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Invitro susceptibility and cure rate of *Staphylococcus aureus* bovine mastitis under treatment with intramammary antimicrobials in the Mid Rift Valley of Ethiopia

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ABSTRACT

Antimicrobial therapy of mastitis is one of the costliest factors for dairy farms. Though various in-vitro susceptibility studies on isolates of *Staphylococcus aureus* from bovine mastitis showed a trend of increasing resistance to antimicrobials, there is still lack of information on the relationship between in-vitro susceptibility results and therapeutic bacteriological cure of the bacteria. This study was conducted with the objectives of characterizing invitro susceptibility of *S. aureus* and its association with bacteriological cure of bovine mastitis caused by *Staphylococcus aureus*. Out of the total 50 isolates, susceptibility range of 66.7- 71.9%, 61.1-65.6% and 59.4- 61.1% was found for amoxicillin, oxacillin and cefoxitin, respectively. Amongst quarters treated with intramammary infusion, higher bacteriological cure (p<0.05) was observed in subclinical mastitis (82.4%) than in clinical mastitis (29.4%). A positive linear relationship (p<0.05) was revealed between in-vitro susceptibility of antibiotics used for the treatment of mastitis (ampicillin and oxacillin) and bacteriological cure of *S. aureus*. Generally, in-vitro susceptibility test result showed a positive linear relationship with bacteriological cure of mastitis though lower cure rate was observed in clinical mastitis than subclinical.

Keywords: Bovine, Meki, *Staphylococcus aureus*, Susceptibility test, Treatment

INTRODUCTION

Bovine mastitis is the single most frequent cause for antibacterial use in dairy herds (USDA, 2008). Mastitis accounts for the largest economic losses on dairy farms in many countries in the world, including the USA, United Kingdom, Europe, Australia and South Africa (Petroviski *et al.*, 2006; Radostit, 2006; Roger and Peter, 2010).

One of the causative agents of mastitis is *Staphylococcus aureus*. *Staphylococcus aureus* causes subclinical, clinical, recurrent and chronic mastitis in dairy cattle and are the most frequently isolated pathogen in subclinical mastitis cases worldwide (Radostit, 2006). One of the factors for the bacteria to be able to cause chronically recurring infections is its ubiquitousness in dairy herds (Bergdoll and Lee Won, 2006).

Staphylococcus aureus is zoonotic and got notoriety due to its ability to evolve new virulent and drug-resistant strains (Chambers, 2001). Development of resistance and the emergence of epidemic strains of the bacterial

pathogens over decades highlighted the adaptability of the bacteria and the remarkable speed of *Staphylococcus aureus* evolution (Enright *et al.*, 2002). This in turn, contributes to the development of resistance to antimicrobial treatment (Olde *et al.*, 2008).

Selection of an appropriate antimicrobial for treatment of mastitis is often based on interpretation of *in-vitro* susceptibility tests (Constable and Morin, 2003), but it is debated that *in-vitro* tests have not been shown to be reliable predictors of treatment outcomes of *Staphylococcus aureus* (Cattell *et al.,* 2001; Apparao *et al.*, 2009). It was suggested that the relationship between clinical and *in-vivo* response depend on several factors such as duration of therapy (Oliver *et al.*, 2004), antimicrobial used (Taponen *et al.*, 2003), strain of the bacteria and duration of infection (Van den Borne *et al.,* 2010b), inherent characteristics of the pathogen, host factors and concentration of the drug (Constable and Morin, 2003) and somatic cell count (SCC) level (Taponen *et al.*, 2003) contribute to outcome of antibiotic treatment. Response to therapy is also related to genotype (Van den Borne *et al.*, 2010a) and regional source of *S. aureus* strains, possibly representing different genetic backgrounds (Bradley and Green, 2009).

Some epidemiological studies on the correlation between *in-vitro* antimicrobial susceptibility of the isolates and the actual bacteriological cure rate after antimicrobial treatment revealed an only moderate result (Sol *et al.*, 2000). Spontaneous bacteriological cure rate of *S. aureus* is reported to lie in the range of 0% to 33% while the expectation for spontaneous cure of other mastitis-causing bacteria revealed quite high response and is presented as *Streptococcus uberis* (89%); *Streptococcus dysgalactiae* (69%) and coagulase negative *Staphylococcus aureus* (CNS) (85%) (McDougall *et al.*, 2007). The bacteriological cure of *S. aureus* under treatment with antibiotics is expected to be 30-60% (Radostit, 2006).

Though the bacteria tend to gain resistance to almost all classes of antimicrobial agents against which it is subjected (Lowy, 2003), various antimicrobials of veterinary importance such as Pen-Strep (procaine penicillin BP 200mg and Dihydrostreptomycin BP 250mg), Procaine Penicillin, Benzathine Penicillin, intramammary infusion (combination of Ampicillin and Cloxacillin), Oxytetracycline (20%) and Oxytetracycline (10%) are commonly used for treatment of bovine mastitis in veterinary clinics in Ethiopia (Alemayehu, 2015).

The response to antimicrobial treatment of *S. aureus* cause infections are affected by untargeted treatment manner, sub-therapeutic doses, repeated use of the drug and inappropriate periods of time for treatment (Guidelines for the prudent use of antimicrobials in veterinary medicine, 2015).

Studies in Ethiopia showed variable resistance of *S. aureus* to different antimicrobials (Deresse *et al.*, 2012; Biniam, 2014; Firaol *et al.*, 2015; Amanu *et al.*, 2016). Thus, resistance frequencies of 68% (Deresse *et al.,* 2012), 93.3% (Fitsum, 2016) and 100% (Biniam, 2014) were reported against penicillin. In the study conducted by Fitsum (2016) and Biniam (2014), 40.0% and 69.2% of the pathogen were resistant to tetracycline, respectively. Girum (2016) revealed resistance of 94% amongst *S. aureus* isolates against tetracycline.

Furthermore, a previous study (Biniam, 2014) showed that the bacteria show a resistance of 35.9% to chloramphenicol, 56.4% to vancomycin, 61.5% to amoxicillin-clavulanic acid and 71.8% to oxacillin. Girum (2016) reported resistance of 96% to vancomycin.

Despite various reports on *in-vitro* resistance profiles of *S. aureus* to various antimicrobials, particularly beta-lactams, there is still lack of information on the relationship of in-vitro result with treatment outcome of mastitis. Therefore, evaluation of antimicrobial susceptibility pattern, characterization of *S. aureus* drug resistance and its association with the bacteriological cure is of paramount importance. Therefore, the present study was conducted with the objectives of determining antibiotic sensitivity of *S. aureus* isolated from mastitic cow milk; evaluating bacteriological cure of bovine mastitis treated with conventional intramammary infusion; and evaluating the relationship between *in-vitro* susceptibility test and therapeutic response of the *Staphylococcus aureus*.

MATERIALS AND METHODS

Study area

The study was conducted in Meki town, East Shoa Zone of Oromia regional state. The town is located on the main road from Addis Ababa to Hawassa at a distance of 134km, and elevation of 1664.88 meters above sea level (masl) with coordinates of 8°9'18.69"N and 38°49'32.79"E (www.distancesto.com). The annual rainfall of the area is about 64% from June to September. Its mean annual temperature is 20.3^0C while average annual precipitation is 774mm. The air relative humidity of the study area is 66% on average (JICA, 2002). The town is surrounded by irrigation based horticulture producing rural villages.

Study animals

The study animals are dairy cows. Meki town contains 146 smallholders (FAO and ILRI, 2016) dairy farms with a total of 4962 cattle. Amongst the total, there are 2455 crossbreed dairy cows, 795 crossbreed heifers and 727 crossbreed calves in the town. The dairy farms feeding are based on supplementation with concentrate and

roughage from field lands. Majority of veterinary service for the dairy farms in the town is based on the veterinary clinic while others rely on home-based service by veterinary professionals. In the study environment (town), dairy cows are intensified and confined in semi-open shaded houses. Majority of cows' bedding is soil and many used concrete. Drug therapy is based on physical examination and of course pathognomonic signs of diseases. The frequently used form of antimicrobials is an injection of oxytetracycline and pen-strep while drugs like intramammary infusion are expensive and not supplied by the government. The most frequently used course of drug administration is one day (Dugda district Livestock Development and Health office, 2017).

Study design

Cross-Sectional study design

In the study, a cross-sectional study design was used to screen animals for cases of mastitis across the selected farms and to perform antimicrobial susceptibility of *Staphylococcus aureus*.

Uncontrolled randomized clinical trial study design

An uncontrolled randomized clinical trial was used to observe the differences in bacteriological performance before and after treatment assuming that the observed difference was due to intervention. Quarters positive for *S. aureus* before treatment were used as self-control by evaluating the presence of the pathogen after treatment (Jeremy, 2000).

Sampling strategy and sample size

First, dairy farms were listed with the help of experts from Dugda district Animal Health and Development Office. Then farm selection was undertaken based on the presence of lactating cows, a number of lactating cows (≥1), herd size and lactation stage (≥2 to ≤5 months), the willingness of farmers to participate in the study and existence of mastitis (clinical mastitis and/or subclinical mastitis) in the herds. Herds with a sufficient number of clinical mastitis cases (if any) took participation in the study to avoid potential problems with sequential testing. Further selection of animals from farms was based on presence of lactating cows, total adult herd size, number of cows currently lactating (lactation between 2-5 months) (atleast one lactating cow), number and percentage of cows with infectious mastitis (the more number of mastitis cows present, the more chance of being selected), cows with no history of antibiotic treatment within 30 days

before the test day and cows with no history of recent vaccination (European Medicine Agency, 2017). To sample milk from the selected cows per farm, a priority criterion (farms with more positive quarters were preferred to those with a single quarter, farms with both clinical and subclinical mastitis were preferred to those with one type of mastitis) was used (Hela, 2012). A maximum of 20% of total cows involved in the study was sampled from each participating farms (Hela, 2012; European Medicine Agency, 2017).

Sample ssize for *Staphylococcus aureus* identification and clinical trial

Assuming expected prevalence of *S. aureus* to be 50% in each clinical and subclinical mastitis (because adjusted sample size from the recent previous prevalence of 46.5% is nearly the same to the adjusted sample size calculated from 50%), the sample size was calculated according to Thrusfield (2005) as follows:

Expected prevalence: Subclinical Mastitis= 50%

Clinical Mastitis= 50%

α= 0.05;

p= 0.5

Then, $n = \dfrac{z^2 pq}{L^2}$

Where q= 1-p.

It was found that sample size was 384 quarters for each mastitis type (clinical and subclinical), but this sample size is larger than the finite population of study units (n= 38 clinically infected quarters for clinical mastitis and n= 78 CMT positive quarters for subclinical mastitis). The sample size calculation was limited to finite population due to intensive and repeated measurement nature of the study.

Therefore, the sample size was adjusted according to OIE Terrestrial Manual (2013) as follows:

nadj= $\dfrac{n \, x \, N}{n + N}$ where nadj is adjusted sample size.

$\text{nadj} = \dfrac{1}{1/n + 1/N} = \dfrac{n \, x \, N}{n + N} = 64.8 \approx 65$, but 64 quarters

of sub-clinically infected were sampled and

$\text{nadj} = \dfrac{1}{1/n + 1/N} = \dfrac{n \, x \, N}{n + N} = 33.7 \approx 34$, but 38 quarters

of clinical mastitis were sampled.

Following identification of *Staphylococcus aureus* (18 clinical and 37 subclinical mastitis positive quarters), 17 quarters from each mastitis with 1 extra quarter for reserve, were assigned to treatment with intramammary infusion (Meltjet, Ashish life science).

Sampling methods and data collection

Sampling methods

Simple random sampling was used for pretreatment sampling from subclinical quarters. Purposive sampling was employed for pretreatment sampling of clinical quarters (due to a small finite population of positive quarters) and all post-treatment samplings.

Screening of animals for mastitis

Animal screening was based on observation of udder (swelling, redness, and soreness) and milk (clots, flakes, watery appearance, blood tinging) (Quinn et al., 2002). Additionally, palpation was used for further clinical examination of quarters. Further screening of animals was based on California mastitis test. After teat cleaning, disinfection, and drying, few streaks of foremilk were discarded. After that, 3ml of milk sample from each quarter was added to each cup of mastitis paddle to an equal volume (3ml) of CMT reagent. Then the paddle was tilted while rotating and observed for gel formation within 10-20 seconds of mixing. The results were recorded as negative, trace, weakly positive and positive (Quinn et al., 2002).

A further animal screening was employed based on the presence of *Staphylococcus aureus* in quarters from both clinical and subclinical mastitis. Only quarters positive for the bacteria in 2 out of 2 or 2 out of 3 consecutive samples were listed for further selection for treatment of the disease (IDF Bulletin 132, 1981; EU Guidelines on Veterinary Medicinal products- volume 7A, 1998). Additionally, quarters of which average SCC >300,000 cells/ml, quarters which didn't receive treatment within 30 days before examination day and the quarter from cows after 30 days of lactation were eligible for the study (IDF Bulletin 211, 1987).

Milk sampling and transportation

Milk sampling before the clinical trial

Milk samples from both clinically infected and apparently healthy (but CMT positive) quarters were taken after washing and disinfecting each quarter with 70% ethanol. All mastitis positive quarters (randomly selected to fit sample size in case of subclinical mastitis and all clinical mastitis), a quarter with CMT level ≥1, were sampled 2 to 3 times (with one day apart). Sampling started by first discarding few streaks of milk from the quarter followed by collecting 15ml of milk into a sterile universal bottle for laboratory analysis. Two to three frequency of sampling was used to increase the sensitivity of *Staphylococcus*

aureus identification by bacteriological culture (94% for two samples and 98% for three) (Thomas and Simon, 2008). After collection, the sample bottles were labeled and carried in the cold ice box to animal health laboratory of Adami Tulu Agricultural Research Center (ATARC) within 4 hours and preserved in a refrigerator at $+4^0C$ until processing within 24 hours of collection (Quinn et al., 2002).

Milk sampling during the clinical trial

Following all the recommended sampling procedure (Quinn et al., 2002), a volume of 15ml milk per treated infected quarter was sampled for cytobacteriological analysis. The sampling took place on day 7th, 14th and 21st of cessation of treatment according to standard sampling procedure (IDF Bulletin 211, 1987; EU Guidelines on Veterinary Medicinal Products- volume 7A, 1998; OIE, 2003). Three milk samples (on day 7th, 14th, and 21st) were used for cytobacteriological analysis in case of subclinical mastitis, but two samples (on 14th and 21st day post-treatment) were used for somatic cell count while all the three samples (on 7th, 14th, and 21st) were used for bacteriological identification for samples from clinical mastitis (IDF Bulletin 132, 1981; IDF Bulletin No. 211, 1987; EU Guidelines on Veterinary medicinal products- volume 7A, 1998; OIE, 2003).

Laboratory analysis of milk samples

Milk cytological analysis for subclinical and clinical mastitis

A volume of 10μl milk sample was put on $1cm^2$ of clean slide and air dried. Then the slide was put in xylene for 2-3 minutes for defatting followed by air drying and then fixed by 95% ethanol for 5 minutes. Then after, it was air-dried and stained by 10% Giemsa solution for 30 minutes. Next, the smear was washed with tap water and observed under oil immersion for leukocyte count. After observing ten fields on a square and counting the number of cells in each field, all number of cells counted per field was added and the total was divided by 10 to get the average number of cells in each field. The average was then multiplied by 5000 (as $1cm^2$ area has 5000 fields). To get the number of cells in 0.01ml of milk, the average number of cells was multiplied by 500,000. For values of SCC between 0 and 200,000 cells/ml, it was interpreted as normal while values more than 200,000 was considered positive. Milk sample containing cell numbers more than 500, 0000 per ml was considered for bacteriological analysis (National mastitis council, 1999). The cytological analysis took place in 2-3 milk samples before treatment and 3 samples after treatment, for each

quarter, in case of subclinical mastitis. The test was applied to only one milk sample per quarter pre-treatment and two samplings post-treatment (sample on 14th and 21st day of treatment cessation) for clinical mastitis (IDF Bulletin 211, 1987; EU Guidelines on Veterinary medicinal products- volume 7A, 1998).

Bacteriological examination of milk samples

Milk samples collected from cows before and after application of treatment were subjected to bacteriological examination. Bacteriological identification was performed by standard culture method followed by biochemical tests and tube coagulase test of the isolates according to Quinn et al. (2002).

Isolation of the *Staphylococcus aureus* started by streaking aliquots of 0.01ml on Baird Parker agar base (Himedia). The inoculum was incubated aerobically at 37°C for 24-48 hrs. This was followed by inoculation on staphylococcus medium No.110 (Oxoid). Typical colonies of *Staphylococcus aureus* were further spread over Mannitol salt agar. Thereafter, it was cultured on purple agar containing 1% maltose (Quinn et al., 2002).

Identification of *Staphylococcus aureus* was made based on colony morphology, Gram stain reaction, shape and arrangements of the bacteria, catalase test and oxidase test, Mannitol sugar fermentation, Coagulase test and 1% maltose fermentation (Quinn et al., 2002).

Antimicrobial susceptibility testing

It is recommended (Taponen et al., 2003) that sensitivity testing should precede treatment, certainly in the case of subclinical mastitis and the choice of inappropriate drugs should not be excused for treatment failure when sensitivity testing is available (Barkema et al., 2006).

A 24-hour colony of *Staphylococcus aureus* was suspended in saline. The inoculum was adjusted to a turbidity equivalent to 0.5 McFarland standards by placing the tubes in front of a white paper with black lines. Following adjustment, containers of disks were removed from the refrigerator and put on the working table for two hours to make it equilibrate with room temperature. After the prepared Mueller Hinton Agar (MHA) plate was placed in the incubator with lids ajar for 10 minutes to make any excess moisture is absorbed into the medium, vortexing the organism suspension and dipping the sterile cotton-tipped swab into the suspension was followed by removing excess liquid by pressing the swab against the side of the tube. Following this, the suspension was streaked on the surface of MHA plate starting at the top of the media. The entire plate was covered by streaking back and forth from edge to edge of the media. The disks were applied within 15 minutes and

6 disks were use per plate. The MHA plates were incubated within 15 minutes (CLSI, 2014).

Based on availability discs of Penicillin, Gentamycin, Kanamycin, Amoxicillin, Bacitracin, Oxacillin, Cefoxitin and Erythromycin were use for antibiotic susceptibility test (CLSI, 2014). After 24hrs of incubation, the inhibition diameter was measured by Caliper and interpreted using standard interpretation zone for disc diffusion (CLSI, 2014; EUCAST, 2017). The measured inhibition diameter for Oxacillin disc was used as screening for *mec*A-mediated Oxacillin resistance and further screening was conducted by using Cefoxitin disc. A diameter of Cefoxitin ≤ 21mm was indicated as *mecA* positive while a diameter of ≥ 22mm was interpreted as *mecA* negative. Cefoxitin is used as a surrogate for *mecA*-mediated oxacillin resistance. Isolates that test as *mecA* positive were recorded as oxacillin resistant (CLSI, 2014).

Trial aapplication

Clinical trials were conducted to demonstrate the therapeutic response of the recommended intramammary drug (Meltjet, Ashish life science) in each target quarters. The manufacturer, Ashish Life Science, recommended treating mastitis quarters with a tube of 5gm Meltjet (a combination of 75mg ampicillin sodium and 200mg Cloxacillin sodium) every 12 hours for 3 days. The recommended treatment regimen (a tube of Meltjet every 12 hours for 3 days) was applied to quarters from which *S. aureus* was identified. Quarters with both sensitive and resistant *in-vitro* antimicrobial sensitivity test results were subjected to the treatment to evaluate the clinical response rate and cure rate of the disease (European Guideline for the testing of Veterinary Medicinal products-volume VII, 1995).

Post-treatment observation and evaluation

Post-treatment follows up and clinical observation

After application of the treatment (Meltjet Intramammary infusion), Animals of which quarters were treated were supervised and clinical observation of quarters and milk, in clinical mastitis, took place every 5 days. The same supervision took place for subclinical mastitis to get any post-treatment complaint.

The choice of the clinical endpoint was critical and post-treatment follow-up was performed to evaluate the outcome or if effects of treatment would have ceased to allow for any relapse to occur (EU Guideline for Veterinary Medicinal Products Volume 7A, 1998). All required information was collected on the prepared data collection sheet.

Evaluation of cure rate

The cure was evaluated between 14 and 28 days of post-treatment. The bacteriological cure was evaluated for each treated infected quarter based on total elimination of the pathogens which were present at the time of treatment or existence of new infection/growth/ of another bacterium in one (last sample) or two last post-treatment samples. Recommended Somatic cell count techniques (IDF Bulletin, 1981) was employed and the actual somatic cell counts were used as a check of the numerical trend between the means of quarter somatic cell counts for "cured" and "not cured" cows.

A quarter was considered cytologically cured if the SCC level was found reduced to <300 000/ml. Mean SCC was calculated from the results of treatment, separately for bacteriologically cured and bacteriologically not cured quarters (EU Guideline on Veterinary Medicinal Products-volume 7A, 1998).

For clinical mastitis, bacteriological status is the key parameter in evaluating the success of treatment. Therefore, the cure was evaluated for each treated infected udder quarter based on the total elimination of the pathogens which was present at the time of treatment. Additionally, clinical cure was evaluated for each infected quarter based on the return to normal of the parameters concerning the animal's general condition, the quality of the milk and the consistency of the udder. A case was regarded as a clinical cure if the milk have a normal appearance and the condition of the udder and the animal's general condition was satisfactory (EU Guideline on Veterinary Medicinal Products- volume 7A, 1998).

Combined cure rates were presented based on individual quarter data (bacteriological cure + quarter milk SCC < 300,000 cells/ml) (IDF Bulletin, 1981). All possible recommended criteria were followed in the cure rate evaluation. Results of bacteriological analyses taken before treatment and after treatment as well as the level of somatic cell counts during post-treatment were reported (EU Guideline on Veterinary Medicinal Products-volume 7A, 1998).

Ethical cconsiderations

The study protocol was reviewed and approved by Adami Tulu Agricultural Research Center. Then the official letter was written by the center to Livestock health and market development office of Dugda district. The letter also contained information about the purpose of the study, the procedure, the risk, benefit and their right. All the information obtained from the study participants was kept confidential.

Data management and analysis

All possible data were collected according to guideline by the international dairy federation and European Medicine Agency (2017). The data were recorded on data collection sheet, coded and fed into Microsoft excel 2016, revised, coded and saved until importation into statistical analysis software. The data were imported and analyzed using SPSS software version 20.0. A descriptive statistical analysis was employed by cross tabulation for the cure rate of the disease; and Correlation for relationship analysis between *in-vitro* susceptibility result and bacteriological cure. Additionally, univariate logistic regression was employed for an association of risk factors with bacteriological cure rate. Chi-square test, Pearson correlation, and odds ratio were amongst values used for analysis output. A *P*-value of ≤ 0.05 was considered statistically significant.

RESULTS

Antimicrobial susceptibility profile of staphylococcus aureus

In this study, *in-vitro* test revealed the highest degree of *Staphylococcus aureus* resistance to penicillin in both clinical (88.9%) and sub-clinical (96.9%) mastitis. The isolates showed a resistance of 28.1- 50% to Gentamycin and 40.6% for Cefoxitin in clinical and sub-clinical mastitis. It was also revealed that the isolates susceptibility to Amoxicillin, Bacitracin, and Kanamycin was 66.7- 71.9%, 66.7% and 81.3%, respectively. Figure 1 and 2 below.

Therapeutic response of teats to intramammary treatment

Out of 17 clinically infected quarters, 8 (47.05%) show clinical cure. Amongst the 17 treated quarters, post-treatment identification revealed an absence of *Staphylococcus aureus* growth in 5(29.4%) of clinical and 14(82.4%) of subclinical mastitis. Moreover, the cytological, bacteriological and cytobacteriological cure rates of treated quarters were significantly higher (p<0.05) in subclinical mastitis as compared with clinical cases. Table 1 below.

Relationship between invitro susceptibility and *staphylococcus aureus* cure rate of quarters

This study revealed the relationship between bacteriological cure and *in-vitro* results of antibiotics used in the treatment. A strong positive linear relationship was revealed for Cefoxitin (x^2=0.824; p=0.000) followed by
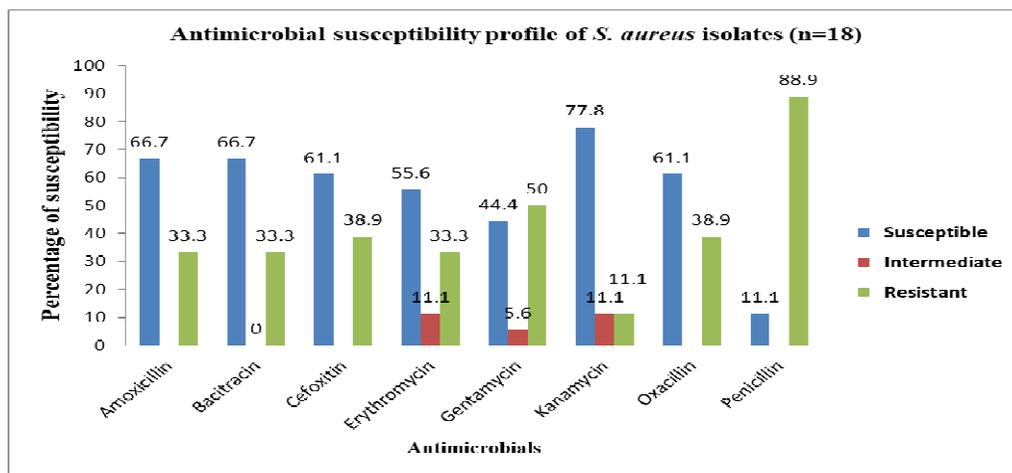
Figure 1. Antimicrobial susceptibility profile of *S. aureus* isolated from clinical mastitis
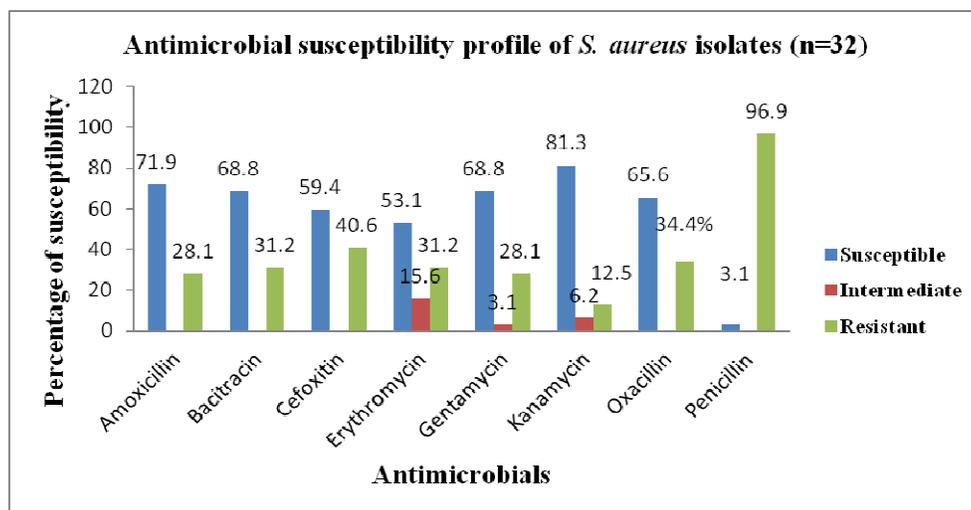

Figure 2. Antimicrobial susceptibility profile of *S. aureus* isolated from sub-clinical mastitis.

Table 1. The post-therapy cure rate of mastitis based on cytological and bacteriological testing

Cure rate criteria	Type of mastitis		Overall cure	Chi-squared Value	p-Value
	Clinical (n=17)	Subclinical (n=17)			
Number of clinical cure (%)	8 (47.05)		8 (47.05)	NA NA	
Number of cytological cure (%)	5(29.4)	12 (70.6)	17 (50.0)	4.339	0.033
Number of bacteriological cure (%)	5(29.4)	14(82.4)	19 (55.9)	6.300	0.010
Number of cytobacteriological cure (%)	3 (17.6)	12(70.6)	15 (44.1)	6.300	0.010

Note: n= sample size, NA= not applicable

Oxacillin (x^2=0.646; p=0.000) and Amoxicillin (x^2=0.471; p=0.005). There was poor and insignificant positive relationship of cure of the pathogen with susceptibility pattern of penicillin (x^2=0.107 vs p=0.547). This show that invitro susceptibility result of penicillin had poor sensitivity to predict for the presence of methicillin-resistant *S. aureus* compared to other antibiotics (Cloxacillin and Cefoxitin), which in turn aids in making decision for

Table 2. Association between in-vitro antimicrobial susceptibility test and bacteriological cure rate

Antimicrobial	Susceptibility Category	The frequency of susceptibility (%)	Cure number (%)	Pearson Correlation	P- Value
Penicillin	Susceptible	3(8.8)	0(0.0)	0.107	0.547
	Intermediate	0 (0.0%)	NA		
	Resistant	31(91.2)	16 (48.5)		
Amoxicillin	Susceptible	21 (61.8)	10 (47.6)	0.471	0.005
	Intermediate	0 (0.0%)	NA		
	Resistant	13 (38.2)	6 (46.2)		
Oxacillin	Susceptible	20 (58.8)	9(50.0)	0.646	0.000
	Intermediate	0 (0.0%)	NA		
	Resistant	14 (41.2)	7(43.8)		
Cefoxitin	Susceptible	20 (58.8)	8 (42.1)	0.824	0.000
	Intermediate	0 (0.0%)	NA		
	Resistant	14 (41.2)	8 (53.3)		

Note: Total number of isolates against which intramammary treatment was provided was 34.

treatment of mastitis. Table 2.

DISCUSSION

Antimicrobial susceptibility profile of *Staphylococcus aureus*

The current *in-vitro* susceptibility test revealed that 53.1-55.6% of *Staphylococcus aureus* isolates were susceptible to Erythromycin. This disagrees with the previous report (88.1%) of Befikadu *et al.* (2017) and the (81.1%) finding of Asmelash *et al.* (2017). The current result argues with the finding (79.5%) of Abo-shama (2014). The difference in the susceptibility to the antibiotic might be associated with environmental variation in the distribution of antimicrobial resistant bacteria. Regarding kanamycin, susceptibility of 77.8- 81.3% isolates is in line with the previous report (100%) by Befikadu *et al.* (2017).

In this study, the susceptibility of the bacterial isolates for penicillin revealed resistance of 88.9% - 96.9% which is agreeable to previous studies conducted in the country and other areas. Befikadu *et al.* (2017) and Asmelash *et al.* (2017) reported 100% resistance of *Staphylococcus aureus* isolates to the antibiotic while Anusha *et al.* (2017) reported susceptibility of 8.47%. In contrary, this finding disagrees with the 45.5% resistance reported by Abo-shama (2014) in Egypt. The increased resistance to penicillin might be due to beta-lactamase production (Roger and Peter, 2010) and frequent use of the sub-therapeutic dose of beta-lactam drugs used as evidence in this study.

Amongst the isolates, 66.7-71.9% was found susceptible to amoxicillin. The current result agrees with Abo-shama (2014) and Hulya *et al.* (2006) who reported resistance of 70.5% in subclinical mastitis and 54.4% for isolates from clinical mastitis, respectively. However, it argues with the findings of Asmelash *et al.* (2017), who

reported 100% resistance in Kombolcha district. The lower level of resistance to the antibiotic might be associated with variation in the degree of antimicrobial use and type of antimicrobials used in the treatment of animals.

In-vitro susceptibility of *S. aureus* isolates show susceptibility of 61.1-65.6% to oxacillin. This is in agreement with Abo-shama (2014) who reported susceptibility of 63.6% to the antibiotic. The susceptibility of 59.4-61.1% by *S. aureus* isolates to cefoxitin is in line with the previous report (52.8%) by Asmelash *et al.* (2017), but lower than 100% (Abo-shama, 2014). The reduced susceptibility of the isolates to the antibiotic in the current study might be associated with the presence of MRSA amongst the bacterial isolates.

Therapeutic response of teats to intramammary treatment

It was revealed that the clinical cure of clinical mastitis was 47.05% and this is comparable to Deluyker *et al.* (1999) who reported a clinical cure of 51.8% following treatment with AMPICLOX.

The current study revealed an overall bacteriological cure of 55.9% (both clinical and sub-clinical mastitis). This finding is in agreement with an expected cure rate of 40-50% (best cure being 65%) regardless of mastitis type (Martin and Andrew, 2004; Radostit, 2006).

In this study, more bacteriological cure (p<0.05) was observed in subclinical mastitis (82.4%) than clinical mastitis (29.4%). The higher cure rate in subclinical mastitis might be due to lower repeated exposure of sub-clinically infected quarters to antibiotic treatment, good ability of the combined drugs (ampicillin sodium and Cloxacillin sodium) to penetrate udder in sub-clinical mastitis and nature of udder pathology in clinical mastitis. Bacteriological cure rate of mastitis is also dependent on

presence of Microabscesses and inaccessibility of the drug to the pathogen (Du Preez, 1988), ineffective drug diffusion, inefficient killing of the bacteria due to L-form of bacteria and biofilm formation (Sandholm *et al.*, 1990; Taponen *et al.*, 2003); and intracellular survival of bacteria and increased antimicrobial resistance (Ziv, 1980; Mestorino, 1993; Radostit, 2006; Cristina *et al.*, 2011).

Bacteriological cure in clinical mastitis (29.4%) agrees with Se´rieys *et al.* (2005) who reported cure rate of 24% following treatment with combination of 200mg of Cloxacillin and 75mg of ampicillin (once a day for 3 days) and the finding (21.7%) of Deluyker *et al.* (1999) under treatment with AMPICLOX.

Drug treatment response depends on drug factors such as the spectrum of activity, route of administration, concentration of the drug that can be maintained at the site of infection, and duration of treatment (Constable and Morin, 2003; Bradley and Green, 2009). It may also be based on a reduction in antibiotic use (and therefore, in the selective pressure to acquire resistance) which in turn benefit the fitter susceptible bacteria, enabling them to outcompete resistant strains over time (Levin *et al.*, 1997).

Relationship between invitro susceptibility and *staphylococcus aureus* cure rate of quarters

In-vitro susceptibility result of antibiotics used in the treatment had a role in predicting bacteriological cure of mastitis. This is supported by previous reports by Owens and Watts (1988) who suggested that herd level knowledge of recent susceptibility patterns may be used to inform treatment. The finding is also supported by Weese *et al.* (2005); Call *et al.* (2008) who reported that antimicrobial susceptibility determined in vitro can be considered as a prerequisite for treatment.

On the contrary, the finding disagrees with other previous reports (Constable and Morin, 2003; Hoe and Ruegg, 2005; Wagner and Erskine, 2006; Apparao *et al.*, 2009) those suggested that susceptibility testing had limited value to inform therapeutic decision making for bovine mastitis. Call *et al.* (2008) also concluded that the efficacy in vitro does not guarantee efficacy *in-vivo* when treating bovine mastitis. The limited role of *in-vitro* susceptibility test result for deciding treatment efficacy might be associated with sensitivity of tests used to evaluate susceptibility of the antibiotics, antibiotics against which susceptibility was performed and use in the treatment of mastitis, prevalence level of resistant bacterial isolates against the drug and sensitivity of laboratory test employed to identify resistant bacteria of consideration. Additionally, animal, environmental and pathogen factors limit the predictive role of *in-vitro* susceptibility test on treatment efficacy.

CONCLUSION AND RECOMMENDATIONS

After *in-vitro* susceptibility test of *S. aureus* isolates, quarters were treated with intramammary infusion and post-treatment analysis of milk samples revealed a more bacteriological cure for subclinical mastitis than clinical mastitis. Correlation analysis revealed strong positive linear relationship (for oxacillin, cefoxitin and amoxicillin) between *in-vitro* susceptibility result and bacteriological cure, but the relatively lower cure was observed in clinical mastitis than subclinical (p≤0.05). Generally, in-vitro susceptibility test was found to be predictive for antibiotics use in the treatment of mastitis.

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

➢ It is recommended to use an intramammary infusion, a drug from a combination of ampicillin and Cloxacillin, for treatment of early mastitis regardless of other factors.

➢ It is recommended to create awareness on the rational use of beta-lactam antibiotics as misuse or overuse of the drugs increases the risk of developing drug resistance.

➢ Further research on evaluation of cure rate under treatment with a combination of antimicrobials from intramammary formulation and systemic administration is advisable.

National surveillance on antimicrobial consumption is needed to identify the status of antimicrobial consumption in the country, particularly dairy farms.

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