Thresholds to determine an IMI at time of calving

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Prefactory note

In the framework of my training Veterinary Medicine at University of Utrecht I should accomplish a master thesis. This project is a part of the research line: The reduction of preventive antibiotic use by selective dry off. This project is conducted by the Ruminant Health Division of the Animal Health Service (GD) in Deventer.

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Abstract

Repeatedly was shown that a cut-off value of approximately 200 000 to 250 000 cells was optimal to distinguish between infected and uninfected quarters in dairy cows (Schukken *et al*., 2003). However, a cut off value shortly after calving was never established. In this study 258 cows were studied. Quarter milk samples were collected at time of calving. The sensitivity (Se) en specificity (Sp) of SCC at several thresholds were relatively low. Se increased when Sp decreased and vice versa. Milk samples with major pathogens have higher SCC compared with those with minor pathogens isolated. Quarter SCC can be used to give an indication of IMI shortly after calving. The choice of definition of Se and Sp will depend on the objectives of study or control program for which the sample was collected.

1. Introduction

Mastitis is worldwide a common disease in dairy cows. Both clinical and subclinical infections have a not to be underestimated impact on the health and well-being of the animal. Udder health problems can lead to great economic losses for dairy farmers due to the quality standards imposed by the consumers. Preferably, the milk should have low somatic cell count (SCC), indicating that milk originates from healthy cows. According to O'Brien *et al* (2009) milk quality can have a significant impact on milk processing efficiency and product quality. The processability of milk decreases when the SCC increases. Moreover there is an increased loss of fat and casein and reduced curd firmness due to increase SCC. High SCC milk may also adversely affect the quality of pasteurised liquid milk and reduced its shelf life.

1.1 Lactogenesis

Before the mammary gland has the ability to secrete milk, it must be transformed from a simple ductal tree to a highly efficient exocrine organ, with expansive lobular-alveolar structures. This transformation is hormonally regulated and begins around mid-pregnancy in most species (McManaman & Neville, 2003). The lactating mammary gland is composed of a branching network of ducts formed of epithelial cells ending in extensive lobulo- alveolar clusters that are the sites of milk secretion. A single layer of polarized secretory epithelial cells surrounds each alveolus within these clusters. In turn the alveoli are surrounded by myoepthelial cells that function in milk ejection, and a vascularized connective tissue stroma that contains lipid-depleted adipocytes and fibroblasts. The milk is ejected through contraction of myoepithelial cells through the alveoli and ducts. This contraction is stimulated by the release of oxytocin from the posterior pituitary as part of a suckling induced neuroendocrine reflex (McManaman & Neville, 2003).

1.2 Dry period

To optimize milk production in the next lactation and to facilitate cell turnover in the bovine mammary gland, the dry period is necessary (Pezeshki *et al*., 2010). According to Hurley (1989), the typical dry period in a dairy cow is characterized by two major phases. The first phase is a period of active involution after cessation of milk removal. During this active involution, stromal area increase and parenchymal area decrease. Moreover, there is an increase in the rate of epithelial cell apoptosis from 2.4% in late lactation to 4.8% after dry off (De Vries *et al.,* 2010). The second phase is a period of redevelopment before the next lactation. This phase starts 3 to 4 weeks before parturition. The luminal area in the mammary tissue increases and the stromal compartments decreases through one week prepartum (De Vries *et al*., 2010).

1.3 Somatic cell count

Somatic cell count (SCC) is the total number of cells per millilitre in milk. Somatic cells are mostly cells of the immune system, 80% in uninfected quarters and 99% in mastitic quarters (Schukken *et al.,* 2003). They are part of the natural defence mechanism and include neutrophil and mammary epithelium and other leucocytes such as T- and B- lymphocytes and macrophages. Somatic cell counts can cover a wide range of values. There is no theoretical maximum and counts as low as 7000 cells/ml has been reported (Dohoo & Meek, 1982). Research from North America and Europe has shown that uninfected quarters have a mean SCC of approximately 70 000 cells/ml (Schukken *et al*., 2003). Usually, an increased SCC indicates an infection of the mammary gland. When a cow gets infected, the resident somatic cells signal to a resting population of white blood cells in the blood stream which results in a massive influx of mostly polymorphonuclear cells into the milk. These polymorphonuclear cells kill bacteria, and when the infection is eliminated within a few weeks cell count of milk returns to normal usually (Schukken *et al., 2003)*. So, the SCC in the milk is the most reliable and useful indicator of intramammary infections (IMI) worldwide (Sheldrake & Hoare, 1981).

According to Dohoo *et al.* (2011), subclinical mastitis implies inflammation within the udder, but not necessarily infection. The inflammatory reaction may be identified by an elevated SCC or another indicator of inflammation. But subclinical mastitis is most often due to a bacterial IMI, so the terms IMI and subclinical mastitis are often used interchangeably. The term IMI indicates specifically an infectious organism in the udder (Dohoo *et al*., 2011). There are also nonbacterial factors that affect SCC, such as age, stage of lactation, stress, management, day-to-day variation, and diurnal variation, but these factors are considered to be less important than IMI status (Olde Riekerink *et al*., 2007). Also seasonal differences are already reported. The SCC is generally the lowest in the winter and highest in the summer (Pyörälä & Taponen, 2009) (figure 1).

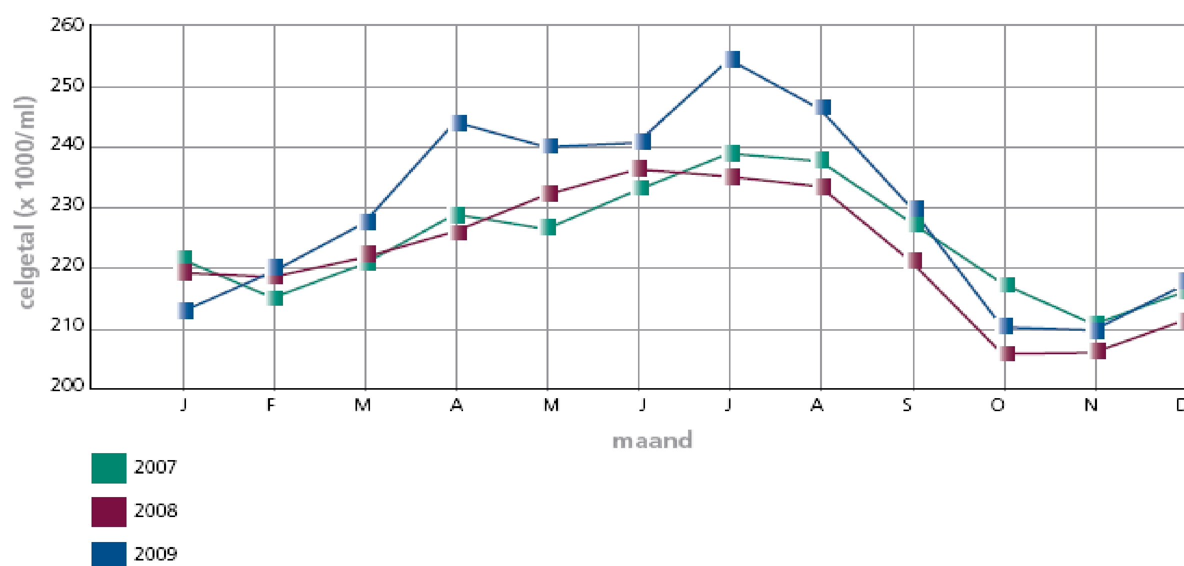

Figure 1: Gradient of the average SCC during the years 2007, 2008 and 2009. (http://www.mcc-vlaanderen.be/content/jaarverslagen)

1.3.1 Gradient SCC during the dry period and lactation

During the mid dry period, milk is considerably reduced. At this moment bovine mammary glands are markedly protected to IMI. On the other hand, the incidence of new IMI is increased when milk accumulates in the glands, i.e. during very early (dry off) and late

(colostrogenesis) phases of the dry period. According to Dohoo and Meek (1982), the somatic cell counts have been found to be elevated immediately after calving, regardless of whether the cow is infected or not. This is also shown by a study by Barkema *et al*. (1999). In that study, farmers were asked to collect foremilk samples of every quarter at the time of calving and each successive milking. The SCC was determined and bacterial analysis was done. Using a natural-logarathmic transformation of the SCC, a normal distribution was approached (Barkema *et al, 1999).*

Table 1. Somatic cell count of 120 udder quarters of 30 Dutch dairy cows after calving, 1997

	Calving	Milkings after calving					
		1	2	3	4	5	6
Ln SCC	6,39	6,75	6,50	5,98	5,55	5,28	4,84
Geometric-mean SCC[a]							
Heifers(n = 13)	602	759	568	259	163	137	87
Cows (n=17)	588	940	754	541	329	259	166
Minimum SCC[a]	4	6	21	31	17	13	13
Median SCC[a]	616	842	567	293	153	129	75
Maximum SCC[a]	13132	10551	10422	8936	7873	9335	9543

[a] x 1000 cells/ml.

Barkema *et al*., 1999,

Table 1 shows an increase of mean Ln SCC from 6.39 at the time of calving to 6.75 at one milking after calving. In the first two weeks after calving the largest reduction of SCC takes place (Dohoo, 1993). Postpartum SCC of culture-negatives quarters of heifers at calving is lower than SCC of culture-positive quarters (Barkema *et al*., 1999). An elevated SCC may also be caused by IMI, because a high percentage of udder quarters is infected at calving (Barkema *et al*., 1999). The SCC declines to normal levels during the first 2-4 weeks of lactation (Dohoo, 1993). Repeatedly was shown that a cut-off value of approximately 200 000 to 250 000 cells was optimal to distinguish between infected and uninfected quarters (Schukken *et al*., 2003). However, a cut off value shortly after calving was never established. The use of SCC during

this period is often not recommended, because SCC is frequently elevated shortly after calving (Barkema *et al*., 1999).

1.4 Aim of the study

The aim of this study was to determine an SCC threshold to identify subclinical mastitis at time of calving caused by minor or major pathogens in Dutch dairy cows, by using the sensitivity (Se) en specificity (Sp) of different thresholds.

2. Materials and Methods

2.1 Study population

This project is a part of the research line: The reduction of preventive antibiotic use by selective dry off, conducted by the Ruminant Health Division of the Animal Health Service (GD) in Deventer. The research was built on a split-udder design. Two quarters (links right or left) serve as negative control and were not treated during the drying off. The other two quarters were treated with antibiotics (Supermastidol). In total, 1030 quarter milk samples from 258 lactating dairy heifers and cows selected between 23 May 2011 and November the 1st and belonging to 30 randomly selected herds. Participation in this study was voluntarily. There were several conditions:

1. the dairy farmers were participant of the MPR
2. the dairy farmer had more than 40 cows
3. Heifers with a SCC < 150.000 cells/ml on the most recent MPR were included
4. Cows with a SCC of < 250.000 cells/ml on most recent MPR were included.

Animals are excluded from participating in cases of damaged teats, general disease or lactating animals with less than four quarters.

2.2 Sample collection

The animals in the test were sampled three times per quarter: at the time of drying off, immediately after calving and 14 days (+/- 7 days) after calving. For this study the second

sample was used on the day of calving. These quarter milk samples were aseptically collected by the farmer while the first streams of milk were discarded. The teat ends were disinfected with cotton swabs sprinkled with 70% alcohol. The first streams of foremilk were discharged, and then milk was collected aseptically from each teat. After collection, milk samples were stored at low temperature until bacteriological culture and SCC tests were performed.

The quarter SCC was determined by using a Fossomatic cell counter (Foss electric, Hillerod, Denmark) and the bacteriological culturing was carried out according to NMC protocols: an inoculums of 0.01 ml of milk will be spread on a 6% sheep blood agar plate and on Edward's medium (Sampimon, *et a.,l* 2009). The sample will be considered to be contaminated if three or more bacterial species were cultured from a sample. *Staphylococcus aureus (*SAU*), Streptococcus agalactiae (*SAG*), Streptococcus dysgalactiae* (SDY)*, Streptococcus uberis* (SUB), *Arcanobacterium pyogenes* (APY), *Pseudomonas aeruginosa* (PSA)*, and Escherichia coli (ECO)* considered being major pathogens. *Corynebacterium bovis* and Coagulase-negative staphylococci, are considered to be minor pathogens (Barkema *et al*., 1999). The quarters were divided into three categories: culture negative, culture with minor pathogens, and culture with major pathogens. If both major and minor pathogens were isolated the cow quarter was classified as infected with the major pathogen.

2.3 Analysis

To approximate a normal distribution, the outcome was the natural logarithm of SCC (lnSCC). The efficacy of the threshold for diagnosing IMI was evaluated using two quality parameters: sensitivity and specificity. The sensitivity of a test is the probability that a truly culture positive animal indeed will be classified as positive using the test. The specificity of a test is the probability that a truly culture negative animal will be classified as culture negative (Schepers, *et al*., 1997). The data were analysed using STATA (StataCorp. TX USA, 2011).

2.4 Se and Sp of SCC Thresholds

To determine the Se, Sp and predictive value, different SCC thresholds were tested. Bacteriological culture was considered to be the reference method to evaluate the SCC thresholds.

3. Results

3.1 General Statistics

Data file 1 (culture negative) consisted of 557 quarters of 30 herds. Data file 2 (minor pathogens) consisted of 117 quarters of 24 herds and data file 3 consisted of 79 quarters of 24 herds. There are 52 contaminated milk samples (5%). These contaminated samples were not included in the statistical analysis.

Table 2 shows the IMI status of the 1030 quarters of 258 cows. Two quarters were missing. The majority of the samples is negative (51,4 %). If there were just some of *Bacillus* spp. or *Enterococcus* spp. present in a sample, the sample was also negative. The prevalence of minor pathogens, C. *bovis* and CNS was relatively speaking high. The prevalence of major pathogens *Arcanobacterium pyogenes* and *Klebsiella* was low.

Table 2. Intramammary infection status of 1030 quarters of 258 cows in early lactation

Pathogen isolated	Number of quarters	% of total quarters	% of infected quarters
No growth	529	51,4	
Bacillus spp.	25	2,4	
Enterococcus spp.	3	0,3	
Major pathogens			
Staphylococcus aureus	9	0,9	4,5
Streptococcus dysgalactiae	11	1,1	5,6
Streptococcus uberis	18	1,7	9,1
Arcanobacterium pyogenes	3	0,3	1,5
Coliformen	10	1,0	5,1
Klebsiella	1	0,1	0,5
Other[1]	23	2,2	11,6
Minor pathogens			
Coagulase-negative staphylococci	84	8,2	42,4
Corynebacterium bovis	28	2,7	14,1
Other[2]	8	0,8	4,0

[1] *Acinetobacte*r spp. (3), *Enterococcus* spp. (19), Ferment (1)

[2] *Aerobacter* spp. (1), *Moraxella* spp. (2), *Sphingomonas paucimobilis* (1), *Enterobateriaceae* (2), *Pantomonis* spp. (1), *Enterobacter cloacae* (1)

Table 3. Overview statistical analysis of Ln SCC

	All samples	Negative samples	Minor pathogens	Major pathogens
N	805	557	117	97
Lo 95% CI	2,94	6,56	6,88	7,57
Median	7,08	6,91	7,22	8,08
Mean	6,9	6,68	7,1	7,84
Up 95% CI	9,21	6,79	7,32	8,11
Minimum	2,2	2,2	3,76	4,38
Maximum	9,21	9,21	9,21	9,21
Kurtosis	3,22	0,14	-0,04	0,4

3.2 Descriptive analysis of SCC

The descriptive statistics are shown in table 3 with on the X-axis the LnSCC (scc2). The geometric mean SCC of all quarters (n=1030) included in the study was 992.000 cells/mL (Ln SCC: 6.90) with a 95% CI of 18.000 – 9.996.000 (Ln SCC: 2.94 – 9.21). The geometric mean SCC of all negative quarters is below the geometric mean SCC of all quarters, 796.000 cells/mL (Ln SCC: 6.68). On the other hand, the geometric mean SCC of all quarters with minor pathogens and major pathogens is higher. The geometric mean SCC of all quarters with minor pathogens is 1.212.000 cells/mL (Ln SCC: 7.1) and the geometric mean SCC of all quarters with major pathogens is 2.540.000 cells/mL (Ln SCC: 7.84). In major-pathogen-positive quarters, Ln SCC was 1.16 Ln units higher than in milk form culture-

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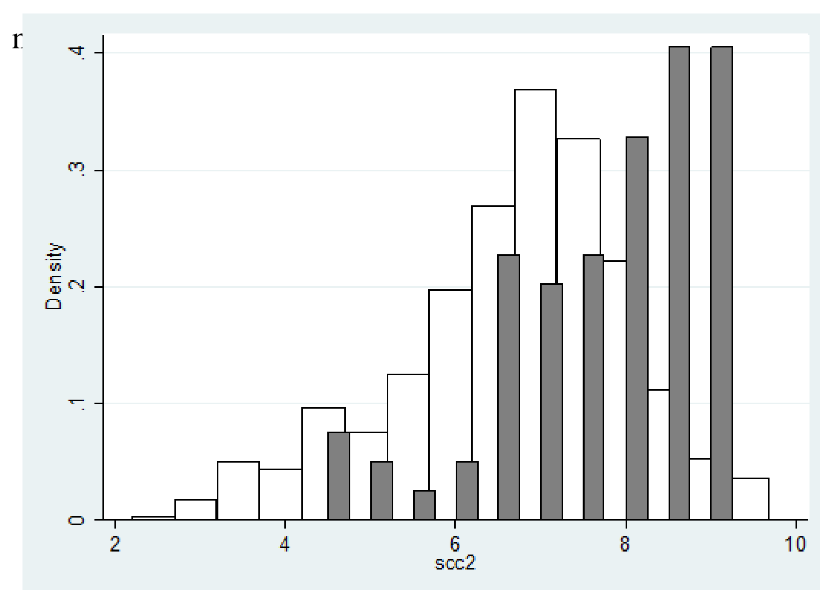

Figure 2: Normal distribution of LnSCC (scc2) of all negative quarters (white) and all major quarters (dark)

3.3 Se and Sp of SCC Thresholds

Figure 2 shows the normal distribution of Ln SCC of all negative quarters and the normal distribution of Ln SCC of all major quarters. On the basis of figure 2 different thresholds were determined. Table 4 shows the sensitivity, specificity, positive predictive value (PVV) and negative predictive value (NPV) of different Ln SCC thresholds to identify subclinical infections caused by major pathogens. Improvements in specificity were related to lower sensitivity. The positive predictive value was relatively low for all of the alternatives discussed in this study with a maximum of 26 %. The negative predictive value was relatively high for all of the discussed alternatives, with a maximum of 96%, what maybe has to do with the fact that most of the samples were negative (51.4%).

Table 4. Sensitivity and specificity of SCC threshold values for identifying major infected quarters around calving

Threshold Ln SCC	Threshold SCC[a]	Sensitivitiy	Specificity	PPV[b]	NPV[c]
4,50	90	0,99	0,07	0,10	0,98
6,50	665	0,88	0,33	0,12	0,96
7,00	1096	0,79	0,51	0,15	0,96
7,70	2208	0,64	0,75	0,21	0,95
7,80	2441	0,60	0,78	0,22	0,95
7,90	2698	0,60	0,82	0,26	0,95
8,00	2981	0,56	0,83	0,26	0,95

[a] * 1000 cells/ml

[b,c] PPV = positive predictive value; NPV = negative predictive value

Table 5. Sensitivity and specificity of SCC threshold values for identifying minor or major infected quarters around calving

Threshold Ln SCC	Threshold SCC[a]	Sensitivitiy	Specificity	PPV[b]	NPV[c]
4,50	90	0,98	0,08	0,26	0,91
6,50	665	0,78	0,34	0,29	0,82
7,20	1340	0,59	0,60	0,33	0,81
7,70	2208	0,46	0,77	0,41	0,81
8,00	2981	0,35	0,85	0,43	0,79

[a] * 1000 cells/ml

[b,c] PPV = positive predictive value; NPV = negative predictive value

Figure 3 shows the normal distribution of Ln SCC of all negative quarters and the normal distribution of Ln SCC (scc1) of all minor and major quarters. On the basis of figure 3 different thresholds are determined. Table 5 shows the Sensitivity, Specificity, positive predictive value (PVV) and negative predictive value (NPV) of different Ln SCC thresholds to identify subclinical infections caused by minor and major pathogens. Improvements in specificity were even this time related to lower sensitivity. The positive predictive value was this time less low compared with the positive predictive values in table 4 for all of the alternatives discussed in this study with a maximum of 43 %. The negative predictive value was relatively less high compared with the negative predictive values in table 4, with a maximum of 91 %.

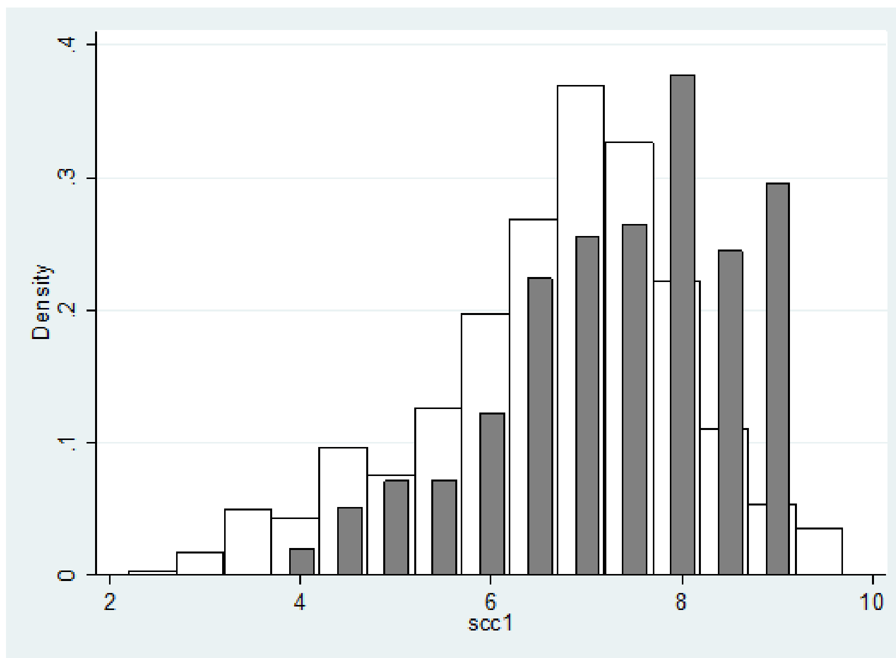

Figure 3: Normal distribution of LnSCC (scc1) of all negative quarters (white) and all infected quarters, major and minor (dark)

4. Discussion

The selection strategy used was a random stratified sampling designed to achieve a representative distribution for herd size and location across the Dutch population of dairy cows. In addition to this test, only cows participated with a SCC lower than 250.000 cells/ml and heifers with a SCC beneath 150.000 cells/ml at the most recent MPR. High percentage of milk samples (5%) were contaminated which could be the result of the fact that the milk samples were collected by farmers. Contamination of samples can be avoided by proper sampling techniques (Sampimon *et al*., 2009).

The research was built on a split-udder design. During this project, two served as negative control and were not treated during the drying off. The other two quarters were treated with antibiotics (Supermastidol). There was no effect detectable on the outcome of this study, because all samples were taken at the moment of calving. It can be assumed that the antibiotics are out, unless the cow calves to early.

In this study, Coagulase-negative staphylococcus (CNS) was the most frequent isolated species followed by *Corynebacterium bovis* (COR). In other studies, CNS was not reported separately. However, CNS is the most frequently isolated group of organisms in almost all recent mastitis surveys. According to Sampimon *et al*. (2009) it is not clear whether IMI with CNS always results in inflammation (Sampimon *et al*., 2009).

In this study, samples with *Bacillus* spp. were marked as negative, because the effect of *Bacillus* spp. seems to be low (Sampimon *et al*., 2009). According to Sampimon *et al.*, (2009), it is not likely that the high prevalence of *Bacillus* spp. is due to contamination . However, the reason of the *Bacillus* spp. in our study is hardly to explain.

Heifers with an elevated SCC postpartum, could suffer from a persistent IMI caused by *Staphylococcus Aureus* (De Vliegher *et al*., 2005). The SCC is elevated, but Staphylococcus Aureus will not be showing in the milk, whereas it is the cause of the elevated cell count. This can cause an increased number of false-negatives.

Although bacteriological culture is considered to be a reliable method for detection of IMI, according to Dohoo *et al*. (2011), many factors can influence the results of bacteriological culture, such as method of sampling, storage condition after collection, microorganism species, number of organisms in each sample, duration of infection, shedding pattern of the organism, medium used for culture, volume of milk plated, and frequency of sampling. If the main goal is identifying as many infections as possible, the use of single milk sample is acceptable (0.01mL) (Dohoo *et al*., 2011). But according to Andersen *et al*., (2010), culturing triplicate milk samples may be considered the golden standard for IMI detection. In this study, the number of herds was limited and the IMI definition was based on a single milk sample. Thus, before the results can be generalized, further research is indicated.

5. Conclusion

Single SCC analysis to classify quarters as uninfected or infected at time of calving may be not useful. The Se en Sp of SCC at several thresholds were relatively low. Sensitivity is increased and Sp is decreased when mammary quarters with major or minor and major pathogens are considered positive. Milk samples with major pathogens have higher SCC compared with those with minor pathogens isolated. The prevalence of minor pathogens was higher than that of major pathogens.

The choice of definition of Se and Sp will depend on the objectives of study or control program for which the sample was collected. Finally, the main objective of choosing a specific SCC threshold is to optimize the efficacy of a mastitis control program. Thus, it is necessary to choose a SCC threshold to minimize IMI diagnostic errors. In practice, SCC cannot be used as a diagnostic tool for IMI early postpartum.

6. Acknowledgment

I want to acknowledge the intensive work carried out by all students, the cooperation of the farmers who were involved in the study, the people who carried out the bacteriological analysis and processing of data. Finally I want to thank DRS. C.G.M. Scherpenzeel and DR. R.G.M. Olde Riekerink for the opportunity to work on this research and Dr. G.A.Hooijer for the supervision from University of Utrecht.

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