Antibiotic susceptibility of *Staphylococcus aureus* from bovine milk samples in Gondar and Bahir Dar region, Ethiopia

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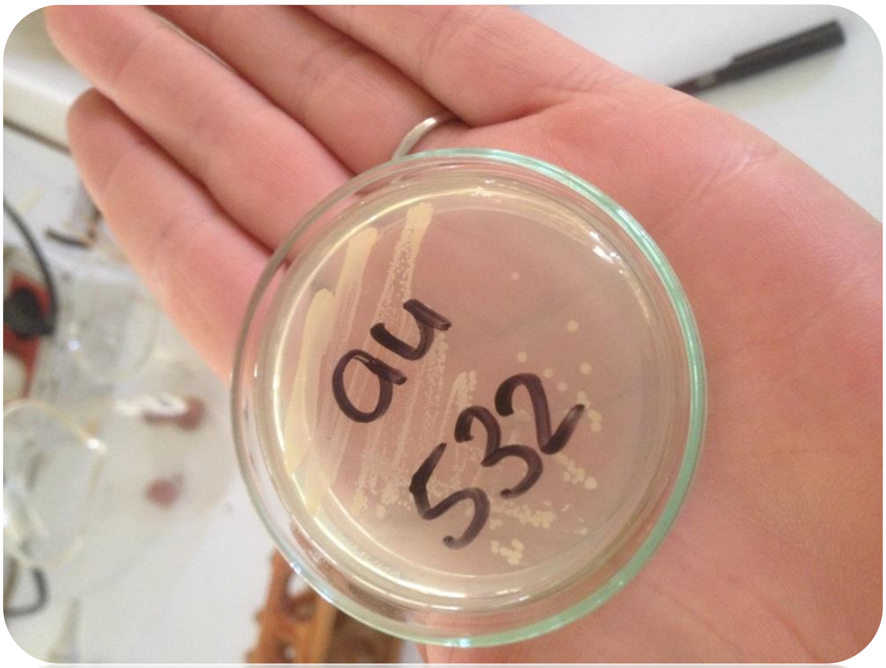

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Content

1. Abstract

In Ethiopia, *Staphylococcus aureus* (*S. aureus*) is an important causative agent of mastitis in dairy cows and therefore can cause significant economic losses to a community. Antibiotic resistance of *S. aureus* is a global problem and a threat to both veterinary and public health. The aim of this study was to estimate the prevalence of antibiotic resistance of *S. aureus* and to identify herd and individual cow risk factors associated with antibiotic resistance. A total of 141 isolates of *S. aureus* were collected from cows around Gondar and Bahir Dar with CMT positive milk, between 2014 and 2016. Samples were tested for antimicrobial sensitivity using disk diffusion with 6 types of antibiotics. The vast majority of strains showed resistance towards penicillin (94%), followed by resistance towards tetracycline (48%) and clindamycin (36%). Penicillin and tetracycline are the most used antibiotics in treatment of mastitis, which is in accordance with these results. Cefoxitin disks were used as an indicator of Methicillin-resistant *Staphylococcus aureus (*MRSA) and were found in 6.4% of the isolates.
Farmer experience in years was positively associated with clindamycin resistance, but no other risk factors were identified. A conclusive explanation of this effect is not yet found.
In conclusion: a high prevalence of antibiotic resistance was seen, including MRSA in milk from Ethiopian cows, which is problematic from an animal health as well as public health perspective. Improvement in knowledge of veterinarians and farmers as well as development of national protocols regulating the use of antibiotics is important to decrease the antibiotic resistance in Ethiopia.

2. Introduction

Mastitis in Ethiopia

Ethiopia relies heavily on livestock for economic stability. Currently, there are an estimated 40 million cattle, 20 million sheep, 16 million goats and over 32 million poultry around the country. The agricultural sector contributes 40 – 50 percent of total GDP, depending upon seasons. Poverty is an enormous problem in Ethiopia. According to the Ministry of Agriculture and Rural Development in 2007, 6 to 13 million people are at risk of starvation every year. Improvements in the livestock-production would tremendously help individual farmers and the country as a whole. However, agricultural growth is declining due to variability in rainfall, prevalence of pests and diseases and impeded trade due to ethnic conflicts (MoARD, 2007).

Mastitis is a specific factor that can affect Ethiopia's agricultural outcomes. Mastitis can reduce production, result in clinical mastitis, culling or death of a cow and costs the farmer money for veterinary services, materials or drugs, therefore leading to economic loss (Hogeveen et al., 2011). Subclinical mastitis reduces milk yield of Ethiopian cows by 17.2% on average, resulting in a loss of around 38US$ per cow per lactation (Mungube et al., 2005). In another study focusing on the economic impact of *S. aureus* caused subclinical mastitis in Ethiopian crossbred cows, the losses are even bigger. An udder quarter estimates to lose 34.5% of its potential milk production and the financial loss per cow per lactation varies between 78 and 150 US$ depending on farm size (Tesfaye et al., 2010)

Prevalence studies on mastitis in dairy cows have been carried out in many regions in Ethiopia. Prevalence of clinical mastitis in herds ranges from 0.9% to 14.9%; subclinical mastitis prevalence ranges from 11.4% to 51% of the sampled cows (Kerro Dego and Tareke, 2003; Lakew et al., 2009; Abebe, 2010; Haftu et al., 2012; Zeryehun et al., 2013; Belayneh et al., 2014; Tolosa et al., 2015). Some studies used a CMT score of 2 and above positive while others used CMT 1 and above as positive, which may explain the range, however, the prevalence of mastitis remains a common problem in Ethiopia.

Prevalence of *S. aureus* and it's susceptibility towards antibiotics

S. aureus is a common causative pathogen of mastitis in Ethiopia. The prevalence of *S. aureus* as the cause of clinical or subclinical mastitis lies between 16.5 (Moges et al., 2011) and 41.5% (Getahun, 2008). Other publications found percentages in between, varying from 21.1 to 41.4% (Kerro Dego and Tareke, 2003; Mekonnen, 2005; Lakew et al., 2009; Haftu et al., 2012; Belayneh et al., 2013; Zeryehun et al., 2013; Belayneh et al., 2014).

Treatment for clinical and subclinical mastitis consists of antibiotic therapy and if necessary symptomatic treatment (Degen, 2015). The most commonly used antibiotics are oxytetracyclin, penicillin-procaine, combination of penicillin and streptomycin and an intramammary combination of cloxacillin and ampicillin (Moges et al., 2011). Sensitivity of the pathogen to the antibiotic treatment is critical for a treatment to be successful (Barkema, 2006). However, due to limitations in laboratory facilities in the field and the financial situation of farmers, the use of antibiograms before starting antimicrobial therapy is rare. It is necessary to chart the incidence of antibiotic resistance and MRSA and to monitor efficacy of locally used antibiotics. MRSA (methicillin resistant *S. aureus*) is a common form of *S. aureus* and a danger to both veterinary and public health (Khameneh et al., 2016).

As found in different publications of antibiotic resistance in Ethiopia, resistance for penicillin is very high. A variety of penicillin's were studied (Benzylpenicillin and ampicillin) and overall resistance varies between 46.6% (Getahun, 2008) to 97.6% (Mekuria, 2013). In research carried out in the Gondar region, resistance for ampicillin was 81.5% (Moges et al., 2011). Other antibiotics with high resistance were tetracycline (0.0%- 73.8%), clindamycin varying from 4 to 88.2%, erythromycin from 4.7 to 58.8% and TMPs from 21.4 to 52.9%. Vancomycin (used as indicator for MRSA) resistance lies between 2.4 and 16% (Mekonnen, 2005; Getahun, 2008; Moges et al., 2011; Sori et al., 2011; Mekuria, 2013).
Possible risk factors for antibiotic resistance have never been studied in Ethiopia.

Purpose of the research

Because there is a lot of resistance of *S. Aureus* in Ethiopia, close monitoring is necessary. In the Gondar region, this monitoring was last done 5 years ago and there is no recent data from the Bahir Dar region. In these regions, resistance testing towards cefoxitin, vancomycin or an equal MRSA detection has not been performed. Finally, risk factors for antibiotic resistance on the farm or cow level have never been evaluated. For that reason, this research will aim to identify the antibiotic susceptibility of *S. aureus* in the regions of Gondar and Bahir Dar, with special attention to existence of MRSA and to identify farm and cow level risk factors for antibiotic resistance.

3. Materials and methods

In this cross-sectional study in the Gondar and Bahir Dar regions in Northern Ethiopia, 141 *S. aureus* isolates were collected and analysed. These samples were collected between October 2014 and February 2016 from small scale farms with 3-20 milking cows (Holstein Friesian, indigenous breeds or crossbreds). Before taking CMT, the udder was cleaned with cotton, about 2 ml of milk from each quarter (except from the dry quarters) was milked into the CMT paddle and CMT fluid of the same amount was added. Subclinical mastitis was defined as CMT score >1, with no clinical signs of mastitis. Clinical mastitis was defined as a warm and swollen udder with pain and/or as visible changes in the milk.

In the sampling of 2014-2015, both CMT positive and negative cows are sampled for bacteriological culture. In the sampling of 2016, only CMT positive (1-3+) quarters were selected for bacteriological culture. Selected quarters were cleaned with cotton and alcohol and milked into a sterile tube. The tubes were stored in a cooler during transport.

A questionnaire, focussing on herd and cow level variables, was carried out during the collection of samples in the cross-sectional study of 2014-2015.

Culturing of isolates

Milk was inoculated on blood agar (with freshly collected sheep blood) and stored in the incubator at 37°C for 24 hours. If no growth occurred, the sample was considered negative. If growth occurred, the colony size, shape, color and haemolysis was recorded after visual inspection. Gram staining was carried out for further identification. In case of a gram positive strain, colonies were cultured on mannitol and examined for growth and colour (Yellow was considered indicative of *S. aureus*). These colonies were then plated on a nutrient agar and used for coagulase and catalase testing. Coagulase slide was the standard method, however with unclear specimens, tube coagulase was performed. Gram positive strains that had yellow growth on mannitol and were positive on coagulase and catalase testing were considered to be *S. aureus.*

Samples from the cross-sectional sampling of 2014-2015 were then used to make isolates. One colony was plated on a nutrient agar and after growth a single colony was put into tryptone Soy Broth with 15% glycerol from Oxoid UK. The tube was vortexed and stored at -20°C. In January and February 2016, the stored isolates were thawed and cultured on nutrient agar by incubating for 24 hours at 37°C. All isolates showed good growth and a few plates had growth of two different cultures. Strains on plates with two cultures were checked on catalase and coagulase activity. If negative on one of the tests, the tested strain was excluded.

Antimicrobial susceptibility testing

All 141 *S. aureus* samples (collected in 2014-2015 and 2016) were tested for antibiotic sensitivity using the disk diffusion method, outlined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2016). A fresh colony from a nutrient agar plate was suspended in a sterile 0.85% NaCl solution to a 0.5 McFarland solution. This was done by visual comparison with a 0.5 McFarland turbidity standard. With a sterile cotton swab, the solution was inoculated semiconfluently on a Mueller Hinton plate (Oxoid, Basingstoke UK). Disks were then placed on the Mueller Hinton agar using a dispenser (Oxoid). Clindamycin and erythromycin disks were placed adjacently in the dispenser to test for macrolide inducible clindamycin resistance (D-test). Disks were placed about 15 mm apart, in concordance with the EUCAST guidelines to place disks 12-20 mm apart (EUCAST, 2016).

The following antibiotic disks (Oxoid, Basingstoke UK) were used:

- P: Penicillin G 1 unit (Cat. Nr. CT0152) in combination with Nitrofecin disk (Cat. Nr. R211667)
- FOX: Cefoxitin 30 ug (Cat. Nr. CT0119)
- DA: Clindamycin 2 ug (Cat. Nr. CT0064)
- E: Erythromycin 15 ug (Cat. Nr. CT0020)
- Te: Tetracyclin 30 ug (Cat. Nr. CT0054)
- SXT: Trimetoprim/sulphametoxazole 1:19 (co-trimoxazole) 1,25/23,75 ug (Cat. Nr. CT0052)

Sensitivity towards benzylpenicilin is used to determine the resistance to penicillin. If a strain is sensitive to a penicillin disk (> 26 mm around disk), beta lactamase activity was tested by doing a nitrocefin test. The nitrocefin disk with saline and cultured bacteria changes in colour: pink for beta-lactamase positive, yellow if not. This disk method combined with sensitivity on Mueller Hinton is a reliable indicator for beta-lactamase in bacteria (Pitkälä et al., 2007). If the zone around penicillin is smaller than 26 mm or the nitrocefin beta lactamase test is positive, we assume the strain is resistant to all penicillin's (Leclercq et al., 2013).
Sensitivity towards cefoxitin is used as an indicator for Methicillin resistance. If a strain is resistant to cefoxitin, it can be reported as resistant to all beta-lactam antibiotics with the exception of antibiotics specifically identified to treat MRSA (Leclercq et al., 2013). Clindamycin and erythromycin were used for individual resistance and put next to each other to determine the presence of an inducible clindamycin resistance. If a strain was resistant to erythromycin and sensitive to clindamycin, but showed growth near the clindamycin disc on the side next to erythromycin, the organism is positive for inducible resistance (D-test positive) (Yilmaz, 2007). 15 mm apart is ideal for detection of inducible clindamycin resistance (O'Sullivan et al., 2006). Tetracycline and TMP/S were used as it allowed for easy comparison with other data and because these antibiotics are widely used.

After 24 hours incubation at 35°C sensitivity was scored by measuring the diameter of the inhibition zone with a calliper. Measurements were performed by the same researcher to avoid interpersonal differences.
The breakpoints from EUCAST for *S. aureus* were taken as leading in deciding whether a strain was called resistant, intermediate or sensitive (EUCAST, 2016).

Antibiotic	Resistant	Intermediate	Sensitive
Penicillin*	< 26 mm		≥ 26 mm
Erythromycin	< 18 mm	18-20 mm	≥ 21 mm
Clindamycin	< 19 mm	19-21 mm	≥ 22 mm
Tetracyclin	< 19 mm	19-21 mm	≥ 22 mm
TMP/S	< 14 mm	14-16 mm	≥ 17 mm
Cefoxitin	< 22 mm		≥ 22 mm

Table 1: Breakpoints for disk diffusion antimicrobial sensitivity testing by EUCAST
***: Strains with growth of ≥ 26 mm were tested with cefinase, if positive the strain is called resistant**

In isolates with suspicious phenotype or double zones, catalase and coagulase testing was done again. Negative isolates on catalase or coagulase were excluded from the study. For the remaining 141 isolates, smallest zones of inhibition were used as recommended (Matuschek et al., 2014).
To check the reproducibility of the test, 13 isolates were cultured twice on Mueller Hinton agar and results from both cultures were compared using a Kappa test in SPSS 22 (IBM Corp, Armonk NY). Only one of the duo tested isolates were included in the resistance pattern results.

Statistical methods

Data of risk factor questionnaires and collected data on antimicrobial resistance were entered in data management software Excel 2010 (Microsoft Corporation, Redmond, WA). The fraction of resistant strains was calculated on this data as $p = \frac{Number\ of\ samples\ resistant}{Total\ number\ of\ samples}$. The Standard Error (SE) and confidence interval were calculated according to Petrie and Watson (Petrie and Watson, 2006).
The 13 samples which were duo-tested (76 individual disks as 2 disks were not attached correctly and therefore not readable) with a Cohens Kappa analysis was carried out with SPSS 22 (IBM Corp, Armonk NY), to measure the repeatability of the test results.

For assessing the association between risk factors measured in the cross sectional study in Gondar and Bahir Dar in 2014-2015 and antimicrobial resistance, chi squared and binary logistic regression were carried out by use of SPSS 22 Breakpoints from EUCAST were used in transforming the antimicrobial sensitivity data into a binary variable, with 0 as sensitive and 1 as resistant or intermediate.

Variables used from the questionnaire were: experience of farmer (in years), size of stable (m^2 per cow), presence of suckling calve, number of cows lactating, average daily milk yield of individual cow, percentage of Holstein blood, mastitis history (yes or no), CMT screening at time of sample and if farmer milks cows with mastitis separately (yes or no). Other variables from the questionnaire were not suitable as they had to little data or spread (all answer the same) or the variable had no logical association with antibiotic resistance.
Because some of the variables had too many different values, groups were made.

Variable	Group	Coding
Experience	≤ 5 years	1
	5 to 15 years	2
	>15 years	3
Stall size	≤3.5 m^2/cow	1
	3.5 to 5 m^2/cow	2
	>5 m^2/cow	3
Average daily milk yield	<8 L/ cow	1
	8 to 13 L/cow	2
Parity	≤3	1
	>3	2
Number of lactating cows	≤3	1
	>3	2

Table 2: Groups and their coding for 5 variables

The chi square test (Pearson's chi square test) was carried out to determine the univariable association between the antimicrobial sensitivity for a specific antibiotic and a variable. If P the value was <0.05, the null hypothesis was rejected and concluded that there is association between antibiotic resistance and the variable.

Secondly, a binary logistic regression was performed which takes more variables into account and weighs them together. Variables with a P value <0.2 in Chi square and no cells of a count <5 were used for binary logistic regression. Homoscedasticity was tested as existence of homoscedasticity is an assumption before the logistic regression is interpretable.

4. Results

Resistance patterns

In figure 1, the resistance patterns of all 141 samples together are given. As shown, almost all the strains (94%) are resistant to penicillin. Tetracycline and clindamycin resistance patterns are also high (48% and 36% respectively).

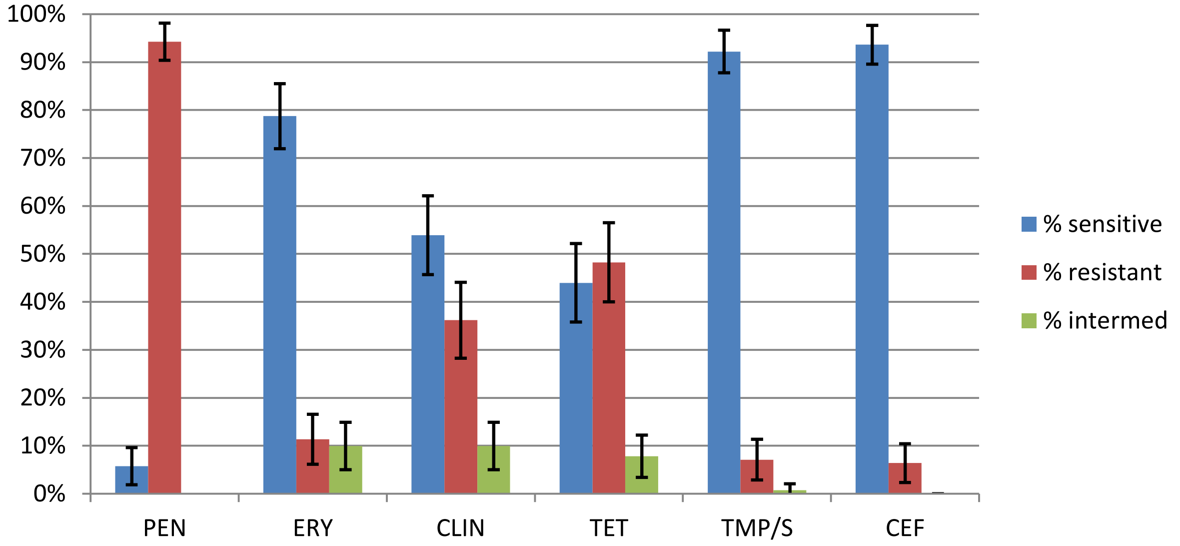

Figure 1: Resistance percentage of 141 S. aureus samples from Gondar and Bahir Dar with error bars representing the 95% confidence interval. Detailed confidence intervals can be found in Appendix A. PEN=penicillin, ERY=erythromycin, CLIN=clindamycin, TET=tetracycline, TMP/S=trimethoprim sulphonamide, CEF=cefoxitin

Susceptibility is similar between Gondar and Bahir Dar. The main difference is located in the clindamycin and tetracycline resistance pattern. Resistance of clindamycin is higher in Gondar (50% resistant in Gondar and 24% in Bahir Dar). Tetracycline resistance is higher in Bahir Dar (64% resistant in Bahir Dar and 28% resistant in Gondar). All data, including confidence intervals for each antibiotic per region can be found in Appendix B.

Double zones of inhibition

In 45% of the Mueller Hinton petridishes one or more antibiotics showed a double zone of inhibition. Out of the 948 zones around an antibiotic-disk (control isolated included), 13.5% of the disks had a double zone around. In 5.7% of all the disks, the double zone was of such a different measure that reading the inner or outer zone would make a difference of being resistant, intermediate or sensitive.

Test repeatability

In 13 isolates, double testing was carried out. For the 76 control disks, the Kappa Value was 0.80, which is considered 'good' according to Petrie and Watson, 2006. The significance is < 0.01. The first and second test results were highly associated. For this reason, we assume this test method is reliable.

Questionnaire associations

Chi square significance was tested for individual variables. The variables of penicillin, TMP/S and cefoxitin in cross-tabs of Chi square had one or more cells with an outcome less than 5, which is the minimum for reliable testing. Therefore, these variables will not be included in binary logistic regression.

		PEN			CLIND			ERY			TETR			TMP/S			CEFOX		
Variable	GROUP	N	%res	Chi	N	%res	Chi	N	%res	Chi	N	%res	Chi	N	%res	Chi	N	%res	Chi
Experience of farmer (years)	≤5	26	100%	0.1*	26	23%	0.004	26	15%	0.28	26	62%	0.28	26	15%	0.04*	26	12%	0.53*
	5-15	44	100%		44	41%		44	32%		44	68%		44	0%		44	5%	
	>15	31	94%		32	66%		32	22%		32	50%		32	9%		32	6%	
N cows lactating on farm	≤3	32	100%	0.84*	32	41%	0.80	32	16%	0.25	32	56%	0.68	32	13%	0.27*	32	3%	0.56*
	>3	69	97%		70	46%		70	29%		70	63%		70	4%		70	9%	
Stall size (m2)	≤3.5	46	96%	0.38*	47	53%	0.18	47	19%	0.25	47	55%	0.55	47	9%	0.32*	47	9%	0.92*
	3.5-5	18	100%		18	56%		18	39%		18	56%		18	6%		18	6%	
	>5	25	100%		25	32%		25	24%		25	68%		25	0%		25	8%	
Average daily milk yield (L)	<8	25	100%	0.62*	25	48%	0.77	25	28%	0.80	25	44%	0.09	25	4%	0.63*	25	0%	0.06*
	8-13	58	97%		58	40%		58	24%		58	69%		58	10%		58	5%	
	>13	24	96%		25	44%		25	20%		25	56%		25	8%		25	16%	
Parity of cow	≤3	65	98%	0.62*	66	45%	0.60	66	26%	0.39	66	52%	0.03	66	9%	1.0*	66	5%	0.42*
	>3	37	95%		37	38%		37	16%		37	73%		37	8%		37	11%	
Holstein Blood Level (%)	25	13	100%	0.76*	13	31%	0.11	13	15%	0.18	13	54%	0.40	13	8%	0.53*	13	0%	0.51*
	38	45	96%		45	31%		45	36%		45	67%		45	4%		45	4%	
	62	37	97%		38	58%		38	16%		38	53%		38	11%		38	11%	
	75	12	100%		12	50%		12	17%		12	67%		12	17%		12	8%	
Calve suckling	No	40	98%	0.76*	41	63%	0.003	41	20%	0.47	41	59%	0.87	41	10%	0.58*	41	12%	0.18*
	Yes	61	98%		61	31%		61	28%		61	62%		61	5%		61	3%	
Separate milking	No	41	98%	1.0*	41	39%	0.60	41	24%	1.00	41	63%	0.76	41	12%	0.20*	41	5%	0.75*
	Yes	58	98%		58	47%		58	26%		58	60%		58	3%		58	9%	
Mastitis history	No	54	96%	1.0*	55	38%	0.45	55	27%	0.58	55	60%	0.97	55	7%	0.95*	55	5%	0.96*
	Yes	53	98%		53	47%		53	21%		53	60%		53	9%		53	8%	
CMT Screening (CMT score)	0	42	95%	0.60*	42	48%	0.74	42	26%	0.51	42	67%	0.67	42	7%	0.90*	42	7%	0.46*
	1	25	96%		25	40%		25	24%		25	52%		25	12%		25	12%	
	2	27	100%		28	43%		28	29%		28	57%		28	7%		28	4%	
	3	13	100%		13	31%		13	8%		13	62%		13	8%		13	0%	

Table 3: P-value (2-sided) of Pearson Chi Square Test, with continuity correction for dichotomous variables (yes/no) and 2 groups. Chi square test with one or more cells with count < 5 are marked with *. PEN=penicillin, ERY=erythromycin, CLIN=clindamycin, TET=tetracycline, TMP/S=trimethoprim sulphonamide, CEF=cefoxitin. N=total number of isolates, %res= percentage resistant strains, Chi= value of chi square test.

As visible in table 3, a significant association is found between clindamycin and level of experience of farmer and presence of a suckling calve and between tetracycline and parity of the cow and tetracycline and daily milk yield.

With binary logistic regression, 65% of samples were included as several records had missing values. According to the binary logistic regression of clindamycin, a significant association was found with experience of farmer of group >15 years of experience. The odds value is 4.39 and confidence level 1.20-16.02. Other groups of variable Experience did not have a significant effect. There is compliance with the assumption of homoscedasticity and this variable was not closely connected to other influencing variables.

A suckling calve and level of Holstein blood proved to be confounders. Lower percentage of suckling calves were observed with higher Holstein blood level. Calve suckling was associated with lower percentage of resistance towards clindamycin and Holstein blood with higher percentage of resistance.

No significant variables were found in logistic regression of tetracycline and erythromycin. Binary logistic regression of penicillin, TMP/S and cefoxitin were not performed due to unilateral distribution of data.

5. Discussion

Resistance patterns

In this study, resistance patterns to 6 antibiotics were recorded. Table 4, outlines several studies of antibiotic resistance towards *S. aureus* in bovine milk samples.

Study	Country	Sample size	PEN (%)	ERY (%)	CLIN (%)	TET (%)	TMP/S (%)	CEF (%)	OX (%)	VAN (%)
Pitkälä, 2004	Finland	196	52.1	1.5	0.5		1.5		4.1	
Peles, 2007	Hungary	59	30.5	0.0		0.0	0.0	0.0		
Thomas, 2015*	Belgium	21	14.3	0.0					0.0	
	Czech	20	55.0	0.0					0.0	
	France	32	37.5	0.0					0.0	
	Germany	42	11.9	4.8					0.0	
	Ireland	38	65.8	0.0					0.0	
	Italy	38	50.0	0.0					0.0	
	Netherlands	21	14.3	0.0					0.0	
	UK	38	31.6	0.0					0.0	
Supré, 2014	Belgium	768		≤ 3.1		≤ 8.2	≤ 1.8	4.4		
Itorre, 2013	Italy	1193	64.5	42.9		25.1	3.4		13.1	
Jamali, 2014	Iran	43	86.0	39.5	34.9	76.7	11.6	16.3	11.6	
Türkyilmaz, 2010	Turkey	93						17.2		
Akkou, 2016	Algeria	67	86.5	4.5	3.0	14.9		0.0		
Silveira, 2014	Brazil	94	89.4	72.3		35.1	6.4		37.2	0.0
Casanova, 2016	Brazil	32				12.5				
Beuron, 2014	Brazil	210	28.6	4.8		9.0	16.7		11.9	
Gao, 2012*	China	53	96.3	66.0	3.8	98.1			0.0	
Zhang, 2016	China	58	91.4		10.3	19.0	1.7			0.0
Makovec, 2003	USA	2124	35.4	6.7		8.6	0.9			
McDougall, 2014*	New Zealand	364	28.0				0.5			
Petrovski, 2015	New Zealand	107	21.6	0.0		0.0			0.0	
	USA	75	36.0						0.0	
Mekonnen, 2005	Ethiopia	17	82.4	≤47.1		≤ 29.4				
Getahun, 2007	Ethiopia	85	46.6	≤ 4.7		≤ 23.3	≤ 38.7			
Sori, 2011	Ethiopia	86	87.2		4.0	0.0				3.0
Moges, 2011	Ethiopia	27	81.5	≤ 48.2	≤11.1	≤ 29.6				
Haftu, 2012	Ethiopia	46	82.4	58.8	88.2		52.9			
Belayneh, 2013	Ethiopia	59	65.0							16.0
Belayneh, 2014	Ethiopia		75.0							15.0
Range (min - max)			**11.9-96.3**	**0.0-72.3**	**0.5-88.2**	**0.0-98.1**	**0.0-52.9**	**0.0-17.2**	**0.0-37.2**	**0.0-16.0**
Median			**53.6**	**4.5**	**7.2**	**17.0**	**2.6**			
Current study	Ethiopia	141	94.2	11.3	36.3	48.2	7.1	6.4		

Table 4: Resistance patterns of *S. aureus* strains isolated from bovine milk samples worldwide. Studies marked with a * are carried out with MIC testing instead of disk diffusion. ≤ means the study provided only susceptibility data so presented numbers are percentages of resistant and intermediate strains. PEN=penicillin, ERY=erythromycin, CLIN=clindamycin, TET=tetracycline, TMP/S=trimethoprim sulphonamide, CEF=cefoxitin, OX=oxacillin, VAN=vancomycin.

Resistance patterns vary substantially between studies (different countries and years). When compared to median values of other studies, this study found higher resistance to penicillin, clindamycin, erythromycin, TMP/S and tetracycline. Resistance percentage towards penicillin and tetracycline is very high in Gondar and Bahir Dar region in Ethiopia, even when compared to other research from Ethiopia.

One reason could be that resistance is spreading over time or that there are different patterns of resistance in different regions in Ethiopia.
Cefoxitin, oxacillin or vancomycin are used as indicators of MRSA (CLSI, 2016). MRSA prevalence in this study was around the mean value of MRSA prevalence worldwide. Compared to western countries such as USA, New Zealand and Europe, where MRSA indicators in milk of mastitis cows are generally zero (Petrovski et al., 2015; Thomas et al., 2015), this resistance pattern is of an emerging size. Taking that into account, a cefoxitin resistance pattern of 6.4% is average in context of other countries, but still an emerging fact worldwide.

Double zones of inhibition

In 45% of the Mueller Hinton petridishes one or more antibiotics showed a double zone of inhibition. Double zones of inhibition can have multiple causes, one possibility is contamination. As nutrient agar petri-dishes were, with a few exceptions, clean and only growing one strain, contamination could have occurred through the use of the NaCl solution or tubes used for making McFarland, cotton used for inoculating on Mueller Hinton, or the Mueller Hinton petri-dishes or agar itself. Other contamination vectors such as sneezing or bacteria from hands from person handling the dishes are illogical as double zones were confluent.
Another way to explain double zones of inhibition is of hetero-resistance of *S. aureus* toward antibiotics such as erythromycin, clindamycin and cefoxitin (Hosbul et al 2013). In the current research, the highest prevalence of double zones were present with erythromycin (26%), followed by clindamycin and tetracycline (18%). Hetero-resistance is also known for *S.* aureus isolates to penicillinase stable penicillin's, such as cefoxitin. Cells can express a susceptible and resistant side and the resistant side could grow more slowly, however, as recommended, growth was performed for 24 hours at 35° so as to minimize heteroresistance (CLSI, 2016).
Antagonists in the Mueller Hinton medium may allow TMP/S to grow inside the zone of inhibition. For that reason, slight growth of 20% or less from the lawn of growth should be disregarded (CLSI, 2016). This slight double zone is not disregarded in this research and as the smallest zone of inhibition is taken, this might have resulted in unfair high resistance for TMP/S.
Subsequently, we can say that it would have been better if regular control of sterility of materials was done. Also quality controls with reference strains would improve the reliability of the outcome (Matuschek et al., 2014). However, Kappa value of the re-tested isolates shows that the outcome of this test (including the double zones) was reliable and therefore could be used.

MRSA

As stated in the discussion around resistance patterns, cefoxitin is an indicator for MRSA, as are cloxacillin, oxacillin and vancomycin. With these disk diffusion methods, the phenotypic behaviour of the strain is visible. Depending on this phenotypic behaviour, the strain is called a MRSA or a MSSA (methicillin sensitive staphylococcus aureus).
MRSA is an emerging problem in both human and veterinary health. MRSA can be divided into different clonal types (CC) and spa-types (ST). The most known livestock-associated MRSA is CC 398 (Vanderhaeghen et al., 2010). CC398 is mainly present in meat producing animals as pigs, broilers and calves but can also be found in less extent in milk from dairy cows (Tenhagen et al., 2014; Dierikx et al., 2016). CC398 and other livestock- associated MRSA's can spread to humans via consumption of animal products or close contact (Fessler et al., 2012).
In the current research, the origin of the cefoxitin resistant isolates is interesting. Cefoxitin and other cephamycin antibiotics such as Cefotetan and Cefmetazole are not used in veterinary medicine in Gondar, Ethiopia according to Moges, 2011 (Moges et al., 2011). The origin of the resistance is therefore questionable. Resistance, however, is not necessarily obtained via selection of bacteria under antibiotic treatment, but can also arise with transfer of resistance genes between bacteria. Strains of *S.* aureus are very diverse and use various mobile genetic elements (MGE) that encode key proteins for antibiotic resistance, adaptation to the host and virulence of the bacteria. These MGE's can transfer horizontally between bacteria (Lindsay, 2014). Also vancomycin or cefoxitin resistance genes could be transferred from enterococci to staphylococci (Weigel et al., 2003). It is also possible that transfer from

human MRSA to the udder of the dairy cow plays a role in this study. Humans can carry MRSA in their nose and hands: In a review article about MRSA prevalence in human hospitals, resistance appears to have risen since 2000 in many African countries (Falagas et al., 2013). In the study performed by Shibabaw in 2013 in Dessie, Ethiopia, 15 out of 118 nasal swabs taken from health care workers were positive for MRSA (12.7%) (Shibabaw et al., 2013). In a study among children and prisoners in Jimma, Ethiopia, a prevalence of 11% (39 MRSA out of 354 nasal swabs) was found (Kejela and Bacha, 2013). In both studies, DNA typing was not done so no information about clonal complex is available. Th high MRSA rate in milk of dairy cows may originate from a human hand colonisation but this is poorly researched in the Ethiopian setting. Still, human MRSA colonisation and transmission to their cat or dog has been shown (Van Duijkeren et al., 2004; Faires et al., 2009) as well as transmission between farmers and their livestock (Harrison et al., 2013). Multi-locus sequence typing of the isolates would likely help determine if these strains are livestock or human associated MRSA's.

Reducing percentage of MRSA's is a big responsibility for both veterinary and human health. Veterinarians and farmers have to evaluate antibiotic treatments routinely and reduce treatment with antibiotics. In case of a low chance of cure (high age, duration of infection, multiple affected quarters and history of mastitis) it could be better to cull or slaughter the animal (Degen, 2015). In every case, antibiotics should be prescribed with care and always given with information for the owner about duration of treatment, dosage and withdrawal period to lower the chance developing resistance.

Risk factors for resistance

A Pearson Chi square test revealed an association between clindamycin and level of experience of farmer and presence of a suckling calve and between tetracycline and parity of the cow. Also significance was found in association between TMP/S and experience of farmer but results are not taken into account as Chi square was unreliable due to extreme unilateral distribution and therefore too low numbers in cells.

The associated variables were only associated with one antibiotic which is hard to explain. The assumption was that for example high parity cows were more likely to have had treatment with antibiotic therapies during their lifetime and therefore be associated with resistance with several antibiotics. However, if this is the case, only resistance to one antibiotic was seen per variable.

In a comparable study from Brazil, use of dry cow treatment, not sending a milk sample for antibiotic testing before giving a treatment and routinely treating of clinical mastitis had a positive Odds ratio with antibiotic resistance. However, also in this research factors were only associated with one or two antibiotics (Beuron et al., 2014).

To ensure that associations between two factors were not the result of confounding, variables were tested together as covariates in a binary logistic regression. In this regression, the only significant association was found between clindamycin and experience of farmer of the highest group (>15 years of experience). Experience of farmer as a covariate, was not influenced by other covariates (change in beta upon exclusion of a variable from the model changed less than 15%).

The covariate 'level of experience (groups)' revealed that the higher the experience in years, the higher the mean prevalence of resistance towards clindamycin. A possible explanation could be that a more experienced farmer is quick to identify mastitis, does not want to wait or treat with non-antibiotics and therefore treats more often with clindamycin. Another reason could be that an experienced farmer does not uses the right dose or not during the minimum amount of days. Clindamycin however, is not a first choice of treatment in Ethiopia as is penicillin or tetracycline (Moges et al., 2011) for mastitis, so it is still doubtable why the effect is seen with clindamycin instead of penicillin or tetracycline. A explanation could be a type 1 error in this study, so further research and a more detailed questionnaire are necessary to determine the origin of this association.

6. Conclusion

The resistance of *S. aureus* to antibiotics is high in this study compared to other studies. Penicillin and tetracycline resistance is abundant and may be associated with the common use of these antibiotics in the treatment of mastitis in Ethiopia. Sample resistance to cefoxitin indicates a high prevalence of MRSA*.* The DNA profile of these isolates may give information about the spread and evolution of MRSA in this region and therefore further research is recommended.

The experience of farmers has a positive association with resistant percentage of *S. aureus* strains towards clindamycin. Possibly, more experience leads to more or less thoughtful use of antibiotics and thereby evolution of resistance but more research is necessary to ascertain this effect.

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7. References

Abebe. 2010. Prevalence of mastitis and brucellosis in cattle in Awassa and the peri-urban areas of two smaller towns. Zoonoses and Public Health. 57:367-374.

Barkema. 2006. The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine staphylococcus aureus mastitis. Journal of Dairy Science. 89:1877-1895.

Belayneh, R., K. Belihu and A. Tesfaye. 2014. Microbiological study on bacterial causes of bovine mastitis and its antibiotics susceptibility patterns in east showa zone, Akaki district, Ethiopia. Journal of Veterinary Medicine and Animal Health. 6:116-122.

Belayneh, R., K. Belihu and A. Wubete. 2013. Dairy cows mastitis survey in Adama town, Ethiopia. Journal of Veterinary Medicine and Animal Health. 5:281-287.

Beuron, D. C., C. S. Cortinhas, B. G. Botaro, S. N. Macedo, J. L. Gonçalves, M. A. V. P. Brito and M. V. Santos. 2014. Risk factors associated with the antimicrobial resistance of staphylococcus aureus isolated from bovine mastitis. Pesquisa Veterinaria Brasileira. 34:947-952.

CLSI. 2016. Performance standards for antimicrobial susceptibility testing. CLSI supplement M100S. Wayne PA: Clinical and Labaratory Standards Institute.

Degen. 2015. Factors affecting the probability of bacteriological cure of bovine mastitis. Tierärztliche Praxis.Ausgabe G, Grosstiere/Nutztiere. 43:222-227.

Dierikx, C. M., P. D. Hengeveld, K. T. Veldman, A. de Haan, S. van der Voorde, P. Y. Dop, T. Bosch and E. van Duijkeren. 2016. Ten years later: Still a high prevalence of MRSA in slaughter pigs despite a significant reduction in antimicrobial usage in pigs the Netherlands. Journal of Antimicrobial Chemotherapy.

EUCAST. 2016. Breakpoint tables for interpretation of MICs and zone diameters. version 6.0.

Faires, M. C., K. C. Tater and J. S. Weese. 2009. An investigation of methicillin-resistant staphylococcus aureus colonization in people and pets in the same household with an infected person or infected pet. Journal of the American Veterinary Medical Assocation. 235:540-543.

Falagas, M. E., D. E. Karageorgopoulos, J. Leptidis and I. P. Korbila. 2013. MRSA in africa: Filling the global map of antimicrobial resistance. PLoS ONE. 8 (7).

Fessler, A. T., Riekerink, R. G. M. O., A. Rothkamp, K. Kadlec, O. C. Sampimon, T. J. G. M. Lam and S. Schwarz. 2012. Characterization of methicillin-resistant staphylococcus aureus CC398 obtained from humans and animals on dairy farms. Veterinary Microbiology. 160:77-84. 34.

Getahun. 2008. Bovine mastitis and antibiotic resistance patterns in selalle smallholder dairy farms, central Ethiopia. Tropical Animal Health and Production. 40:261-268.

Haftu, R., H. Taddele, G. Gugsa and S. Kalayou. 2012. Prevalence, bacterial causes, and antimicrobial susceptibility profile of mastitis isolates from cows in large-scale dairy farms of northern Ethiopia. Tropical Animal Health and Production. 44:1765-1771.

Harrison, E. M., G. K. Paterson, M. T. G. Holden, J. Larsen, M. Stegger, A. R. Larsen, A. Petersen, R. L. Skov, J. M. Christensen, A. Bak Zeuthen, O. Heltberg, S. R. Harris, R. N. Zadoks, J. Parkhill, S. J.

Peacock and M. A. Holmes. 2013. Whole genome sequencing identifies zoonotic transmission of MRSA isolates with the novel mecA homologue mecC. EMBO Molecular Medicine. 5:509-515.

Hogeveen, H., K. Huijps and T. J. G. M. Lam. 2011. Economic aspects of mastitis: New developments. New Zealand Veterinary Journal. 59:16-23.

Kejela, T. and K. Bacha. 2013. Prevalence and antibiotic susceptibility pattern of methicillin-resistant staphylococcus aureus (MRSA) among primary school children and prisoners in Jimma town, southwest Ethiopia. Annals of Clinical Microbiology and Antimicrobials. 12 (11).

Kerro Dego, O. and F. Tareke. 2003. Bovine mastitis in selected areas of southern Ethiopia. Tropical Animal Health and Production. 35:197-205.

Khameneh, B., R. Diab, K. Ghazvini and B. S. Fazly Bazzaz. 2016. Breakthroughs in bacterial resistance mechanisms and the potential ways to combat them. Microbial Pathogenesis. 95:32-42.

Lakew, M., T. Tolosa and W. Tigre. 2009. Prevalence and major bacterial causes of bovine mastitis in Asella, south eastern Ethiopia. Tropical Animal Health and Production. 41:1525-1530.

Leclercq, R., R. Cantón, D. F. J. Brown, C. G. Giske, P. Heisig, A. P. Macgowan, J. W. Mouton, P. Nordmann, A. C. Rodloff, G. M. Rossolini, C. Soussy, M. Steinbakk, T. G. Winstanley and G. Kahlmeter. 2013. EUCAST expert rules in antimicrobial susceptibility testing. Clinical Microbiology and Infection. 19:141-160.

Lindsay, J. A. 2014. Staphylococcus aureus genomics and the impact of horizontal gene transfer. International Journal of Medical Microbiology. 304:103-109.

Matuschek, E., D. F. J. Brown and G. Kahlmeter. 2014. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. Clinical Microbiology and Infection. 20 (4): 255-266.

Mekonnen. 2005. Antimicrobial susceptibility profiles of mastitis isolates from cows in three major Ethiopian dairies. Revue De Médecine Vétérinaire. 156:391-394.

Mekuria. 2013. Identification and antimicrobial susceptibility of staphylococcus aureus isolated from milk samples of dairy cows and nasal swabs of farm workers in selected dairy farms around Addis Ababa, Ethiopia. African Journal of Microbiology Research. 7:3501-3510.

MoARD. 2007. Livestock development master plan study
phase I report – data collection and analysis. Volume 5- Policy and institutions:.

Moges, N., Y. Asfaw, K. Belihu and A. Tadesse. 2011. Aantimicrobial susceptibility of mastitis pathogens from smallholder dairy herds in and around Gondar, Ethiopia. Journal of Animal and Veterinary Advances. 10:1616-1622.

Mungube, E. O., B. -. Tenhagen, F. Regassa, M. N. Kyule, Y. Shiferaw, T. Kassa and M. P. O. Baumann. 2005. Reduced milk production in udder quarters with subclinical mastitis and associated economic losses in crossbred dairy cows in Ethiopia. Tropical Animal Health and Production. 37:503-512.

O'Sullivan, M. V. N., Y. Cai, F. Kong, X. Zeng and G. L. Gilbert. 2006. Influence of disk separation distance on accuracy of the disk approximation test for detection of inducible clindamycin resistance in staphylococcus spp. Journal of Clinical Microbiology. 44:4072-4076.

Petrie, A. and P. Watson. 2006. Statistics for Veterinary and Animal Science. 2nd ed. Blackwell Publishing, Oxford, UK.

Petrovski, K. R., A. Grinberg, N. B. Williamson, M. E. Abdalla, N. Lopez-Villalobos, T. J. Parkinson, I. G. Tucker and P. Rapnicki. 2015. Susceptibility to antimicrobials of mastitis-causing staphylococcus aureus, streptococcus uberis and str. dysgalactiae from New Zealand and the USA as assessed by the disk diffusion test. Australian Veterinary Journal. 93:227-233.

Pitkälä, A., L. Salmikivi, P. Bredbacka, A. -. Myllyniemi and M. T. Koskinen. 2007. Comparison of tests for detection of ß-lactamase-producing staphylococci. Journal of Clinical Microbiology. 45:2031-2033.

Shibabaw, A., T. Abebe and A. Mihret. 2013. Nasal carriage rate of methicillin resistant staphylococcus aureus among dessie referral hospital health care workers; Dessie, northeast Ethiopia. Antimicrobial Resistance and Infection Control. 2 (25).

Sori, T., J. Hussien and M. Bitew. 2011. Prevalence and susceptibility assay of staphylococcus aureus isolated from bovine mastitis in dairy farms of Jimma town, south west Ethiopia. Journal of Animal and Veterinary Advances. 10:745-749.

Tenhagen, B. A., B. Vossenkuhl, A. Kasbohrer, K. Alt, B. Kraushaar, B. Guerra, A. Schroeter and A. Fetsch. 2014. Methicillin-resistant staphylococcus aureus in cattle food chains - prevalence, diversity, and antimicrobial resistance in Germany. Journal of Animal Science. 92:2741-2751.

Tesfaye, G. Y., F. G. Regassa and B. Kelay. 2010. Milk yield and associated economic losses in quarters with subclinical mastitis due to staphylococcus aureus in ethiopian crossbred dairy cows. Tropical Animal Health and Production. 42:925-931.

Thomas, V., A. De Jong, H. Moyaert, S. Simjee, F. El Garch, I. Morrissey, H. Marion and M. Vallé. 2015. Antimicrobial susceptibility monitoring of mastitis pathogens isolated from acute cases of clinical mastitis in dairy cows across Europe: VetPath results. International Journal of Antimicrobial Agents. 46:13-20.

Tolosa, T., J. Verbeke, Z. Ayana, S. Piepers, K. Supre and S. Vliegher. 2015. Pathogen group specific risk factors for clinical mastitis, intramammary infection and blind quarters at the herd, cow and quarter level in smallholder dairy farms in Jimma, Ethiopia. Preventive Veterinary Medicine. 120:306-312.

Van Duijkeren, E., M. J. H. M. Wolfhagen, A. T. A. Box, M. E. O. C. Heck, W. J. B. Wannet and A. C. Fluit. 2004. Human-to-dog transmission of methicillin-resistant staphylococcus aureus. Emerging Infectious Diseases. 10:2235-2237.

Vanderhaeghen, W., K. Hermans, F. Haesebrouck and P. Butaye. 2010. Methicillin-resistant staphylococcus aureus (MRSA) in food production animals. (special issue: Epidemiology of methicillin-resistant staphylococcus aureus with emphasis on veterinary and community settings.). Epidemiology and Infection. 138:606-625. 195 ref.

Weigel, L. M., D. B. Clewell, S. R. Gill, N. C. Clark, L. K. McDougal, S. E. Flannagan, J. F. Kolonay, J. Shetty, G. E. Killgore and F. C. Tenover. 2003. Genetic analysis of a high-level vancomycin-resistant isolate of staphylococcus aureus. Science. 302:1569-1571.

Yilmaz. 2007. Detection and prevalence of inducible clindamycin resistance in staphylococci. Journal of Medical Microbiology. 56:342-345.

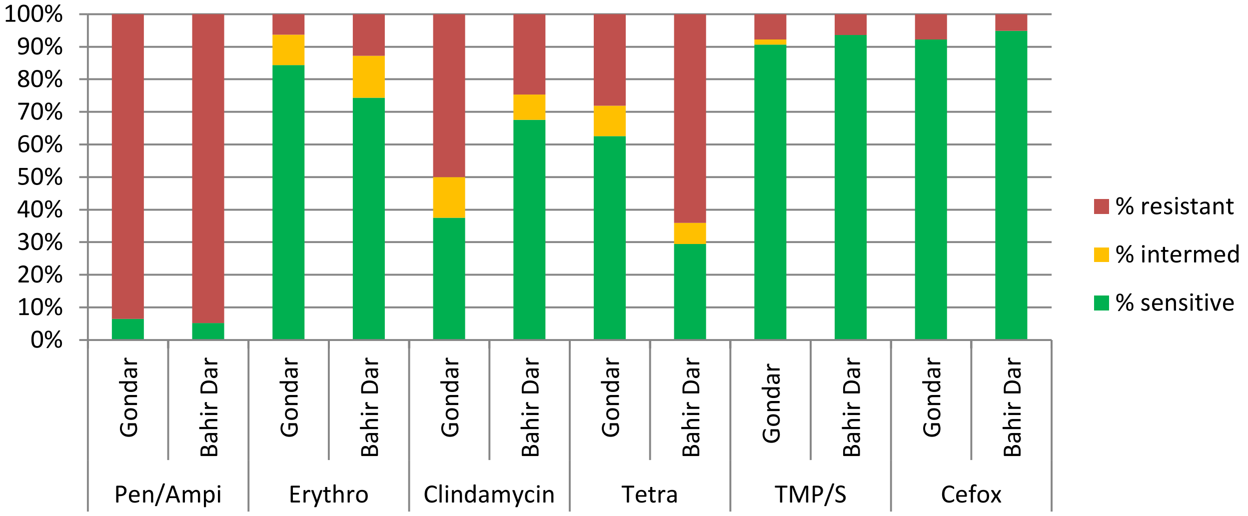
Zeryehun, T., T. Aya and R. Bayecha. 2013. Study on prevalence, bacterial pathogens and associated risk factors of bovine mastitis in small holder dairy farms in and around Addis Ababa, Ethiopia. Journal of Animal and Plant Sciences. 23:50-55.

Appendix A

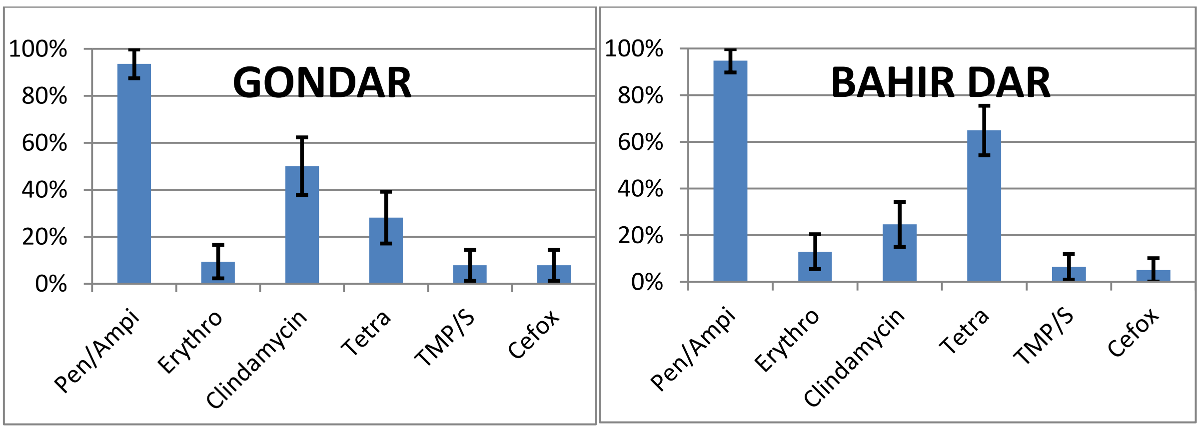
Antibiotic	Resistance%	SE	95% Confidence interval
Penicilin	94.2	0.0198	0.904 – 0.981
Erytromycin	11.3	0.0267	0.061 – 0.166
Clindamycin	36.2	0.0405	0.282 – 0.441
Tetracyclin	48.2	0.0421	0.400 – 0.565
TMP/S	7.1	0.0216	0.029 – 0.113
Cefoxitin	6.4	0.0206	0.023 – 0.104

Standard Error (SE) and Confidence interval per antibiotic.

Appendix B



Gondar and Bahir Dar percentages of resistant, intermediate and sensitive strains



Gondar and Bahir Dar resistance percentages with confidence level error bars.