An exploration study of CNS during the dry period in Dutch dairy cows.

Research survey drs T.J. Wessels

Faculty of Veterinary Medicine

Utrecht University, the Netherlands

Table of content

Abstract

Coagulase-negative *Staphylococcus* (CNS) is a minor pathogen, but the prevalence is increasing in cows with a low somatic cell count (SCC) all over the world. And the overall SCC is lowering, because of well performed mastitis control programs. The bacterium is isolated from healthy and sick cows. In this study the risk and the presence of CNS was observed at the beginning and at the end of the dry period.

Part of a dry cow project (whereby farmers could voluntary participate) of the GD (Animal Health Service in the Netherlands), from 445 healthy cows throughout the Netherlands, with a low SCC, milk samples were collected before (D-sample) and after (K-sample) the dry period. From entering the dry period until 100 days in milk the cows were observed for the development of a clinical mastitis (CM).

In total 863 quarter samples were used for this study. With a CNS prevalence of 18,77%, there was no significant correlation between infection with CNS during the dry period and the development of a CM. With an relative risk (RR) smaller than 1, the risk of developing a CM for a quarter with CNS during the dry period was smaller than a quarter without it.

Because samples were taken before and after the dry period, it was also possible to look at the CNS infection development at quarter level during the dry period and the risk of developing a CM. In total 162 quarters were CNS positive, from which 21 were persistent, 50 fainted and 91 gained. So at the end of the dry period there were more quarters with CNS than at the start. But there was no significant correlation between CM and the CNS infection development.

Although the prevalence was high by cows with a low SCC, the risk of CM was low . Because CNS is not a homogeneous group, more strain specific research is needed for understanding and controlling CNS associated CM. This is important because of the risk of using antibiotic treatments without any sense especially regarding the problem of antibiotic resistance.

Key words: CNS, coagulase negative *Staphylococcus*, prevalence, persistence, dry period, Dutch dairy cattle

Abbreviation key:

BMSCC	Bulk milk somatic cell count
CI	Confidence interval
CM	Clinical mastitis
CNS	Coagulase-negative *Staphylococcus*
CNSdp	CNS associated during the dry period
DIM	Days in milk
DP	Dry period
D-Sample	Milk sample taken at the start of the dry period
GD	Animal Health Service Centrum in the Netherlands
K-Sample	Milk sample taken at calving

MPR	Dutch milk production registration system
OR	Odds Ratio
SCC	Somatic cell count
SCM	Subclinical mastitis
UGCN	Udder Health Centrum in the Netherlands

1. Introduction

Mastitis is a worldwide problem in dairy cattle, resulting in significant production loss, less animal welfare and with economic consequences. Mastitis causes a decrease in milk production and milk quality (Oliver *et al*, 2003; Sampimon *et al*, 2009).

Mastitis is the result of the interaction of a pathogen and the mammary gland of a host. The presence of a pathogenic bacterium in the mammary gland can result in mastitis (Piessens *et al*, 2011). Therefore, the mastitis pathogens have been classified on their pathogenicity in two groups: major pathogens and minor pathogens. Major pathogens have a high potential to cause clinical mastitis (CM) and elevated somatic cell count (SCC). Clinical signs are a calorous, ruborous, dolorous and/or tumorous udder, sereus or mucopurulent milk and a rising body temperature. Minor pathogens are bacteria mainly associated with subclinical mastitis (SCM), showing only an elevation of SCC or mild clinical signs.

Beside their pathogenicity pathogens can also be classified according to their origin: from the environment and/or from the animal/the mammary gland (Quin *et al*, 2002).

1.1 Coagulase-Negative *Staphylococci*

Staphylococci are gram positive, facultative anaerobic, catalase positive, salt tolerant cocci and occur worldwide as skin commensal on animals and humans, but most species are animal specific colonizers. The genus can be divided on their ability to coagulate rabbit plasma into a positive and a negative group, also known as Coagulase-Positive *Staphylococci* (CPS) and Coagulase-Negative *Staphylococci* (CNS)(Taponen *et al*, 2009; Piessens *et al*, 2011). The virulence is thought to be correlated with the production of coagulase (Quin *et al*, 2002).

The group of CNS is known as a cow related minor pathogen. Infections with CNS are mostly found in heifers around calving (Schukken *et al*, 2009) and can persist for months throughout the lactation period. Clinical signs are mild with a slightly increased SCC. Taponen *et al* (2006) found in 51% of CNS mastitis clinical signs without elevated body temperature. Other authors believe CNS have a protective function (sometime with a higher milk yield) and others found more pathogen types (Almeida *et al*, 2001; Schukken *et al*, 2009; Piessens *et al, 2011;* Piepers *et al*, 2012).

New research showed that the prevalence of CNS in mastitis is negatively correlated with the bulk milk somatic cell count (BMSCC). Schukken (2009) found a CNS prevalence of 17,9% in herds with a BMSCC less than 200.000 cells/ml. Because of the lowering trend in the national BMSCC, the influence of CNS will probably increase. From several cases of

mastitis 49 CNS types had been isolated. Normally, further differentiation of CNS types is not carried out, but most common are *S. chromogenes*, *S. simulans* and *S. epidermidis* (Simojoki *et al*, 2009).

Taponen *et al.* found in 2006 that *S. chromogenes* mostly occurs in heifers and *S. simulans* mostly in higher parities. In a study with artificial infected cows, they all developed mastitis with mild clinical signs, production decrease of 16,3% without elevated body temperature (Simojoki *et al*, 2009).

1.2 Reason for this Research

Because of well performed mastitis controlling programs the BMSCC has decreased in many countries and CNS is becoming a predominant pathogen. Therefore the importance of CNS control cannot be denied (Sampimon *et* al, 2009; Schukken *et al*, 2009; Taponen *et al*, 2006; Supré, 2011; Simojoki, 2009; Piepers *et al*, 2012). Still, there is a lack of species specific impact. Moreover, CNS have been isolated from healthy and sick cows (Supré *et al*, 2011), contributing to the diversity of opinions and the creation of a knowledge gap.

1.3 Problem description

The objective of this study was to explore the CM risk and the presence of CNS around the dry period (DP) in Dutch dairy cattle with a low BMSCC. Delineations toward the dry period were justified because CNS is most prevalent in milk samples around calving and in the early lactation (Sampimon *et* al, 2009; Supré *et al*, 2011).

1.4 Hypothesis

H_0 = There is no significant evidence for developing a clinical mastitis correlated to Coagulase-Negative *Staphylococci* presence during the dry period in Dutch dairy cattle.

H_1 = There is significant evidence for developing a clinical mastitis correlated to Coagulase-Negative *Staphylococci* presence during the dry period in Dutch dairy cattle.

1.5 Research questions

This research contributed to answer the following questions: What was the prevalence of CNS in healthy cows during the dry period? Were CNS infections persistent during the dry period? What was the risk for clinical mastitis (CM) when CNS had been detected? And would it be relevant to differentiate CNS types for future mastitis research?

2. Methodology

This observational, longitudinal, cohort study involved a milk sample survey and was conducted throughout the Netherlands with help of GD (Animal Health Service in the Netherlands) professionals. It was part of a national dry cow project of the GD. For this project, the GD used a random split udder design. When the cows entered the dry

period, one half (left or right) of the udder was injected with an injector with antibiotics and the other stayed untouched. This was done single-blinded, so the farmers did not know which side was treated or not. The untouched side was used for this research.

2.1 Farm enrolment

Non-organic cattle farms all over the Netherlands with milk production registration (MPR) could voluntary participate in the selective dry cow project of the GD.

2.2 Animal enrolment

The main inclusion criteria of the cows in this study were: healthy cows with four healthy quarters, without clinical signs of mastitis. Otherwise a disbalance in the udder could influence the outcomes.

Secondly, because CNS is mostly found in herds with low BMSCC, a low SCC at the last MPR was a prerequisite for participation. The cut-off value of the SCC for heifers was lower than 150.000 cells/ml and for older cows lower than 250.000 cells/ml. From every farm a maximum of 20 cows could participate.

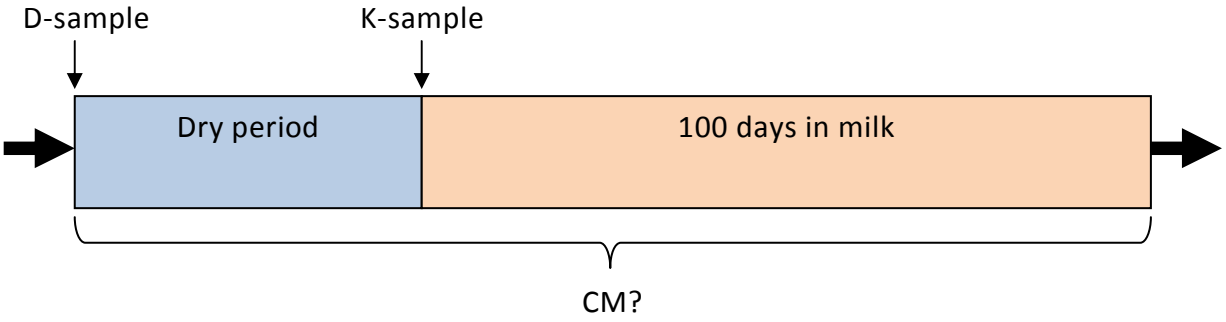

Figure 1: Schematic presentation of research steps per cow, from entering the dry period untill 100 days in milk.

2.3 Sample strategy

Milk samples were taken at the moment of entering the dry period (D-Sample) and at the moment of calving (K-Sample, also known as colostrums) with at least 6 weeks in between, see also figure 1. The samples were taken at normal milking times and aseptic as possible. This was done according to the milk sample protocol of the Udder Health Centre in the Netherlands (UGCN), see also appendix 1 (is in Dutch). This protocol is for right handed people:

Step 1: Clean the udder with a clean piece of fabric per cow.
Step 2: Flare the first milk from the udder.
Step 3: Clean the teat with wadding well drained in 70% alcohol and let it dry. Start with the teat most far away and work towards yourself.
Step 4: Take your milk tube with the identification number of the cow and the quarter to sample written on it. Start sampling at the teat most nearby front and work contrary as in step 3.

Step 5: Open the tube by holding the cover in the left index finger and place the tube quickly horizontal as possible between dumb and the other 3 fingers of the left hand.
Step 6: Again flare the first milk away and fill the tube for 2/3 with milk. Close it as quick as possible.
Step 7: Write the cow ID number, quarter and date on the tube.
Step 8: Store the tube in the fridge at -18°Celsius or put it on cold transport at 4°Celsius.

All samples were taken with gloves, these were cleaned with water and alcohol when visual contaminated or replaced when damaged or excessive contaminated. A teat dip or spray was allowed afterwards, but not teat sealers like Orbeseal©.

2.4 Laboratory technique

All samples were cultured at the GD Laboratory according to the guidelines of the National Mastitis Council with a phenotypic tests (blood agar, Gram stain, Catalase and a tube coagulase test), see appendix 2 for further details (Hogan *et al*, 1999).

2.5 Statistical analysis

Missing data were excluded. For the hypothesis, the significance level was set on $P < 0,05$ for rejecting the H0. The calculation was done in STATA (Stata 2009) with a qualitative two-tailed chi- square test. Additional, the 95% confidence interval and odds ratios (OR) were calculated.

2.6 Practical site

The D-Samples were taken when the cows entered the dry period by a professional sampler instructed by the GD. This was done at least five weeks before calving and the samples were immediately transported at 4°C to the GD laboratory in the Netherlands. The sampler had also a closer look to the cow criteria with an individual cow related protocol.

The follow-up samples (K-sample) were taken by the farmer because of logistic issues. The K-sample needed to be taken within 12 hours after calving from the first colostrum and was frozen at -18°C before cold transport to the lab.

All samples were taken in the milkpit, in the milkrobot or at the feedgate. And from the point of entering the dry period until 100 days in milk (DIM) the farmer observed the cow for CM signs. Every change in the udder and/or milk was individually registrated as CM.

3. Results

In total 445 cows were sampled from November 2011 until February 2012. Two quarters per cow meaning 890 samples in total. From these samples, 27 were excluded because: the cows had been sold, were not pregnant, did not calf, had low milk production and inadequate sampling. In total 863 quarters were included for this research.

As shown in table R1, CNS prevalence was 18,77% and was detected in 162 samples around the dry period. Four quarters were associated with CM and 158 were not. In total 48 quarters did show a CM. The Chi^2 calculated P-value was 0,057 with a confidence interval (CI) from 0,027 until 0,193, an Odds Ratio (OR) of 0,378 and the Relative Risk (RR) of the disease was 0,393.

Table R1: CNS and CM

	CM	No CM	Total
CNS	4 (0,46%)	158 (18,31%)	162 (18,77%)
No CNS	44 (5,1%)	657 (76,13%)	701 (81,23%)
Total	48 (5,56%)	815 (94,44%)	863 (100%)

An overview of the numbers and percentages of quarters with or without CNS and clinical mastitis (CM) sampled before and after the dry period (P = 0,057; CI 95% = 0,027-0,193; Pearson Chi^2 = 3,632; OR = 0,378; RR = 0,393).

Table R1 does not tell anything about the development of a CM and the influence of CNS infection development, therefore it is important to compare the samples taken before and after the dry period (at calving) of the same cow as shown in table R2. According to the data, 21 samples were CNS persistent, 91 samples gained a bacterial CNS invasion during the dry period, 50 samples fainted the bacterial CNS during the dry period and 701 samples were negative at both moments.

Table R2: CNS infection development and CM

		CM	No CM	Total
Negative	(-|-)	44 (91,67%)	657 (80,61%)	701 (81,23%)
Fainted	(+|-)	2 (4,17%)	48 (5,89%)	50 (5,79%)
Gained	(-|+)	2 (4,17%)	89 (10,92%)	91 (10,54%)
Persistent	(+|+)	-	21 (2,58%)	21 (2,43%)
Total		48 (100%)	815 (100%)	863 (100%)

An overview of the numbers and percentages of quarters with or without CNS and clinical mastitis (CM) sampled before and after the dry period (P = 0,25; degrees of freedom = 3; Pearson Chi^2 = 4,1116). The CNS infection development is divided in four different CNS characters: Negative (no CNS before and after dry period), Fainted (CNS before dry period but not after), Gained (no CNS before dry period but positive after) and Persistent (CNS before and after the dry period).

Comparing the influence of the CNS character and the prevalence of CM no quarter with a persistent CNS got a CM. The four CNS quarters with CM from table R1 belong to the fainted and the gained group (2 quarters per group). In both groups, one quarter had a CM in the lactation and the other quarter had a CM in the dry period. In line with table R2, a multiple logistic regression analysis is performed using CM as the dependent variable, see Table R3 and R4. For this analysis, 21 samples were excluded, for the same reasons as before. Only the negative group (the referent group) of CNS had a significance of 0,000. See also appendix 3 for more calculations.

Table R3: Logistic regression of CNS infection development and CM

	Coef.	Std. Err.	Z	P>[z]	Lower 95% CI	Upper 95% CI
Negative (-|-)	-2.703494	0.155722	-17.36	0.000	-3.008704	-2.398285
Fainted (+|-)	-1.091995	0.7317686	-1.49	0.136	-2.526235	0.3422453
Gained (-|+)	-0.4745594	0.7382971	-0.64	0.520	-1.921595	0.9724764
Persistent (+|+)	0	-	-	-	-	-

A logistic regression table with CM as dependent variable and the CNS infection development as explanatory variables (Number of quarters = 842; LR Chi^2 = 3,40; Log likelihood = -182,4; Pseudo R2 = 0,0092). The CNS infection development is divided in four different CNS characters: Negative (no CNS before and after dry period), Fainted (CNS before dry period but not after), Gained (no CNS before dry period but positive after) and Persistent (CNS before and after the dry period).

Table R4: Calculated exponential coefficients and CI

	Exp(Coef.)	Lower Exp 95% CI	Upper Exp 95% CI
Negative (-|-)	0,062767498	0,047034201	0,083303567
Fainted (+|-)	0,251242793	0,074039352	0,58473583
Gained (-|+)	0,383537664	0,127683809	0,725612822
Persistent (+|+)	0,5	-	-

Exponential coefficients and CI calculated from table R3.

4. Discussion

In total 445 cows were sampled during this study by a lot of different people. Farmers could voluntary participate the study of the GD. The farms were not at random selected throughout the Netherlands. The mean and the reference range of the cows per farmer was unknown. Twenty cows from one farm or four cows per farm can make a difference in the results, because of a strong farm influence and the possibility of CNS clusters on a farm (Simojoki *et al*, 2011). Beside the environment, other biological covariations could have occurred, like age, milk production, SCC at getting dry and genetics.

It is known that an older cow and a high milk production at the start of the dry period give a higher risk of mastitis. Although the SCC was checked on the MPR, the SCC level at drying off was not certain, because of the fact a MPR is taken with an interval of 4 to 6 weeks. Although the SCC was measured by the GD, it was not included in this study,

because of the high SCC in the K-samples. So, the influence of CNS on a SCM level was not determined and the risk could not be analyzed.

Beside biological covariations, there could also have been technical errors, which can be divided in two groups: Human and instrumental errors. Human errors could have been occurred during cow selecting, the sampling, the storing, the transport, the research in the lab and during the statistical analysis.

During the cow selecting, there were several inclusion criteria that had to be fulfilled. A correct MPR SCC and judging the clinical signs were depending on the quality of the judger. A person could have made a wrong interpretation (a mistake called poor precision), but there could also have been a difference in the interpretation between persons (a mistake called poor accuracy). A good work protocol, well prepared persons and adequate instructions increase the precision and accuracy. Whether the GD workers and farmers worked consequently and properly determined for a great part the quality of the samples. Out of this data set it was not known how many of the negative cultures were a mixed flora. Because the GD is a professional research organisation, these human errors were expected to be minimal.

The instrumental errors could have occurred throughout the process. From the alcohol in the start until the laboratory equipment. Beside the colony forming units depended phenotyping laboratory techniques, there are much better genotyping techniques (Api Staph ID, MALDI-TOF and AFLP) which are more precise and make the identification of the different CNS strains much easier, because CNS is not an homogeneous group (Piessens *et al*, 2011).

The correlation between CNS and CM was not significant, but there was a change of making a Type 2 error (accepting H_0 when H_0 is false). Because of the low OR and RR, the risk of a CM in a quarter with CNS associated during the dry period is lower than a quarter without CNS. Having a closer look at the CNS character with the mastitis development there was also no significant correlation. Of the 21 persisting CNS quarters, none did develop a CM. Uncertain is how many really persisted, because there was no strain identification. It could also be a gainted and fainted quarter.

From the 162 CNS associated quarters, 71 quarters with CNS entered the dry period and 112 quarters (21 plus 91) were CNS positive afterwards, only 50 fainted. So the prevalence of CNS increased during the dry period, but it was unknown what happened with these CNS during the lactation. This negative cure rate during the dry period was controversially to the findings of Dufour *et al* in 2012. The only significant correlation with CM development was found in the CNS negative group. Beside the poor significance levels, the R square (the multiple correlation coefficient) of the logistic regression was 0,0092, meaning 0,92% of the variation in CM was dependent on the variable CNS in this study group.

But CNS is a heterogeneous group, that can cause CM. Strain specific pathogenicity is still unknown, but is needed for evidence based treatment of a quarter with CNS and preventing unneeded use of antibiotics.

5. Conclusion

A quarter, with a low SCC on the last MPR before the dry period with intramammary CNS during the dry period, had less risk in developing a CM compared to a quarter without CNS.

6. Acknowledgements

First of all, I want to thank my supervisors Dr. G.A. Hooijer and Drs. C.G.M. Scherpenzeel for given me this research opportunity. Although it was a long journey to find my own way in the research world, I knew I could always ask my supervisors. Looking back in time, I have experienced a lot about the difficulties of a good research. Even when you think you are doing well, there always will be (human) errors which could and could not have been overseen. A next research would be much easier and of higher quality.

Secondly, I want to thank the Dutch Health Service in the Netherlands for participating in their research project and giving me their trust, the responsibility, the (financial) care and the time for doing my own thing. The 13 weeks in Deventer felt as very pleasant to me and I met a lot of nice people. Although it was hard work to collect all samples, the friendly attitude gave me a lot of energy every day.

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At last, I want to thank all the other people at the faculty of Veterinary Medicine, at the Dutch Health Service in the Netherlands and close to me, which I have not mentioned by name. All together they helped me directly and indirectly with this research. In the 13 weeks of research and during the ending stage, it always felt good I could lean back on them.

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Appendices

Appendix 1: UGCN milk sample strategy (in Dutch)

Appendix 2: Laboratory techniques (Hogan *et al*, 1999).

Page 180

Blood-Esculin Agar Plates

Ingredients

Trypticase or Tryptic Soy Agar Powder ..... 40 g
Esculin ..... 1 g
Purified (distilled or deionized) water ..... 1000 ml

Procedure

1. Mix thoroughly and heat with frequent agitation; boil for 1 minute to completely dissolve the agar powder.
2. Dispense into 200-ml aliquots using 250-ml flasks with vented stoppers or aluminum foil.
3. Sterilize by autoclaving at 15 lb pressure (121 C) for 15 minutes.
4. Place sterile media in a water bath set at 45 to 50 C for 1 hour. NOTE: If agar is being prepared for later use, it may be stored at room temperature for up to 5 days; otherwise, it should be refrigerated at approximately 6 C.
5. After 1 hour, add defibrinated bovine blood (or washed bovine erythrocytes or bovine blood) to a final concentration of 5% and swirl gently. Mix well and pour into petri plates using aliquots of 12 to 14 ml for each 100 x 15-mm plate. The yield is approximately 15 to 18 plates/200 ml of sterile media. NOTE: If bubbling occurs upon pouring, use pourite or similar additive (1 drop/L of media), or flame surface of liquid medium in petri dishes briefly with a Bunsen burner.
6. Allow agar to solidify, and incubate inverted at 37 C for 18 to 24 hours to reduce excess moisture and to check sterility of the medium.
7. Store inverted in a refrigerator at approximately 6 C until use.
8. Use within 2 weeks because plates become dehydrated and do not support growth of some microorganisms.

Advantages

Use of blood-esculin agar plates permits esculin hydrolysis by streptococci on primary isolation.

Limitations

Alpha hemolysis of some *Streptococcus agalactiae* isolates may be confused with esculin splitting. Blood-esculin agar plates are not readily available commercially.

Quality control of media preparation

Dehydrated media should be purchased in sufficient quantities so that one lot can be used over a long period of time (6 to 12 months). Each lot should be checked for its ability to produce expected reactions according to the American Public Health Association's Standard Methods for the Examination of Dairy Products. Each bottle of dehydrated media should be labeled indicating the date received and opened, and stored in a cool, dry location protected from light, or in a refrigerator or freezer if recommended by the manufacturer. Expired media should be discarded, or if any change in color or texture is noted. Complete mixing of media is necessary prior to autoclaving.

Quality control of blood-esculin agar plates

Each lot of newly prepared blood-esculin agar plates should be tested with quality control microorganisms to insure that the media will support growth and provide correct differential reactions for hemolysis and utilization of esculin.

Suggested quality control microorganisms:

Streptococcus dysgalactiae ATCC 27957 to insure proper growth of fastidious streptococci.

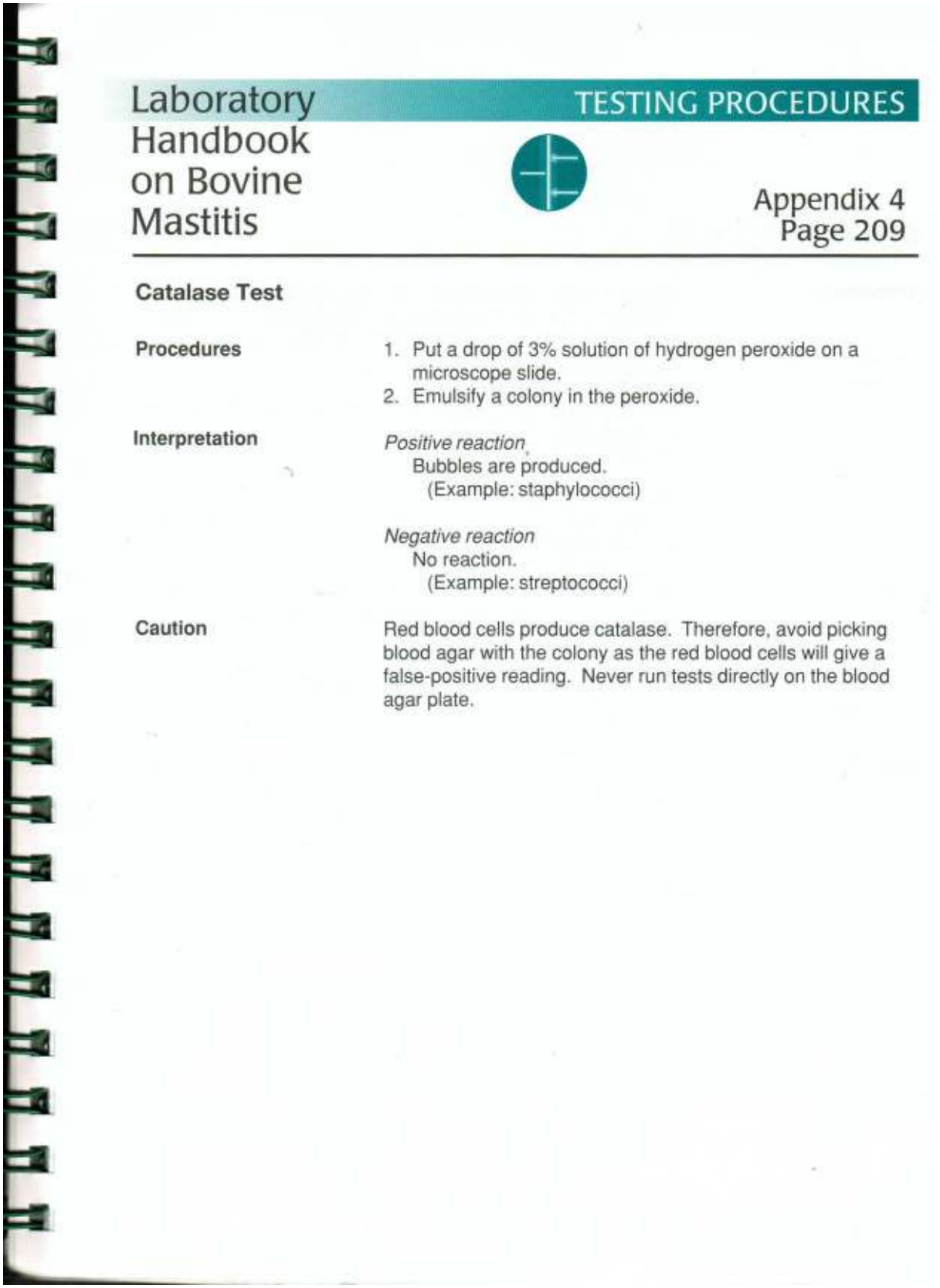
Streptococcus uberis ATCC 27958, esculin positive to insure proper esculin reaction.

Staphylococcus aureus ATCC 29749, alpha and beta toxin positive to insure correct hemolytic patterns.

Page 182

Note

Blood-esculin agar plates can be prepared from small batches of agar as described above or from large batches of media that are prepared in commercial agar sterilizers and automated plate pourers according to the manufacturers' instructions.



Laboratory Handbook on Bovine Mastitis

TESTING PROCEDURES

Appendix 4
Page 209

Catalase Test

Procedures

1. Put a drop of 3% solution of hydrogen peroxide on a microscope slide.
2. Emulsify a colony in the peroxide.

Interpretation

Positive reaction
Bubbles are produced.
(Example: staphylococci)

Negative reaction
No reaction.
(Example: streptococci)

Caution

Red blood cells produce catalase. Therefore, avoid picking blood agar with the colony as the red blood cells will give a false-positive reading. Never run tests directly on the blood agar plate.

Coagulase Tube Test

Inoculation

1. Inoculate 0.5 ml coagulase plasma with a heavy inoculum of staphylococci from a 24-hour plate culture. Use a wire loop or the end of a sterile applicator stick for inoculation.
2. Incubate tubes at 37 C in a water bath or an incubator for up to 24 hours.

Interpretation

Positive reaction
Semi-solid to solid gelling evident when tube is tipped.
(Example: *Staphylococcus aureus*)

Negative reaction
Liquid state after 24 hours incubation.
(Example: *Staphylococcus chromogenes*)

Note

The inoculum must be staphylococci of one type for uniform test results. If the culture is greater than 24 hours old, a colony should be picked and placed in Trypticase Soy Broth overnight. From the overnight culture, inoculate a coagulase tube with 0.05 ml of broth culture.

A positive test can appear within 2 to 4 hours or may take up to 24 hours. Positive results can be recorded when evident. Some *S. aureus* produce the enzyme staphylokinase that may lyse the clot if allowed to incubate too long. All negative tests must remain incubated for a total of 24 hours. Coagulase-positive and coagulase-negative controls of staphylococci should be run with each test to determine the stability of plasma.

Laboratory Handbook on Bovine Mastitis

STAINS

Appendix 5
Page 221

Gram Stain

Prepare dry smear

Make a slide from a PURE culture by mixing a small amount with a small drop of sterile distilled water or sterile broth. Mark slide with wax pencil to locate smear. Air dry and fix by lightly passing slide through flame, being careful not to burn. Flamed slide should be able to be held on wrist without feeling too hot.

Staining

1. Apply crystal violet to smear by flooding slide for 30 to 60 seconds.
2. Wash off with tap water.
3. Apply Gram's iodine for 30 to 60 seconds. Drain - do not wash.
4. Decolorize by continual gentle rinsing with 95% alcohol just until color is no longer present in run off.
5. Rinse with tap water.
6. Apply safranin for approximately 1 minute.
7. Rinse away stain with tap water.
8. Blot dry with bibulous paper and examine.

Caution

ALWAYS RUN CONTROLS with known Gram-positive (staphylococcus - blue) and Gram-negative (coliform - red) organisms.

Stains should always be kept in brown bottles to prevent deterioration by light. Stains must be free of sediment and should be filtered every 2 to 3 weeks to avoid the danger of confusion with artifacts. ALWAYS USE FRESH CULTURES as old cultures may give erroneous results.

Note

Prepared solutions of stains are available commercially.

Appendix 3: Calculations

3.1 STATA© and other calculations

RECODE of cns	km 0	1	Total
0	657	44	701
1	158	4	162
Total	815	48	863

Pearson chi2(1) = 3.6320 Pr = 0.057

p = odds of CM to occur in the group with CNS (exposed group)
= 4/162

q = odds of CM to occur in the group without CNS (unexposed group)
= 44/701

OR = (p/(1-p))/(q/(1-q)
= ((4/162)/(158/162))/((44/701)/(657/701))
= (4/158)/(44/657)
= 0,3780

RR = p/q
= (4/162)/(44/701)
= 0,393378

cns	Freq.	Percent	Cum.
-1	50	5.79	5.79
0	701	81.23	87.02
1	91	10.54	97.57
2	21	2.43	100.00
Total	863	100.00	

cns	km 0	1	Total
-1	48	2	50
0	657	44	701
1	89	2	91
2	21	0	21
Total	815	48	863

Pearson chi2(3) = 4.1116 Pr = 0.250

Logistic regression — Number of obs = 842
LR chi2(2) = 3.40
Prob > chi2 = 0.1829
Log likelihood = -182.40578 — Pseudo R2 = 0.0092

km	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
cns						
1	-1.091995	.7317686	-1.49	0.136	-2.526235	.3422453
2	0	(empty)				
3	-.4745594	.7382971	-0.64	0.520	-1.921595	.9724764
_cons	-2.703494	.155722	-17.36	0.000	-3.008704	-2.398285

Odds = e^coef/(1+e^coef)

Table R4: Calculated exponential coefficients and CI

	Exp(Coef.)	Lower Exp 95% CI	Upper Exp 95% CI
Negative (-|-)	0,062767498	0,047034201	0,083303567
Fainted (+|-)	0,251242793	0,074039352	0,58473583
Gained (-|+)	0,383537664	0,127683809	0,725612822
Persistent (+|+)	0,5	-	-

Exponential coefficients and CI calculated from logistic regression table.

Lin regr Y = 0,06

3.2 Win episcoop© calculations