci, hemolytic on sheep blood agar, catalase and coagulase-positive, oxidation negative and as one which produced yellow pigment on mannitol salt agar [19]. Samples were considered positive for S. aureus when at least one colony was identified as S. aureus.

Antibiotic susceptibility test: Antibiotic susceptibility tests were carried out on 42 randomly selected S. aureus isolates and their susceptibility profile was determined using Kirby-Baur disc diffusion method on Mueller-Hinton agar, following the procedures described by Quinn et al. [19]. Selected isolates were first cultured into nutrient broth overnight. A suspension of each test isolate was prepared in 0.9 % saline water to a turbidity equivalent to a 0.5 McFarland standard. Each suspension was streaked onto Mueller-Hinton agar, catalase and coagulase [18] and oxidation tests. Finally, Erythromycin (15µg), Vancomycin (30µg), Chloramphenicol (30µg), Tetracycline (30µg), Kanamycin (30µg), Sulphamethoxazole (25µg) and Penciling (10units) were positioned onto the plates, using sterile forceps. Inoculated plates were incubated at 37°C for 24hr. The inhibition zone was then recorded as the diameter of the zone surrounding the individual disk in which bacterial growth was absent. Based on this, the isolates were defined as resistant, intermediate and susceptible according to the guide lines of Quinn et al. [19].

Data Management and Analysis: All the data obtained and collected during the study period, risk factors, including age, parity, lactation stage, housing and husbandry and the laboratory results were coded and entered into Microsoft excel database system and subjected to descriptive statistics and chi-square in order to assess the magnitude of the difference of comparable variables using SPSS program version 20 software of computer program. The prevalence was calculated by dividing the number of positive animals for S. aureus to the total number of mastitis positive animals examined times 100%. Chi- square was used to know the existence...
of an association between *S. aureus* mastitis positivity and the risk factors. Statistically significant associations between variables were considered to exist if the p-value is less than 0.05.

**RESULTS**

**Prevalence of Mastitis:** Out of 384 cross breed lactating dairy cows examined for mastitis using CMT screening test and clinical examination, 230(59.9%), 195(50.8%) subclinical and 35(9.1%) clinical were found to be positive (Table 1).

**Prevalence of *S. aureus***: *S. aureus* was isolated from (N=92) 47.2% and (N=15) 42.9% of the sub-clinical and clinical cases, respectively. The overall prevalence of *S. aureus* recorded in this study was (N=107) 46.5% as shown in Table 1.

During the course of study risk factors that can have a possible association with the prevalence of *S. aureus* in mastitis cows were collected and analyzed accordingly to determine the association between considered risk factors and occurrence of *S. aureus*. As a result age, parity number and lactation stage were found to be statically significant (p<0.05) with the prevalence of *S. aureus* increases as long as these risk factors increases. However, housing and husbandry were found to be statically insignificant as illustrated in Table 2.

**Antimicrobial Susceptibility Test:** Forty two (42) randomly selected *S. aureus* isolates were subjected to antimicrobial susceptibility test to seven different antimicrobial drugs. In this study, *S. aureus* were found to be highly susceptible and resistant to kanamycin (100%) and pencilin G (100%), respectively as it has been indicated in Table 3.

### Table 1: Prevalence of *Staphylococcus aureus* in sub-clinical and clinical mastitis cases

<table>
<thead>
<tr>
<th>Forms of Mastitis</th>
<th>No of animals examined</th>
<th>No of <em>S.aureus</em> isolated</th>
<th>prevalence</th>
<th>x²</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-clinical</td>
<td>195</td>
<td>92</td>
<td>47.2%</td>
<td>3.84</td>
<td>0.00</td>
</tr>
<tr>
<td>Clinical</td>
<td>35</td>
<td>15</td>
<td>42.9%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>230</td>
<td>107</td>
<td>46.5%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2: Associated risk factors with the prevalence of *S. aureus* in mastitis cows.

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Total animal found positive</th>
<th><em>S.aureus</em> isolated</th>
<th>prevalence</th>
<th>x²</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>3-5</td>
<td>64</td>
<td>22</td>
<td>34.4%</td>
<td>53.5</td>
</tr>
<tr>
<td></td>
<td>6-9</td>
<td>119</td>
<td>56</td>
<td>47.1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;9</td>
<td>47</td>
<td>29</td>
<td>61.7%</td>
<td></td>
</tr>
<tr>
<td>Parity number</td>
<td>1-2</td>
<td>92</td>
<td>36</td>
<td>39.1%</td>
<td>42.6</td>
</tr>
<tr>
<td></td>
<td>3-4</td>
<td>83</td>
<td>40</td>
<td>48.2%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;4</td>
<td>55</td>
<td>31</td>
<td>56.4%</td>
<td></td>
</tr>
<tr>
<td>Lactation stage</td>
<td>&lt;3</td>
<td>93</td>
<td>34</td>
<td>36.6%</td>
<td>82.2</td>
</tr>
<tr>
<td></td>
<td>3-6</td>
<td>59</td>
<td>25</td>
<td>42.4%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;6</td>
<td>78</td>
<td>48</td>
<td>61.5%</td>
<td></td>
</tr>
<tr>
<td>Housing</td>
<td>concrete</td>
<td>165</td>
<td>81</td>
<td>49.1%</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>Muddy</td>
<td>65</td>
<td>26</td>
<td>40.0%</td>
<td>0.252</td>
</tr>
<tr>
<td>Husbandry</td>
<td>intensive</td>
<td>140</td>
<td>74</td>
<td>52.9%</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>Semi-intensive</td>
<td>90</td>
<td>33</td>
<td>36.7%</td>
<td>0.055</td>
</tr>
</tbody>
</table>

### Table 3: Summary on the result of antimicrobial susceptibility test

<table>
<thead>
<tr>
<th>Antimicrobials used with their concentration</th>
<th>Susceptible (%)</th>
<th>Intermediate (%)</th>
<th>Resistant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin 15µg</td>
<td>88.1</td>
<td>11.9</td>
<td>0</td>
</tr>
<tr>
<td>Vancomycin 30µg</td>
<td>92.9</td>
<td>7.1</td>
<td>0</td>
</tr>
<tr>
<td>Chloramphenicol 30µg</td>
<td>90.5</td>
<td>9.5</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline 30µg</td>
<td>14.3</td>
<td>35.7</td>
<td>50</td>
</tr>
<tr>
<td>Kanamycin 30µg</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sulphamethoxazole 25µg</td>
<td>83.31</td>
<td>4.3</td>
<td>2.4</td>
</tr>
<tr>
<td>Pencilin G 10unitis</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>
DISCUSSIONS

In this study, the prevalence of *Staphylococcus aureus* was found, significantly associated with types of mastitis and more frequently isolated in sub clinical mastitis than the clinical cases which was in agreement with the finding of [20] in Addis Ababa city and [21] in Kenya and with other similar studies that proved *S. aureus* is the principal causative agent of subclinical mastitis [6, 22], but disagreed with *S. aureus* isolated in clinical more than subclinical mastitis, [23]. Despite the fact, poor treatment successes and limited dry cow therapy may cause this disagreement.

The overall prevalence of *S. aureus* in study population and study area were found to be 46.5% which was in line with the prevalence (47%) reported from dairy farms of Holota town, central Ethiopia [24] and 48.3% from Addis Ababa city Ethiopia [20] but, higher when compared with the reports of 39.1% [25], 35.7% [26] and 39.4% [27], in Oromia regional state, in Asella, southern Ethiopia. On the other hand, a higher prevalence than the present result was reported in Ethiopia [28] and from dairy cows in Kenya [21]. The possible explanation for the variability in occurrence of *S. aureus* in mastitis cows among different reports, might be that *S. aureus* is a contagious pathogen transmitted from one cow, infected to another healthy individual by contact with animals during unhygienic milking procedure; hence, differences in farm management practices, environmental inconsistency and difference in awareness between dairy producer, how the disease transmit, may bring this difference [6].

The present study demonstrated that the prevalence of *S. aureus* is not associated with housing condition and husbandry system. This result agrees with the findings of Legesse *et al.* [20] from Addis Abeba Ethiopia, but, as opposed to this, reports from Adama, Ethiopia, reported that a high proportion of *S. aureus* was isolated from CMT positive cows kept in poor housing (muddy) conditions compared to concrete floor houses [26]. This variation may be attributed from management and herd size differences since *S. aureus* is a contagious pathogen that can be transmitted from cow to cow easily through the environment (floor) in which cows are kept close-proximity and unhygienic milking procedures, via milker’s hand, utensils, towels, through flies and cross-suckling that are used as a source of infection[6].

In this study, the susceptibility of *S. aureus* was found to be 88.1% to Erythromycin, 92.9% to Vancomycin, 90.5% to Chloramphenicol, 14.3% to Tetracycline, 100% to Kanamycin, 88.3% to Sulphamethoxazole and 0% to Peniciln G. The antimicrobial susceptibility tests carried out in this study revealed that the existence of low susceptibility of *S. aureus* to commonly used antimicrobials. The average susceptibility (67.7%) of *S. aureus* to all antimicrobials tested in this study is in accordance with 69.4% [26] reported from Adama, but, disagrees with other existing reports of 73.6% Marama *et al.* [34] at Holota, western Ethiopia and 62.7% by Mekonnen *et al.*[4] in Ethiopia dairy farms. This variation among these different reports could be arised, from the difference in resistance level, pathogen strain, an antimicrobial used and their concentration, as well as, other related factors that can affect the zone of inhibition during antimicrobial susceptibility test.

The current study demonstrated low susceptibility of *S. aureus* to penicillin G compared to other. This is in accordance with the findings that reported 0% susceptibility of *S. aureus* to penicillin G [34] and Tassew *et al.* [23] in holota area and around kombucha respectively, apart from, these findings, Adera *et al.*[26] around Adamahas also recorded 5.6% susceptibility to this drug. Besides this, the present study has indicated low susceptibility of *S. aureus* to tetracycline which is in parallel with different reports 7.5% susceptibility [23] and 73.8% resistance [10] around Addis Ababa. The mean susceptibility of *S. aureus* to the remaining antibiotics in this study was 92% which was in line with other studies like [34].

In general, the current study was able to show that susceptibility of *S. aureus* in the study area to commonly used antimicrobials, penicillin G and Tetracycline was very low. The possible justification for this could be the development of the alarming level of resistance of *S. aureus* due to the regular use of these commonly used antibiotics for the treatment of cows that may result in the spread of resistant strains in the study area. This result is in accordance with reports from earlier studies in other countries, suggesting a possible development of resistance from the prolonged and indiscriminate use of some antimicrobials [35, 36].

Here resistance to Penicillin G has a great concern, because, this antibiotic represents the main antibiotic group recommended for Staphylococcal mastitis infection. Antibiotic resistance is carried on plasmids and transposons that can pass from one Staphylococcal species to another [37]. The resistance of *S. aureus* to penicillin may be attributed to the production of beta lactamase enzyme that hydrolysis beta lactam ring of
penicillin and inactivates it and other closely related antibiotics. It is believed that around 50% of mastitis causing S. aureus strains produces beta-lactamase [38]. The low susceptibility of S. aureus isolates to this drug is evident.

CONCLUSIONS

In conclusion, the overall prevalence of S. aureus in the study area was 46.5%, which was 40 and 6.5% subclinical and clinical cases respectively. Occurrence of subclinical mastitis was more prevalent in the study farm (40%) which might indicate dairy farm owners, managers and veterinary professionals give due attention for clinical mastitis than subclinical infection which gives very little emphasis to the status of the subclinical mastitis. Among the different risk factors considered age, parity and lactation stage were found to be significantly associated with the prevalence of S. aureus in lactating dairy cows in the study area. The susceptibility of randomly selected isolates was also tested for some commonly used antimicrobial agents, as a result, it was observed that the isolates have very low susceptibility, 0% to penicillin G, followed by 14.2% to tetracycline but, good susceptibility was observed to the rest antibiotics tested. Therefore, regular testing for the detection of subclinical mastitis and proper treatment of the clinical cases together with the appropriate treatment of cows during dry and lactation period should be practiced and use of kanamycin should be encouraged to treat mastitis caused by S. aureus infected cows in the study area.

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