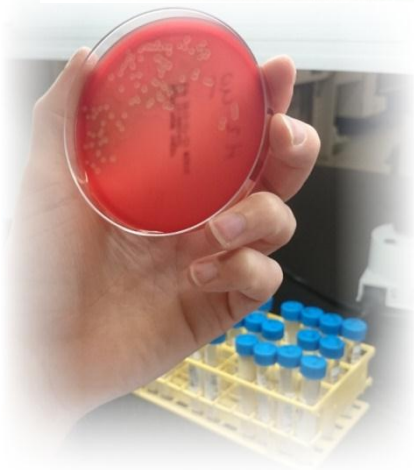

USE OF ANTIBIOTICS FOR IMPROVING UDDER HEALTH IN RELATION TO ANTIMICROBIAL RESISTANCE DEVELOPMENT IN DAIRY CATTLE

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Summary

A high use of antibiotics is usually associated with increased levels of antimicrobial resistance (**AMR**). Although AMR in mastitis pathogens is generally not considered as a major problem, monitoring AMR levels in these organisms remains important, since most antibiotics that are used in Dutch dairy cows are for udder-related indications.

Little information is available on the effect of the treatment and treatment route on AMR development in dairy cows. Therefore, the general goal of these three field studies was to assess the effect of intramammary (**IMM**) applied antibiotics on AMR levels in major mastitis pathogens and coagulase-negative staphylococci (**CNS**) in milk samples and *Escherichia coli* in feces samples. This general goal was more specified into three different studies. In the first study, the effect of antibiotics used to cure clinical mastitis (**CM**) during lactation on AMR development in major mastitis pathogens and CNS was assessed. In the second and third study, the focus was on dry cow treatment (**DCT**). The effect of DCT on AMR levels in major mastitis pathogens and CNS was evaluated in study two, whereas study three focused on the relation between DCT and β-lactam resistance in fecal *E. coli*. Additionally, in part four, available monitoring data is presented on AMR levels in mastitis pathogens from Dutch dairy cattle.

AMR development in mastitis isolates in relation to clinical mastitis treatment

Aseptic milk samples were obtained from 74 individual cows in 11 Dutch dairy herds. These 74 cows had a total of 96 quarters with CM. Both the CM quarter and the contralateral quarter were sampled before and after treatment. Farmers were asked to record additional data on the cow's disease and treatment history.

Of the obtained isolates, minimal inhibitory concentrations (**MIC**) were determined. *S. aureus* (n=38), CNS (n=45), *S. uberis* (n=37) and *E. coli* (n=22) were the most frequently isolated organisms from all samples. Due to low numbers, statistical analysis was limited to descriptive statistics. MIC-50 and MIC-90 values were used to compare groups of organisms isolated before and after treatment. Results were very variable; both increases and decreases were found. One farm might have significantly influenced the results, as all six multiresistant *S. aureus* strains were obtained from there. Unfortunately, numbers were too small to conclude whether or not the application route has an effect. Also, 16 isolates were obtained from the same quarter before *and* after treatment. Though, no indication for AMR development due to antibiotic use during lactation was found in this study. For mastitis pathogens, monitoring AMR on herd level seems to be more reflective of the situation in practice regarding AMR development.

AMR development in bacteria isolated from milk samples in relation to dry cow treatment

Ten dairy farmers took quarter milk samples from 49 cows that were dried off with benzathine cloxacillin and also from 30 cows that were dried off without antibiotics for control. The quarters were sampled before drying off (n=132) and post calving (n=273), when the milk withdrawal time was over. Unfortunately, due to practical reasons, a considerable amount of samples from before drying off was missing. Major pathogens, such as *S. aureus* (n=7) and *S. uberis* (n=4) were seldom isolated. Most commonly isolated were CNS (n=50) and *Corynebacterium* spp. (n=71). Again, MIC-values were determined of the isolated organisms. Although numbers in this study were too low for definitive conclusions, values for the MIC-50 and MIC-90 in the DCT-group were equal or even lower compared to the control group. For more definitive conclusions, using a similar study design with a larger number of cows seems suitable.

AMR development in E. coli isolated from feces samples in relation to dry cow treatment

Of the same 79 cows as described above, feces samples were collected before drying off and post calving. These samples were tested for presence of ESBL/AmpC *E. coli*, and the proportion of ampicillin-resistant *E. coli* was determined.

The latter was determined by replicating ±90 colonies onto Mueller-Hinton agar plates (Central Veterinary Institute, Lelystad, the Netherlands) with and without ampicillin (16 mg/L), and after overnight incubation at 37 °C growth results were compared. Ampicillin-resistance was uncommon: in 38 of the 48 samples with ≥ 10 isolates, no ampicillin-resistance was found.

Two out of 85 tested samples were positive for ESBL-suspected isolates. There was no growth in the semi-quantitative test, which indicates presence in low numbers. Subtyping of these two isolates was performed by specific susceptibility testing, micro-array assay and sequencing techniques. This resulted in one CTX-M-1 ESBL *E. coli* and one *AmpC*-positive *E. coli*.

An effect of DCT was not shown in this study. Based on these results, β-lactam-resistance in fecal *E. coli* of dairy cattle does not seem to be a problem at the moment, nor an emerging problem.

Trends in AMR in mastitis pathogens from Dutch dairy cattle over the years

The aim of this study was to create an overview of occurrence and trends in antimicrobial resistance in staphylococci, streptococci and coliforms for the period 2002-2014. Data from GD Animal Health and the Central Veterinary Institute (**CVI**) were combined. In general, AMR levels in the major pathogens *S. aureus, S. uberis, S. dysgalactiae, E. coli* and *Klebsiella* spp. were low for the tested antibiotics, often lower than 10%. β-lactam resistance in CNS, however, is substantial (51% penicillin-resistance and 23% oxacillin-resistance in 2014), although there seems to be a decreasing trend. Trends, however, have to be interpreted with care, due to changes in methods of susceptibility testing and progressing insights. The extend of these effects will be further analyzed in the upcoming months.

List of used abbreviations:

(**ADDD**) Animal Defined Daily Dosage
(**AMR**) Antimicrobial resistance
(**AMT**) Antimicrobial treatment
(**AST**) Antimicrobial susceptibility testing
(**CFU**) Colony forming units
(**CLSI**) Clinical Laboratory Standards Institute
(**CM**) Clinical mastitis
(**CNS**) Coagulase-negative staphylococci
(**CVI**) Central Veterinary Institute
(**DCT**) Dry cow treatment
(**ESBL**) Extended-spectrum β-lactamase
(**GD**) GD Animal Health
(**KNMvD**) Royal Dutch Veterinary Association
(**IMM**) Intramammary
(**IMI**) Intramammary infection
(**MALDI-TOF**) Matrix-assisted laser desoption/ionization time-of-flight mass spectrometry
(**MIC**) Minimum inhibitory concentration
MIC-50 MIC-value where 50% of the tested isolates are inhibited
MIC-90 MIC-value where 90% of the tested isolates are inhibited
(**SCC**) Somatic cell count
(**TMP/S**) Trimethoprim/sulfonamide(s)

General introduction

General background

Antimicrobial resistance is a worldwide problem and is an important cause of non-successful antimicrobial therapies (Oliver and Murinda, 2012). A lot of research on this subject has already been done. In general, high antibiotic use rates have been related to an increased prevalence of AMR (Levy and Marshall, 2004). To preserve effective drugs for human use, in 2011, the Health Council in the Netherlands published that antimicrobial use in livestock industries should be reduced (Gezondheidsraad, 2011). Subsequently, different livestock production sectors in the Netherlands implemented changes in their protocols on antibiotic use and preventive management in animals. Hence, farmers are only allowed to use antibiotics under strict conditions, where antibiotic use and prescription by veterinarians is regulated, with the intention to decrease antibiotic use. Furthermore, the antibiotics usage is monitored by the SDa: the Netherlands Veterinary Medicines Authority (SDa, 2014a). The monitoring distinguishes antibiotic use between animal species and as well by antibiotic preference groups (based on their ability to induce ESBL/AmpC resistance (SDa, 2014b, Speksnijder et al., 2015). Based on this monitoring, farmers and veterinarians receive a benchmark regarding their antibiotic use and prescription, respectively (Speksnijder et al., 2015). This insight makes farmers and veterinarians aware of their antibiotic management, which should stimulate prudent use. The dairy sector aims to reduce antibiotic usage by increasing preventive health management and monitoring antibiotic use. Also, more specifically, an important goal is to (strongly) reduce the use of specific types of antibiotics, such as third and fourth generation cephalosporins (Lam, 2013) and dry cow antibiotics (Scherpenzeel et al., 2014).

Antibiotic therapies can be administered to dairy cattle by topical, oral, parenteral, intrauterine and, in particular, by IMM administration, where IMM application is the most commonly used route in adult animals. In the Netherlands, for example, 69% of all antibiotics used on dairy farms was applied IMM in 2013 (based on Animal Defined Daily Dose (**ADDD**): 1,95 ADDD was applied IMM, out of a total of 2,84 ADDD) (SDa, 2014b). These IMM administrations include treatment of mastitis during lactation and DCT applied at drying off. Both are intended to cure intramammary infections (**IMI**), which is one of the most important economic and animal welfare problems in the dairy sector worldwide (Hogeveen et al., 2011).

Antimicrobial resistance and mastitis

A possible association between use of antibiotics and AMR in mastitis pathogens has been assessed by multiple studies (Oliver and Murinda, 2012, White and McDermott, 2001). These studies often used herds as the experimental unit, for example comparing organic herds to conventional herds (Pol and Ruegg, 2007, Roesch et al., 2006, Tikofsky et al., 2003). Another study compared primiparous cows to multiparous cows (Rajala-Schultz et al., 2004). Some significantly increased odds-ratio's for occurrence of AMR have been found, for example for IMM administration of pirlimycin, or systemic administration of penicillin (Pol and Ruegg, 2007, Saini et al., 2012). However, when looking at AMR patterns over time, linear trend analysis showed no effect on AMR or even increased susceptibility of major mastitis pathogens for multiple antibiotics (Erskine et al., 2002). Specified antimicrobial use was not always taken into consideration, but if so, this was also translated to use of antibiotics at herd level, for instance by calculating ADDD (Saini et al., 2012, 2013). The previously mentioned field studies are confounded by the fact that science-based evidence on changes in AMR, based on susceptibility testing before and after drug administration is lacking (Erskine et al., 2004, Oliver and Murinda, 2012). Specific studies assessing the effect of antimicrobial treatment (**AMT)** (drug type, application route, therapy duration) on AMR development at cow level are rare. One study assessed the effect of DCT on AMR development in CNS (Rajala-Schultz et al., 2009). They found increased odds ratios for β-lactam resistance, but only

in treated cows with high somatic cell counts at the moment of drying off and not in treated cows with low somatic cell counts. As far as to our knowledge, no such data exist for major mastitis pathogens at the moment.

The effect of IMM application of antibiotics on AMR development could be smaller than the effect of parenteral treatment. Bacterial exposure in the udder to IMM administered antimicrobials is limited, especially when compared to the exposure of gastrointestinal flora after, for example, parenteral treatment. The udder tissue is an environment with few bacteria, besides the strain that causes the infection. Moreover, the bovine udder is separated from the body through the blood-milk barrier. Diffusion across this barrier depends on the pharmacokinetic properties, such as ionization and lipid solubility (Kietzmann and Bäumer, 2008). Exchange of specific antibiotics between the udder tissue and systemic circulation in healthy cows, therefore, is limited, which was shown for various antibiotics (Erskine et al., 2003, Kietzmann et al., 2010, Lainesse et al., 2012, Zonca et al., 2011). However, this might not always be the case in cows affected by mastitis, where bacteria and inflammation impair the blood-milk barrier (Lainesse et al., 2012). This is clearly seen after IMM administration of for example florfenicol and gentamicin (Soback et al., 1995, Sweeney et al., 1996). This may lead to exposure of intestinal bacteria to residues from IMM applied antibiotics, although concentrations will likely not reach the levels of parenteral administration.

As a consequence of the udder being a separated part of the body, development of intestinal AMR due to IMM antibiotic treatment likely is limited. AMR development in the gastrointestinal tract and in the udder likely is influenced by the application route of the antibiotics. In practice, however, specific knowledge on this subject is missing. Therefore, in the antibiotic use guidelines made by the Royal Dutch Veterinary Association (**KNMvD**), no specific distinction is made between different routes of administration of antimicrobial substances. In these guidelines, all antimicrobial substances have been classified by the KNMvD's Veterinary Antimicrobial Policy Working Group (WVAB) into three classes, based on the risk of developing ESBL/AmpC-producing bacteria (KNMVD, 2014, Speksnijder et al., 2015). Multiple studies showed that the use of specific categories of antibiotics contributes more to the presence of ESBL at dairy farms than other categories (Snow et al., 2012). Thus, resistance levels may depend on the antibiotic type. More knowledge is needed on the importance of different factors of the antibiotic therapy on the resistance development within a cow.

Hypothesis

We hypothesize that due to differences in numbers of bacteria and dependent on the route of application of antibiotics, the degree of AMR development differs between bacteria in the udder and gastrointestinal tract. In this study, we will focus on the consequences of IMM application of antibiotics on AMR development in bacteria in the udder and feces. The purpose is to quantify the presence and relative AMR development contribution of each of these pathways. Not all these pathways can be studied at once, thus, at first three subjects will be studied: a possible relationship between IMM (and additional parenteral) *mastitis* treatments during lactation and AMR (chapter 1) and a possible relationship between IMM *dry cow treatment* and AMR (chapter 2 and 3). In chapter 1 and 2 we will focus on udder pathogens, while in chapter 3 we will focus on ampicillin resistant and/or ESBL-producing *E. coli* in fecal samples.

Possible routes of AMR development in relation to AMT are visualized in figure 1. In chapter one, route B and possibly D (in the case of additional parenteral treatment) will be assessed. Chapter two will evaluate route D as well, for DCT, whereas chapter three focuses on route C. The effect of not-IMM applied antibiotics on fecal bacteria (route A) will not be discussed in this study.

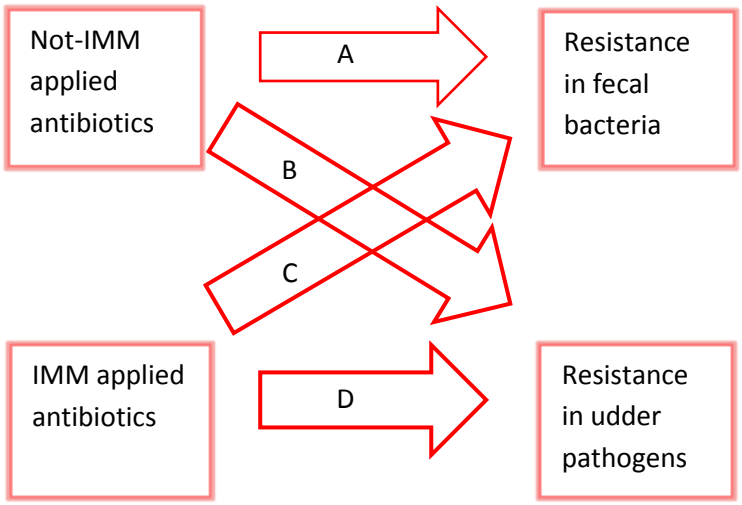


Figure 1: Possible ways of AMR development related to different antimicrobial treatment routes in dairy cows.

Development of AMR in samples for individual animals, if present, will eventually lead to changing AMR patterns at herd and national level. For that reason, available national (monitoring) data on AMR levels in mastitis pathogens will be presented and discussed in chapter 4.

1 AMR development in clinical mastitis isolates in relation to mastitis treatment

Clinical mastitis is one of the major problems on dairy farms, and most antibiotics in Dutch dairy herds are used for udder related indications (SDa, 2014b). These antibiotics are often used for intramammary treatment, with sometimes additional parenteral treatment.

The goal of this chapter is to study the AMR development within mastitis pathogens after IMM and possible parenteral treatment in case of clinical mastitis. The following subjects will be discussed:

- AMR levels in pathogens isolated from milk samples
- Possible differences in AMR levels in pathogens recovered before treatment and after treatment: is there a 'treatment effect'?
- Possible differences in AMR development related to administration route (IMM treatment compared to IMM and parenteral treatment combined)
- Possible differences in AMR development related to the type of antibiotic that was used.

As the probability of finding major pathogens in the same quarter before *and* after treatment might not be very high, we will focus on both major pathogens and CNS. CNS are often considered as commensals, and therefore might be recovered more often. Because of exposure to antibiotics during mastitis treatment, CNS found after treatment might show higher levels of AMR, or possibly a shift could occur in CNS subspecies that recolonize the teat skin. They might function as AMR sentinels or even as AMR gene reservoirs. Therefore, possible differences in CNS characteristics pre- and post-treatment will be analyzed.

1.1 Materials and methods

Sample collection

Eleven different dairy farmers were asked to sample each mastitis cow from October 2014 to June 2015. Each clinical mastitis cow that was treated with antibiotics, was sampled aseptically twice at two different times: two samples before the cow was treated with antibiotics and two samples after the milk withdrawal period. At each sampling moment, either the two rear or the two front quarters were sampled: the mastitis quarter and its healthy mirror quarter (the latter is the within-cow control sample). All obtained samples were stored at -18°C until collected, and then transferred to -80°C until further analysis. Additionally, the farmer was asked to record the disease and treatment history of the participating cows.

Herds

The selected herds have a conventional milking system and were regular dairy herds. They were selected for another study, based on their claw health statuses: the expected prevalence of claw disorders on the participating farms lies above the average prevalence on Dutch dairy farms. Other selection criteria were a size of 60 to 90 cows (which excludes smaller and larger farms) and the location: most herds are located in the eastern part of the Netherlands (Overijssel and Gelderland).

Treatment

Quarters affected by clinical mastitis will be treated according to the obligatory farmer's own herd treatment plan, as discussed with their veterinary practitioner. This plan indicates which antibiotics should be used in case of clinical mastitis and other diseases. This indication is based on the farm-specific situation, to achieve optimal treatment results and prudent antibiotic use.

Sample analysis

On all obtained samples, bacteriological culturing was performed, based on National Mastitis Council recommendations (NMC, 1999). 10 µl of milk was plated out on sheep blood agar (bioTRADING, Mijdrecht, the Netherlands). Afterwards, these agar plates were incubated aerobically at 37 °C. Eventual growth was examined after 24 and 48 hours. Identification of obtained isolates was performed by matrix-assisted laser desoption/ionization time-of-flight mass spectrometry (MALDI-TOF; Bruker Daltonics, Bremen, Germany). Somatic cell count of each milk sample was determined by SomaScope LFC 600 HP(Delta Instruments, Drachten, the Netherlands) based on the flowspectometry method.

The identified major pathogens as well as CNS were tested for susceptibility to various antibiotics. MIC-values were determined for the most common antibiotics by the broth microdilution method (Wellinghausen et al., 2007). The panel for gram-positive bacteria contained penicillin, oxacillin, clindamycin, erythromycin, neomycin, kanamycin, streptomycin and trimethoprim/sulfamethoxazole (**TMP/S**). For gram-negative bacteria, ampicillin, cefotaxim, marbofloxacin, neomycin, kanamycin, streptomycin and TMP/S were tested.

Depending on the isolate, 54,5-218 µl of a 0,5 McFarland suspension was mixed with 12 ml of MH II bouillon (bioTRADING, Mijdrecht, the Netherlands). This suspension was incubated in MIC-determination panels for gram-negative and gram-positive bacteria (Micronaut panel E1-061-200 and panel E1-062-200 respectively, Merlin, Bornheim-Hesel, Germany) at 35 °C for 21 ± 3 hours. MIC-determination was performed by standardized reading of these plates (Micronaut Skan, Merlin, Bornheim-Hesel, Germany). Breakpoints, based on criteria from Clinical Laboratory Standards Institute (**CLSI**), were used to categorize bacteria into susceptible and non-susceptible isolates (the latter group containing both 'intermediate' and 'resistant' isolates). MIC-dilution ranges and corresponding clinical breakpoints for various isolates are shown in **table 1.1**. When comparing MIC-values from isolates, a one-step shift in MIC-values is considered lab variation, especially when dealing with a small number of isolates.

Table 1.1 Antimicrobial concentration ranges and MIC-breakpoints (based on CLSI data) used for Gram-positive and Gram-negative organisms, to categorize susceptible and non-susceptible isolates.

Gram-positive organisms		**Clinical breakpoints**			
Antimicrobial agent	**Concentration range**	***S. aureus***	**CNS**	***S. uberis***	***S. dysgalactiae***
Clindamycin	0,125 - 4	≥1	≥1	≥1	≥1
Erythromycin	0,25 - 8	≥1	≥1	≥0,5	≥0,5
Kanamycin	1 - 64	≥32	≥32	≥32	≥32
Neomycin	2 - 16	≥16	≥16	≥16	≥16
Oxacillin	0,25 - 8	≥2	≥0,5	n.a.	n.a.
Penicillin G	0,0625 - 4	≥0,25	≥0,25	≥0,25	≥0,25
Streptomycin	2 - 32	≥16	≥16	n.a.	n.a.
Trimethoprim/sulfam.	0,125/2,375 - 4/76	≥4/76	≥4/76	≥4/76	≥4/76

Gram-negative organisms		
Antimicrobial agent	**MIC dilution range**	***E. coli***
Ampicillin	0,5 - 64	≥16
Cefotaxim	0,03125 - 4	≥2
Kanamycin	1 - 64	≥32
Marbofloxacin	0,125 - 4	≥2
Neomycin	1 - 32	≥16
Streptomycin	2 - 64	≥16
Trimethoprim/sulfamethoxazole	0,25/4,75 - 8/152	≥4/76

Processing results: definitions and statistical analysis

Analysis was performed at the quarter level. Clinical mastitis cases that recurred within 14 days were considered as one case if the same quarter was affected, and as a new case if another quarter was affected.

Statistical analysis, due to the limited number of positive samples, was limited to descriptive statistics. To compare different groups, MIC-50 and MIC-90 were used. The MIC-50 is the MIC where 50% of the isolates are inhibited, whereas the MIC-90 inhibits 90% of the isolates.

1.2 Results

General description

In total, 83 unique cows were studied. Nine cows, however, did not meet our inclusion criteria (due to receiving no (five) or unknown (four) antimicrobial treatment), and were excluded from further analysis. The 74 cows that were left had 90 cases of mastitis in a total of 96 quarters. 14 of these cows had multiple mastitis cases, and eight cows had multiple affected quarters at the same time. In the milk samples of these clinical quarters, *Streptococcus uberis* and *Staphylococcus aureus* were the most frequently found major pathogens (**table 1.2**). 'Other' isolated micro-organisms include *Serratia* spp., *Pasteurella multocida*, *Enterococcus* spp., *Corynebacterium* spp., *Trueperella pyogenes*, *Bacillus* spp. and yeasts. All data are presented in table 1.2, irrespective of whether or not all samples intended were actually collected.

Table 1.2 Results of bacteriological culturing of milk samples from quarters with clinical mastitis and control quarters, with samples taken both before and after treatment. Only data from cows that met our inclusion criteria are shown (displayed as % (n)).

Group	**Mastitis quarters**		**Control quarters**		**Total**
Sampling moment	Before	After	Before	After	
Number of quarters	96	72	81	63	312
Bacteriologically positive quarters	88.5 (85)	37.5 (27)	28.4 (23)	14.3 (9)	46.1 (144)
No growth	9.4 (9)	43.1 (31)	40.7 (33)	58.7 (37)	35.3 (110)
Contaminated samples	2.1 (2)	19.4 (14)	30.9 (25)	27.0 (17)	18.6 (58)
# *S. aureus*	20.8 (20)	16.7 (12)	7.4 (6)	0	12.2 (38)
# CNS	15.6 (15)	11.1 (8)	12.3 (10)	19.0 (12)	14.4 (45)
# *S. uberis*	29.2 (28)	9.7 (7)	2.5 (2)	0	11.9 (37)
# *S. dysgalactiae*	7.3 (7)	0	0	0	2.2 (7)
# *E. coli*	19.8 (19)	4.2 (3)	0	0	7.1 (22)
# Other	9.4 (9)	4.2 (3)	9.9 (8)	11.1 (7)	8.7 (27)
Total isolates (n)	98	33	26	19	176

MIC-overview

Overall AMR levels of major pathogens and CNS are summarized in **table 1.3**. Clinical breakpoints obtained from CLSI were used to differentiate between susceptible and non-susceptible isolates (the latter containing both intermediate and resistant isolates). Six multiresistant *S. aureus* strains were isolated, that were often resistant against clindamycin, erythromycin, kanamycin, penicillin, oxacillin and sometimes TMP/S. These six multiresistant strains were all obtained from four cows from one farm, and contributed significantly to the AMR levels that are shown in table 1.3. In CNS, erythromycin resistance stands out, followed by β-lactam and clindamycin resistance. In streptococci, macrolide resistance is very high (especially in *S. uberis*), but all isolates are fully

susceptible to penicillin. All *E. coli* are fully susceptible to the antibiotics tested, except for two TMP/S resistant isolates.

Table 1.3 Percentages of non-susceptible isolates for various antibiotics, recovered from 312 milk samples. The data in this table are purely based on obtained MIC-values; no expert rules or additional test results were processed in the displayed data (for instance, oxacillin resistant CNS are obviously penicillin resistant as well). An empty spot means the antibiotic was not tested.

	S. aureus	CNS	*S. uberis*	*S. dysgalactiae*	*E. coli*
	n=38	n=45	n=37	n=7	n=22
Penicillin	23,7	11,1	0	0	
Oxacillin	15,8	33,3	-	-	
Clindamycin	10,5	15,6	10,8	14,3	
Erythromycin	28,9	53,3	8,1	0	
Kanamycin	10,5	4,4	94,6	28,6	0
Neomycin	0	2,2	100,0	42,9	0
Streptomycin	0	2,2	-	-	0
Trim./sulfon.	5,3	4,4	0	0	9,1
Marbofloxacin					0
Ampicillin					0
Cefotaxime					0

MIC-50 and MIC-90 distributions of isolates before and after treatment are shown in **table 1.4** (CM quarters) and **table 1.5** (control quarters). The full MIC-distribution of all isolates can be found in **appendix 1**. Again, only data from isolates of cows that met the inclusion criteria are shown. Of one CNS (in a control quarter from the 'after' group), no MIC-values could be determined due to lack of growth during AST, which explains the difference between numbers of CNS in table 1.2 and table 3.

When comparing MIC-50/90 values of isolates from CM quarters, obtained before and after treatment, increasing values are found for *S. aureus*. Although penicillin and neomycin values do not change, MIC-50 values for oxacillin and MIC-90 values for oxacillin, clindamycin, erythromycin, kanamycin, streptomycin and TMP/S increase substantially. No decreasing values were observed. MIC-50/90 values for CNS are very variable. Decreasing MIC-90 values are found for penicillin, oxacillin, clindamycin, erythromycin and streptomycin, whereas MIC-50 values increased for clindamycin and erythromycin.
In *S. uberis* isolates, little change was observed, as only the MIC-50 for oxacillin and MIC-90 for clindamycin decreased. Other values were the same before and after treatment. *E. coli* values also were unaltered, except for a small increase in MIC-90 for cefotaxime.

In the control quarters, no major pathogens were found in the samples taken after treatment. For CNS, MIC-90 values for penicillin, oxacillin, clindamycin and TMP/S increased.

A possible treatment effect can only be assessed for CNS. No major pathogens were found in the control quarters after treatment, so differences in MIC-values due to treatment cannot be assessed. For CNS in the CM group, the MIC-90 for penicillin, erythromycin, streptomycin and TMP/S were lower as compared to the CNS in the control group, as well as the MIC-50 for clindamycin.

Table 1.4 MIC-50 and MIC-90 distributions of bacteria isolated from clinical mastitis quarters before and after antimicrobial treatment.

Isolate		***S. aureus***		**CNS**		***S. uberis***		***S. dysgalactiae***		***E. coli***	
Sampling moment		Before (n=20)	After (n=12)	Before (n=15)	After (n=9)	Before (n=28)	After (n=7)	Before (n=7)	After	Before (n=19)	After (n=3)
Penicillin	MIC-50	<=0,0625	<=0,0625	<=0,0625	<=0,0625	<=0,0625	<=0,0625	<=0,0625			
	MIC -90	>4	>4	0,5	0,125	0,125	0,125	<=0,0625			
Ampicillin	MIC-50									2	2
	MIC -90									4	4
Oxacillin	MIC-50	<=0,25	1	<=0,25	<=0,25	1	<=0,25	<=0,25			
	MIC -90	1	>8	1	0,5	1	1	<=0,25			
Cefotaxim	MIC-50									0,0625	0,0625
	MIC -90									0,125	0,25
Clindamycin	MIC-50	<=0,125	<=0,125	<=0,125	0,25	<=0,125	<=0,125	<=0,125			
	MIC -90	<=0,125	>4	2	1	4	<=0,125	<=0,125			
Erythromycin	MIC-50	0,5	0,5	0,5	1	<=0,25	<=0,25	<=0,25			
	MIC -90	1	>8	>8	8	<=0,25	<=0,25	<=0,25			
Kanamycin	MIC-50	2	2	<=1	<=1	64	64	16		2	2
	MIC -90	4	>64	<=1	<=1	>64	64	>64		4	4
Neomycin	MIC-50	<=2	<=2	<=2	<=2	>16	>16	8		<=1	<=1
	MIC -90	<=2	<=2	<=2	<=2	>16	>16	>16		<=1	<=1
Streptomycin	MIC-50	4	4	<=2	<=2	>32	>32	8		4	4
	MIC -90	4	8	4	<=2	>32	>32	>32		8	8
Trimethoprim/	MIC-50	<=0,125/2,375	<=0,125/2,375	<=0,125/2,375	<=0,125/2,375	<=0,125/2,375	<=0,125/2,375	<=0,125/2,375		<=0,125/2,375	<=0,125/2,375
sulfamethoxazole	MIC -90	<=0,125/2,375	>4.76	<=0,125/2,375	0,25/4.75	<=0,125/2,375	<=0,125/2,375	<=0,125/2,375		<=0,125/2,375	<=0,125/2,375
Marbofloxacin	MIC-50									<=0,125	<=0,125
	MIC -90									<=0,125	<=0,125

Table 1.5 MIC-50 and MIC-90 distributions of bacteria isolated from control quarters before and after antimicrobial treatment.

Isolate		***S. aureus***		**CNS**		***S. uberis***	
Sampling moment		Before (n=6)	After	Before (n=9)	After (n=11)	Before (n=2)	After
Penicillin	MIC-50	<=0,0625		<=0,0625	<=0,0625	<=0,0625	
	MIC -90	<=0,0625		<=0,0625	>4	<=0,0625	
Oxacillin	MIC-50	<=0,25		<=0,25	<=0,25	<=0,25	
	MIC -90	1		<=0,25	0,5	1	
Clindamycin	MIC-50	<=0,125		<=0,125	<=0,125	<=0,125	
	MIC -90	<=0,125		0,25	1	<=0,125	
Erythromycin	MIC-50	0,5		1	1	<=0,25	
	MIC -90	0,5		>8	>8	<=0,25	
Kanamycin	MIC-50	<=1		<=1	<=1	64	
	MIC -90	2		<=1	<=1	64	
Neomycin	MIC-50	<=2		<=2	<=2	>16	
	MIC -90	<=2		<=2	<=2	>16	
Streptomycin	MIC-50	4		<=2	<=2	>32	
	MIC -90	4		4	4	>32	
Trimethoprim/	MIC-50	<=0,125/2,375		<=0,125/2,375	<=0,125/2,375	<=0,125/2,375	
sulfamethoxazole	MIC -90	<=0,125/2,375		<=0,125/2,375	4/76	<=0,125/2,375	

Persistent infections

In 19 CM quarters, the same pathogen was found both before and after AMT. These data are presented in **Appendix 2**. *Staphylococcus aureus* was found in seven quarters, *S. uberis* in six quarters, and *E. coli* in three quarters. Regarding CNS presence in the affected quarters, CNS was isolated twice before as well as after treatment, although not exactly the same strain. Once, *S. hominis* was found after treating a quarter in which *S. chromogenes* was isolated before, and once S. xylosus was isolated after treatment of a quarter which yielded *S. epidermidis* before treatment. When taking the one-step lab variation into account, little shift in MIC-values occurred. MIC's of one *S. aureus* increased for oxacillin, clindamycin, erythromycin, TMPS/S and kanamycin. In *S. uberis* isolates, kanamycin MIC's both increased and decreased once, and in another isolate the MIC of TMP/S increased.

When looking at the results of cows that were excluded from the study, three persistent infections were found. These all came from clinical mastitis quarters that were not treated with antibiotics. One quarter yielded *K. pneumoniae* twice, another quarter *S. dysgalactiae* and in a third quarter *S. simulans* was isolated both before and after treatment. These isolates are marked with an asterisk (*) in table 4 and 5. Although these isolates were not treated with antibiotics, an increase in TMP/S MIC value for *S. simulans* (see table 4) and a decrease in MIC-value for cefotaxim in *K. pneumoniae* (see table 5) were found.

In the control quarters, no persistent infections with major pathogens were found. Regarding CNS, *S. capitis* was once isolated from the same quarter before *and* after treatment, and *S. hyicus* in another. These data are also shown in **Appendix 2**. No shift in MIC-values seemed to occur. Also, in three quarters persistent infections with *Corynebacterium bovis* were found.

Treatment and drug type

Overall, 96 clinical mastitis cases were treated with antibiotics; intramammary antibiotics were used in all 96 cases. Most commonly used was the combination of amoxicillin/clavulanic acid (Avuloxil ®) (n=55), followed by a cefalexin/kanamycin combination (Ubrolexin ®) (n=33). Occasionally, a lincomycin/neomycin combination (Albiotic ®) was used (n=2). In the remaining cases, a combination of multiple injectors was used (n=6).

Additional parenteral antimicrobial treatment was used in 47 out of 96 cases. TMP/S was the most commonly used drug type (n=30), followed by tylosin (n=11) and penethamate (n=3). Combinations of multiple parenteral antibiotics occurred as well (n=3). There was a remarkable difference between farmers regarding the frequency of using additional parenteral treatment, varying from 0% to 84% of their cases. Parenteral treatment was not used as an antimicrobial treatment as such, without intramammary treatment.

Regarding supportive treatment, most commonly used were anti-inflammatory drugs (NSAID, corticosteroids), in 24 out of 96 cases. Other, less commonly used therapies comprise propylene glycol, vitamin E/selenium, calcium bolus, pyrogenium and udder mint cream.

Unfortunately, subdividing isolates based on the application route or based on the drug type that was used, yielded such small sample sizes that no tendencies could be observed. Therefore, data are not shown.

1.3 Discussion

General description

In the current study, *S. aureus*, *S. uberis* and *E. coli* were the most isolated major pathogens. CNS were also often isolated, but CFU/ml were considerably lower compared to quarters positive for major pathogens. CNS were often isolated in quantities around 100 CFU/ml, whereas quantities of major pathogens such as *S. aureus* and *S. uberis* often exceeded 1000 or even 10.000 CFU/ml. To distinguish between 'real' CNS IMI and transient infections or contamination, often, higher thresholds (i.e. ≥ 300 cfu/ml) or consecutive positive samples are used as criteria (Oliveira et al., 2013). In this study, all isolated CNS in quantities ≥ 100 cfu/ml were included, since also transient CNS could be exposed to the treatment and can carry resistance genes as well.

AMR levels in streptococci and *E. coli* for the most commonly used antibiotics are low. β-lactam antibiotics remain very effective for these pathogens. In CNS, AMR levels are higher, especially for β-lactam antibiotics and erythromycin (33% and 53%, respectively). Although based on MIC-values penicillin resistance in CNS seems lower than oxacillin resistance, this is unlikely as isolates that are oxacillin-resistant due to, for example, *mecA* gene presence are penicillin-resistant as well (Brakstad and Mæland, 1997). Thus, we have to realize that the percentage of penicillin resistance is probably underestimated. Additional tests (such as Nitrocefin-testing or cefoxitin disk diffusion testing) and expert rules may increase the accuracy of susceptibility (EUCAST, 2015). Such data are available, but were not processed in the data in this study, because the focus of this study was on (changes in) MIC-values and not on clinical treatment success rate. Furthermore, the AMR levels for CNS, streptococci and *E. coli* from this study are similar to AMR data available from GD Animal Health (GD, 2015), who monitors AMR levels in isolates cultured from milk samples acquired from all over the Netherlands.

AMR levels in *S. aureus* are much higher compared to the monitoring data. This can be explained by the presence of the multiresistant strains in our study; if these isolates are excluded, AMR levels (9% non-susceptible for penicillin and 19% for erythromycin) come close to the available monitoring data (GD, 2015). The cure rate of a *S. aureus* IMI is limited; the pathogen is often still isolated after treatment, in our data in at least eight out of 22 cases. To indicate a quarter as cured, ideally multiple milk samples should be analyzed, because of the intermittent shedding pattern of *S. aureus* (Zadoks et al., 2002). This could mean that more than those eight *S. aureus* IMI are not actually cured. The intermittent shedding pattern may explain the surprising finding that quite a few quarters (n=5) tested positive for *S. aureus* after treatment, while being negative beforehand. Also, all control quarters that tested positive before treatment (n=6), tested negative afterwards, although no IMM

treatment was used in those quarters. Three of those cows were, however, additionally treated parenterally for CM in the mirroring quarter, which may have had a curing effect as well.

Comparison of MIC-50 and MIC-90 values of different groups of isolates

When all pre-treatment isolates are compared to all post-treatment isolates, numbers of isolates in the CM quarters vary between one and 30 isolates, and in the control quarters between zero and ten. Usually, to obtain useful MIC-50 and MIC-90 data, more isolates are required (Schwarz et al., 2010), since, in the case of low numbers, single isolates have a strong impact on final results.

In this study, the isolated *S. aureus* show very variable AMR patterns, with a tendency to increasing MIC-values for most antibiotics. The presence of the multiresistant *S. aureus* has a large share in this finding: the two multiresistant *S. aureus* (out of a total of 20 isolates) that were isolated before treatment are not included in the MIC-90-value, whereas the four (out of 12) multiresistant *S. aureus* recovered after treatment strongly influence that value. All multiresistant, MRSA suspected isolates were obtained from one dairy farm. This seems to be consistent with the current idea that in most herds one predominant strain of *S. aureus* is present, that may be accompanied with several other strains (Cremonesi et al., 2014). Such findings confirm the need for regular bacteriological culturing of milk samples, and thereafter sensitivity testing, since many of the most commonly used antibiotics to treat CM do not work against these multiresistant *S. aureus* infections. Note that in this study nine (of which six were multiresistant) out of 38 *S. aureus* isolates were submitted by one particular farm. Hence, this farm might have signiticantly influenced the results.

CNS were isolated in at least 50 out of 346 quarter milk samples. Usually, CNS show high resistance against multiple antibiotics, especially β-lactams (GD, 2015). However, variation between subspecies is high (Sampimon et al., 2011). In combination with low numbers of isolates, very variable results can occur. This may explain, for example, the unexpected shifts in MIC-90 values for oxacillin and penicillin in the treated (decrease) and control (increase) group, despite most quarters were, and were not, treated with some β-lactam antibiotic, respectively. The total amount of CNS that were isolated was, unfortunately, lower than expected at the start of the study.

Although the number of isolated streptococci and *E. coli* from samples after treatment was low, MIC-50/90 values were constant or even tended to decrease. Combined with the low overall AMR levels found in these isolates in this study and in monitoring data (GD, 2015), there seems to be no indication of emerging resistance in these pathogens due to antibiotics use.

Unfortunately, analysis for possible effects of application route or the drug type yielded such small sample sizes per organism that MIC-data were very variable and no tendencies could be observed.

Persistent infections

Ideally, the pathogens that that caused the clinical mastitis are absent after a successful treatment. Then, in theory, no AMR can develop in the concerned pathogen. However, a successful treatment is not always achieved, due to various reasons, such as biofilm production, intracellular hiding or AMR (Barkema et al., 2006). In this case, the specific pathogen has been exposed to antibiotics, and is still present in the udder afterwards. Such persistent infections were found in 16 out of 96 CM quarters in the current study. If bacteria are still present in spite of treatment, AMR may develop in the mastitis pathogens itself, due to treatment of CM.

In one out of seven evaluated *S. aureus* strains, multiresistance developed (the shaded SAU, appendix 2.1). After treatment, one multiresistant strain was cultured. But, coincidentally, a similar multiresistant *S. aureus* had been isolated from the same quarter during an earlier case of clinical mastitis. It is unlikely that such extreme change in resistance is due to mutations of a single strain; a co-infection with two (or more) strains is more likely (Martinez and Baquero, 2000, Schultze, 1983). Because only one out of many colonies that were cultured on agar was tested for MIC's, presence of

this resistant strain may have been missed in the sample containing the susceptible isolate. This indicates that reisolation of bacteria per se is only weak evidence for persisting infections, since reinfection or recolonization can also occur. Genotyping of isolated strains can partly solve this problem, but this was not performed in the current study.

Overall, if we disregard the one-step lab-variation in MIC-values, little change in MIC-values in the pairs of isolates was found. And, if looking at possibly relevant changes, there seem to be as much increasing as decreasing MIC-levels. This could indicate infection with multiple strains at once, but also a dynamic situation around the expression of AMR genes in isolates, or influences due to the unavoidable lab variation. This is supported by the fact that also MIC-variation has been found in the isolates that were not exposed to antibiotics.

Lessons learned

In this study, we tried to investigate the effects of antimicrobial treatment of CM on AMR development in mastitis pathogens and CNS. In the results that were found, no indication of AMR development was found. In the case of the *S. aureus* isolate where AMR development seemed to occur (marked in table 1.6), there are other explanations possible that should not be forgotten. Hence, the results of this study have to be interpreted with care, because of high variability in results. This is mainly due to the variety of isolates that were recovered from the milk samples, which resulted in low numbers of different, individual pathogens. Another concern is the quality and reliability of the samples: although farmers were instructed how to sample milk aseptically, relatively many samples were contaminated, especially after treatment (up to 30%). This obviously reduced the reliability and usefulness of the results for our study. Moreover, some of the obtained background information on sampling, disease and treatment was inaccurate, which may have caused uncertainties in the data. Therefore, for future studies, specific attention should be given to selection and instruction of participating farmers, and it may be necessary to clearly demonstrate and practice aseptic milk sampling instead of providing an instruction form.

Evaluating AMR development at cow level turned out to be very difficult due to high variability in results and low prevalences. To obtain useful results, numerous CM cases would have to be included in the study. Additionally, to study the effect of AMR development in individual cows, only unsuccessful treatments can be evaluated, which is only a part of all treatments applied. Using experimental study designs could help clarifying the effects of unsuccessful treatments, but those results do not directly reflect the situation in practice.

To evaluate actual AMR development in CM pathogens in practice, herd level data or even national level data are more reflecting the situation. For example, monitoring AMR in several herds for a longer period of time (Erskine et al., 2002), such as a few years or even decades could be related to the use of specific antibiotics or different ways of treatment. These observational studies will provide risk factors on herd level, rather than causal relationships. Additionally, in the Netherlands, only specific antibiotics can be evaluated, as there is a restricted choice in antibiotics for farmers. A real comparison between effects of different drug types therefore will be difficult.

Field studies like the one described in this chapter focus on possible short term consequences of antimicrobial treatment on AMR development. Eventual long term consequences over the years, on herd level or even nationwide, will require a different study design, which ideally consists of monitoring AMR levels of randomly taken mastitis isolates over a prolonged period. Such monitoring data, although not fully random, are available from different sources, such as the GD Animal Health 2007 until present) (GD, 2015) and the Central Veterinary Institute (MARAN-reports; 2002-2008) (CVI, 2009). These (preliminary) data will be discussed in chapter 4.

2 AMR development in bacteria isolated from milk samples in relation to dry cow treatment

In addition to treatment of CM, Dry Cow Treatment (DCT) also accounts for a substantial part of AMU. In the Netherlands, selective DCT is applied to reduce AMU (Scherpenzeel et al., 2014). DCT contains long-acting formulations of antimicrobials, as opposed to the short-acting CM formulations. The prolonged exposure of bacteria to antibiotics leads to higher IMI cure rates compared to treatment during lactation (Royster and Wagner, 2015). A better cure may lead to less AMR development in mastitis pathogens compared to a, from a bacteriological point of view, possibly less effective lactational treatment. Aiming for successful treatment by using optimal therapy dosage and duration are an important part of prudent use of antibiotics (FVE, 2014). After a while, however, concentrations of DCT antimicrobials may drop to subtherapeutic levels due to slow drug release rates (Sun et al., 2004). This might lead to increased selective pressure in commensal flora, such as CNS. For these reasons, DCT is not comparable to lactational treatment regarding AMR development, and was studied separately.

This part of the study will address the same issues regarding AMR development as in chapter one:

- AMR levels of pathogens and CNS isolated from milk samples
- Possible differences in AMR levels in pathogens and CNS that were either exposed or not exposed to DCT: is there a 'treatment effect'?

Since IMM benzathine cloxacillin is the only treatment applied in the cows evaluated in this chapter, differences between different drugs cannot be assessed. The same, obviously, is the case for application route.

2.1 Materials and methods

Sample collection

Ten participating dairy farmers took quarter milk samples from a total of 49 cows that were dried off with antibiotics (benzathine cloxacillin) and also from a total of 30 cows that were dried off without antibiotics. The latter is the control group. The quarter samples were taken aseptically and twice per cow: once short before the cow was dried off, and once post-partum, right after the milk withdrawal time was over. Whether or not cows received DCT, was decided by the farmer and the local veterinarian, based on the guideline 'Selective DCT' of the Royal Dutch Veterinary Association (KNMvD, 2013). The obtained samples were stored at -18°C, and within 14 days shipped to -80°C until they were all collected for analysis. Additionally, the farmer was asked to record disease and treatment history of the participating cows.

Note: the sample size as described in the general introduction (samples of 60 cows that received DCT and samples of 30 control cows), that was initially aimed for, was not fully achieved. There were not enough individual DCT cows available before the submission deadline for this report was due. Also, for practical reasons, some cows were included in the study that had been dried off already, which resulted in missing samples from before drying off. Numbers of available samples are described in table 2.1.

Herds

The ten conventional herds used in this study were selected in another study that was conducted at GD. On average, herds consisted of 110 cows older than two years. The farms were selected based

on the willingness of the farmer to participate and were, for practical reasons, situated in the area of Deventer (the Netherlands).

Sample analysis

Bacteriological culturing was performed on all obtained samples, and the identified major pathogens as well as CNS were tested for susceptibility (MIC-value) against the most common antibiotics by the broth microdilution method. Testing procedures were the same as described in paragraph 1.1.

Processing results: definitions and statistical analysis

Analysis was performed at the quarter level. When comparing MIC-values from isolates, it has to be kept in mind that a one-step shift in MIC-values is considered lab variation. Due to limited numbers of samples, only descriptive statistics are presented. To compare different groups, MIC-50 and MIC-90 were used.

2.2 Results

General description

In total, 405 quarter samples of 79 cows were analyzed. 49 cows were dried off with DCT, and 30 without. Of these 405 milk samples, 132 samples were taken at drying off, and 273 after calving, when the milk withdrawal period was over. The sample distribution over the groups and isolated organisms are summarized in **table 2.1**. Of the quarters before drying off, 81,2% had bacterial growth, against 24,5% of the quarters post calving (of the quarters dried of with DCT, 24,1% was bacteriologically positive post calving, for control quarters without DCT, this was 25,2%). *Corynebacterium* spp. were the most commonly isolated bacteria, followed by CNS and *S. aureus*. Incidentally, *S. uberis* was isolated. Samples at calving seem to be more often contaminated than samples at drying off.

Table 2.1 Isolates in % (n) from quarter milk samples, taken before drying off and post calving, from 30 cows that did not receive DCT (control quarters) or 49 cows that did receive DCT (DCT quarters).

	Quarters of control cows (% (n))		**Quarters of DCT cows (% (n))**		**Total (% (n))**	
	Drying off	Calving	Drying off	Calving	Drying off	Calving
Number of quarters	48	107	84	166	132	273
# *S. aureus*	6,3 (3)	1,9 (2)	2,4 (2)	0	3,8 (5)	0,7 (2)
# CNS	22,9 (11)	6,5 (7)	20,2 (17)	9,6 (16)	20,5 (27)	8,4 (23)
# *S. uberis*	0	0,9 (1)	3,6 (3)	0	2,3 (3)	0,4 (1)
# *Corynebacterium* spp.	41,7 (20)	5,6 (6)	52,4 (44)	0,6 (1)	48,5 (64)	2,6 (7)
# Other	4,2 (2)	0,9 (1)	1,2 (1)	3,0 (5)	2,3 (3)	2,2 (6)
# Total isolates	34	15	68	24	102	39
# Bact. positive	62,5 (30)	14,8 (16)	71,1 (59)	17,5 (29)	67,4 (89)	16,5 (45)
# No growth	35,4 (17)	74,8 (80)	25,0 (21)	75,9 (126)	28,8 (38)	75,5 (206)
# Contaminated	2,1 (1)	10,3 (11)	4,8 (4)	6,6 (11)	3,8 (5)	8,1 (22)

Other isolates were *Enterococcus* spp., other *Streptococcus* spp., *Trueperella pyogenes*, *Citrobacter* spp. and yeasts. Some quarters yielded more than one isolate.

MIC-overview

In **table 2.2**, the percentages of non-susceptible CNS isolates are shown for various antibiotics, based on CLSI clinical breakpoints, as shown in **table 1.1** (chapter 1). Overall resistance against

erythromycin (31%) and β-lactam antibiotics (19% for penicillin and 25% for oxacillin) is most commonly found. The data that are shown are purely based on MIC-values; no expert rules were applied.

Table 2.2 The percentage of non-susceptible CNS isolates are shown for various antibiotics, based on CLSI clinical breakpoints. Isolates are split up between test groups and sampling moment. Trim./sulfom. = Trimethoprim/sulfamethoxazole

	DCT		**Control**		**Total**
	Drying off (n=17)	Calving (n=14)	Drying off (n=11)	Calving (n=6)	
Penicillin	23.5 %	14.3 %	9.1 %	33.3 %	18.8 %
Oxacillin	23.5 %	28.6 %	9.1 %	50.0 %	25.0 %
Clindamycin	11.8 %	0	9.1 %	0	6.3 %
Erythromycin	29.4 %	42.9 %	0	66.7 %	31.3 %
Kanamycin	0	0	0	0	0
Neomycin	0	0	0	0	0
Streptomycin	0	0	0	16.7 %	2.1 %
Trim./sulfom.	0	0	0	0	0

In **table 2.3** and **table 2.4**, the MIC-50 and MIC-90 distributions of isolates from control cows and cows with DCT are shown, respectively. Isolates are split up between pre-drying off (Drying off) and post-calving (Calving) samples. The number of CNS in these tables differs from the number of CNS table 2.1, because some CNS failed to grow during (repeated) sensitivity testing. Especially for *S. aureus* and *S. uberis*, the number of isolates is very low. Full MIC-distributions are added in **appendix 3.**

Table 2.3 The MIC-50 and MIC-90 distributions of isolates recovered from control cows.

		S. aureus		CNS		*S. uberis*	
		Drying off	Calving	Drying off	Calving	Drying off	Calving
	n	**3**	**2**	**11**	**6**	**0**	**1**
Penicillin	MIC-50	<=0,0625	<=0,0625	<=0,0625	0,125		<=0,0625
	MIC-90	1	<=0,0625	0,125	0,25		
Oxacillin	MIC-50	<=0,25	<=0,25	<=0,25	<=0,25		<=0,25
	MIC-90	<=0,25	<=0,25	<=0,25	1		
Clindamycin	MIC-50	<=0,125	<=0,125	<=0,125	0,25		0,25
	MIC-90	<=0,125	<=0,125	<=0,125	0,5		
Erythromycin	MIC-50	0,5	0,5	<=0,25	1		<=0,25
	MIC-90	0,5	0,5	0,5	>8		
Kanamycin	MIC-50	2	<=1	<=1	<=1		>64
	MIC-90	2	2	<=1	<=1		
Neomycin	MIC-50	<=2	<=2	<=2	<=2		>16
	MIC-90	<=2	<=2	<=2	<=2		
Streptomycin	MIC-50	4	<=2	<=2	<=2		>32
	MIC-90	4	4	4	<=2		
Trim. /	MIC-50	<=0,125/2,375	<=0,125/2,375	<=0,125/2,375	<=0,125/2,375		<=0,125/2,375
sulfam.	MIC-90	<=0,125/2,375	<=0,125/2,375	<=0,125/2,375	<=0,125/2,375		

Table 2.4 The MIC-50 and MIC-90 distributions of isolates recovered from DCT cows.

		S. aureus		CNS		*S. uberis*	
		Drying off	Calving	Drying off	Calving	Drying off	Calving
	n	**2**	**0**	**17**	**14**	**3**	**0**
Penicillin	MIC-50	<=0,0625		<=0,0625	<=0,0625	<=0,0625	
	MIC-90	<=0,0625		0,5	0,25	<=0,0625	
Oxacillin	MIC-50	<=0,25		<=0,25	<=0,25	0,5	
	MIC-90	<=0,25		0,5	0,5	1	
Clindamycin	MIC-50	<=0,125		<=0,125	<=0,125	0,25	
	MIC-90	<=0,125		0,5	0,25	>4	
Erythromycin	MIC-50	0,5		0,5	0,5	<=0,25	
	MIC-90	0,5		1	8	<=0,25	
Kanamycin	MIC-50	2		<=1	<=1	16	
	MIC-90	2		<=1	<=1	32	
Neomycin	MIC-50	<=2		<=2	<=2	>16	
	MIC-90	<=2		<=2	<=2	>16	
Streptomycin	MIC-50	8		<=2	<=2	32	
	MIC-90	8		<=2	<=2	>32	
Trim. /	MIC-50	<=0,125/2,375		<=0,125/2,375	<=0,125/2,375	<=0,125/2,375	
sulfam.	MIC-90	<=0,125/2,375		<=0,125/2,375	<=0,125/2,375	<=0,125/2,375	

Since the number of both *S. aureus* and *S. uberis* isolates post calving is very low, only CNS was evaluated. The number of CNS cases, unfortunately, was also too low for definitive conclusions.

In the control group, when comparing isolates before and after drying off, both the MIC-50 and MIC-90 values for penicillin, clindamycin and erythromycin increased. For oxacillin, only the MIC-90 value increased, but the MIC-90 decreased for streptomycin. The increase in the MIC-50 and MIC-90 for erythromycin is most remarkable; a fourfold and eightfold increase, respectively.

In the group that was dried off with benzathine cloxacillin, fewer differences were found. All MIC-50 values were constant. Only the MIC-90 for erythromycin increased, whereas MIC-90 values for penicillin and clindamycin were halved.

To evaluate differences that occur due to DCT, MIC-50 and MIC-90 values of the post-calving DCT and control group can be compared. In general, values of the DCT-group seem to be equal or even lower (MIC-50 for penicillin, erythromycin and clindamycin, and MIC-90 for clindamycin, erythromycin and oxacillin).

Persistent infections

From 25 cows, samples before *and* after DCT were present. In neither the DCT group nor the control group indications for persisting infections were present. No quarters contained similar isolates before and after the dry period.

2.3 Discussion

In this study, major pathogens were seldom isolated from milk samples. And when such isolates were found, they seemed to be fully susceptible, except for one penicillin-resistant *S. aureus*, one clindamycin-resistant *S. uberis* and high resistance against macrolides in *S. uberis* in general. No isolates were recovered from the same quarter before and after the dry period, neither in the DCT group nor in the control group. In CNS, the most commonly found AMR was against β-lactam antibiotics and erythromycin. This is in agreement with monitoring data as reported by GD Animal Health (GD, 2015) and field studies (Sampimon et al., 2011), where especially the high β-lactam resistance and presence of genes such as mecA stand out. Although these CNS are not always

associated with udder pathogenicity (Piessens et al., 2011), we evaluated them as they might function as a reservoir for AMR genes (Rajala-Schultz et al., 2009, Sampimon et al., 2011).

Although quantities are small, there was no indication for increased resistance due to DCT in this study. If isolates from drying off and post calving are compared, small increases and decreases are seen in both the DCT and control group. In the DCT-group, MIC-50 were constant, and MIC-90 either increased (for erythromycin) or decreased (penicillin and clindamycin). Contrary to our expectations, MIC-50/90 values of CNS from the control cows, that received *no* DCT, tended to increase for penicillin, oxacillin (MIC-90 only), erythromycin and clindamycin after the dry period. CNS, however, is a group that consists of various *Stapylococcus* spp. When quantities are low, variability in compositions of the CNS groups in the study can highly affect theMIC-50/90, due to differences in AMR levels in CNS species (Sampimon et al., 2011). Another study, that assessed the effect of DCT on AMR in CNS in the US, therefore used 752 cows (460 CNS isolates), as opposed to 79 cows (48 CNS isolates) in our study (Rajala-Schultz et al., 2009).

When comparing the MIC-50 and MIC-90 values in the post-calving groups, the values of the DCT-group in our study were equal or often lower than the values in control cows. In 1983, Schulze (Schultze, 1983) studied the effect of DCT with procaine penicillin G and dihydrostreptomycin on AMR-levels in major pathogens and CNS. AMR levels in major pathogens were not affected by the DCT; as they already showed resistance before treatment was applied. In persistent *S. epidermidis* IMI (n=20) however, AMR did seem to increase, from 14/20 to 18/20 penicillin resistant isolates post calving., and dihydrostreptomycin from 12/20 to 18/20 resistant isolates. This seems to be in line with the results of Rajala-Schultz et al. (2009) , where the effect of DCT (either benzathine cloxacillin or cephapirin benzathine) was assessed. They found that isolates from 'high risk cows' (cows with a SCC > 200.000 cells/ml or a history of CM during lactation) that received DCT had higher odds of being non-susceptible for oxacilin, penicillin & novobiocin combination and sulfadimethoxine, but not for penicillin itself. In treated low-risk cows, no effect was seen. The results from this study do not support these findings. It might, however, be interesting to re-evaluate the effect of Selective DCT on AMR development in CNS with a larger sample size in the Netherlands, since the 'high risk cows' are the exact cows that are to be treated with antibiotics.

Overall, from our data, no indications for increasing AMR in CNS due to DCT are present. MIC-50 and MIC-90 values both increase and decrease, which may indicate a dynamic situation around AMR presence. However, the size of this study is too small to draw definitive conclusions. Based on the observed AMR levels and other studies, prudent use of antibiotics, for example by using selective DCT, seems to be wise after all.

3 AMR development in *E. coli* isolated from feces samples in relation to dry cow treatment

Intramammary applied antibiotics are intended to work locally, but an effect on bacteria in the intestines cannot be excluded. AMR in these bacteria, especially ESBL-production is of great interest, and because most AB in dairy are used IMM the relation between this type of IMM AB use and ESBL in faecal bacteria is of interest. For other exposure routes, such as oral 'treatment' (for example waste milk feeding in calves), associations with presence of ESBL-*E. coli* have been described already (Brunton et al., 2014). IMM application, however, likely has a different effect, due to the blood-udder barrier. The objective of this study was to investigate if cows that are dried off with DCT develop more AMR (ampicillin resistance or ESBL-production) in fecal *E. coli* than cows that are dried off without DCT.

3.1 Materials and methods

Sample collection

Rectal feces samples were collected by the same ten farmers and from the same cows as described in chapter 2 (feces samples from a total of 49 cows that were dried off with benzathine cloxacillin, and, for control, feces samples from a total of 30 cows that were dried off without antibiotics). Feces sampling occurred twice: once in the week before drying off and once directly after calving. These fecal samples were mixed with an exactly known amount (~1,5 ml) of glycerolpeptone buffer to protect the bacteria during freezing, and then stored at -18°C. Within 14 days the samples were collected and shipped to -80°C (at GD Animal Health, Deventer) without thawing. Additionally, the farmers were asked to record disease and treatment history of the participating cows.

The sample size that was initially aimed for, as described in the general introduction (samples of 60 cows that received DCT and samples of 30 control cows) was not achieved. For practical reasons, some cows were included in the study that had been dried off already. Therefore, some samples from before drying off were missing. Also, not all samples were available at the moment of testing, which is why differences in sample size occurred between the tests for ESBL-presence (n=85) and the determination of ampicillin-resistant proportion of *E. coli* (n=98).

Bacteriological analysis

From the collected feces samples, the proportion of β-lactam resistant bacteria was determined at GD Animal Health, Deventer. ESBL presence was tested at the CVI, Lelystad, according to international standards (DTUFood, 2014). Obtained ESBL-producing *E. coli* were also further subtyped on gene level by micro-array, PCR and sequencing methods.

1. Proportion of ampicillin resistant E. coli

To determine the proportion of ampicillin resistant *E. coli* in the feces samples, about 100 cfu are needed. Therefore, dilutions of the fecal suspension were made. This was done by repeatedly diluting 20 µl of the fecal suspension into 180 µl of pepton saline, until dilutions of 10^{-1} to 10^{-4} were obtained. Then, 100 µl of each dilution (10^{-1} to 10^{-4}) was plated out on MacConkey agar (Oxoid Ltd, Hampshire, United Kingdom) and incubated overnight at 37 °C.

The next day, of each sample, up to 90 different colonies from the MacConkey agar plates that morphologically resembled *E. coli* were each suspended in 100 µl of Mueller-Hinton II broth (bioTRADING, Mijdrecht, the Netherlands) in separate wells of a 96-wells microtitre plate (VWR International B.V., Amsterdam, the Netherlands). For positive growth controls, two reference *E. coli*

were used: *E. coli* ATCC 25922 (no growth on ampicillin plate) and *E. coli* cefotaxime resistant (ctxR) (growth on ampicillin plate). The last two spots were used as a negative growth control.

Using a 96 pins replicator (Genetix Limited, Hampshire, UK), the suspensions were replicated on two Mueller-Hinton agar plates (Central Veterinary Institute, Lelystad, the Netherlands). One of these plates contained 16 mg/L ampicillin, whereas the other did not. These plates were incubated overnight at 37 °C.

On day three, the plates were evaluated visually for growth. The amount of CFU grown on both plates resulted in a percentage of ampicillin resistant *E. coli*.

2. Qualitative ESBL-analysis

To determine whether or not ESBL-producing *E. coli* are present, a swab with a sample of the feces solution was incubated overnight in 10 ml of MH-broth (Remel Inc., Lenexa, USA), at 37 °C. The next day, 10 µl of the enrichment was plated out on both MacConkey agar and MacConkey agar + 1 mg/L Cefotaxime (Central Veterinary Institute, Lelystad, the Netherlands). These plates were incubated overnight at 37 °C. On day 3, growth was evaluated.

Colonies on the MacConkey agar + 1 mg/L cefotaxime that showed the growth characteristics of *E. coli* were confirmed to be *E. coli* by MALDI-TOF. These *E. coli* were further tested and subtyped (see 3.1.4).

3. Semi-quantitative ESBL-analysis

The next step was to quantify the amount of ESBL-producing *E. coli* in the feces samples that were positive in 3.1.2. This was done using the 'running-drop method'. Dilutions up to 10^{-4} were made (as described under 3.1.1). Next, 10 µl of each dilution were pipetted on both TBX-agar and TBX-agar containing 1 mg/L cefotaxime (CVI, Lelystad, the Netherlands) at an angle of 45°. This way, the drop will run down to form a line in which individual CFU can be counted. TBX-agar plates were incubated overnight at 37°C, and CFU were counted the next day. Quantitative information on presence of ESBL-*E. coli* can be deducted from comparing CFU counts from TBX-agar plates with and without cefotaxime.

4. ESBL-typing and sequencing

The ESBL-suspected isolates obtained from the qualitative ESBL-analysis (3.1.2) were further analyzed to determine the mechanism of resistance. This was performed in multiple ways: Broth microdilution test, a micro-array assay and PCR combined with sequencing.

- *A. Broth microdilution test*

Using the broth microdilution method, MIC-values for different antibiotics were determined, whether or not combined with clavulanic acid. The tested antibiotics and dilution ranges are shown in **table 3.1**. Procedures are the same as described under paragraph 1.1, but other MIC-panels (EUVSEC and EUVSEC 2, Sensititre, Trek Diagnostics, Cleveland, OH, USA) were used. The EUVSEC panel contains a general antibiotics suited for *E. coli*, whereas the EUVSEC2 panel specifically differentiates between various presumptive ESBL/AmpC-producing *E. coli (2013/652/EU, 2013)*. Differentiation between ESBL- and AmpC-based was possible based on whether or not clavulanic acid had an effect, as Amp-C-strains will not be inhibited by clavulanic acid.

Table 3.1 Tested antibiotics and dilution ranges for panels EUVSEC and EUVSEC2, respectively (c.a. = clavulanic acid). The Cut-off values shown are based on EUCAST epidemiological cut-off values (ECOFF) (2013/652/EU, 2013)

EUVSEC	
Antibiotics	Dilution range
Ampicillin	1-64
Azithromycin	2-64
Cefotaxime	0,25-4
Ceftazidime	0,5-8
Chloramphenicol	8-128
Ciprofloxacin	0,015-8
Colistin	1-16
Gentamicin	0,5-32
Meropenem	0,03-16
Nalidixic Acid	4-128
Sulfamethoxazole	8-1024
Tetracycline	2-64
Tigecycline	0,25-8
Trimethoprim	0,25-32

EUVSEC2		
Antibiotics	Dilution range	ECOFF
Cefepime	0,06-32	> 0,125
Cefotaxime	0,25-64	> 0,25
Cefotaxime+c.a.	0,06/4-64/4	NA
Cefoxitin	0,5-64	> 8
Ceftazidime	0,25-128	> 0,5
Ceftazidime+c.a.	0,12/4-128/4	NA
Ertapenem	0,015-2	> 0,06
Imipenem	0,12-16	> 0,5
Meropenem	0,03-18	> 0,125
Temocillin	0,5-64	NA

- *B. Micro-array assay*

Secondly, a micro-array assay (CT 101, Check-points, Wageningen, the Netherlands) was performed. Purified DNA (DNeasy Blood & Tissue Kit, Qiagen) from the obtained isolates was multiplied by using PCR-techniques: first DNA ligation, followed by PCR and hybridization and finally detection by placing the micro-array tube in the Check-points Tube Reader (Check-points, Wageningen, the Netherlands). Detection is based on a reaction of hydrogen peroxide reacting with substrate, which can be seen as colored spots (Check-points, 2012). Beta-lactamase genes that were tested for are TEM, CMY, SHV, KPC, NDM-1 and CTX-M1, -M2 and -M9 groups.

- *C. PCR and sequencing*

The third method of identifying resistance genes was sequencing. Using this method, genes can be identified and subtyped very accurately, and eventual mutations can be found as well. To find out which genes were present that had to be sequenced, first, PCR and electrophoresis were used to test the isolates for *ampC*, CTX-M1 *group*, CMY and TEM-genes. These results were combined with the micro-array assay results, to identify the genes which had to be sequenced.

Sequencing was performed by (ABI PRISM 310 Genetic Analyzer, Thermofisher, Foster City, USA), and results were analyzed by Sequencher 5.2.3 (Gene Codes Corporation, Ann Arbor, USA).

Data analysis

Analysis was performed at cow-level. Due to a limited number of samples, only descriptive statistics were used.

3.2 Results

1. Proportion of ampicillin resistant E. coli

To determine the proportion of ampicillin-resistant *E. coli* in the feces samples, the number of colonies on both plates with and without ampicillin were counted. Results from samples before and after calving are shown in **table 3.2**. From before drying off, 19 feces samples were available, against 79 from post calving. Six out of 19 of the samples taken before drying off and 27 out of 79 samples from post calving did not show growth on MacConkey agar. Also, 17 out of 79 samples from post calving yielded ≤10 CFU.

Table 3.2 Percentage ampicillin-resistant *E. coli* from feces samples of dairy cows before **drying off** and post **calving**. The latter group is split up for cows that received **DCT**, and **control** cows, that did not receive DCT.

Resistance	**Drying off - total**		**Calving - total**		**Calving - control**		**Calving - DCT**	
level	%	n (n=19)	%	n (n=79)	%	n (n=28)	%	n (n=51)
0%	84,6	11	34,2	27	25,0	7	39,2	20
1-3%	15,4	2	6,3	5	0,0	0	9,8	5
4-6%	0,0	0	2,5	2	0,0	0	3,9	2
72%	0,0	0	1,3	1	0,0	0	2,0	1
No growth	46,2	6	34,2	27	35,7	10	33,3	17
≤ 10 isolates	0,0	0	21,5	17	39,3	11	11,8	6

When the feces of the DCT-group and control group are compared, ampicillin-resistant isolates are only found in samples from the DCT-group (both before and after drying off).

In the samples from before drying off, two out of seven samples of DCT cows were positive, and 0 out of four control cows. Post-calving, 28 samples were available in the control group, of which seven samples contained >10isolates, 11 samples <10 isolates and ten showed no growth. There were no ampicillin-resistant *E. coli* isolated. In the DCT-group , 51 samples were available, of which 28 samples contained >10isolates, six <10 isolates and 17 had no growth. Ampicillin-resistant isolates were only found in samples that yielded >10isolates (eight out of 28).

From eight cows, both samples were available *and* positive for growth. Three cows were from the control group, and these isolates were all susceptible for ampicillin. In the DCT group, four out of five cows did not carry ampicillin resistant *E. coli* at either time point, but one cow harboured ampicillin-resistant *E. coli* before drying off and post calving (3,3% and 1,1%, respectively). No development of ampicillin-resistance within the dry period in fecal *E. coli* was found.

2. Qualitative ESBL-analysis

Out of all 85 samples tested for ESBL-presence (19 samples before drying off and 66 post calving), only two samples were positive for ESBL-producing *E. coli* (0 out of 19 before drying off and two out of 66 (cow 36 and cow 38) post calving). These two isolates were further analyzed (see 3.2.4.). Of the two ESBL-positive samples, the corresponding fecal samples from before drying off were not available. From 16 cows, both the feces sample before and after treatment were complete, but no indications for ESBL-*E. coli* were found in these samples.

3. Semi-quantitative ESBL-analysis

The running drop tests were performed on dilutions of two samples (36 and 38) to quantify the amount of ESBL-suspected isolates in the feces sample. The test lanes on regular TBX-agar plates (without cefotaxime) were positive for *E. coli* growth. On the TBX-agar plates containing cefotaxime, no growth occurred, even in the lane containing pure solution. This is illustrated in **figure 3.1**.

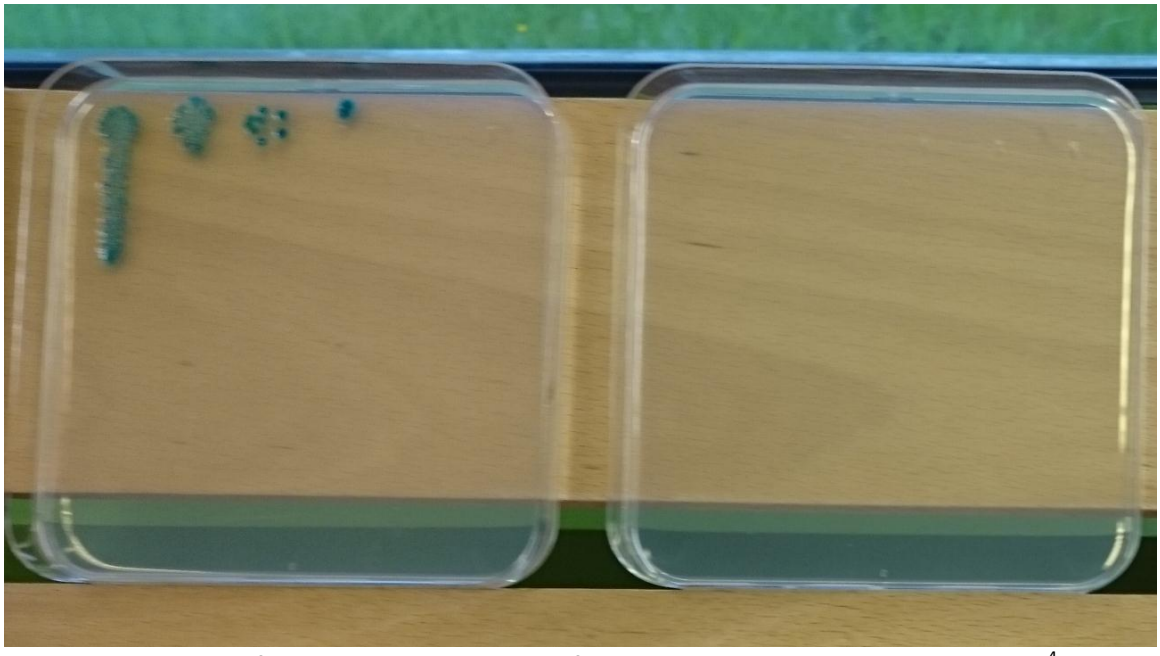


Figure 3.1 Example of the running drop test performed on isolate 38: dilutions up to 10^{-4} were pipetted under an angle of 45 ° on a TBX agar plate (left) and on a TBX agar plate containing 1 mg/L cefotaxime (right). On the right plate, no growth occurred, which means no ESBL/AmpC *E. coli* were found.

4. ESBL-typing and sequencing

The ESBL-suspected isolates from the two samples (36 and 38) were further analyzed to determine their mechanism(s) of resistance by broth microdilution sensitivity testing, micro-array assay and resistance gene sequencing.

- *A. Broth microdilution test*

MIC-distributions of both isolates are shown in **table 3.3** (EUVSEC2 ESBL-differentiation panel). From the resistance patterns shown by the microbouillon dilution test, isolate 36 was identified as a classic ESBL-type and 38 as an AmpC type, since clavulanic acid effectively lowered the MIC for both cefotaxime and ceftazidime in isolate 36, whereas isolate 38 was not affected.

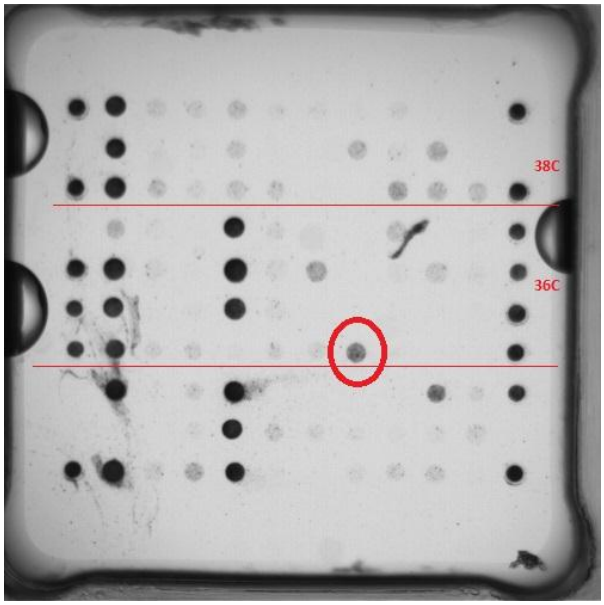
Table 3.3 MIC-distributions of suspected ESBL-producing *E. coli* isolates 36 and 38 for various β-lactam antibiotics by using (ESBL-differentiating) AST panel EUVSEC2 (Sensititre). C.a. = clavulanic acid, NA = not available.

Sample	36 MIC	36 S/I/R	38 MIC	38 S/I/R
Cefepime	8	R	0,12	S
Cefotaxime	**32**	**R**	**2**	**R**
Cefotaxime+c.a.	**<=0.06/4**	**S**	**2/4**	**R**
Cefoxitin	4	R	64	R
Ceftazidime	**1**	**R**	**4**	**R**
Ceftazidime+c.a.	**0.25/4**	**S**	**4/4**	**R**
Ertapenem	<=0.015	S	0,03	S
Imipenem	0,25	S	0,25	S
Meropenem	<=0.03	S	<=0.03	S
Temocillin	4	NA	16	NA

- *B. Micro-array*

The result of the micro-array assay indicated that isolate 36 was a member of the CTX-M1 group. Isolate 38 turned out to be negative for all genes that were tested in the array. Results are shown in **figure 3.2**.

Figure 3.2 Result of the array. The upper panel are the results of isolate 38, whereas the middle panel shows the results of isolate 36. The spot encircled is an indication for the presence of a gene from the CTX-M1 group. The other coloured spots are mainly control spots.



- *C. PCR and sequencing*

Electrophoresis results indicated that isolate 36 was a member of the CTX-M1-group. 38C was negative for the SHV- and CMY-genes. Sequencing classified isolate 36 positive for CTX-M-1 (as part of the CTX-M1 group), whereas isolate 38 was sequenced to look for mutations in the promotor-region of the chromosomally located *ampC*-gene. At the deadline for submitting this report, results of isolate 38 were not available yet due to technical problems.

3.3 Discussion

The aim of this study was to investigate the effect of IMM applied DCT (benzathin cloxacillin) on the development of ampicillin-resistance and/or ESBL-production in *E. coli*. Overall, very little β-lactam-resistance was found in the fecal samples. In 80% of the cows, no indication for ampicillin-resistance was present. In the cows that were positive, ampicillin-resistance varied between 1-6% with one outlier at 72%. This is in agreement with data available from monitoring studies, such as MARAN, who reported an average of ampicillin resistant *E. coli* from slaughtered adult dairy cattle as <4% in 2014 (D.J. Mevius, 2015).
ESBL-producing *E. coli* were scarcely found. 98% of the feces samples were negative. Only two isolates, one CTX-M-1 ESBL *E. coli* and one *AmpC*-positive *E. coli* were found after enrichment culturing. These isolates were not cultured on the quantifying 'running-drop' test, indicating quantities <1000 CFU/g feces, since growth in the lane with undiluted feces suspension would correspond to ~10^3 CFU/g. According to other studies, ESBL-prevalence in adult dairy cattle is low as well. MARAN reported 6% ESBL-prevalence in slaughtered dairy cows in 2014, which was comparable to results from previous years (D.J. Mevius, 2015). Studies in other countries report

slightly higher ESBL-prevalence in dairy cattle (DANMAP, 2011, Schmid et al., 2013), but with great variation between herds (Snow et al., 2012). Compared to adult cows, calves are more likely to carry ESBL *E. coli* (Schmid et al., 2013), and significant associations with the use of 3rd/4th generation cephalosporins are found (Randall et al., 2014). The minimized use of this type of antibiotics in the Netherlands could contribute to the low prevalence of ESBL *E. coli* in Dutch dairy cattle.

Remarkable was that three feces samples showed no growth on MacConkey agar when performing the qualitative and quantitative ESBL-analysis, although they did show growth on regular HIS-agar. Later on, when determining the proportion of ampicillin-resistant isolates, 6/19 drying-off samples and 27/79 calving-samples did not show growth on MacConkey agar, and 17/79 calving samples yielded <10 isolates. This could be due to freezing/thawing of samples: for practical reasons, after performing the qualitative and quantitative analysis, samples had to be frozen again. In spite of the addition of glycerol peptone, which is supposed to protect bacteria during freezing by prohibiting crystallization (Ternent et al., 2004), the viability of bacteria in these samples decreased strongly. This led to loss of results when assessing the ampicillin-resistance percentage in the *E. coli*, due to samples suddenly being growth negative despite being positive for growth before. Moreover, a lot of data from samples before drying off was missing as well, due to the formerly mentioned practical reasons regarding participating cows in the study.

All in all, the amount of missing data turned out to be a lot higher than expected afterwards. Nevertheless, the available data still indicate that, based on these results, β-lactam-resistance in fecal *E. coli* of dairy cattle does not seem to be a problem at the moment, and neither seems to be an emerging problem. Ampicillin-resistance is low, and ESBL-*E. coli* are rarely found, or if present, in very low quantities. These data provide no indications that IMM application of benzathin cloxacillin is a risk factor for β-lactam-resistance development in fecal *E. coli* of dairy cattle. This is in accordance with evidence that cloxacillin resorption in the udder is minimal (Kietzmann et al., 2010). Moreover, given the fact that cloxacillin actually has a narrow antimicrobial spectrum (Bradley et al., 2011) these results can be explained . All *E. coli* are naturally resistant against narrow spectrum β-lactams, and therefore, cloxacillin use will not select for ampicillin-resistant or ESBL / AmpC *E. coli*.

4 Trends in antimicrobial resistance in mastitis pathogens from Dutch dairy cattle over the years

These data were presented as a poster at the XV. Middle European Buiatric Congress / 10th ECBHM symposium in Maribor, Slovenia, June 10th-13th. A PDF-file of the poster will be enclosed as Appendix. The aim of this study was to create an overview of occurrence and trends in antimicrobial resistance (**AMR**) in staphylococci, streptococci and coliforms for the period 2002-2014. The results are still preliminary; data will be further analyzed in the upcoming months, with the intention to be submitted for publication. Topics and questions that require further attention are, for example, the extent of the effect of changes in susceptibility testing methods over the years, addition of other available data (national and international) and discuss the advantages, disadvantages and possible enhancements of the current monitoring system.

Note: due to limited space on the poster, the legend for all figures is just shown at figure 1.

Background

Due to public health concerns about AMR, antibiotic use in Dutch livestock has been reduced. International studies, however, are inconclusive with respect to the effect of (intramammary) antibiotic use on the development of AMR in mastitis pathogens (Oliver and Murinda, 2012). To evaluate the effect of (reduced) antibiotic use on AMR in mastitis pathogens in the Netherlands, AMR has to be monitored.

Materials and Methods

The Dutch monitoring data on AMR in mastitis pathogens from GD Animal Health (**GD**) and Central Veterinary Institute (**CVI**) were combined to create an overview of occurrence and trends in AMR in staphylococci, streptococci and coliforms for the period 2002-2014.

The CVI data (2002-2008) are a result of testing approximately 100 randomly taken isolates of species yearly, delivered by GD and which were send in by farmers and veterinarians for microbiological culturing of milk samples.
The GD data (2007-present) are based on all isolates from the routine milk culturing. The number of tested GD isolates varied between 200 and 2000 per species per year.
Both datasets were combined to create an overview as complete as possible of occurrence and trends in AMR.

Results

For *S. aureus*, AMR levels are low and stable, as is shown in **Fig. 1**. Also, resistance percentages against neomycin, kanamycin, streptomycin and TMP/S never exceeded 4% (data not shown). In 2007-2011, only resistance data of penicillin-resistant *S. aureus* isolates were reported. Therefore, they are not included in **Fig. 1**. In this subpopulation of 2007-2011, resistance against pirlimycin varied between 4-11%, which seems to be higher.

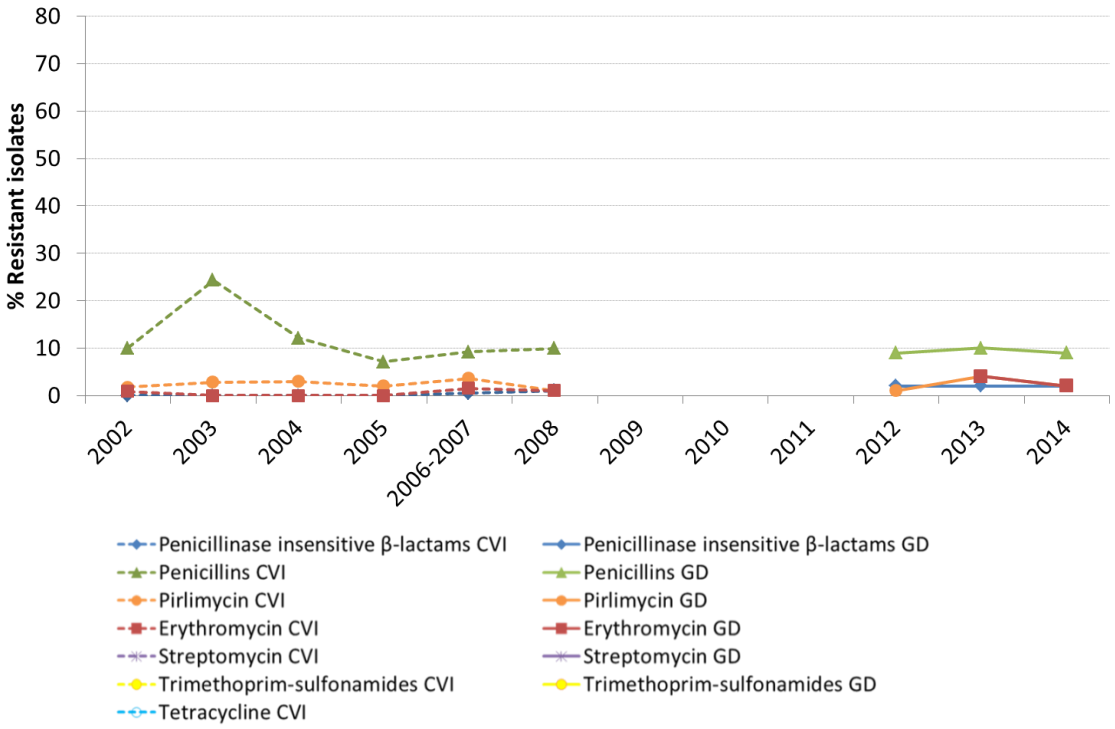


Fig. 1. *Resistance levels over the years in Staphylococcus aureus isolates*

In CNS (**Fig. 2**), resistance for β-lactams seems to decrease, although they vary a lot. The variation in the other antibiotics is limited. Resistance levels of neomycin, kanamycin and TMP/S, which are not shown, varied around 1%.

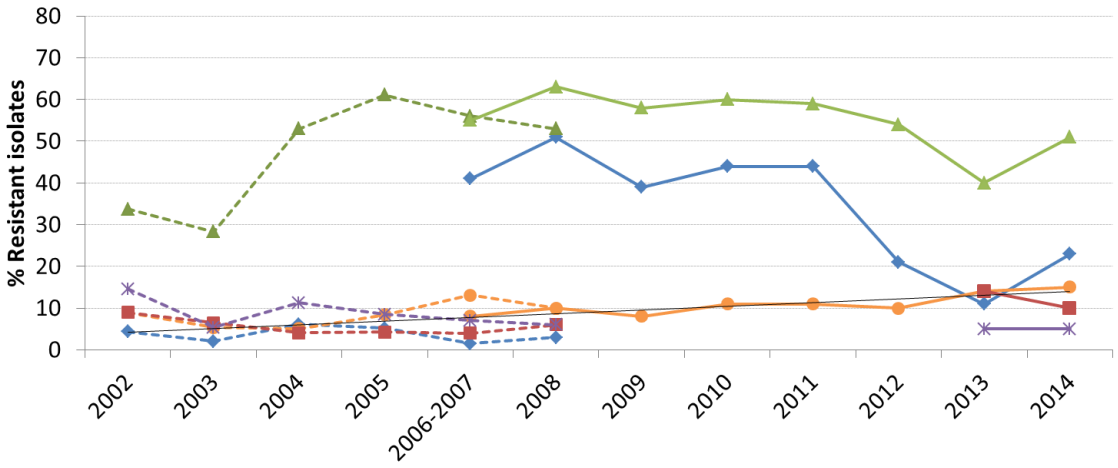


Fig. 2. *Resistance levels over the years in coagulase-negative staphylococci*

For *Streptococcus dysgalactiae* and *Streptococcus uberis*, data are shown in **Fig. 3** and **Fig. 4**, respectively. As shown, resistance against penicillin is very rare. Pirlimycin resistance seems to slightly increase, whereas resistance against tetracycline is invariably high.

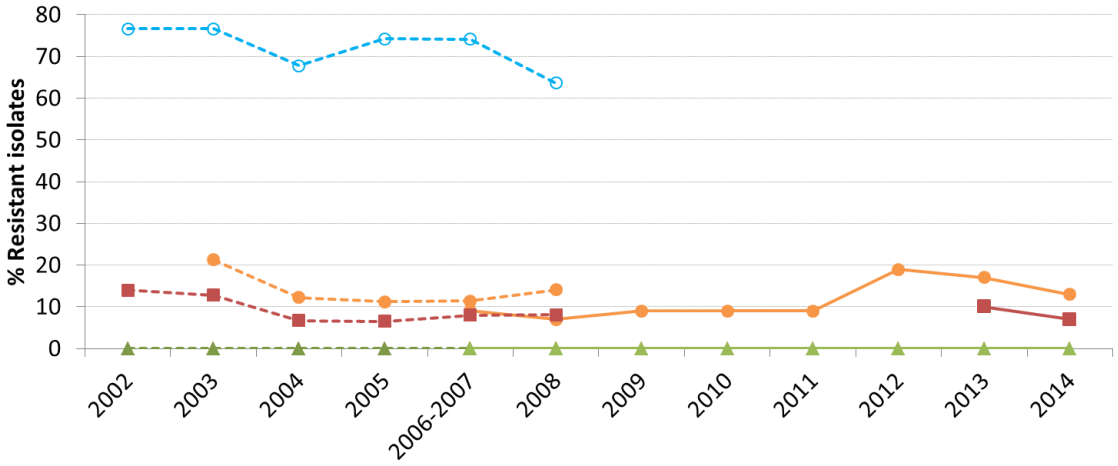


Fig. 3. *Resistance levels over the years in Streptococcus dysgalactiae isolates*

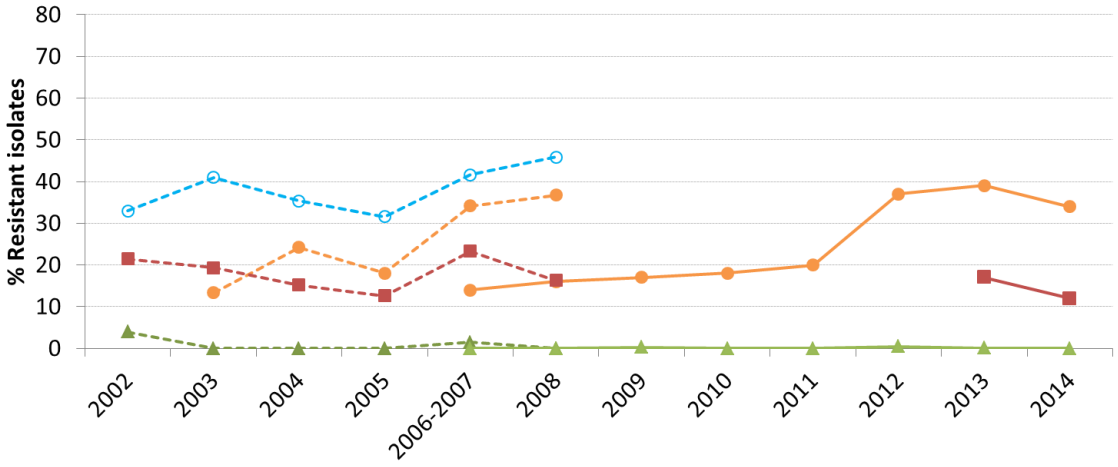


Fig. 4. *Resistance levels over the years in Streptococcus uberis isolates*

For *E. coli* (**Fig. 5**), resistance levels do not vary much over the years. AMR data for *Klebsiella* spp. specifically were only available from GD, and were low for all antibiotics tested (**Fig. 6**). The intrinsic resistance of *Klebsiella* spp. against ampicillin was consequently 100% and therefore not shown. Fluoroquinolone resistance is rarely found in *E. coli* or *Klebsiella* spp. However, since 2007, occasionally a presumptive ESBL-isolate has been isolated from mastitis cases (<1% of all coliforms analyzed).

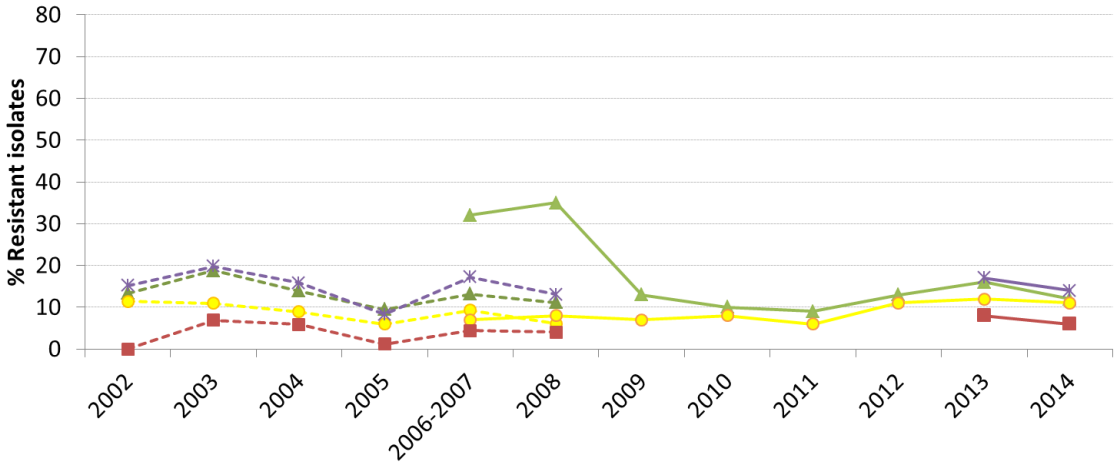


Fig. 5. *Resistance levels over the years in Escherichia coli isolates*

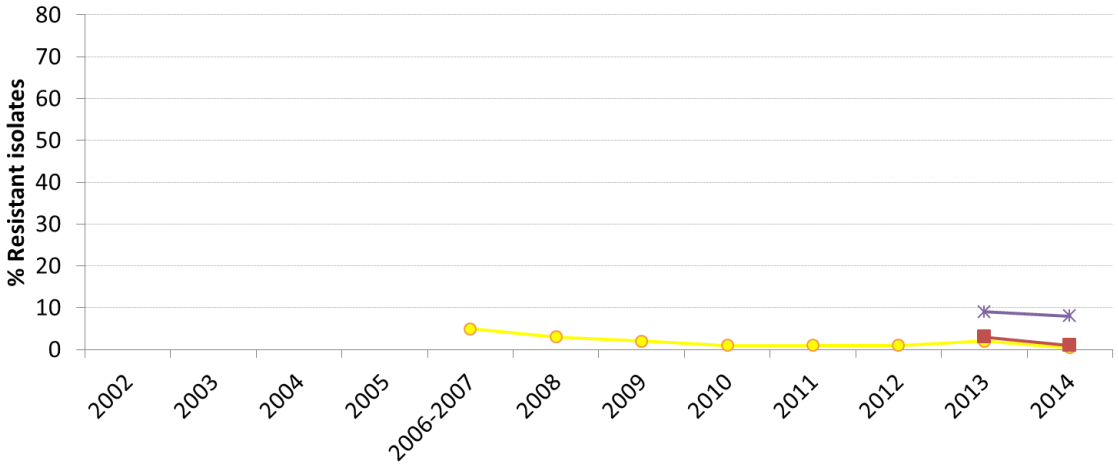


Fig. 6. *Resistance levels over the years in Klebsiella* spp.

Discussion and conclusions

In general, resistance levels seem stable in the long run, despite some year-to-year variation. These variations can partly be explained by a relatively low sample size in the CVI data (n~100). Sample sizes of the GD data were larger. The variability in the results of the CNS is most intriguing. We have to realize however, that CNS is a group of different species.

Possible trends should be interpreted carefully. Progressing insights and changes in testing methods may explain differences in resistance levels that have occurred. An example of this is the classification of penicillin resistance based on *bla*$_z$-presence in CNS in the CVI data, since 2004. This leads to strongly increased resistance levels, as compared to 2002-2003. Changes in testing methods also explain the additional data from GD for certain antibiotics, since 2013.

Obviously, differences in selection of samples and used methods occur between laboratories and in time, which may lead to a certain bias. Nevertheless, AMR in mastitis pathogens seems stable and low over time. In CNS resistance against β-lactams even seems to decrease. Resistance against pirlimycin in CNS and streptococci may need further attention, but it's test results are known to be difficult to interprete.

Monitoring AMR remains important. To avoid bias, however, active monitoring studies would be preferred in the future.

General discussion

The general results of this study provide no indications for AMR development. The data are very variable, and numbers are too low for statistical analysis. Although the study was not designed as a pilot study, the results can be seen as such. To obtain statistically significant results, sample sizes would have to be a lot larger. Unfortunately, there were a lot of missing samples (especially chapter 2 and 3) or contaminated samples (chapter 1). Because we had to fit these projects in ongoing projects within a short time span, we were limited in our study design and implementation. Advantages of combining projects are reduction of costs and time needed, and no extra recruitment. of farmers is needed. Disadvantages are limitations in study design and the risk of asking too much effort from the farmers. Retrospectively, these limitations caused a larger loss of sample than expected at the start of the study.

Due to the many missing samples and low amounts of isolates in the results, in-depth statistical analysis would be difficult and probably in vain. Therefore, it was decided to stick to descriptive statistics, analyse possible trends and focus on possible improvements in the study designs. Overall, this study provided useful insights in the possibilities and concerns of assessing AMR in major and minor udder pathogens in relation to different treatments. Down below, recommendations for future study designs have been made for each of the three studied subjects.

When the AMR development situation in major mastitis pathogens is assessed, comparison of samples before and after treatment has, retrospectively, a few important disadvantages. It would require a very large amount of samples to get sufficient numbers of isolates, which is very pricey. First, the large variety of mastitis-causing pathogens is a problem. Secondly, the isolates that would be compared would come from treated, but non-cured quarters, which is actually a small subpopulation of the total amount of isolated pathogens, since bacteriological cure obviously is the goal of the treatment. This may lead to overestimated or even false conclusions regarding impact. Another concern is the lack of a reliable within-cow control group, since those control quarters are not affected by CM, and, moreover, it cannot be guaranteed that such control quarters are not exposed to antibiotics, especially in the case of parenteral treatment. For major pathogens, a more useful study design would be following 'sentinel' herds over time: monitoring AMR in CM isolates over time, combined with accurate (quarter-level) data on use of specific types of antibiotics and application routes (Pol and Ruegg, 2007, Saini et al., 2012, 2013) . Another possibility is nation-wide monitoring, which is preferably performed by active monitoring studies using randomly selected CM cases (Erskine et al., 2002). These studies, however, are expensive, so data from laboratories that process a sufficient share of the nationwide total could be a useful alternative to monitor trends (GD, 2015). Changes in testing methods during such monitoring studies should be watched carefully, as they could potentially lead to false conclusions if the impact is not properly assessed. To evaluate whether or not fluctuations over the years in AMR levels in isolates from the monitored herds or laboratory data are statistically significant, a two sample test of proportions can be used. With this test, the proportion of AMR between the years are compared, taken into account the number of samples included. An alternative could be a Chi-square test in which the ratio of AMR positive and negative results are compared between different years.

Regarding DCT, to use cloxacillin as a first preference DCT is wise, since it does not select for ampicillin-resistant or ESBL/AmpC *E. coli* in intestinal flora, which our data supported, although this result was not statistically significant. Considering this, retrospectively, a non-inferiority study design would be more suitable, since no effect is expected. A possible future study design would be to compare the proportion of ESBL/AmpC *E. coli* positive cows between the DCT and control groups. Assuming an average of 2% positive cows, with $\alpha = 0.05$, 80% and a 5% non-inferiority limit, every group (DCT and control) should consist of 97 cows (SealedEnvelopeLtd., 2012). This might be a

different situation for DCT containing , for instance, cefquinome, as residues of 3rd/4th generation cefalosporines in waste milk have been associated with increased ESBL *E. coli* prevalence (Randall et al., 2014). Cefquinome, therefore, is considered third preference, and use has been practically nullified in the Netherlands (SDa, 2014b).

Major pathogens isolated from the udder around the dry period seem to play no significant role, since numbers of isolated pathogens are very low, and IMI cure rate is high as well (Bradley et al., 2011). But, when looking at CNS, the situation might be different. CNS are considered as teat skin commensals, and will be exposed prolongedly to (after a while subtherapeutical) levels of DCT antibiotics. Although we didn't find effects in CNS in this study, possibly due to small sample size, data from Rajala-Schultz et al. (2009) reported increased odds for DCT treated 'high risk cows' to have an IMI with a non-susceptible CNS, whereas no effect was found in isolates from treated low-risk cows. High risk cows, in short, were defined as cows with a SCC > 200.000 cells/ml or a history of CM during the last lactation. In the Netherlands, Selective DCT is applied (KNMvD, 2013, Scherpenzeel et al., 2014) to reduce the use of antibiotics, with the intention to decrease the bacterial exposure and thus AMR development. When using Selective DCT, however, the 'high risk cows' are the exact cows that are treated with DCT. Further research is needed to quantify the effect of Selective DCT on AMR development in CNS in the Netherlands. To do so, when using a similar study design, a sample size of at least 200 isolates per group would be recommended, to find a 10% difference in percentages of resistant isolates between groups (for various antibiotics tested) with 95% confidence and 80% power (WinEpi2.0, 2010). If these data are translated to numbers of participating cows, considering the low prevalences of 20% CNS positive quarters at drying off and 8% CNS positive quarters post calving found in the current study, at least 1000 and 2500 quarter milk samples per group would have to be collected, respectively.
Resulting, a logistic regression model with a logit link function and an independent correlation structure can be used to evaluate the effect of the DCT. To adjust for variables such as multiple observations within cows and herds, a generalized estimation equation model can be developed, using a binary distribution, as was described by (Rajala-Schultz et al., 2009). Using this model, the likelihood of an isolate being non-susceptible can be assessed for every antimicrobial that was tested for. In this model, AMR should be included as dependent variable and DCT could, amongst others, be included as independent variable to evaluate the effect of DCT on AMR, represented by Odds ratios. An interesting, additional question would be whether or not increased odds ratios could decrease again after being non-exposed for a while. Therefore, a third sampling moment could be added, some time after calving, to see if CNS AMR patterns have normalized again.

General conclusion

In the three studies that were performed, no indications for AMR development in either major mastitis pathogens, CNS or fecal *E. coli* were found in relation to CM treatment or DCT. General AMR levels of isolates corresponded largely to available monitoring data, and generally remain the same over the years. Recommendations for future research have been made: to reflect the situation in practise, AMR levels in mastitis pathogens ideally should be monitored on herd and national level. Although results from this study did not indicate AMR development in CNS due to DCT, this might have to be reassessed for the Dutch situation and moreover in relation to selective DCT, using a larger sample size.

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Gevolgde cursussen en andere wetenschappelijke activiteiten

Wetenschappelijke activiteiten

- Bezoek en mondelinge presentatie op een meeting van het ESBL-attributieproject
- Bezoek Veterinary Science day 2014, Driebergen
- Bezoek GGL-congres 2014, Doorn
- Deelname aan verscheidene 'mastitis meetings', waar interessant mastitisgerelateerd onderzoek bediscussieerd wordt.
- Bezoek en posterpresentatie op het XV. Middle European Buiatric Congress / 10th ECBHM congress in Maribor, Slovenië

Gevolgde cursussen

- "Modern Methods in Data Analysis", Utrecht University (4,5 EC)
- "Presenting in English" (in de komende maanden)
- "Writing for publication" (in de komende maanden) (3 EC)

Appendices

Appendix 1 Full MIC-distribution of isolates in CM quarters and control quarters, both before and after treatment

Appendix 1.1 Full MIC-distribution of Gram-positive isolates recovered from CM quarters, split up between isolates from before and after quarter treatment with antibiotics. SAU = *S. aureus*, SUB = *S. uberis*, SDY = *S. dysgalactiae.*

			Penicillin								Oxacillin							Clindamycin							Erythromycin							Kanamycin								Neomycin					Streptomycin						Trimethoprim/sulfam.						
Isolate	Sampling moment	n	<=0,0625	0,125	0,25	0,5	1	2	4	>4	<=0,25	0,5	1	2	4	8	>8	<=0,125	0,25	0,5	1	2	4	>4	<=0,25	0,5	1	2	4	8	>8	<=1	2	4	8	16	32	64	>64	<=2	4	8	16	>16	<=2	4	8	16	32	>32	<=0,125/2,375	0,25/4.75	0,5/9,5	1/19	2/38	4/76	>4/76
SAU	Before	20	17							3	12	4	2	1			1	19						1	2	11	6				1	8	9	2					1	20						18	2					19		1				
SAU	After	12	9							3	3	1	5				3	9						3	2	6	1				3	3	6						3	12						8	4					9		1				2
CNS	Before	15	9	4			1			1	9	4	2					9	3	1		2			4	5	1	1			4	14		1						15					11	3	1				15						
CNS	After	9	7	1	1						7	1	1					4	2	1	1	1			3	2		1		2	1	9									9						8		1				8	1					
SUB	Before	28	24	4							11	2	14	1				24					1	3	25						3				2			5	17	4					2	26				1	3	24	26	1	1				
SUB	After	7	6	1							4		3					7							7													3	4						7						7	6		1				
SDY	Before	7	7								7							6						1	7										3	2				2		1	3		3			2	2	1		2	7						
SDY	After	0																																																								

Appendix 1.2 Full MIC-distribution of *E. coli* (ECO) recovered from CM quarters, split up between isolates from before and after quarter treatment with antibiotics.

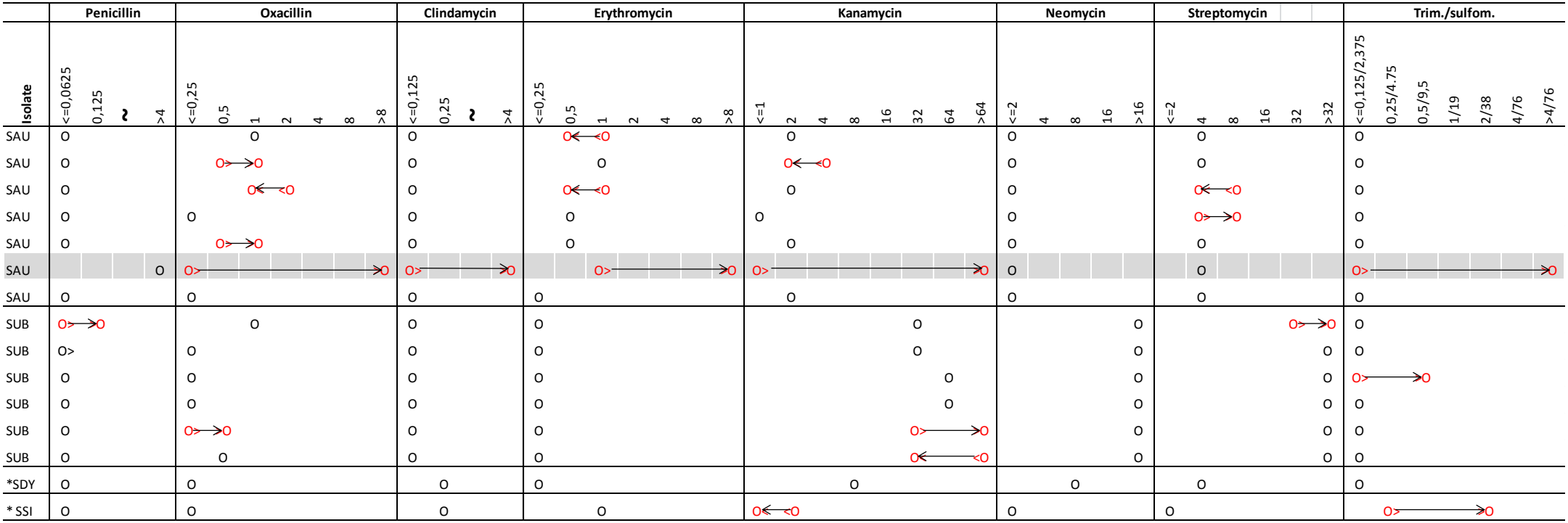
			Ampicillin								Cefotaxim								Marbofloxacin							Kanamycin							Neomycin							Streptomycin							Trimethoprim/sulfam.							
Isolate	Sampling moment	n	<=0,5	1	2	4	8	16	32	64	>64	<=0,03125	0,0625	0,125	0,25	0,5	1	2	4	>4	<=0,125	0,25	0,5	1	2	4	>4	<=1	2	4	8	16	32	64	>64	<=1	2	4	8	16	32	>32	<=2	4	8	16	32	64	>64	<=0,25/4.75	0,5/9,5	1/19	2/38	4/76	8/152	>8/152
ECO	Before	19		3	10	6						1	14	3	1						19							2	12	4	1					18	1						3	13	3					18						1
ECO	After	3		1	1	1							2		1						3								2	1						3							1	1	1					2					1	

Appendix 1.3 Full MIC-distribution of isolates recovered from <u>control</u> quarters, split up between isolates from before and after treatment of the corresponding CM quarter. SAU = *S. aureus*, SUB = *S. uberis*, SDY = *S. dysgalactiae.*

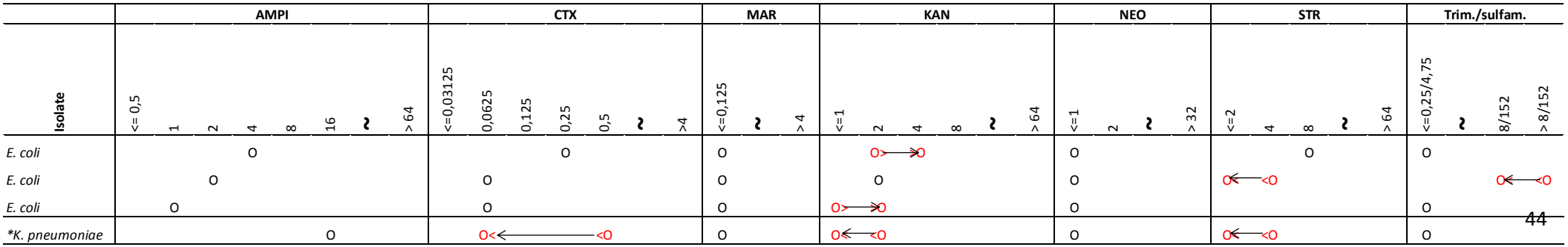
			Penicillin								**Oxacillin**								**Clindamycin**								**Erythromycin**								**Kanamycin**								**Neomycin**								**Streptomycin**								**Trimethoprim/sulfam.**							
Isolate	**Sampling moment**	**n**	<=0,0625	0,125	0,25	0,5	1	2	4	>4	<=0,25	0,5	1	2	4	8	>8		<=0,125	0,25	0,5	1	2	4	>4		<=0,25	0,5	1	2	4	8	>8		<=1	2	4	8	16	32	64	>64	<=2	4	8	16	>16		<=2	4	8	16	32	>32		<=0,125/2,375	0,25/4.75	0,5/9,5	1/19	2/38	4/76	>4/76	
SAU	Before	6	5					1			4			1			1		6								1	5							4	2							6							5	1							6							
SAU	After	0																																																																		
CNS	Before	9	9								8	1							4	4		1					2		4			1	2		8					1			8	1					7	2							8			1				
CNS	After	11	7	2						2	5	5					1		6	3		1	1				1	3	2			1	4		10							1		10					1		9	1					1		9					1	1	
SUB	Before	2	2								1		1						2								2													2													2								2							
SUB	After	0																																																																	
SDY	Before	0																																																															
SDY	After	0																																																															

Appendix 2 Changes in MIC-values in isolates that were recovered both before and after treatment in the same quarter

Appendix 2.1 MIC-values of gram-positive pathogens isolated from CM quarters before *and* after treatment in the same quarter. O indicates no change, whereas an arrow indicates a shift in susceptibility, either an increase or a decrease. Isolates from quarters marked with an * (SDY, SSI) were not treated with antibiotics. SAU = *S. aureus*, SUB = *S. uberis*, SDY = *S. dysgalactiae*, SSI = *S. simulans*.



Appendix 2.2 MIC-values of gram-negative pathogens isolated before *and* after treatment in the same quarter. O indicates no change, whereas an arrow indicates a shift in susceptibility, either an increase or a decrease. The infection with *K. pneumoniae* was not treated with antibiotics.



Appendix 2.3 MIC-values of gram-positive pathogens isolated from control quarters before *and* after treatment in the same quarter. O indicates no change, whereas an arrow indicates a shift in susceptibility, either an increase or a decrease.

	Penicillin			Oxacillin					Clindamycin				Erythromycin					Kanamycin			Neomycin			Streptomycin				Trim./sulfam.		
Isolate	<=0,0625	≥	>4	<=0,25	0,5	1	≥	>8	<=0,125	0,25	≥	>4	<=0,25	0,5	1	≥	>8	<=1	≥	>64	<=2	≥	>16	<=2	4	≥	>32	<= 0,125/2,375	≥	>4/76
** S. hyicus*	O				O				O←→O					O←→O				O			O			O				O		
** S. capitis*	O			O					O←→O					O				O			O				O			O		

Appendix 3 Full MIC-distribution of isolates in DCT quarters and control quarters, both before drying off and post calving

Appendix 3.1 Full MIC-distribution of isolates recovered from DCT quarters, split up between isolates from before drying off and after calving. SAU = *S. aureus*, SUB = *S. uberis*, SDY = *S. dysgalactiae.*

			Penicillin								Oxacillin								Clindamycin								Erythromycin								Kanamycin								Neomycin								Streptomycin								Trimethoprim/sulfam.							
Isolate	Sampling moment	n	<=0,0625	0,125	0,25	0,5	1	2	4	>4	<=0,25	0,5	1	2	4	8	>8		<=0,125	0,25	0,5	1	2	4	>4		<=0,25	0,5	1	2	4	8	>8		<=1	2	4	8	16	32	64	>64	<=2	4	8	16	>16		<=2	4	8	16	32	>32		<=0,125/2,375	0,25/4.75	0,5/9,5	1/19	2/38	4/76	>4/76	
SAU	Drying off	2	2								2								2									2								2								2									2								2							
SAU	Calving	0																																																																								
CNS	Drying off	17	9	4	1	1		2			13	2	2						12	1	2	1			1		5	7	3			1	1		17								17								16	1							17							
CNS	Calving	14	9	3	1					1	10	4							10	4							2	6	2	1		1	2		14								14								12	2							13	1						
SUB	Drying off	3	3								1	1	1						1	1					1		3								1				1	1			1				2			1		1			3							
SUB	Calving	0																																																																				

Appendix 3.2 Full MIC-distribution of isolates recovered from control quarters, split up between isolates from before drying off and after calving. SAU = *S. aureus*, SUB = *S. uberis*, SDY = *S. dysgalactiae*

			Penicillin								Oxacillin								Clindamycin								Erythromycin								Kanamycin								Neomycin								Streptomycin								Trimethoprim/sulfam.							
Isolate	Sampling moment	n	<=0,0625	0,125	0,25	0,5	1	2	4	>4	<=0,25	0,5	1	2	4	8	>8		<=0,125	0,25	0,5	1	2	4	>4		<=0,25	0,5	1	2	4	8	>8		<=1	2	4	8	16	32	64	>64	<=2	4	8	16	>16		<=2	4	8	16	32	>32		<=0,125/2,375	0,25/4.75	0,5/9,5	1/19	2/38	4/76	>4/76	
SAU	Drying off	3	2				1				3								3								1	2							1	2							3								1	2							3							
SAU	Calving	2	2								2								2									2								1	1							2								1	1							2							
CNS	Drying off	11	8	2				1			10				1				10					1			5	6							11								11								9	2							11							
CNS	Calving	6	2	2	1			1			3	1	1	1					1	2	3							2	1			1	2		6								6								5				1				5		1					
SUB	Drying off	0																																																																				
SUB	Calving	1	1								1									1							1														1														1								1							