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Burden and Antimicrobial Resistance of *S. aureus* in the Hawassa Milk Shed, South Ethiopia

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Abstract: *Staphylococcus aureus* is a frequent colonizer of human and several animal species, including dairy cows. It is the most common cause of intramammary infections in dairy cows. Its public health importance increases in line to the continuous emergence of drug-resistant strains. The aim of this study was to determine the burden and drug resistance pattern of *S. aureus* in Hawassa milk shed. This study was done on 529 lactating cows and 95 dairy farms. Based on CMT results and clinical examination, the prevalence of mastitis were 74.7% at herd level and 62.6% at cow level. A total of 172 milk samples collected from mastitic cows, revealed *S. aureus* in 88 (51.2%) samples. Gram staining, oxidase, catalase, haemolysis and coagulase tests were employed for bacterial identification. In the *in vitro* antimicrobial susceptibility test, *S. aureus* was noted to be resistant to Ampicillin (84.1%), Penicillin (81.8%), Tetracycline (36.4%) and Amoxicillin-Clavulanic acid (34.1%), while it was found to be susceptible to Ceftriaxone (100%), Gentamicin (100%), Streptomycin (88.6%), Erythromycin (88.6%), Nitrofurantoin (72.7%) and Cefotaxime (72.7%). Eighteen different antimicrobials resistance patterns were observed and 95.5% *S. aureus* isolates were found to be multi-drug resistant involving two & more antimicrobials. In conclusion, this study has shown *S. aureus* is an important cause of mastitis and has developed multi-drug resistance against various commonly used antimicrobials. Multi-drug resistant *S. aureus* can spread from animal to human through consumption of raw milk and its products having significant role in public health. Therefore, care has to be taken during choice of treatment for mastitis especially those caused by *S. aureus*.

Key words: Antibiotics · *S. aureus* · Milk

INTRODUCTION

Staphylococcus aureus is the most predominant cause of mastitis in dairy cows in Ethiopia [1]. The Bacterial contamination of milk from an infected udder may render it unsuitable for human consumption by causing food poisoning or providing a vehicle for the spread of zoonotic diseases to humans [2]. In Ethiopia, *Staphylococcus* species presence of an alarming level of resistance isolates [3, 4]. Infection with antimicrobial-resistant bacteria has been known to be associated with frequent treatment failure and increased severity of the disease [5]. Resistant bacteria from food animals may be passed through the food chain to humans resulting in resistant infections [6].

Antibiotic-resistant *S. aureus* isolates pose a severe challenge to both veterinary and health professions and dairy cattle producers because they have a negative impact on therapy [7]. The usage of antibiotics correlates with the emergence and maintenance of antibiotic-resistant traits within pathogenic strains [8]. These traits are coded for by particular genes that may be carried on the bacterial chromosome, plasmids, transposons or on gene cassettes that are incorporated into integrons [9], thus are easily transferred among isolates. Multiple antibiotic resistant *S. aureus* strains have been isolated from milk obtained from cattle, beef and human samples in many parts of the world [10]. The prevalence of antibiotic resistance usually varies between isolates from the different sampled stations and even between isolates from different herds on the same farm [11].

The bacterium is so resistance against most antibiotics across the cow-pens that most of treatments protocols used against this pathogen fail to research. Various antibiotics in treatment of mastitis caused by *S. aureus* have been evaluated up to now. With regard to

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the fact that the probability of resistant strains occurrence and their incidence against common antibiotics has been reported [12, 13].

Determination of levels of *S. aureus* and an evaluation of the antibiotic-resistant Pattern of the isolates could serve as a tool for determining the hygiene standards implemented during milking. Data on antibiotic resistance could also be used to characterize these opportunistic pathogens, which may further limit the risks associated with the consumption of contaminated milk and its products [14]. Thus, the aim of this study was to isolate *S. aureus* from milk obtained from farm level collected milk and further characterization of their antimicrobial resistance patterns to eleven selected antibiotics.

MATERIALS AND METHODS

Study Area: This study was conducted between December 2015 and May 2016 in dairy herds found at Hawassa milk shed. The milk shed includes Hawassa city and its adjacent towns which supply milk for the big market at Hawassa. For this study, Hawassa city and two of the towns (Wondo Genet and Arsi Negelle) that are supplying milk for Hawassa were purposively selected because of their relatively larger potential for dairy cattle population. Hawassa is located at 275 km south of Addis Ababa, the capital. The city is situated at an elevation of 1708 m above sea level and located at 70 3' north latitude and 380 29' east longitudes. The annual rain fall and temperature varies from 800 to 1000 mm and 20.1-25°C, respectively. Wondo Genet is located at 30 km west of Hawassa. It is situated about 1723 m above sea level at latitude of 705' N and longitude of 380 37' E. The town receives an average annual rainfall of 1372 mm. The mean annual temperature is 19°C. Arsi Negelle town is found in the West Arsi zone of the Oromia regional state at a distance of 225 km from Addis Ababa. The town is situated about 2043 m above sea level at latitude of 70 21' N and longitude of 380 42' E. The average annual temperature of the area varies from 10 to 25°C while rainfall varies between 500 and 1000 mm.

Study Population and Farm Type: A total of 95 dairy herds selected randomly from the dairy farms present in the study areas were included in the study. The herd size of the selected farms varied from four to 112 cattle of which two to 57 were lactating cows. A total of 529 lactating cows were selected. Most of them (84.3%) were cross-breeds (Holstein-Friesian x Zebu) whilst few were local (Borana and Arsi breeds) (15.7%). With regard to management, 35 (36.8%) of the herds were managed intensively while 60 (63.2%) herds were semi-intensive. The intensively managed cattle were kept in-doors and received concentrate feeds in addition to hay and crop residues (such as corn stalks, wheat/barley straw and other leftovers from grain threshing). On the other hand, the semi-intensively managed cattle grazed freely on pasture but received supplementary feeds in the morning and evening when they were milked. All cows were hand milked twice daily, in the morning and evening. The milk yield of the cows ranged from four to 14 lit per day for cross breeds while two to four liters for local breeds.

Study Design and Sample Size: A cross-sectional study design was followed to address the objective of the study. The sample size was estimated following the method described by Thrusfield [15] for simple random sampling with 95% confidence level and 5% absolute precision, considering an optimum expected herd level prevalence of 50%. Accordingly, the sample size was computed to be 384 herds. This sample size would be acceptable if the size of the study population were large in relation to the sample. However, since the number of dairy farms in the region registered and known by the respective district veterinary departments was smaller (only 126) than the calculated sample size, an adjustment was made based on the formula recommended for a small population size [15].

$$n\ adj = \frac{N \times n}{N + n}$$

where n is the sample size based on an infinite population and N is the size of the study population. Thus, the required sample size, nadj, was calculated to be 95. Consequently, proportional allocation was made for each district in the milk shed and 40 herds each was considered for Hawassa and Arsi Negele and the remaining 15 allotted for Wondo Genet. In each district, respective herd sampling frame was constructed in collaboration with district veterinary department and herds were picked randomly using computer generated random numbers. Before data collection, a support letter written by the respective district veterinary department was sent to the identified farms requesting them to collaborate in the study. Fortunately, all of the farm owners who were randomly selected showed willingness to participate in the study. In each herd a minimum of 10% of the lactating cows were picked at random following the same procedure used for herd selection. Eventually a total of 529 lactating cows were included in the study.

Physical Examination of the Udder and Milk: According to Radostitis *et al.* [16], the lactating cows were clinically observed for the manifestation of general clinical signs related to udder and presence of any gross abnormalities like fibrosis, inflammatory swellings, pain, visible injury or lesion and atrophy of the tissue. The size and consistency of mammary quarters were inspected for the presence of any abnormalities, such as disproportional symmetry, pain upon palpation and blindness. Physical appearance of milk including color, odor, consistency, specific gravity, viscosity and appearance of milk secretion from each mammary quarter with the presence of clots, flakes, blood and watery secretions were also used for screening of presence of clinical mastitis.

California Mastitis Test: The California mastitis test (CMT) was used as a screening test for sub-clinical mastitis. It was carried out according to the procedure described by Quinn *et al.* [17]. A squirt of milk, about 2 ml from each quarter was placed in each of four shallow cups in the CMT paddle. An equal amount of the commercial CMT reagent was added to each cup. A gentle circular motion was applied to the mixtures in a horizontal plane for 15 s. The CMT results were scored as 0 (negative), trace, 1(weak positive), 2 (distinct positive) and 3 (strong positive) based on gel formation. All CMT scores of 0 and trace were considered as negative while CMT scores of 1, 2 and 3 were considered indicators of sub clinical mastitis. Positive cows were defined as having at least one quarter with CMT score of 1+.

Isolation and Identification of *S. Aureus*: For identification of the causative microorganisms, milk samples were collected from about 50% of CMT positive cows, randomly selected from all mastitis cases, assuming that the causative organisms within a herd are similar and also to reduce the time, labor and cost burdens. In case where only one mastitis case found in a farm, that positive cow was directly sampled. CMT negative cows (0 and trace scores) were not included in milk sampling. During sampling, milk was collected from all quarters of CMT positive cows at the same time as the CMT was done. Milk sampling was carried out following aseptic procedures as described by National Mastitis Council [18]. The time chosen for milk sample collection was before milking. Udder and especially teats were cleaned and dried before sample collection. Each teat end was scrubbed vigorously with a pledged of cotton moistened with 70% ethyl alcohol. Recontamination of teats during scrubbing was avoided by scrubbing carefully, the teats on the far side of the udder first, then those on the near side. A separate pledged of cotton was used for each teat. Teats towards sample collection were sampled first and then the far ones. The first few streams of milk were discarded and approximately 10 ml of milk was collected into horizontally held sterile universal sample collection bottle. After collection, the samples were labeled and placed in icebox and transported to the microbiology laboratory of the School of Veterinary Medicine, Hawassa University. The samples were immediately cultured or stored at 4°C for a maximum of 24 h until cultured on standard bacteriological media. To comply with specific objective of the study, bacteriological examination was focused only on identification and isolation *S. aureus,* which is the most common cause of contagious mastitis in dairy herds worldwide. The isolation of *S. aureus* was carried out according to the standard microbiological procedures described by National Mastitis Council [18]. One standard loop (0.01 ml) of milk sample was streaked on 7% sheep blood agar (Oxoid, Hamp shire, England) using the quadrant streaking method for each cow. In case of refrigerated milk samples, as bacteria might be concentrated in the cream layer and held with in clumps of fat globules, dispersion of fat and bacteria was accomplished by warming the samples at 25°C for 15 min before plating on blood agar. The inoculated plates were then incubated aerobically at 37°C for 24 to 48 h. When growth was not observed after incubation for 24 to 48 h, the milk sample was re-inoculated on an enriched tryptone soya broth (Oxoid, Hampshire, England) to amplify the bacterial growth. The plates were examined for growth, morphologic features such as colony size, shape, color and hemolytic characteristics. Presumptive colonies were selected and sub cultured on nutrient agar (Oxoid, Hampshire, England) and incubated aerobically at 37°C for 24-48 h to get a pure culture. After incubation, colonies were identified according to their Gram reaction (Gram-positive or Gram-negative), cellular morphology (coccus or rod) and arrangements of the bacteria and the catalase test. The Gram and catalase-positive cocci were characterized for mannitol fermentation on mannitol salt agar (Oxoid, Hampshire, England), which was followed by tube coagulase test. Samples were considered positive for S. aureus when at least one colony was identified as *S. aureus*.

Antibiotic Susceptibility Test: Antimicrobial susceptibility test was conducted on randomly selected *S. aureus* isolates (n = 44) from a total of 88 isolates. The isolates were tested for 11 antimicrobials using the Kirby-Bauer disk diffusion method [17, 19]. In brief, *S. aureus* isolates were grown overnight on blood agar

(Oxoid, Hampshire, England) at 37°C and the colonies were transferred to a tube containing 4-5 ml of in tryptone soya broth (Oxoid, Hampshire, England). The inoculated broth is incubated at 35-37°C until a slight turbidity appears; usually within 2-8 hours. The turbidity of broth was adjusted by comparison with McFarland turbidity standard. The suspension (100 µl) was spread plated on Mueller Hinton agar (HIMEDIA, India) by using swab. Then, the antibiotic disk was transferred aseptically on to the surface of the inoculated medium and incubated further at 35°C, for a period of 24 hours. *S. aureus* ATCC 25923 was used as control. The following antimicrobial disks (HIMEDIA, India) with their corresponding concentrations were used: Amoxicillin-Clavulanic acid (AC) (30 µg), Ampicillin (10µg), Cefotaxime (30 µg), Ceftriaxone (30 µg), Erythromycin (15 µg), Gentamicin (10 µg), Kanamycin (5 µg), Nitrofurantoin (100 µg), Penicillin (10 µg), Streptomycin (10 µg), Tetracycline (10 µg). Interpretation of the test results: sensitive (S), intermediate sensitive (I) and resistant (R) was based on CLSI criteria. Activity of 11 antibiotics to each bacterial species was analyzed.

RESULTS

Prevalence of Mastitis: Table 1 illustrates the prevalence of clinical and subclinical mastitis at herd and cow level. Based on CMT results and clinical examination, 74.7% of the herds and 62.6% of the cows were positive for mastitis. Sub-clinical mastitis was the predominant type of mastitis observed at herd and cow levels. Of the total cows with subclinical mastitis, 35.2, 49.5 and 15.3% were weak, distinct and strong CMT positives, respectively.

Isolation of *S. Aureus* in Tested Milk Samples: A total of 172 milk samples collected from 18 clinical and 154 sub-clinical mastitic cows in 71 herds were cultured on blood agar media. Accordingly, growth of different groups of bacteria was observed in 170 (98.8%) samples. However, since the objective was to isolate *S. aureus,* further bacteriological tests were conducted by taking only those colonies presumptive of this species and leaving others. Finally, *S. aureus* was detected in 51.2% (88/172) of the milk samples cultured. The proportion of isolation from milk of clinically and sub-clinically affected cows was 22.2 and 54.5%, respectively. At herd level, *S. aureus* was isolated from 73.2% of the herds affected with mastitis (Table 2).

***In vitro* Antimicrobial Susceptibility Test:** From the total of 88 isolates of *S. aureus* obtained from clinical and subclinical mastitis, antimicrobial susceptibility tests were performed on 44 isolates using 11 antibiotics. Accordingly, *S. aureus* was found to be highly susceptible to Ceftriaxone and Gentamicin (100%) followed by Erythromycin (88.6%), Streptomycin (88.6%), Cefotaxime (72.7%) and Nitrofurantoin (72.7%). However these isolates were highly resistant to Ampicillin (84.1%) and Penicillin (81.8%) followed by Tetracycline (36.4%) and Amoxicillin-Clavulanic acid (AC) (34.1%). This result showed that Ceftriaxone, Gentamicin, Erythromycin and Streptomycin were the most effective antibiotics on *S. aureus* (Table 3).

Eighteen different antimicrobials resistance patterns were observed. Most of the isolates of *S. aureus* (95.5%) were found to be multi-drug resistant involving two and more antimicrobials and few isolates of *S. aureus* (4.5%) were found to be resistance to one antimicrobial. The predominant multi-drug resistance patterns for *S. aureus* were Amp, Pen, Strep; Amp, Amoxy, Pen, Strep and Amp, Pen, Strep, Tetra in 6 (13.64%), 7 (15.91%) and 8 (18.18%) isolates, respectively (Table 3).

Table 1: Prevalence of clinical and subclinical mastitis at herd, cow and quarter levels based on CMT

	Clinical Mastitis		Sub-clinical Mastitis		
Observation	No. examined	+ve cases	No. examined	+ve cases	Over all prevalence
Herd level	95	18 (18.9%)	95	71 (74.7%)	71 (74.7%)
Cow level	529	18 (3.4%)	529	313 (59.2%)	331(62.6%)

Table 2: Isolation of *S. aureus* in mastitic milk samples from dairy herds at Hawassa milk shed

Clinical mastitis		Sub-clinical mastitis			
No. cultured	+ve cases	No. cultured	+ve cases	Total cultured	Total positive (%)
18	4(22.2%)	154	84(54.5%)	172	88 (51.2%)

Table 3: *In vitro* antimicrobial susceptibility test result

		Range of disk diffusion inhibition zone diameters (mm)		
Antimicrobial agent	Disc content	Resistant (%)	Intermediate (%)	Susceptible (%)
Ampicillin	(10µg)	84.1	_	15.9
Amoxicillin-Clavulanic acid	(30 µg)	34.1	_	65.9
Cefotaxime	(30 µg)	0	27.3	72.7
Ceftriaxone	(30 µg)	0	0	100
Erythromycin	(15 µg)	2.3	9.1	88.6
Gentamicin	(10 µg)	0	0	100
Kanamycin	(5 µg)	9.1	36.4	54.5
Nitrofurantoin	(100 µg)	0	27.3	72.7
Penicillin	(10 µg)	81.8	_	18.2
Streptomycin	(10 µg)	0	11.4	88.6
Tetracycline	(10 µg)	36.4	13.6	50
Mean		28.2	11.2	60.6

Table 3: Percentage and frequency of antimicrobials resistance pattern of *S. aureus* (n=44) for selected antimicrobials agents

No. of Antimicrobials	Name of Antimicrobials	Frequency	Percentage
1	Strep	2	4.55
2	Amp, Strep	2	4.55
	Amp, Amoxy	1	2.27
	Amp, Strep	1	2.27
	Amp, Pen	1	2.27
	Pen, Strep	1	2.27
3	Amp, Pen, Strep	6	13.64
	Pen, Strep, Tetra	4	9.09
	Amp, Amoxy, Pen	2	4.55
	Amp, Pen, Tetra	1	2.27
	Amp, Amoxy, Strep	1	2.27
4	Amp, Amoxy, Pen, Strep	7	15.91
	Amp, Pen, Strep, Tetra	8	18.18
	Amp, Kan, Pen, Strep	2	4.55
	Amp, Amoxy, Strep, Tetra	1	2.27
5	Amp, Amoxy, Kan, Pen, Strep	2	4.55
	Amp, Ery, Pen, Strep, Tetra	1	2.27
	Amp, Amoxy, pen, Strep, Tetra	1	2.27

Amp= Ampicillin; Amoxy=Amoxicillin-Clavulanic acid; Ery=Erythromycin; Kan=Kanamycin; Pen=Penicillin; Strep=Streptomycin; Tetra=Tetracycline

DISCUSSION

According to this finding of the study, *S. aureus* was isolated from 51.2% mastitic milk samples and 73.2% herds affected with mastitis. The cow-level finding is comparable to a previous report of 51.7% from northern part of Ethiopia [20] and also other mastitis studies in the country which recorded *S. aureus* as the predominant agent [21-24]. Apart from Ethiopia, *S aureus* has also been reported as the chief aetiological agent of mastitis in cattle by many studies from African and Asian countries [25]. *S. aureus* and other contagious microorganisms are usually found on the udder or teat surface of infected cows and are the primary source of infection between uninfected and infected udder quarters, usually during milking. Even though in all the observed herds milkers' used to wash their hands before milking, they do it only before milking the first cow.

The antimicrobial susceptibility tests carried out in this study indicated the existence of susceptibility and resistance of *S. aureus* to some of the antimicrobials. The average susceptibility (60.6%) of *S. aureus* to all antimicrobials tested in this study is comparable to the existing reports of 62.7% by Mekonnen *et al.* [26] and 58.3% by Abera *et al*. [21] in Ethiopia.

In this study *S. aureus* was found resistant to Ampicillin (84.1%) and Penicillin (81.8%). This is in agreement with the findings of Mekonnen *et al.* [26] and Sori *et al.* [27] in Ethiopia who reported 82.4% resistance to Ampicillin and 87.2% to Penicillin,

respectively. The high resistance of *S. aureus* to Penicillin and Ampicillin may be attributed to the production of beta-lactamase, an enzyme that inactivates penicillin and other closely related antibiotics. It is believed that around 50% of mastitis causing *S. aureus* strains produce beta-lactamase [28].

All of the 44 *S. aureus* isolates tested were found to be 100% susceptible to Ceftriaxone and Gentamicin. This study revealed that Ceftriaxone and Gentamicin are the most effective antimicrobials against *S. aureus* among all the tested antimicrobials. This is in quite agreement with Sori *et al*. [27] who reported 100% susceptibility of *S. aureus* to Gentamicin in bovine. Chandrasekaran *et al.* [29] in India have also reported that *S. aureus* was 71.2% susceptible to Gentamicin and 69.2% to Ceftriaxone. The second most effective drugs in the present study were Erythromycin (88.6%) and Streptomycin (88.6%). Similar findings have been reported by Abera *et al*. [21] in which Streptomycin (86.1%) was found to be effective antibiotic. Cefotaxime (72.7%) and Nitrofurantoin (72.7%) were also the third most effective drugs observed.

This study showed that 95.5% *S. aureus* isolates were multi-drug resistant that is alarming. This was comparable to Memon *et al.* [30] who reported that 100% of isolates were multi-drug resistant in China. In present study, few isolates showed similar antimicrobial resistance patterns, which could be due to identical strains or the dissemination of the same strains among animals. The resistance of *S. aureus* to antimicrobial agents has been extensively documented and it contributed significantly to the treatment failure [31-33]. The incidence of multi-drug resistant of *S. aureus* is higher which might be due to indiscriminate use of antibiotics and intra-mammary preparations containing combinations and broad spectrum antibiotics. In fact, *S. aureus* pathogens have many characteristics that make them difficult targets for antimicrobial therapy [34]. The previous studies regarding antibiotic resistance in *S. aureus* revealed that antibiotic resistance genes are often located on plasmids and transposons, which can easily pass from one staphylococcal species to another [35]. Multi-drug resistant *S. aureus* can spread from animal to human through consumption of raw milk and its products having significant role in public health.

CONCLUSION AND RECOMMENDATIONS

S. aureus is an important cause of mastitis which was isolated from more than half of mastitic cows tested. The distribution of *S. aureus* and its resistance to the commonly used antimicrobials in the selected dairy farms indicate the economic impact of the organism as a cause of bovine mastitis. Moreover, the isolation of *S. aureus* in observed proportion indicates the higher public health risk due to consumption of raw milk and its products. A large proportion of the isolates obtained were multi-drug resistant. This demonstrated the existence of alarming levels of resistance of *S. aureus* to commonly used antimicrobial agents. Hence the high level of MAR *S. aureus* and the implications thereof warrant further investigation. One of the aspects that need to be investigated is the cause of the observed resistance phenotypes. Furthermore, impacts and dynamics of genetic antibiotic determinants should also be investigated using molecular methods.

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