



# Factors Associated with Bacteriological Cure of *S. aureus* in Bovine Mastitis in Mid Rift Valley of Ethiopia

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## Abstract

A study was conducted with the objective of evaluating risk factors those play role in the treatment failure of bovine mastitis. During the study, animals were randomly selected from California mastitis test positive cases and assigned to treatment with intramammary infusion (Meltjet, Ashish life science). Both bacteriological and cytological analysis took place to observe change in the somatic cell count and bacteriological status, before and after treatment. The study revealed that bacteriological cure was influenced by poor bedding hygiene, rough floor surface, soil floor, previous treatment for mastitis, poor animal body hygiene, previous cure failure, multiple parity and late lactation stage. Biofilm formation and presence of MRSA were amongst the other contributory pathogen factors for bacteriological cure failure. Overall, bacteriological cure rate was influenced by bacteriological, environmental and pathogen factors in the study.

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**Keywords:** Risk factors; Cure rate; Staphylococcus aureus; Bovine mastitis; Meki.

## Introduction

Bovine mastitis is the single most frequent cause for antibacterial use in dairy herds (USDA, 2008). The disease 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 [1,2,3]. One of the causative agents of the disease is *Staphylococcus aureus*. *Staphylococcus aureus* causes subclinical, clinical, recurrent and chronic mastitis in dairy cattle and is the most frequently isolated pathogen in subclinical mastitis cases worldwide [2]. One of the factors for the bacteria to

be able to cause chronically recurring infections is its ubiquitousness in dairy herds [4].

*Staphylococcus aureus* is zoonotic and got notoriety due to its ability to evolve new virulent and drug resistant strains [5]. 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 the bacterial evolution [6]. This in turn contributes for the resistance to antimicrobial treatment [7].



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Antimicrobial use is the principal contributing factor to the emergence and dissemination of antimicrobial resistance among bacterial pathogens and commensals that have food animal reservoirs (Codex Committee on Food Hygiene, 2001). Development of antibacterial resistance has raised serious concerns worldwide from both human health and animal health safety perspectives, putting their use in food-producing animals under constant scrutiny over the years [8].

Increasing prevalence of Antimicrobial Resistance (AMR) and the associated negative health outcomes have led to intense examination of the factors promoting the emergence and dissemination of resistance among pathogens in humans and animals [9]. Antimicrobial Use (AMU) in human and veterinary medicine is the main driver for emergence of resistance in bacteria (Levy and Marshall, 2004). This exposure over longer duration changes the microbial ecology in each environment such that resistant strains become dominant in the bacterial population [10].

Selection of an appropriate antimicrobial for treatment of mastitis is often based on interpretation of *in-vitro* susceptibility tests [11], but it is debated that *in-vitro* tests have not been shown to be reliable predictors of treatment outcomes of the bacteria (Cattell *et al.*, 2001)[12]. It was suggested that relationship between clinical and *in-vivo* response depends on several factors such as duration of therapy [13], antimicrobial used [14], strain of the bacteria and duration of infection [15], inherent characteristics of the pathogen, host factors and concentration of the drug [11] and Somatic Cell Count (SCC) level [14] contribute to outcome of antibiotic treatment. Response to therapy is also related to genotype [15] and regional source of *S. aureus* strains, possibly representing different genetic backgrounds [16].

Some epidemiological studies on correlation between *in-vitro* antimicrobial susceptibility of the isolates and the actual bacteriological cure rate after antimicrobial treatment revealed only moderate result [17]. 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%) [18]. The bacteria tend to gain resistance to almost all classes of antimicrobial agents against which it is subjected [19].

With various modified versions of antimicrobials, treatment of disease caused by *S. aureus* has been made possible. 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 bovine mastitis in animal clinics in Ethiopia [20]. The response to antimicrobial treatment of *S. aureus* caused infections is affected by untargeted treatment manner, sub-therapeutic doses, repeated use of drug and inappropriate periods of time for treatment [21].

Studies in Ethiopia showed variable resistance of *S. aureus* to different antimicrobials [22,23,24,25]. Thus, resistance frequencies of 68% [22], 93.3% [26] and 100% [23] were reported against penicillin. In the study conducted by Fitsum [26] and Biniam [23], 40.0% and 69.2% of the pathogen were resistant to tetracycline, respectively. Girum [27] revealed resistance of 94% amongst *S. aureus* isolates against tetracycline.

Furthermore, previous study [23] showed that the bacteria showed resistance of 35.9% to chloramphenicol, 56.4% to vancomycin, 61.5% to amoxicillin-clavulanic acid and 71.8% to oxacillin. Girum [27] 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 relationship of invitro result with treatment outcome of mastitis. Therefore, evaluation of antimicrobial susceptibility pattern, characterization of *S. aureus* drug resistance and its association with bacteriological cure is of paramount importance. Therefore, the present study was conducted with the following objectives:

- To evaluate factors associated with therapeutic cure of commonly used antibiotics (intramammary infusion) in the treatment of mastitis.

## 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.distance.to.com) (Figure 1). The area gets about 64% of annual rainfall from June to September. Its mean annual temperature is 20.3°C while average annual precipitation is 774 mm. 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 .

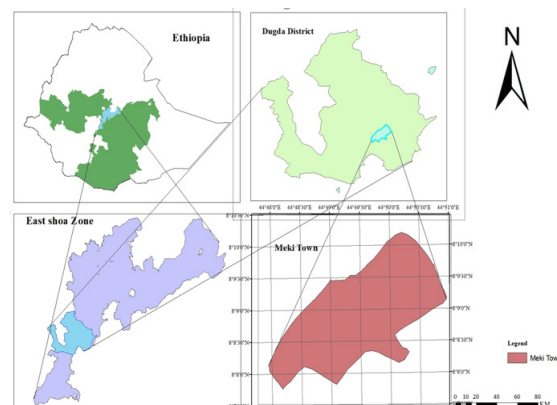


Figure 1: Map of study area (GIS- Ethiopia).

### Study animals

The study animals are dairy cows. Meki town contains 146 small holders (FAO and ILRI, 2016) dairy farms with a total of 4962 cattles. Amongst the total there are 2455 crossbreed dairy cows, 795 crossbreed heifers and 727 cross breed calves in the town. The dairy farms feeding is based on supplementation with concentrate, and roughage from field lands. Majority of veterinary service for the dairy farms in the town is based on veterinary clinic while others rely on home based service by veterinary. In the study environment (town) dairy cows are intensified and confined in semi open shaded houses. Majority of cows' bedding are soil, and many used concrete. Most of the farms have no well drained system. Drug therapy is based on physical examination and of course pathognomonic signs of diseases. The frequently used form of antimicrobials is injection of oxytetracycline and penstrep while drugs like intramammary infusion is 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 present study a cross-sectional study design was employed to screen animals for mastitis cases across the selected farms and to perform antimicrobial susceptibility of *Staphylococcus aureus*.

### Uncontrolled randomized clinical trial study design

Uncontrolled randomized clinical trial was employed for observed 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 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 farms were selected based on presence of lactating cows, number of lactating cows ( $\geq 1$ ), herd size and lactation stage ( $\geq 2$  months), willingness of farmers to participate in the study and existence of mastitis (clinical mastitis and/or subclinical mastitis) in the herds. Herds with sufficient number of clinical mastitis cases (if any) took participation in the study to avoid potential problems with sequential testing. 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), number and percentage of cows with infectious mastitis, cows with no history of antibiotic treatment within 30 days before the test day and cows with no history of recent vaccination. To sample milk from the selected cows per farm, a priority criterion (farms with more positive quarters were preferred to those with single quarter, farms with both clinical and subclinical mastitis than farms with one type of mastitis) was used [28]. Only  $\leq 20\%$  of cows per participating farms, of which quarters were involved in the treatment, was included in the study [28].

### Sample size 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 recent previous prevalence of 46.5% is nearly the same to the adjusted sample size calculated from 50%), sample size was calculated according to Thrusfield [29] as follows:

Expected prevalence: Subclinical Mastitis= 50%

Clinical Mastitis= 50%

$\alpha = 0.05$ ;

$p = 0.05$

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

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, sample size was adjusted according to OIE Terrestrial Manual [30] as follows:

$$\text{nadj} = \frac{n \times N}{n + N} \text{ where nadj is adjusted sample size.}$$

$\text{nadj} = \frac{1}{1/n + 1/N} = \frac{n \times N}{n + N} = 64.8 \approx 65$ , but 64 quarters of sub-clinically infected were sampled and

$\text{nadj} = \frac{1}{1/n + 1/N} = \frac{n \times N}{n + N} = 33.7 \approx 34$ , 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 small finite population of positive quarters).

#### 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) [31]. Additionally, palpation was used for further clinical examination of quarters. Sequential screening of animals was based on California mastitis test. After teat cleaning, disinfection and drying, few streaks of foremilk was discarded. Then after, 3 ml of milk sample from each quarter was added to each cup of mastitis paddle and an equal volume (3 ml) of CMT reagent was added to the cups (manufacturer). Then the paddle was tilted while rotating and observed for gel formation within 10-20 seconds of mixing. Results were recorded as negative, trace, weakly positive and positive [31].

Further animal screening was employed based on presence of *Staphylococcus aureus* in quarters from both clinical and subclinical mastitis. Only quarters positive for the bacteria in 2 or 2 out of 3 consecutive samples were listed for further selection for treatment of the disease [32].

#### Milk sampling and transportation

##### Milk sampling before 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), quarter with CMT level  $\geq 1$ , 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 sterile universal bottle for laboratory analysis. A 2 to 3 frequency of sampling was used to increase sensitivity of *Staphylococcus aureus* identification by bacteriological culture (94% for two samples and 98% for three) [33]. After collection, the sample bottles were la-

beled and carried in cold ice box to animal health laboratory of Adami Tulu Agricultural Research Center (ATARC) within 4 hours and preserved in refrigerator at +4°C until processing within 24 hours of collection [31].

### Milk sampling during clinical trial

Following recommended procedure [31], sampling took place on day 7<sup>th</sup>, 14<sup>th</sup> and 21<sup>st</sup> of cessation of treatment according to standard sampling procedure [34,35,36]. All the three milk samples were used for cytobacteriological analysis (sub-clinical mastitis) two samples (on 14<sup>th</sup> and 21<sup>st</sup> for somatic cell count) and all the three samples (on 7<sup>th</sup>, 14<sup>th</sup> and 21<sup>st</sup>) were used for bacteriological identification for samples from clinical mastitis [32,34,36].

### Bacteriological examination of milk samples

Milk samples collected from cows before treatment and after application of treatment were subjected to bacteriological examination. Different treatment recommendations for different groups or species of bacteria suggest that treatment decisions should be guided by culture results [37,38]. Bacteriological identification was performed by standard culture method followed by biochemical tests and tube coagulase test of the isolates according to Quinn *et al.* [31].

Isolation of *Staphylococcus aureus* started by streaking aliquots of 0.01 ml on Baird parker agar base (Himedia). Then 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. Then after, it was cultured on purple agar containing 1% maltose [31].

Identification of the bacteria 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 [31].

### Antimicrobial susceptibility testing

It is recommended that sensitivity testing should precede treatment [14] and accordingly standard antibiotic sensitivity test was performed [39] for Penicillin, Gentamycin, Kanamycin, Amoxicillin, Bacitracin, Oxacillin, Cefoxitin and Erythromycin.

### Recording of risk factors

Recording of risk factors those play role in the failure of bacteriological cure of the disease took place during the study period. The factors included bedding hygiene, floor surface, floor nature, previous treatment for mastitis, keeping milking order, milker's hygiene, previous cure failure, parity, lactation stage and number of infected quarters. Amongst the risk factors information concerning bedding hygiene, floor surface, floor nature, milker's hygiene and number of infected quarters were recorded based on visual observation of the researcher while previous treatment for mastitis, keeping milking order, previous cure failure, parity and lactation stage was taken from the farm owner. Additionally, pathogen risk factors such as biofilm formation and presence of MRSA were recorded from laboratory results during identification of the pathogen. Hygiene related risk factors were categorized according to Food Standards Agency (2013).

### Trial application

Clinical trials were conducted to demonstrate the therapeutic response of the recommended intramammary drug (Meltjet, Ashish life science) in each target quarters. Mastitis positive quarters were treated for 3 days as per recommendation of the manufacturer.

### Post-treatment observation and evaluation

#### Post-treatment follows up and clinical observation

After application of the treatment (Meltjet Intramammary infusion), treated animals were supervised and clinical observation took place every 5 days.

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 [35].

### Evaluation of cure rate

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.

For clinical mastitis, bacteriological status is the key parameter in evaluating success of treatment. Therefore, cure was evaluated for each treated infected udder quarter based on the total elimination of the pathogens which were 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 had a normal appearance and the condition of the udder and the animal's general condition was satisfactory [35].

### Ethical considerations

The study protocol was reviewed and approved by Adami Tulu Agricultural Research Center. Then 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 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 was imported and analyzed using SPSS software version 20.0. Descriptive statistical analysis was employed by cross tabulation for 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 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  $\leq 0.05$  was considered statistically significant.

## Results

### Quarter level prevalence of *Staphylococcus aureus*

The overall prevalence of coagulase positive *Staphylococcus aureus* among mastitic quarters was 53.9%. The study also revealed a prevalence of 57.8% in clinical and 47.4% in sub-clinical mastitis, which is not statistically different ( $p>0.05$ ) (Table 1).

**Table 1:** Prevalence of *S. aureus* and other bacteria in clinical and sub-clinical mastitic quarters.

Bacterial species	Types of mastitis		Total (n=102)	Chi-squared value	p-value
	Clinical (n= 38)	Sub-clinical (n= 64)			
<i>S. aureus</i>	18 (47.4)	37 (57.8)	55 (53.9)	1.047	0.31
CNS	3 (7.9)	11 (17.2)	14 (13.73)		
Other bacteria	17 (44.7)	16 (25.0)	33 (32.4)		

**Note:** n: sample size

**Table 2:** Prevalence of methicillin resistant *S. aureus* in clinical and subclinical mastitis.

Mastitis Type	Number of MRSA isolates (%)	Chi-squared value	p-Value
Clinical (n=18)	7 (38.9)	0.613	0.434
Subclinical (n=32)	9 (28.13)		
Total (n=50)	16 (32.0)		

**Note:** n: sample size

### Prevalence of MRSA isolates from mastitic milk

Cefoxitin based screening showed that, methicillin resistant *S. aureus* was found in 32% of *Staphylococcus aureus* isolates with no statistically significant difference ( $p>0.05$ ) in the prevalence of the bacteria between the type of mastitis (Table 2).

### Therapeutic response of teats to intramammary treatment

Out of 17 clinically infected quarters, 8 (47.05%) showed clinical cure. Amongst the 17 treated quarters, post treatment identification revealed absence of *Staphylococcus aureus* growth in 5 (29.4%) of clinical and 14(82.4%) of subclinical mastitis. Moreover, the cytological, bacteriological and cyto-bacteriological cure rates of treated quarters was significantly higher ( $p<0.05$ ) in subclinical mastitis as compared with clinical cases (Table 3).

**Table 3:** 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 cyto-bacteriological cure (%)	3 (17.6)	12(70.6)	15 (44.1)	6.300	0.010

**Note:** n: sample size, NA: not applicable

### Association of risk factors with bacteriological cure rate

The current study showed that, except Milker's hygiene, all environment related risk factors had strong association ( $p\leq 0.05$ ) with bacterial cure rate of infected quarters. Particularly, floor surface and previous treatment of herd for mastitis showed highly significant ( $p=0.000$ ) association. Thus, udder of animals from farms with no history of previous treatment for mastitis had 34.5 times the chance of being cured for *S. aureus* infection than those with history of antimicrobial therapy (OR=34.5; CI=5.0-250;  $p=0.000$ ). Moreover, quarters from rough floor surface had 76.9 times the chance of bacteriological cure failure than those from smooth surface (OR=76.9; CI=6.9-1000;  $p=0.000$ ) (Table 4).

Based on Uni-variable logistic regression analysis of host related risk factors, quarters with previous treatment for mastitis had 35 odds of failure for bacteriological cure as compared with those not treated before (OR=35; CI=5.1- 241.6;  $p=0.000$ ). Moreover, the chance of failure to cure for antimicrobial therapy in quarters harboring biofilm forming *S. aureus* and MRSA was 11.3 and 8.7 times, respectively than those quarters not having biofilm forming organism and MRSA (OR=11.3; CI=2.3-57.2;  $p=0.003$  and OR=8.7; CI= 1.8- 42.6;  $p= 0.008$ ) (Table 5).

**Table 4:** Uni-variable logistic regression analysis of environmental factors in relation to bacteriological cure of *S. aureus* infected quarters

Variables	Categories	Total number examined	Total number cured (%)	Crude odds ratio (CI)	p-value
Bedding hygiene	Dirty	18	4 (22.2)	1	0.001
	Clean	16	14 (87.5)	24.4 [3.9,166.7]	
Floor surface	Rough	18	3 (16.7)	1	0.000
	Smooth	16	15 (93.8)	76.9 [6.9-1000]	
Floor nature	Soil	19	5 (26.3)	1	0.002
	Concrete	15	13 (86.7)	18.2 [2.9-111.1]	
Previous treatment for mastitis	Yes	15	2 (13.3)	1	0.000
	No	19	16 (84.2)	34.5 [5.0-250]	
Keeping milking order	No	24	10 (41.7)	1	0.05
	Yes	10	8 (80)	5.6 [0.9-32.3]	
Milker's hygiene	Poor	6	2 (33.33)	1	0.300
	Good	28	16 (57.14)	2.7 [0.4-16.9]	

**Table 5:** Uni-variable logistic regression analysis of host and pathogen factors in relation to bacteriological cure of *S. aureus* infected quarters

Variables	Categories	Total number examined	Total number cured (%)	Crude odds ratio (CI)	p-value
Animal body hygiene	Dirty	24	9 (37.5)	1	0.017
	Clean	10	9 (90)	15.0 [1.6,142.8]	
Previous treatment for mastitis	Yes	17	3 (17.6)	1	0.000
	No	17	15 (88.2)	35.0 [5.1, 241.6]	
Previous cure failure	Yes	13	1 (7.7)	1	0.001
	No	21	17 (80.9)	51.0 [5.0, 515.1]	
Parity	Multiparous	21	6 (28.6)	1	0.003
	Primiparous	13	12 (92.3)	30.0 [3.2, 284.3]	
Lactation stage	Late	11	1 (9.1)	1	0.028
	Mid	10	6 (60)	15 [1.3, 167.6]	
	Early	13	11 (84.6)	55 [4.3,703.4]	
Number of infected quarters	Single	8	6 (75)	1	0.013
	≥3	10	1 (10)	27.0 [1.9, 333.3]	
	Two	16	11 (68.8)	1.7 [0.2,9.3]	
Biofilm formation	Yes	18	5 (27.8)	1	0.003
	No	16	13 (81.3)	11.3 [2.3,57.2]	
Presence of MRSA	Yes	19	6 (31.6)	1	0.008
	No	15	12 (80)	8.7 [1.8, 42.6]	

## Discussion

### 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.* [40] who reported a clinical cure of 51.8% following treatment with AM-PICLOX.

The current study revealed an overall bacteriological cure of 55.9% (both clinical and sub-clinical mastitis). This finding is in agreement with expected cure rate of 40-50% (best cure being 65%) regardless of mastitis type [41,2].

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 [42]; ineffective drug diffusion, inefficient killing of the bacteria due to L-form of bacteria and biofilm formation [43,14], and intracellular survival of bacteria and increased antimicrobial resistance [44,2,45]. Bacteriological cure in clinical mastitis (29.4%) agrees with the finding (21.7%) of Deluyker *et al.* [40] under treatment with AMPICLOX.

Drug treatment response depends on drug factors such as spectrum of activity, route of administration, concentration of the drug that can be maintained at the site of infection, and duration of treatment [11,16]. It may also be based on 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 [46].

#### Association of risk factors with bacteriological cure rate

The present study showed a statistically significant ( $p \leq 0.05$ ) association of bacteriological cure failure with quarters managed under dirty bedding, rough floor and soil floor than those managed under clean bedding, smooth surface and concrete floor. Additionally, udders found in farms with previous history of mastitis therapy, farms not keeping milking order and poor milker's hygiene had high chance of failure to be cured from *S. aureus* than the other categories. The study also revealed that, in quarters: with previous treatment for mastitis; with previous cure failure; from multiparous animals; from late lactation stage; from udder with multi-quarter infection; harboring biofilm forming *S. aureus* and MRSA the chance of failure for bacteriologic cure rate was significantly higher ( $p \leq 0.05$ ) than the other categories.

These findings are in agreement with other studies. Thus, Piepers *et al.* [47] reported that, increased rate of infection under unhygienic condition decreases cure rate of the disease. The lower cure rate with poor bedding and animal body hygiene might be due to high exposure to the pathogen in the presence of the risk.

According to Roger and Peter [3], previous unsuccessful treatment for mastitis can reduce cure rate as poor as 6%. This is further supported by various authors [48,49,11,49,16] who suggested low cure rate in the presence of previous failure of cure. Moreover, previous treatment for mastitis was one of the factors affecting bacteriological cure of the disease and this might be due to more cure in cows experienced the disease for the first time in the lactation [50]. The reduced cure rate in quarters with previous history of mastitis is related to increased potency of teats and subsequent increase in degree and frequency of exposure.

The decrease in bacteriological cure had significant association with increasing parity which is supported by Michael [51] and Pinzon-sanchez and Reugg [50]. Michael [51] also suggested that multiple quarter's infection is associated with a decreased probability of cure. In line with previous reports [17,11,52,16], bacteriological cure rate was declined with increased lactation stage and location of quarter.

There was lower bacteriological cure rate in udder harboring biofilm forming *S. aureus*. According to Amorena *et al.* [53], biofilm compromises the ability to deliver antibiotics to the biofilm-embedded bacteria and cells of *S. aureus* at the inner biofilm layers tend to remain intact after antibiotic treatment. Efficacy of antimicrobial treatment also depends on inherent characteristics of the pathogen such as virulence and antibiotic susceptibility [54,51].

#### Conclusion

This study was conducted with the objectives of evaluating risk factors associated with therapeutic cure of commonly used intramammary infusion antibiotics in the treatment of sub-clinical mastitis. The study started with dairy farm selection followed by selection of lactating cows after which screening took place for presence of subclinical mastitis. California mastitis test for subclinical mastitis was employed to identify animals and quarters with mastitis. Animal inclusion/exclusion was based on recommended criteria and those animals fulfilling criteria were selected by simple randomization. Then after, each quarter was sampled 3 times before treatment for cytobacteriological analysis. A milk sample of 15ml was aseptically sampled at every sampling time, transported according to recommendation and somatic cell count was performed under light compound microscope while bacteriological culture employed for isolation and identification of the bacteria. After identification, 18 *S. aureus* isolates from clinical and 32 *S. aureus* from subclinical mastitis were subjected to invitro susceptibility test on Mueller Hinton agar and diameter of inhibition. Additionally, cefoxitin disc was used for identification of methicillin resistant *S. aureus* (MRSA) from *S. aureus* isolates. A total of 10 animals (17 quarters) were assigned to treatment with meltjet (Ashish lifescience) intramammary infusion every 12 hours for 3 days. All quarters were sampled 3 times post-treatment (7<sup>th</sup> day, 14<sup>th</sup> day and 21<sup>st</sup> day) and milk samples were analyzed for somatic cell count. Bacteriological identification of the pathogen was also employed to evaluate bacteriological cure. In addition to this, animals were regularly supervised every 3 days for clinical observation and any additional complaint by the farmer. During the study risk factors assumed to be predictors of cure failure were carefully observed, recorded and saved until finalization of laboratory results. The risk factors included bedding hygiene, floor surface, floor nature, previous treatment for mastitis, milker's hygiene, keeping milking order, animal body hygiene, previous cure failure, lactation stage, parity, number of infected quarters, biofilm formation and presence of MRSA.

#### Conflict of interest

The authors declared that they have no potential conflict of interest with respect to the authorship and/or publication of this article.

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#### Authors' contributions

AEN made the conceptualization, designed the methodology and critically reviewed the final draft. AAF was involved in designing the work, document revision and supervision. EGL revised the document. AEN collected and processed samples, analyzed the data with AAF and wrote the first draft. All authors approved the final version for submission.

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