Antimicrobial sensitivity of coagulase negative *Staphylococcus* species isolated from bovine milk samples in Gondar and Bahir Dar area, Ethiopia

Janke Maat
3515745
January – June 2016

Supervisors:
Utrecht University Gerrit Koop
University of Gondar Sefinew Alemu Mekonnen

Contents

1. Abstract

Coagulase-negative staphylococci (CNS) are increasingly isolated from intramammary infections in bovine milk samples worldwide. In North West Ethiopia, 150 crossbreed dairy farms were sampled and a questionnaire was performed on farm and cow level factors. Out of 1523 samples, 496 samples were identified as CNS (33% of quarters). Of this sample set, 102 CNS strains were tested for antimicrobial sensitivity using disc diffusion. The strains were most resistant to penicillin (75%), tetracycline (31%) and clindamycin (23%). Cefoxitin resistance was 10%, which indicates methicillin resistance. Resistance to erythromycin and TMPS was lower with 10% and 6% respectively. 47% of strains were resistant to one or more antibiotics. Farms with a history of mastitis and treatment for mastitis in the last year had higher odds of resistance to penicillin than other farms. There was also a positive correlation between herd size and resistance. This could be because of more use of antibiotics on these farms, but that should be further analyzed together with dose and frequency of treatment. In conclusion, resistance in CNS isolated from milk samples in Gondar and Bahir Dar area is high. Adequate use of antibiotics and more studies on risk factors can help prevent further development of antimicrobial resistance.

Abbreviations

AMR/S	antimicrobial resistance/sensitivity
95% CI	95% confidence interval
CNS	Coagulase-negative staphylococci
DA	clindamycin
E	erythromycin
FOX	cefoxitin
OR	odds ratio
P	benzylpenicillin
SCC	Somatic Cell Count
SXT	trimethoprim-sulphamethoxazole
Te	tetracycline
TMPS	trimethoprim-sulpha combinations
UoG	University of Gondar
ZOI	zone of inhibition

2. Introduction

Ethiopia ranks 5th in the world in the number of dairy farms, with around 2.5 million farms on a human population of 75 million in 2005. Milk consumption is low compared to other countries, at less than 30 kg per head per year. It is however expected that increasing income levels, population growth and urbanization will stimulate the demand for dairy products (Knips, 2005). Mekonnen et al (2006) state that the future milk production in Ethiopia has to increase 4% per year to meet the demand. It is expected that the increasing demand for dairy products to urban centers will rely mainly on smallholder dairy farms. More than 50% of smallholder dairy farms are less than 6 years old, indicating dairy farming is an upcoming business. Both economic analysis and the perception of farmers indicated mastitis as a major health constraint in dairy farming in Ethiopia. (Mekonnen et al, 2006)

Mastitis in Ethiopia

Several studies on the prevalence of mastitis in Ethiopia have been done. In the southern regions prevalences have been reported in a wide range from 30-61% at quarter level. (Abebe et al, 2010; Dego et al, 2003; Lakew et al, 2009; Tolosa et al, 2013 and 2015). In the central region studies report a quarter prevalence of subclinical mastitis between 10% and 43% (Getahun et al, 2008; Tesfaye et al, 2010). In the north, where also this study was performed, prevalences of 15% and 18% were reported by Almaw et al (2008) and Haftu et al (2012). The preliminary data of this research revealed a prevalence of subclinical mastitis of 39% on quarter level.

Staphylococci often cause intramammary infections in Ethiopia. *S. aureus* was isolated from 11% and 29% of clinical cases and 20% to 43% of subclinical cases. (Haftu et al, 2012; Lakew et al, 2009; Mekonnen et al, 2005) These studies found coagulase-negative staphylococci (CNS) in 4%-8% of clinical and 10%-24% of subclinical cases. CNS species were most frequently isolated from samples from intramammary infections by Tolosa et al (2013) and Almaw et al (2008), with a very high prevalence of 75% resp. 50%.

Also in Europe, CNS are increasingly found in bovine milk samples. In The Netherlands for example, the number of CNS isolates in milk samples of cows with subclinical mastitis increased from 16% (1999) to 42% (2004) (Sampimon et al, 2007). Other studies in Belgium, Poland, Germany and England show prevalences of CNS between 15% and 47% in subclinical mastitis and between 14% and 16% in clinical mastitis (Piepers et al, 2007; Sztachanska et al, 2016; Tenhagen et al, 2006; Bradley et al, 2007).

It is difficult to compare the findings between studies, because of varying definitions of (sub)clinical mastitis, sample selection and study design. It seems however clear that the prevalence of intramammary infections caused by CNS is increasing and CNS are regarded as the main causative agent of subclinical mastitis on dairy farms that have controlled major pathogens. (Schukken et al, 2009)

CNS and mastitis

Coagulase-negative staphylococci are a large group of bacteria consisting of 40 different species and subspecies of which at least 24 have been cultured from bovine milk (Vanderhaeghen et al, 2015). The most commonly isolated species differ between countries and herds, but overall the following species are most frequently isolated: *S. chromogenes*, *S. simulans*, *S. xylosus*, *S. haemolyticus* and *S. epidermidis* (Supré et al, 2011; Raspanti et al, 2016; Schmidt et al, 2015). CNS mainly cause subclinical mastitis, but reports are available on CNS causing severe clinical signs, even up to 50% of CNS intramammary infections. (Jarp et al, 1991; Taponen et al, 2006)

CNS infected cows have a slight increase in somatic cell count (SCC) compared to cows infected with major pathogens (Schukken et al, 2009). Intramammary infection caused by CNS species can however increase somatic cell count (SCC) up to 1.277.000 cells/ml, with an estimated effect of 475.000 cells/ml (Djabri et al, 2002). Certain CNS species (*S. chromogenes, S. simulans* and *S. xylosus*) could cause similar SCC levels as *S. aureus.* (Schukken et al, 2009) S*. chromogenes* caused an average of 226.000 cells/ml where *S. xylosus* had an average of 85.000 cells/ml. (Supré et al, 2011) Despite the slight or more significant increases in SCC there is not much evidence for substantial milk losses due to CNS intramammary infection (Schukken et al, 2009; Tomazi et al, 2015; Hertl et al, 2014), though some challenge experiments were able to show a reduction in milk yield (Piccart et al, 2015; Simojoki et al, 2009).

Antimicrobial sensitivity

Antimicrobial resistance (AMR) is a global problem and a threat to both veterinary and public health. The reduced sensitivity of bacteria threatens the effective prevention and treatment of infection and increases morbidity and mortality in common bacterial infections (WHO, 2014; Klevens et al, 2007). There are indications that the use of antibiotics stimulates the selection for resistance genes in bacteria (Thomas et al, 2015; Rolain et al, 2013). It has also been found that reducing the use of antimicrobials reduces resistance in certain bacterial strains (Seppälä et al, 1997). In the dairy sector worldwide, mastitis is an important indication for antimicrobial treatment and thereby a risk factor for developing resistance (Oliver and Murinda, 2012; MARAN, 2005).

A study in the Netherlands found that that CNS were more often resistant to all tested antimicrobials compared to *S. aureus* (MARAN, 2007). Other studies in Finland, Portugal and Switzerland conclude the same and also that CNS develop more often multiresistance (Corti et al, 2003; Nunes et al, 2007; Taponen et al, 2009). In general, most European studies report high resistance of CNS to penicillin, erythromycin and clindamycin. (Taponen et al, 2016; Moser et al, 2013; Persson et al, 2011; Kalmus et al, 2011)
Only three studies investigated antimicrobial sensitivity in coagulase-negative staphylococci in Ethiopia. Haftu et al (2012) studied sensitivity of 9 CNS strains against 6 antimicrobials in Mekelle, Northern Ethiopia. Getahun et al (2008) studied antimicrobial sensitivity against 8 antimicrobials in Selale, central Ethiopia on 24 strains of *S. epidermidis*. Mekonnen et al (2005) studied 15 strains of CNS against 6 antimicrobials on three government dairy farms around Debre Zeit, central Ethiopia. Haftu et al (2012) found very high resistance levels against clindamycin, erythromycin and TMPS (67%). Getahun et al (2008) found high penicillin (47%) and tetracycline (31%) resistance. Mekonnen et al (2005) also found high resistance to erythromycin (60%) and tetracycline (80%).

It is relevant to study the antimicrobial sensitivity of CNS species for several reasons. Firstly, as CNS are an increasingly important mastitis pathogen, it is important to know antimicrobial sensitivity to make therapy decisions and adjust guidelines for treatment of eg. clinical mastitis cases and persistent infections (Sawant et al, 2009; Taponen et al, 2006). Resistant CNS strains are a direct concern for animal health, but also a zoonotic risk, especially in countries were human-livestock contact is intense (Mekonnen et al, 2006). Furthermore, it is known that resistance genes like the SCCmec cassette encoding for methicillin resistance can cross-over to other staphylococci, for example *S. aureus* (MRSA) and *S. epidermidis* (MRSE). In this way CNS can act as a reservoir for resistance with consequences for both animal and public health. (Leonard et al, 2011; Holmes and Zadoks, 2011; Schoenfelder et al, 2016)

Studies looking for risk factors of antimicrobial resistance are rare. Rajala-Schultz et al (2009) found a correlation between increasing age of cow, dry cow treatment and positive clinical mastitis history and antimicrobial resistance. Clinical mastitis history is usually correlated to a higher resistance than subclinical isolates. (Bansal et al, 2015) Some studies have been done on differences in resistance patterns on organic and conventional farms (Pol and Ruegg, 2007; Roesch et al, 2006). No studies have been found on correlations between other farm characteristics and resistance, or more detailed factors on cow level. No studies on risk factors for AMR in bovine milk samples have been done in Ethiopia. Knowledge about risk factors for antimicrobial resistance is important to reduce and prevent resistance.

Considering the high prevalence of CNS in intramammary infections worldwide, the need to know resistance patterns of mastitis pathogens and risk factors for AMR and the lack of studies on this topics in Ethiopia, the objectives of this study are as follows:

To assess 1) the antimicrobial sensitivity of CNS strains isolated from bovine milk samples of in Gondar and Bahir Dar area, Ethiopia and 2) to search for risk factors for antimicrobial resistance at cow and farm level.

3. Materials and Methods

CNS samples identified in the course of a cross sectional study in Gondar and Bahir Dar area, Ethiopia from October 2014 to June 2015 were used. Among these were also samples taken at the farm of University of Gondar (UoG). For the study 150 dairy farms were randomly selected and visited for sampling and performing questionnaires. Quarters of each lactating cow in each herd were screened for mastitis by clinical examination and California mastitis test (CMT) at the time of sampling. Samples were taken from all quarters, independent of CMT or mastitis status.
Milk samples were stored in an icebox containing ice pack and frozen at -20°C until further processing. After thawing, the milk was inoculated on sheep blood agar, incubated aerobically at 37°C and examined for bacterial growth after 24hrs of incubation. The culture was considered negative if no growth occurred after 24 hours of incubation. When growth occurred, primary identification was done based on colony size, shape, color, hemolytic characteristics, Gram staining and catalase production. Biochemical tests were performed after subculturing an isolated colony on nutrient agar. Isolates were prepared by subculturing a single colony on nutrient agar for 24 hours. Then a large quantity of culture was put into tryptone soy broth with 15% glycerol (Oxoid, Basingstoke UK). This was mixed by vortex and stored at -20°C.

The questionnaire focused on herd level variables and cow level variables. Herd level variables were eg. level of education of the farmer, farming experience, number of milking herd, membership to dairy association, dairy management training, role of advisors, stall type, bedding, history of mastitis in last year, treatment, milking method, deworming and vaccination. The cow level variables were eg. mastitis and treatment history, pregnancy, history of lameness, daily milk yield, days in milk (DIM), parity, body condition and common dairy health problems (dystocia, retained placenta, hypocalcemia, ketosis and abortion).

Culturing of isolates

From the 150 sampled farms, a total of 1523 samples were collected. Out of these, 496 samples were identified as CNS (33% of quarters). Because of limited research materials, 187 of these isolates (80 Gondar, 51 Bahir Dar and 56 UoG farm) were randomly selected and used for the current study. 63 samples had one or more double ZOI and were excluded from the sample set. 124 samples remained, of which 102 samples (37 Gondar, 31 Bahir Dar, 34 UoG farm) had data from the questionnaires and were used for further analysis. The isolates were regrown aerobically on nutrient agar (Oxoid, Basingstoke UK) for 24hrs at 37°C. When insufficient growth was recorded after 24h, samples were incubated for another 24h. All cultures with growth of two types of colonies were tested for catalase and coagulase to confirm colonies were CNS. Cultures with three or more types of colonies were considered contaminated and excluded from further analysis.

Antimicrobial susceptibility testing

The disk diffusion method as stated by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) was used. (EUCAST, 2015) From an overnight growth on nutrient agar, some separate colonies were suspended in a sterile 0.85% NaCl solution to a 0.5 McFarland solution. This was done by visual comparing 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). Discs were then placed on the Mueller Hinton agar by using a dispenser (Oxoid). Clindamycin and erythromycin discs were placed adjacently in the dispenser to test for macrolide inducible clindamycin resistance. In this way, discs were placed about 15 mm apart, which is in line with the EUCAST advice to place discs 12-20 mm apart (EUCAST, 2015)

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

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

These antibiotic discs were selected as recommended to determine resistance patterns in veterinary important staphylococci by the EUCAST guidelines (EUCAST, 2015).

Interpretation of results

After 16-24h of incubation at 35°C, zones of inhibition were measured by using a caliper. Zone diameters were measured from the back above a dark background in a bright environment to the closest millimeter. Separate colonies within the distinct zone of inhibition were ignored. Zone diameters were compared to the EUCAST criteria for disc diffusion (EUCAST, 2016). The breakpoints for the used antibiotic discs are as follows:

Antibiotic	**Disc content**	**Susceptible**	**Resistant**
P: Benzylpenicillin	1 unit	≥26*	<26*
FOX: Cefoxitin	30 µg	≥25	<25
DA: Clindamycin	2 µg	≥22	<19
E: Erythromycin	15 µg	≥21	<18
Te: Tetracyclin	30 µg	≥22	<19
SXT: Trimetoprim/sulfamethoxazole	1,25 – 23,75 µg	≥17	<14

* No reliable method is available for detecting penicillinase production in CNS; breakpoints for *S. aureus* and *S. lugdenensis* have been used in this research.

Besides the diameter of the zone of inhibition (ZOI) there were three outcomes possible for each antibiotic: Susceptible (SENS), Resistant (RES) and Intermediate (INT; only for DA, E, Te and SXT). The ZOI diameter was only used to analyze if certain diameters were more frequent than others in histograms. Further analysis was done with SENS, RES and INT outcomes.

Samples with one or more diameter zones missing, because of failing to dispend the disk (blockade in the disk dispenser) or antibiotic disc tilted on the side, were excluded from further analyses. Also excluded were samples with growth of differently shaped or colored colonies (contamination) and samples with no semiconfluent growth. Strains with a double zone of inhibition were still interpreted for further analysis of the cause of the double ZOI. In scattterplots it was analyzed if certain combinations of double ZOI were found more often than others.

For the interpretation of results, the EUCAST expert rules in microbial susceptibility testing were used (Leclerq et al, 2013) as following:

- If strains were resistant to cefoxitin (mecA-gene indicator), they were reported as resistant to all beta-lactams except those specifically licensed to treat infections caused by methicillin-resistant staphylococci. If resistant to cefoxitin strains were also reported resistant to carbapenems and cephalosporins.
- If resistant to penicillin G or if beta-lactamase is detected (by nitrofecin test), they were reported resistant to all penicillins, regardless of MIC, except the isoxazolyl-penicillins (oxacillin, cefoxitin) and combinations with beta-lactamase inhibitors.

- If susceptible, intermediate or resistant to erythromycin, then report the same category of susceptibility to azithromycin, clarithromycin and roxithromycin.
- If resistant to erythromycin but susceptible to clindamycin, then test for macrolide-lincosamide-streptogramin B (MLSb) resistance (D-test). If positive, then report as resistant to clindamycin.
- If resistant to erythromycin strains were also reported resistant to azithromycin, clarithromycin and roxithromycin.

Statistical analysis

Descriptive statistics and statistical analysis was done in IBM SPSS Statistics 22.0. Correlations between resistances against two or more antibiotics (multiresistance) were searched through Chi-squared test. Differences in resistance pattern between herd and cow level factors from the questionnaires were tested for statistical significance using the Chi-squared test. Outcomes were reported as significant when $P<0.05$. Associations were quantified using the odds ratio and 95% confidence interval. When more than two covariate variables were present logistic regression was used to determine the odds ratio.

4. Results

The dataset of 102 samples was taken from 67 different cows from 40 different farms of Gondar area (37), Bahir Dar area (31) and the UoG farm (34). There were no CNS samples of quarters with clinical mastitis. Farms had on average 35 cows (range 2 – 80) and 31% had ≤6 cows (32 farms).

Resistance data

Figure 1 shows the percentage of resistance per area. Overall, especially the amount of resistance for penicillin and tetracycline is high with 75% and 31% respectively. Overall cefoxitin resistance is 10%. Between the areas there appear to be some differences, for example in Gondar area, penicillin and erythromycin resistance seems lower compared to the other areas, whereas Bahir Dar has high tetracycline resistance and the UoG farm high penicillin resistance. However, only in tetracycline significant differences were found between the study areas (p=0.007).

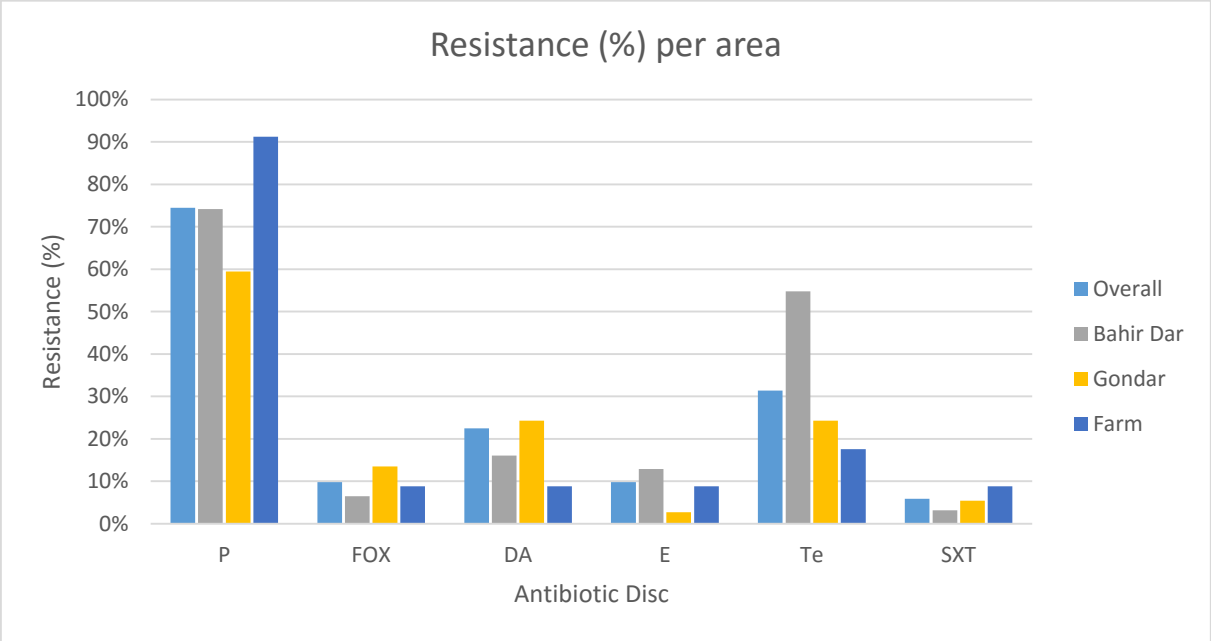


Figure 1. Resistance (%) per antibiotic disk per area. P: benzylpenicillin; FOX: cefoxitin, DA: clindamycin; E: erythromycin; Te: tetracycline, SXT: trimethoprim-sulphamethoxazole.

Histograms of diameters of zones of inhibitions were made (Appendix 1) to analyze their distribution. Several peaks can be seen in for example the chart of tetracycline (figure 2), but they are not centered on the cut-off value.

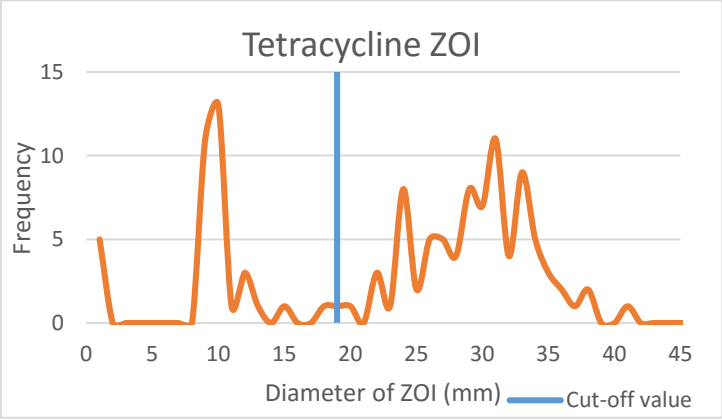


Figure 2. Histogram of ZOI of tetracycline.

Multiresistance

Of all samples 48 (47%) were resistant to one or more antibiotics and 6 samples (6%) were resistant to two or more antibiotics. No samples were panresistant, 12% of samples were pansusceptible. Table 1 shows which combinations of antibiotic resistance were found frequently. For example, all cefoxitin resistant strains were also resistant to penicillin ($p<0.05$). A high concordance between tetracycline and penicillin (81%) and cefoxitin and erythromycin (88%) was found.

Table 1. Frequency of multiresistance between antibiotic discs.

	P	FOX	DA	E	TE	SXT	TOTAL
P	X	10 (13%) [a]	12 (16%) [a]	1 (1%)	26 (34%)	6 (8%)	76 (100%)
FOX	10 (100%)[a]	X	5 (50%) [a]	7 (70%)	1 (10%)	1 (10%)	10 (100%)
DA	12 (71%) [a]	5 (29%) [a]	X	3 (18%)	3 (18%)	1 (6%)	17 (100%)
E	1 (13%)	7 (88%)	3 (38%)	X	4 (50%)	0 (0%)	8 (100%)
TE	26 (81%)	1 (3%)	3 (9%)	4 (13%)	X	1 (3%)	32 (100%)
SXT	6 (100%)	1 (17%)	1 (17%)	0 (0%)	1 (17%)	X	6 (100%)

[a] Significance ($p<0.05$) P: benzylpenicillin; FOX: cefoxitin, DA: clindamycin; E: erythromycin; Te: tetracycline, SXT: trimethoprim-sulphamethoxazole

Correlations

Thirteen correlations were found between factors from the questionnaire and resistance against penicillin. No correlations were found with resistance against the other antibiotic discs. A herd size smaller than 6 cows had lower odds of resistance to penicillin which is in line with lower odds of resistance in farms with less than 3 lactating cows. Farms with a veterinarian or dairy management training had higher odds of resistance to penicillin compared to farms without a vet or training. Checking for mastitis, experience of mastitis in the last year and treatment history for mastitis were more prone to resistance to penicillin. All significant correlations and their ORs are shown in table 2.

Table 2. Correlations between questionnaire factors and penicillin resistance.

Factor	P-value	Odds Ratio	95% confidence interval
Herd size	0,001	4,7 (>6 vs ≤6)	1,8 – 12,5
Number of lactating cows	0,002	4,4 (>3 vs ≤3)	1,7 – 11,1
Dairy management training by government / NGO	0,011	3,3 (yes vs no)	1,3 – 8,3
Presence of veterinarian	0,000	7,14 (yes vs no)	2,5 - 20
Tick infestation present	0,038	2,77 (no vs yes)	1,04-7,38
Use of deworming	0,048	3,5 (yes vs no)	1,0 – 12,5
Washing the stall	0,005	3,7 (yes vs no)	1,4 – 9,1
Experience of mastitis on farm in last one year	0,000	7,7 (yes vs no)	2,8 – 20
Treatment history for mastitis on farm	0,027	3,7 (yes vs no)	1 – 14,3
Checking for mastitis	0,05	10 (yes vs no)	0,97 – 100
Repeated mastitis infection	0,007	3,6 (yes vs no)	1,41 – 9,1
Frequency of repeated mastitis infection	0,014	6,2 (>3x vs 2x)	1,45 – 25
Milking mastitis quarters	0,045	2,9 (yes vs no)	1 – 8,3

Double zones of inhibition

During interpretation 120 double zones of inhibition (at 63 samples) were found out of a total of 1.122 zones (11%). These double ZOI were evenly distributed over different antibiotic discs, ranging from 7-13% per disk. In the university farm 4% of the zones had a double zone of inhibition, in Bahir Dar 10% and in Gondar 20%.

In 40% of the cases the different zones of inhibition had no consequences for reporting a strain resistant, intermediate or sensitive, and in 60% it made a difference in outcome. When a double ZOI made no difference in outcome, the sample was still used for analysis. In the scatterplots (Appendix 2) of the double ZOI no cluster formation was found. As an example, figure 3 shows the scatterplots of erythromycin and tetracycline.

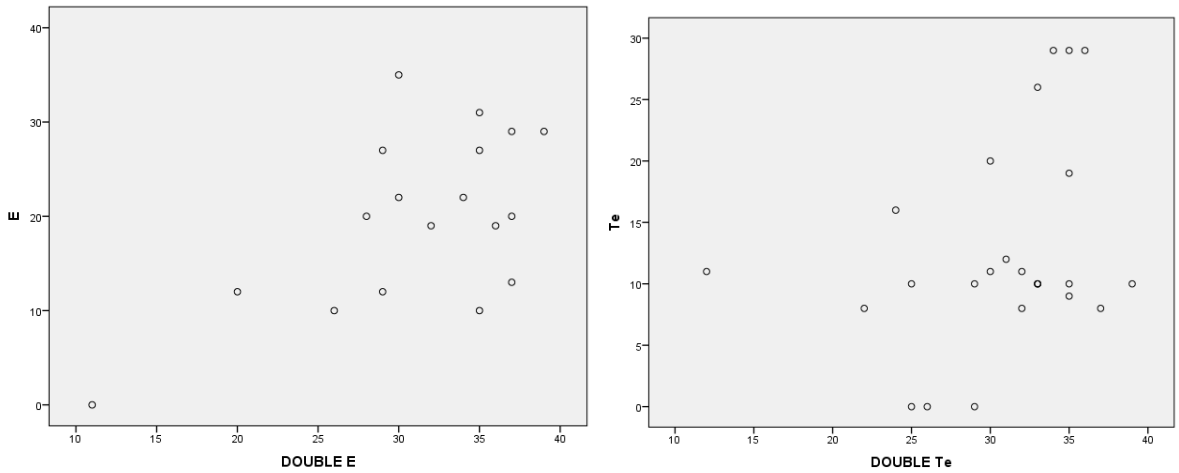


Figure 3. Scatterplots of both double ZOI of erythromycin and tetracycline.

5. Discussion

The objectives of this study were to assess antimicrobial sensitivity of CNS from bovine milk samples and find correlations between management practices, individual cow factors and resistance patterns.

Interpretation and comparison with other studies

Table 3 summarizes results from studies that investigated phenotypic resistance of CNS isolated from bovine milk samples as compared to the findings in the current study. When studies investigated antimicrobial sensitivity of CNS on species level, the total sensitivity of all tested CNS species was used in this table (Taponen et al, 2016; Sampimon et al, 2011). Getahun et al (2008) studied AMS of *S. epidermidis* as only CNS species, but was still included in the comparison because of the limited information on AMS of CNS in Ethiopia.

Table 3. Comparison of phenotypic AMS results of CNS isolated from bovine milk samples.

Study	Country	Sample size	P	FOX	OX	DA	E	Te	SXT
Rajala-Schultz et al, 2009	USA	460	22%				20%	18%	
Sawant et al, 2009	USA	168					8%		
Pol and Ruegg, 2007	USA	294	15%			2%	16%	13%	
Ruegg et al, 2015	USA	51	10%*		2%*		84%*	28%	
Sampimon et al, 2011	Netherlands	170	30%		28%*		7%	11%	
Piessens et al, 2012	Belgium	82					23%		
Botrel et al, 2010	Germany	417	35%	3%			11%	7%	
Roesch et al, 2006	Switzerland	158	40%			5%	3%	1%*	
Moser et al, 2013	Switzerland	120	39%	6%			5%		
Bal et al, 2010	Turkey	100	58%			14%	20%	14%	
Taponen et al, 2016	Finland	68	37%	7%	9%	4%	0%	13%	
Pitkälä et al, 2004	Finland	335	32%		10%	0%	5%	9%	2%
Bengtsson et al, 2009	Sweden	56	13%				4%	5%	7%
Persson et al, 2011	Sweden	95	37%				2%	1%*	
Kalmus et al, 2011	Estonia	80	34%			18%	15%	12%	3%
Raspanti et al, 2016	Argentina	219	52%				29%	30%	
Gentilini et al, 2002	Argentina	123	28%				6%		
Schmidt et al, 2015	SA	102	37%	0%*		0%*	0%*	9%	1%*
Bansal et al, 2015	India	58	76%*						
Kudinha and Simango, 2002	Zimbabwe	131	8%				0%*	18%	
Mekonnen et al, 2005	Ethiopia	15					60%	80%*	
Haftu et al, 2012	Ethiopia	9				67%*	67%		67%*
Getahun et al, 2008	Ethiopia	24	47%			5%		24%	
	AVERAGE	145	34%	4%	12%	13%	18%	17%	16%
Maat and Overvliet, 2016	Ethiopia	102	**75%**	**10%***		**23%**	10%	31%	6%

P: benzylpenicillin; FOX: cefoxitin, OX: oxacillin, DA: clindamycin; E: erythromycin; Te: tetracycline, SXT: trimethoprim-sulphamethoxazole

* Highest or lowest value found

Most European studies find high resistance of CNS to penicillin, erythromycin and clindamycin (Taponen et al, 2016; Moser et al, 2013; Persson et al, 2011; Kalmus et al, 2011). Resistance against penicillin (75%) and clindamycin (23%) is in this study also in the high range and above the average of studies found. In clindamycin, only the study of Haftu et al (2012) found higher AMR but this study had a limited samples size of 9 (6 resistant strains). Compared with Getahun et al (2008), equally high resistance to tetracycline and a higher penicillin resistance (47% vs 75%) was found. Tetracycline (80%) and erythromycin (60%) was found very high in Mekonnen et al (2005) with 15 samples. This study found AMR on the low side of the range of reported data regarding to erythromycin and TMPS.

The resistance percentage of cefoxitin (10%) is higher in this study than in other published data. An overall cefoxitin resistance of 10% is worrying because of the indicator function for methicillin resistance and the *mecA* gene. In other studies, instead of cefoxitin oxacillin was used. It was found that phenotypic AMS testing with oxacillin can lead to both an under- and overestimation of the presence of the *mecA* gene. (Sampimon et al, 2011; Moon et al, 2007; Cauwelier et al, 2004) Recent guidelines therefore advice the use of cefoxitin (EUCAST, 2016). Thus, the oxacillin results have to be interpret with care.

Table 3 shows that a wide range of AMR of CNS has been found worldwide. This can be explained by a different history of samples, for example sampled animals, subclinical versus clinical samples and differences in CMT or SCC cut-off value. Also, it is known that prevalence of CNS species differs on country and herd level and that different CNS species have different resistance patterns. For example, resistance to some antimicrobials is more common in *S. epidermidis* and *S. chromogenes*. (Taponen et al, 2016; Sampimon et al, 2011; Sawant et al, 2009) It would therefore be interesting to have more studies testing AMS on CNS species level. Also different methods of AMS testing and breakpoints can explain varying resistance data. (Rajala-Schultz et al, 2009)

This makes comparing and interpretation of studies difficult. However, it seems that the results from this study are in general on the high side of what has been found before. This is supported by the fact that almost half (47%) of the samples were resistant to one or more antibiotics, and 6% to two or more. This is higher than found in studies in The Netherlands, Finland and Sweden. Pansusceptibility in this study was 12%, where in Finland this was 32% and in The Netherlands even 49%. (Taponen et al, 2016; Sampimon et al, 2011; Bengtsson et al, 2009; Waller et al, 2011) The prevalent combinations of multiresistance found in this study (penicillin and tetracyclin, 81% and cefoxitin and erythromycin, 88%) were not found in other studies. The 100% multiresistance between cefoxitin and penicillin was expected from the interpretation guidelines. (Leclerq, 2013)

Risk factors for resistance

The second objective in this study was to search for associations between cow and farm factors from the questionnaires and resistance against antibiotics, as information on this is limited.

Cow factors

Instead of age of cow, parity was recorded in this study. No correlation between parity and resistance was found, as opposed by Rajala-Schultz et al (2009). This could be because fertility is a problem in dairy farms in Ethiopia and parity and age are probably less correlated than in eg. the USA due to long intercalving time. (Mekonnen et al, 2006) No CNS samples were isolated of cows with clinical mastitis, so this correlation could not be assessed. Dry cow treatment was positively correlated with resistance development (Rajala-Schultz, 2009) and also in the current study, treatment history for mastitis was correlated with higher penicillin resistance odds (3.7).

If a farm had had mastitis in the last year and repeated mastitis infections they had higher odds to develop resistance (7.7 and 3.6). In this cases the 95% CI was also wide (1.45 – 25 resp 2.8 – 20) which indicates that the correlation is not very strong. The same was found for checking for mastitis, with an OR of 10 and a very wide 95% CI of 0.97-100. When mastitis quarters were milked, there were higher odds for resistance (2.9).

Farm factors

Correlations were found between herd size and number of lactating cows and resistance. Farms with more than 6 cows and more than 3 lactating cows had higher odds for penicillin resistance (4.7 and 4.4). This could be because large scale farms have more financial means to treat cows with antibiotics, which is a hypothetic reason for developing antimicrobial resistance (Rolain et al, 2013; Thomas et al, 2015).However, this has to be confirmed with the Ethiopian situation. Along the same lines, farms where a veterinarian was present had higher odds (7.14) of developing resistance. This factor was however not a clear factor, because some farmers would answer that they had a vet when they asked advice from one regularly, and others when they asked one eg. once a year. 53 farmers answered yes on this question and 49 famers no. The 95% confidence interval was wide on this correlation (2.5 – 20) which makes the correlation weak. Paradoxically, farmers with a dairy management training were more likely to have resistance on their farm (3.3). This could be because those farmers know the possibilities for treating mastitis with antibiotics and use it more often to treat their cows. No correlation was found between education level and resistance.

Paradoxically, washing the stall regularly and deworming gave higher odds for resistance (3.7 resp. 3.5), though they would be regarded as good management practices. Another interesting correlation was between tick infestation and resistance, where tick infestation lead to lower resistance (2.77). It could be that there is a relation between the use of antiparasitics and resistance development, but no studies on this have been found.

Explanation of correlations

All these correlations could be explained by the chance of treating animals with antibiotics and therefore selecting for resistance genes. However, no correlation was found between treatment for lameness and resistance, although lameness could also be treated with antibiotics. Also, outcomes field studies on the relation between antibiotic use on farms and resistance development are not consistent (Roesch et al, 2006; Pol and Ruegg et al, 2007). More studies between the frequency and dosage of antibiotics and development of resistance should be done, instead of the indirect factors as incidence of mastitis or treatment of mastitis in the last year. From personal communications with veterinarians and farmers, it is expected that antibiotic treatment is not always done adequately (low dose, short treatment period), which should also be investigated more. For some correlations, eg. tick infestation and deworming, no explanation for a causal connection has been found.

Only correlations were found with certain factors and penicillin resistance, not for the other antibiotics tested. This is probably because penicillin has by far the highest resistance rate (75%), which gives a higher power to find a correlation.

Discussion of methods

In this study the disc diffusion method was used for antimicrobial sensitivity testing. This method has been criticized for detecting methicillin-resistance in CNS because of a large number of false-negative results by Martin and Cunha, 2007. Also, cefoxitin resistance was relatively high (10%) and disc diffusion would only underestimate the problem. Also, EUCAST (2016) states that disk diffusion is more reliable than MIC determination for detection of penicillinase producers, which is one of the most important mechanisms of resistance for staphylococci.

Disc diffusion is suitable for most bacterial pathogens, many antimicrobial agents can be tested and it requires no special materials so is also suitable for analysis in labs with limited equipment. (Matuschek et al, 2014; EUCAST, 2015)

The limited availability for veterinary relevant pathogens is a problem. For CNS species there is no breakpoint for resistance against benzylpenicillin, as there is no reliable method to detect penicillinase production in CNS currently. (EUCAST, 2016) To solve this, the breakpoints for *S. aureus* and *S. lugdenensis* were used (<26mm) which is not very accurate. For cefoxitin the breakpoint of 25mm for CNS species other than *S. lugdenensis* and *S. saprophyticus* was used. Both species have been found in milk samples before, though rarely. (Bochniarz et al, 2014) Chromogenic cephalosporin-based beta-lactamase tests (nitrofecin disks) do not reliably detect penicillinase in staphylococci. It is however not known if this would give an over- or underestimation of the resistance. (EUCAST, 2016)
It was considered to test for resistance against streptomycin, as this is a drug often used in the treatment of mastitis in Ethiopia. However, no breakpoints are available for the combination of streptomycin and staphylococci. It was also considered to test for vancomycin resistance, but because of the low prevalence of strains resistant against this compound and because of limited space for discs on the petri dishes it was decided to leave vancomycin out for this study (EUCAST, 2016).

Double zones of inhibition

Double zones of inhibition can have different causes, of which the most important one is contamination. In this study contamination could also be in the isolates with more than one subspecies of CNS, as different CNS species have different resistance patterns. (Sampimon, 2009; Vanderhaeghen, 2015) In the case of cefoxitin, clindamycin and erythromycin heterogeneous resistance has been described in staphylococci indicating a resistant subpopulation (Hosbul et al, 2013; Antunes et al, 2007). Also, some antibiotic-bacteria combinations can give double ZOI in susceptible strains, but this has not been reported in staphylococci (Matuschek et al, 2014).

The spread between zones of inhibition per antibiotic disc in the scatterplots was very wide. In Gondar area (the samples tested in January) the amount of double ZOI were higher (20%) than in Bahir Dar (10%) and the UoG farm (4%). This could indicate that contamination mostly happened in the beginning of the study. No other cluster forming of double ZOI have been found in batches of petri dishes, agar or sterile saline whatsoever. Considering the circumstances in the lab, there are many steps in the process where contamination was possible but it is hard to say exactly where.

Testing materials on sterility should have been done at least every batch of sterilization of petri dishes and agar, cotton swabs and saline. Another recommendation would be to do routine quality controls daily with reference strains to control the process (Matuschek et al, 2014).

Conclusion

Compared to other studies the resistance figures in this study were among the highest that have been found for CNS isolated from bovine milk, especially for penicillin, cefoxitin and clindamycin. Farms with more than 6 cows had higher risk for penicillin resistance, as had mastitis history in the last year and treatment for mastitis. These correlations could be related to the use of antibiotics and selection for resistance genes, but more studies should be done on the exact role of treatment dose and frequency in developing resistance. Also adequate use of antibiotics can help prevent the development of more antimicrobial resistance.

6. Acknowledgements

The author would like to thank Gerrit Koop and Sefinew Mekonnen for the opportunity to perform this interesting study in Ethiopia and providing samples and data. Special thanks go to Wudu Jemburu and Firdyawukal Abuhay for the supervision at University of Gondar and the veterinary lab. Also, the support of Hans Vernooij with the statistical analysis is much appreciated.

7. References

Abebe, G.; Ike, A. C.; Siegmund-Schultze, M.; Mané-Bielfeldt, A.; Valle-Zárate, A.; Prevalence of mastitis and brucellosis in cattle in Awassa and the peri-urban areas of two smaller towns, Zoonoses and Public Health, 57(5):367-74, 2010.

Almaw, G.; Zerihun, A.; Asfaw, Y.; Bovine mastitis and its association with selected risk factors in smallholder dairy farms in and around Bahir Dar, Ethiopia, Tropical Animal Health and Production, 40(6):427-32, 2008.

Antunes, A. L. S.; Secchi, C.; Reiter, K. C.; Perez, L. R. R.; De Freitas, A. L. P.; d'Azevedo, P. A.; Evaluation of oxacillin and cefoxitin disks for detection of resistance in coagulase negative staphylococci, Memorias do instituto Oswaldo Cruz, 102 (6), 719-723, 2007.

Bal, E. B.; Bayar, S.; Bal, M. A.; Antimicrobial susceptibilities of coagulase-negative staphylococci (CNS) and streptococci from bovine subclinical mastitis cases, Journal of Microbiology, 48(3):267-74, 2010.

Bansal, B. K.; Gupta, D. K.; Shafi, T. A.; Sharma, S.; Comparative antibiogram of coagulase-negative Staphylococci (CNS) associated with subclinical and clinical mastitis in dairy cows, Veterinary World, 8(3): 421-426, 2015.

Bengtsson, B.; Unnerstad, H. E.; Ekman, T.; Artursson, K.; Nilsson-Ost, M.; Waller, K. P.; Antimicrobial susceptibility of udder pathogens from cases of acute clinical mastitis in dairy cows, Veterinary Microbiology, 126(1-2): 142-9, 2009.

Bochniarz, M.; Wawron, W.; Szczubial, M.; Production of slime by coagulase-negative staphylococci (CNS) isolated from clinical and subclinical mastitis in cows, Polish Journal of Veterinary Science, 17 (3), 447-52, 2014.

Botrel, M. A.; Haenni, M.; Morignat,E.; Sulpice, P.; Madec, J. Y.; Calavas, D.; Distribution and antimicrobial resistance of clinical and subclinical mastitis pathogens in dairy cows in Rhone-Alpes, France, Foodborne Pathogens and disease, 7(5):479-87, 2010.

Bradley, A. J.; Leach, K. A.; Breen, J. E.; Green, L. E.; Green, M. J.; Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales, The Veterinary Record, 160(8):253-7, 2007.

Cauwelier, B.; Gordts, B.; Descheemaecker, P.; Van Landuyt, H.; Evaluations of a disk diffusion method with cefoxitin (30 ug) for detection of methicillin-resistant *Staphylococcus aureus*, Europe Journal of Clinical Microbiology of Infectious Diseases, 23:389-392, 2004.

Corti, S.; Sicher, D.; Regli, W.; Stephan, R.; Current data on antibiotic resistance of the most important bovine mastitis pathogens in Switzerland, Schweizer Archiv für Tierheilkunde, 145(12):571-5, 2003.

Dego, O. K.; Tareke, F.; Bovine mastitis in selected areas of southern Ethiopia, Tropical Animal Health and Production, 35(3):197-205, 2003.

Djabri, B.; Bareille, N.; Beaudeau, F.; Seegers, H.; Quarter milk somatic cell count in infected dairy cows: a meta-analysis, Veterinary Research, 33(4):335-357, 2002.

EUCAST, Antimicrobial susceptibility testing, version 5.0, January 2015.

Gentilini, E.; Denamiel, G.; Betancor, A. Rebuelto, M.; Rodriguez Fermepin, M.; De Torrest, R. A.; Antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine mastitis in Argentina, Journal of Dairy Science, 85(8):1913-7, August 2002.

Getahun, K.; Kelay, B.; Bekana, M.; Lobago, F.; Bovine mastitis and antibiotic resistance patters in Selalle smallholder dairy farms, central Ethiopia, Tropical Animal Health and Production, 40 (4):261-8, 2008.

Haftu, R.; Taddele, H.; Gugsa, G.; Kalayou, S.; Prevalence, bacterial causes, and antimicrobial susceptibility profile of mastitis isolates from cows in large-scale dairy farms of Northern Ethiopia, Tropical Animal Health Production, 44 (7):1765-71, 2012

Hertl, J. A.; Schukken, Y. H.; Welcome, F. L.; Tauer, L. W.; Gröhn, Y. T.; Pathogen-specific effects on milk yield in repeated clinical mastitis episodes in Holstein dairy cows, Journal of Dairy Science, 97(3):1465-80, 2014.

Holmes, M. A.; Zadoks, R. N.; Methicillin resistant S. aureus in human and bovine mastitis, Journal of Mammary Gland Biology and Neoplasia, 2011.

Hosbul, T.; Bozdogan, B.; Haznedaroglu, T.; Ozyurt, M.; Heterogenous macrolide resistance in methicillin-resistant *Staphylococcus aureus* isolates: investigation of resistance mechanisms and clonality Mikrobiyoloji bulteni, 47(2):211-22, 2013

Jarp J.; Classification of coagulase-negative staphylococci isolated from bovine clinical and subclinical mastitis, Veterinary Microbiology, 27(2):151-8, 1991.

Kalmus, P.; Aasmae, B.; Karssin, A.; Orro, T.; Kask, K.; Udder pathogens and their resistance to antimicrobial agents in dairy cows in Estonia, Acta Veterinaria Scandinavica, 53:4, 2011.

Klevens, R. M.; Morrison, M. A.; Nadle, J.; Petit, S.; Gershman, K.; Ray, S.; Harrison, L. H.; Lynfield, R.; Dumyati, G.; Townes, J. M.; Craig, A. S.; Zell, E. R.; Fosheim, G. E.; McDougal, L. K.; Carey, R. B.; Fridkin, S. K.; Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States, JAMA, 298(15):1763-71, 2007.

Knips, V.; Developing countries and the Global Dairy Sector, PPLPI Working paper No 30, FAO, December 2005.

Kudinha, T.; Simango, C.; Prevalence of coagulase-negative staphylococci in bovine mastitis in Zimbabwe, Journal of Southern African Veterinary Association, 73(2):62-5, 2002.

Kudinha, T.; Simango, C.; Prevalence of coagulase-negative staphylococci in bovine mastitis in Zimbabwe, Journal of the South African Veterinary Association, 73(2):62-5, 2002.

Lakew, M.; Tolosa, T.; Tigre, W.; Prevalence and major bacterial causes of bovine mastitis in Asella, South Eastern Ethiopia, Tropical Animal Health and Production, 41 (7):1525-30, 2009.

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

Leonard, F. C.; Markey, B. K.; Methicillin-resistant *Staphylococcus aureus* in animals – a review, Veterinary Journal, 175(1):27-36, 2008.

MARAN 2005, Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2005.

MARAN 2007, Montioring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2006/2007.

Martins, A.; Cunha, M. L. R. S.; Methicillin resistance in *Staphylococcus aureus* and Coagulase-Negative Staphylococci: epidemiological and molecular aspects, Microbiology and Immunology, 51(9): 787-795, 2007.

Matuschek, E.; Brown, D. F. J.; Kahlmeter, G.; Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories, Clinical Microbiology and Infection, 20(4):0255-0266, 2014.

Mekonnen, H. M.; Asmamaw, K.; Courreau, J. F.; Husbandry practices and health in smallholder dairy farms near Addis Ababa, Ethiopia, Preventive Veterinary Medicine, 74(2-3):99-107, 2006.

Mekonnen, H.; Workineh, S.; Bayleyegn, M.; Moges, A.; Tadele, K.; Antimicrobial susceptibility profiles of mastitis isolates from cows in three major Ethiopian dairies, Revue Medical Veterinaire, 156(7):391-394, 2005

Moon, J. S.; Lee, A. R.; Kang, H. M.; Lee, E. S.; Kim, M. N.; Paik, Y. H.; Park, Y. H.; Joo, Y. S.; Koo, H. C.; Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea, Journal of Dairy Science, 90(3):1176-85, 2007.

Moser, A.; Stephan, R.; Ziegler, D.; Johler, S.; Species distribution and resistance profiles of coagulase-negative staphylococci isolated from bovine mastitis in Switzerland, Schweizer Archif für Tierheilkunde, 155(6):333-8, 2013.

Nunes, S. F.; Bexiga, R.; Cavaco, L. M.; Vilela, C. L.; Technical note: antimicrobial susceptibility of Portuguese isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis* in subclinical bovine mastitis, Journal of Dairy Science, 90(7):3242-6, 2007.

Oliver, S. P.; Murinda, S. E.; Antimicrobial resistance of mastitis pathogens, The Veterinary Clinics of North America, Food Animal Practice, 28(2):165-185, 2012.

Persson, Y.; Nyman, A. K.; Gronlund-Andersson, U.; Etiology and antimicrobial susceptibility of udder pathogens from cases of subclinical mastitis in dairy cows in Sweden, Acta Veterinaria Scandinavia, 8(53):36, 2011.

Piccart, K.; Piepers, S.; Verbeke, J.; De Sousa, N. M.; Beckers, J. F.; De Vliegher, S.; Milk prolactin response and quarter milk yield after experimental infection with coagulase-negative staphylococci in dairy heifers, Journal of Dairy Science, 98(7):4593-600, 2015.

Piepers, S.; De Meulemeester, L.; De Kruif, A.; Opsomer, G.; Barkema, H. W.; De Vliegher, S.; Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium, Journal of Dairy Research, 74(4):478-83, 2007.

Piessens, V.; De Vliegher, S.; Verbist, B.; Braem, G.; Van Nuffel, A.; De Vuyst, L.; Heyndrickx, M.; Van Coillie, E.; Characterization of coagulase-negative stahylococcus species from cows' milk and environment based on bap, icaA, and mecA genes and phenotypic susceptibility to antimicrobials and teat dips, Journal of Dairy Science, 95(12):7027-38, 2012.

Pitkälä, A.; Haveri, M.; Pyörälä, S.; Myllys, V.; Honkanen-Buzalski, T.; Bovine mastitis in Finland 2001 – prevalence, distribution of bacteria, and antimicrobial resistance, Journal of Dairy Science, 87(8): 2433-2441, 2004.

Pol, M.; Ruegg, P. L.; Relationship between antimicrobial drug usage and antimicrobial susceptibility of gram-positive mastitis pathogens, Journal of Dairy Science, 90(1):262-73, 2007.

Rajala-Schultz, P. J.; Torres, A. H.; Degraves, F. J.; Gebreyes, W. A.; Patchanee, P.; Antimicrobial resistance and genotypic characterization of coagulase-negative staphylococci over the dry period, Veterinary Microbiology, 134(1-2):55-64, 2009.

Raspanti, C. G.; Bonetto, C. C.; Vissio, C.; Pellegrino, M. S.; Reinoso, E. B.; Dieser, S. A.; Bogni, C. I.; Larriestra, A. J.; Odierno, L. M.; Prevalence and antibiotic susceptibility of coagulase-negative *Staphylococcus* species from bovine subclinical mastitis in dairy herds in the central region of Argentina, Revista Argentina de Microbiología, 48(1):50-6, 2016.

Roesch, M.; Perreten, V.; Doherr, M. G.; Schaeren, W.; Schallibaum, M.; Blum, J. W.; Comparison of antibiotic resistance of udder pathogens in dairy cows kept on organic and on conventional farms, Journal of Dairy Science, 89(3):989-97, 2006.

Rolain, J. M.; Diene, S. M.; Kempf, M.; Gimenez, G.; Robert, C.; Raoult, D.; Real-time sequencing to decipher the molecular mechanism of resistance of a clincial pan-drug-resistant Acinetobacter baumanni isolate from Marseille, France, Antimicrobial agents and chemotherapy, 75(1):592-6, 2013.

Ruegg, P. L.; Oliveira, L.; Okwumabua, O.; Phenotypic antimicrobial susceptibility and occurence of selected resistance genes in gram-positive mastitis pathogens isolated from Wisconsin dairy cows, Journal of Dairy Science, 98(7):4521-34, 2015.

Sampimon O. C.; Lam, T. J. G. M.; Mevius, D. J.; Schukken, Y. H.; Zadoks, R. N.; Antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine milk samples, Veterinary Microbiology, 150(1-2), 173-179, 2011.

Sampimon, O. C.; Barkema, H. W.; Berends, I. M. G. A.; Sol, J.; Lam, T. J. G. M.; Prevalence and herd-level risk factors for intramammary infection with coagulase-negative staphylococci in Dutch dairy herds, Veterinary Microbiology, 134(1-2):37-44, 2009

Sampimon, O. C.; Vernooij, J. C.; Mevius, D. J., Sol, J.; Gevoeligheid voor diverse antibiotica van coagulase negatieve staphylococcen, geisoleerd uit melkmonsters van Nederlands rundvee, Tijdschrift voor Diergeneeskunde, 132(6):200-4, 2007.

Sawant, A. A.; Gillespie, B. E.; Oliver, S. P.; Antimicrobial susceptibility of coagulase-negative *Staphylococcus* species isolated from bovine milk, Veterinary Microbiology, 134(1-2):73-81, 2009.

Schmidt, T.; Kock, M. M.; Ehlers, M. M.; Diversity and antimicrobial susceptibility profiling of staphylococci isolated from bovine mastitis cases and close human contacts, Journal of Dairy Science, 98(9):6256-69, 2015.

Schoenfelder, S. M.; Dong, Y.; Feßler, A. T.; Schwarz, S.; Schoen, C.; Köck, R.; Ziebuhr, W.; Antibiotic resistance profiles of coagulase-negative staphylococci in livestock environments, Veterinary Microbiology, Epub ahead of print, 2016.

Schukken, Y. H.; González, R. N.; Tikofsky, L. L.; Schulte, H. F.; Santisteban, C. G.; Welcome, F. L.; Bennett, G. J.; Zurakowski, M. J.; Zadoks, R. N.; CNS mastitis: Nothing to worry about?, Veterinary Microbiology, 134(1-2):9-14, 2009.

Seppälä, H.; Klaukka, T.; Vuoppio-Varkila, J.; Muotiala, A.; Helenius, H.; Lager, K.; Huovinen, P.; The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland. The New England journal of medicine, 337(7):441-6, 1997.

Simojoki, H.; Orro, T.; Taponen, S.; Pyörälä, S.; Host response in bovine mastitis experimentally induced with *Staphylococcus chromogenes*, Veterinary Microbiology, 134(1-2):95-9, 2009.

Supré, K.; Haesebrouck, F.; Zadoks, R. N.; Vaneechoutte, M.; Piepers, S.; De Vliegher, S.; Some coagulase-negative *Staphylococcus* species affect udder health more than others, Journal of Dairy Science, 94(5), 2329-40, 2011.

Sztachanska, M.; Baranski, W.; Janowski, T.; Pogorzelska, J.; Zdunczyk, S.; Prevalence and etiological agents of subclinical mastitis at the end of lactation in nine dairy herds in North-East Poland, Polish Journal of Veterinary Sciences, 19(1):119-24, 2016.

Taponen, S.; Nykäsenoja, S.; Pohjanvirta, T.; Pitkälä, A.; Pyörälä, S.; Species distribution and in vitro antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine mastitic milk, Acta Veterinaria Scandinavica, 58:12, 2016

Taponen, S.; Pyörälä, S.; Coagulase-negative staphylococci as cause of bovine mastitis – not so different from *Staphylococcus aureus*?, Veterinary Microbiology, 134(1-2), 29-36, February 2009.

Taponen, S.; Simojoki, H.; Haveri, M.; Larsen, H. D.; Pyörälä, S.; Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP, Veterinary Microbiology, 115(1-3):199-207, 2006.

Tenhagen, B. A.; Koster, G.; Wallmann, J.; Heuwieser, W.; Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany, Journal of Dairy Science, 89(7):2542-51, 2006.

Tesfaye, G. Y.; Regassa, F. G.; Kelay, B.; 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 (5):925-31, 2010.

Thomas, V.; De Jong, A.; Moyaert, H.; Simjee, S.; El Garch, F.; Morrissey, I.; Marion, H.; Vallé, M.; 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(1):13-20, 2015.

Tolosa, T.; Verbeke, J.; Ayana, Z.; Piepers, S.; Supre, K.; De Vliegher, S.; 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 (3-4), 306-12, 2015.

Tolosa, T.; Verbeke, J.; Piepers, S.; Supre, K.; De Vliegher, S.; Risk factors associated with subclinical mastitis as detected by California Mastitis Test in smallholder dairy farms in Jimma, Ethiopia using multilevel modelling, Preventive Veterinary Medicine, 112(1-2), 68-75, 2013.

Tomazi, T.; Gonçalves, J. L.; Barreiro, J. R.; Arcari, M. A.; Dos Santos, M. V.; Bovine subclinical intramammary infection caused by coagulase-negative staphylococci increases somatic cell count but has no effect on milk yield or composition, Journal of Dairy Science, 98(5):3071-8, 2015.

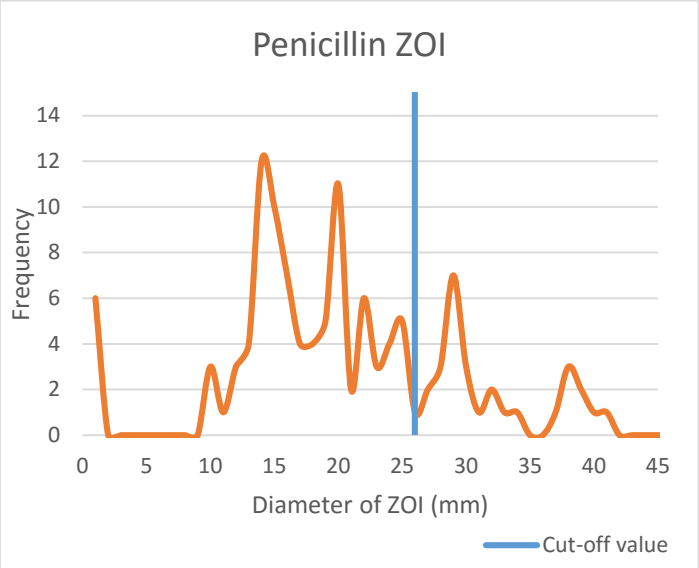
Vanderhaeghen, W.; Piepers, S.; Leroy, F.; Van Coillie, E.; Haesebrouck, F.; De Vliegher, S.; Identification, typing, ecology and epidemiology of coagulase negative staphylococci associated with ruminants, Veterinary Journal, 203(1): 44-51, 2015.

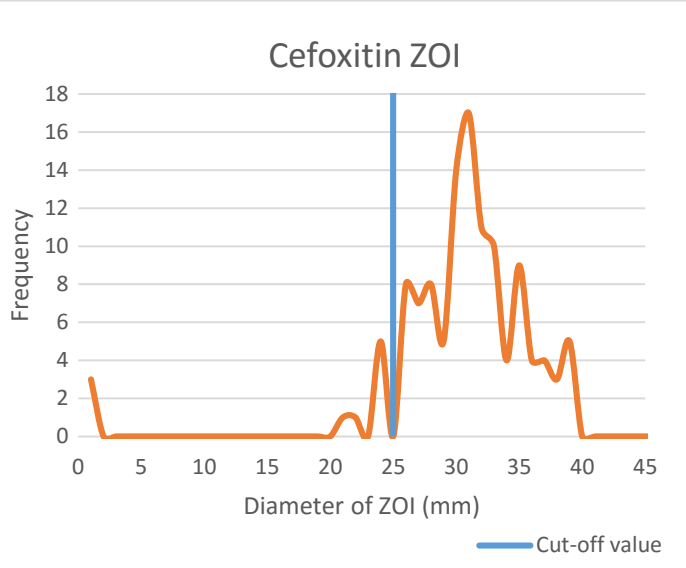
Vanderhaeghen, W.; Piepers, S.; Leroy, F.; Van Coillie, E.; Haesebrouck, F.; De Vliegher, S.; Invited review: Effect, persistence, and virulence of coagulase-negative *Staphylococcus* species associated with ruminant udder health, Journal of Dairy Science, 97(9):5275-93, 2014.

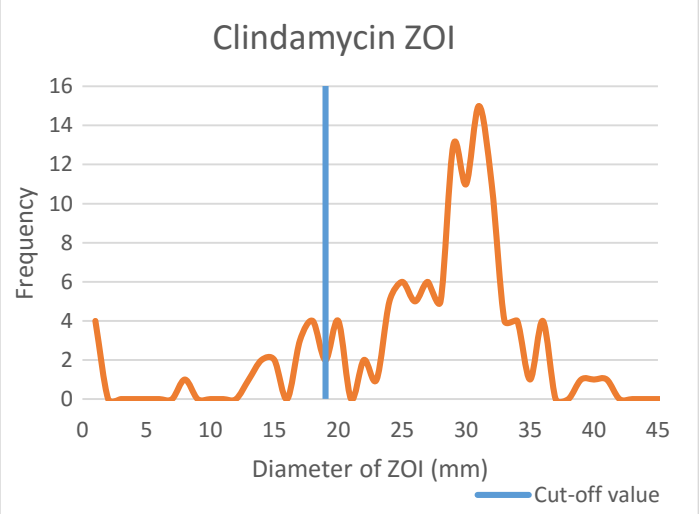
Waller, K. P.; Aspán, A; Nyman, A.; Persson, Y.; Andersson, U. G.; CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis, Veterinary Microbiology, 152(1-2):112-6, 2011.

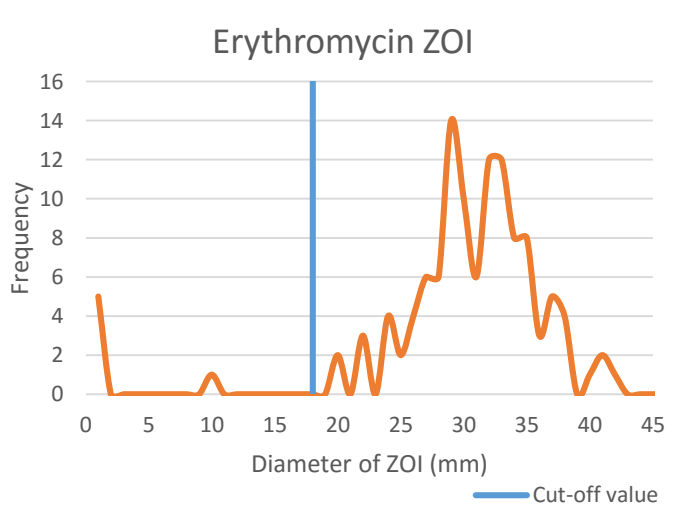
WHO, Antimicrobial resistance: global report on surveillance, 2014.

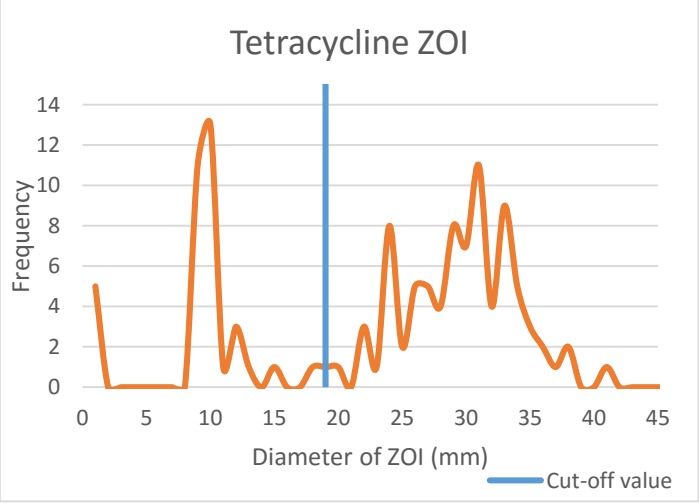
Appendix 1 – Distribution of diameters per antibiotic disc

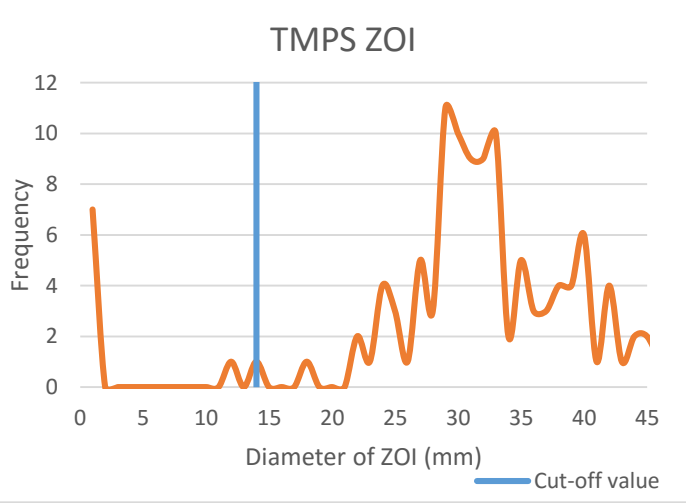












Appendix 2 - Scatterplots of double ZOI