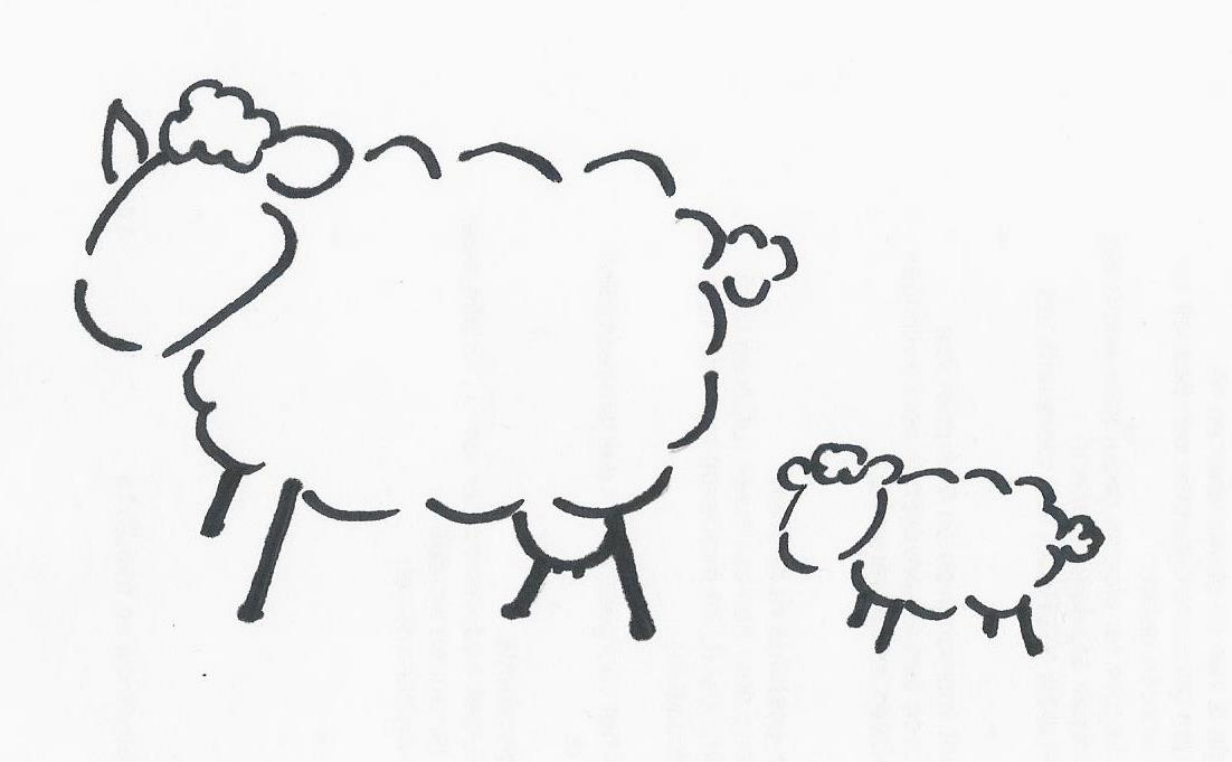
Mastitis in sheep



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Abstract

The two types of mastitis (clinical and subclinical) are mostly caused by micro-organisms. The most important pathogens are *Staphylococcus aureus, Mannheimia haemolytica* and *Coagulase-Negative Staphylococci* (CNS). Much is unknown about intramammary infections in meat sheep. For this study fifteen farms were selected to be visited, on each farm fifteen meat ewes were sampled for bacterial culture (BC) and somatic cell count (SCC), each udder half was sampled separately. The total number of milk samples was 388. The aim of the study was to examine if SCC could be used as a diagnostic analyze method for intramammary infection (IMI). The SCC-samples were compared to the bacterial culture samples. The results shows that the SCC-test with a threshold value of 36,500 cells/ml had a sensitivity (Se) of 88% and a specificity (Sp) of 25%. The SCC-test with a threshold value of 293,000 cells/ml had a specificity of 90% but a sensitivity of 37%. So SCC has no threshold value with a high sensitivity and high specificity, so it cannot be used as a diagnostic test for intramammary infections. When the *Staphylococcus aureus* and *Mannheimia haemolytica* samples were analyzed, the sensitivity was 81% with a specificity of 53% for a threshold value of 80,500 cells/ml. SCC is a better test for the detection of major pathogens (*Staphylococcus aureus* and *Mannheimia haemolytica*) than for intramammary infections, but the sensitivity and specificity are not almost the same as bacterial culture. The same kind of test were done with *Escherichia coli* and *Enterococcus faecalis*, when the Se was high, the Sp was low and vice versa, so it is an unusable test too.

Keywords

Meat sheep, somatic cell count, intramammary infection, mastitis

Introduction

Mastitis is the inflammatory response of the mammary tissue, most frequently caused by micro-organisms.[1-6] Most of the time mastitis is observed soon after lambing, 2-4 weeks post-partum or soon after weaning (post-weaning-mastitis).[3] Mastitis can be divided into two types: clinical and subclinical mastitis. Clinical mastitis shows clinical signs, in the acute phase redness, heat, pain, asymmetry and swelling of the mammary gland and changes in the milk can be seen, like watery, bloody, clumpy or purulent secretion and discoloration. The ewe gets a fever, anemia and becomes lethargic. The ewe reduces her eating behavior, changes its posture and locomotion to relieve the udder. Because of the pain the ewe avoids suckling by the lamb. In severe cases paralysis of the ewe may occur.[1,2,7-14] In the chronic phase, the udder becomes atrophic and loses its function. Abscesses can be found, the teat and udder become swollen and pus can be seen in the milk and teat canal.[10,12,13] Mostly chronic mastitis occur post-weaning.[10,12,13] Because of the changes in the milk, lambs grow suboptimal and get hungry because of the milk-changes.[15,16] The kind of cells will change, normally macrophages are the most common cells in milk, in the acute phase of subclinical mastitis neutrophils will rise abnormally and in the chronic phase lymphocytes rise abnormally.[16] Subclinical mastitis shows no clinical signs, however the milk contains bacteria as well.[1,10,12]

Mastitis is a very important disease in sheep.[2,17,18] Mastitis is important for three reasons: economic, hygienic and legal problems. The economic aspect is most important for meat sheep. Some economic aspects are the mortality of animals, treatment costs, reduced quantity and quality of milk.[18] About five till ten percent of the

lambing mortality is caused by mastitis and ten percent of the ewe mortality.[10,17] Hygienic and legal aspects are important for milk-sheep. Importance of mastitis in hygienic perspective is mostly applicable to milk sheep, it is a risk of infection of consumers by consuming infected milk. There are definitions of bacteriological milk quality in the law, this is the legal aspect of mastitis.[18]

Mastitis is a highly multifactorial disease with several manifestations.[19] Influences like rainy weather, animal lying on dirty, cold and wet ground, soiled wet bedding, high prevalence of mastitis in the herd and udder lesions are risk factors for getting mastitis.[1,5,7,13,14,17] Nutrition also has a predisposing effect on mastitis, for example hypocalcemia.[8,20] Not only calcium is an important nutrient, magnesium and copper levels also have an influence on the frequency of mastitis.[20]

There are many bacterial species causing mastitis. *Staphylococcus aureus, Mannheimia haemolytica* and *Coagulase-Negative Staphylococci* (CNS) are most important bacteria.[7,10,13] Other pathogens are *Streptococcus spp., Corynebacterium pyogenes, Pseudomonas spp., Escherichia coli, Listeria spp., Salmonella spp., Klebsiella spp., Clostridium perfrigens C, Mycoplasma spp.* and *Enterobacteriaceae. S. aureus* and *M. haemolytica* cause most frequently clinical mastitis. *CNS* are important pathogens for subclinical mastitis.[7,10,13,14,21-24]

Subclinical mastitis detection is very hard, only bacteria in the milk is an indication for subclinical mastitis. Also clinical mastitis is not easy to detect, certainly in a big herd, because the symptoms can be minimal, like only milk changes, or the udder is not inspected regularly. Most of the time the farmer inspects the udder just after lambing, during the suckling period the udder will not be observed frequently, because the lamb suckles the ewe, not the farmer as in case by milk-sheep. When the ewe or lambs start showing obvious symptoms the farmer will inspect the udder again.

In other countries then the Netherlands, some studies have been performed on Somatic Cell Count (SCC) in milk ewes as a diagnostic method for finding intramammary infections. This studies concluded: Somatic Cell Count in milk increases when bacteria can be found in the milk. These studies have been performed with milk sheep, not with meat breeds.[1,3,5,10-12,14,15,21,22]

If SCC is a good test, it will be more practical, cheaper and can be quickly used by the farmers. Several days are needed for bacteriological examination, while SCC can be quickly measured and the results will be announced at the same day. In addition to periodic measurements, cases of subclinical mastitis can be detected and so the risk of infection is lowered. It is easy for the farmer to take SCC samples. If a lot of ewes have udder problems because of intramammary infections, the farmers are willing to invest some time to reduce the problems and costs of any intramammary infections. A good SCC test requires a high sensitivity, so most ewes with intramammary infection are detected. Detection of intramammary infections is important because infected ewes are a risk for other ewes and the effects of an infection can be decreased, by treating these animals or to eliminate them. In addition the test requires a relatively high specificity, so animals are not treated unnecessarily against intramammary infections or will be eliminated unjustified. Unnecessary treatment introduce more bacterial immunity, so bacteria respond no longer to antibiotics. Could SCC detect intramammary infections of meat ewes in the first three weeks after lambing? So has SCC approximately the same sensitivity and specificity as a bacterial culture (gold standard), so it can be used as a more practical diagnostic test to detect any intramammary infections? Or can SCC be used as a diagnostic test to detect major pathogens like *Staphylococcus aureus* or *Mannheimia haemolytica*?

Materials and methods

Experiment overview

Fifteen sheep farms were enrolled in this study. The selected farms were located throughout The Netherlands. Farmers could sign up themselves by the NSFO (breeding association for goats and sheep in the

Netherlands). Fifteen ewes, most of them were Texelaar-ewes, were selected on each farm, resulting in 225 (meat)ewes used for this study. The samples were taken in a period of 0-3 weeks after lambing. The fifteen ewes on each farm were selected by the farmer because of their different parity (5 first, 5 second and 5 third). The samples were taken in a period of six weeks during February-April, but all of the ewes were 0-3 weeks post-partum. Two hours before the visit, lambs and ewes were housed separately, so there was enough milk in the udder for sampling. During the visit the udder was physically examined and then a 10 mL milk sample for Somatic Cell Count (SCC-samples) and a 2 mL milk sample for bacteriological examination (BE-samples) were taken. SCC samples and BE-samples were taken from each mammary gland (left and right udder half). Some ewes did not give enough milk for the 10 ml SCC samples, some of the BE-samples were contaminated and at some farms more ewes were sampled than the fifteen selected ewes, so the total number of useful samples eventually became 388.

Collection of samples

The left udder was sampled first. The first two squirts of milk were discarded and then ±10 mL was milked into this SCC tube. The BE-sample was taken after the SCC-sample. First the teat end was disinfected with alcohol (isopropyl 70%) and cotton wool. The sterile milk-tube was opened horizontal and ±2 ml milk was sampled, without touching the teat-end or milk-tube inside. The sample has to be taken sterile to reduce the number of contamination. After taken the SCC- and BE-sample, the right udder was sampled at the same way. All samples will be cooled during transportation in a Styrofoam box with ice-elements.

Somatic cell count (SCC)

In the SCC-tube is 0,05 mL preservative added (sodium azide 5%), so the number of cells will be stable and the milk won't spoil. The Somatic Cell count was done external with a Delta instruments Combiscope 600 by the Vereniging voor Veehouderijbelangen – "Veluwe-IJsselstreek" in Nunspeet (NL). Due to the geographical spread of the farms, the SCC-samples were not taken every day to the laboratory in Nunspeet. All samples were brought within three days to the laboratory. During these days, the samples were cooled at 5 degrees Celsius.

Bacteriological examination

All the BE-samples were tested for presence of bacteria and the kind of bacteria. On the first day (day of sampling) 10 µL was inoculated on a half sheep blood agar, so each sheep has his own sheep blood agar plate (left udder sample and right udder sample on the same agar). The plate was placed in a 5%-CO_2 incubator with a temperature of 37°C. The remainder of the BE-samples milk were placed in the incubator too.

After 24 hours (day 2, 24h after sampling) all the plates were examined. On the plates with bacteria (positive agars), the bacteria colonies were described (number of types colonies, number of colonies, color, hemolytic or not, shape and size). The positive agars were placed in the incubator for another 24 hours. When there was no bacteria-growth (negative agars), the agars were placed in the incubator again. The incubated milk of these samples was inoculated on a half sheep blood agar (10µl) again and placed in the incubator for 24 hours.

After another 24 hour (day 3, 48 hours after sampling) the bacteria colonies were described too (number of types colonies, number of colonies, color, hemolytic or not, shape and size). The negative agars after 48 hours were called 0. The positive agars after 48 hours were called 1. Agars with 3 or more types of bacteria colonies were excluded from the study, because its possible contaminated.

Bacterial identification

The bacteria on a positive plate were identified. This was performed by the MALDI-TOF in the University Medical Centre of Utrecht (UMC Utrecht). In order to make use of the MALDI-TOF the bacteria were put on a MALDI-TOF plate. Each sample had its own

spot on the MALDI-TOF plate, one plate has 96 spots on it. In addition, each sample was tested in duplicate, in order to increase reliability. The first four spots were sampled with two known test-colonies, *Escherichia coli 25922* and *Staphyloccus (pseud)intermedius 2081218007*. These spots were used as a control group, when these spots were tested correct, the other spots will be tested correct too.
After placing a few bacteria on the MALDI-TOF plate with a sterile skewer, each spot was dripped with 10 µL of formic acid. When this drop was dried by air, 10 µL matrix was dripped on the spot too. After drying everything, the plate can be analyzed by the MALDI-TOF. The results were given in an Excel sheet. Each spot had a top 10 of results and a worth of reliability, when this worth is >2.0 the results are reliable. A worth <1.7 is unreliable and a worth between 1.7 and 2 is reliable for the genus of a bacteria, not the subspecies, for example *Staphylococcus spp.*. If the results were below 1.7, the bacteria were placed on the MALDI-TOF again, with a maximum of three times. When the results were not above 1.7 after three times, the bacteria will be noticed as unknown.

Data analyses

The results of the clinical observations, bacteriological examination and somatic cell count were presented in an Excel file, each ewe udder halve had his own row and sample-number.
To say something about the diagnostic value of Somatic Cell Count (SCC), the bacterial culture result was compared with the SCC of the samples. A ROC-curve shows the sensitivity against 1-specificity. Also the area under the curve is calculated. When the area under de curve (AUC) is 0,5 the test is worthless. When the AUC is 1, it is the perfect test.

Results

Bacteria culture results

Table 1 shows the results of bacteria growth and SCC. During the visits there were 461 udder halves sampled for milk and 11 udder halves had no milk in it. Not every milk sample had a SCC value, because the ewe did not give enough milk for it. 44% of the BE-samples were negative, so there were no bacteria found after 48 hours. In 14 BE-samples the kind of bacteria could not be identify. The samples without milk, samples with a mixed culture and samples without a SCC were excluded from the study.
The mean of SCC was also given in Table 1 per bacteria species, with a standard deviation and minimal and maximal value per species.

Somatic cell count results

The test properties of SCC have been tested with a receiver operating characteristic curve (ROC-curve) (Figure 1). In this test the positive or negative bacteriological examination was compared with the level of SCC. In the study were 192 positive samples with a known SCC found and 196 negative samples with a known SCC. So 84 of the samples were missing, because mixed cultures, no milk and no SCC were excluded from the study. The area under the curve (AUC) for this test was 0,660 with a 95% confidence interval (CI) of 0.606-0.714. For a "high" sensitivity of 88%, the specificity was 0.25 with a SCC threshold value of 36,500 cells/mL, so the specificity would be 25%. For a high specificity of 90%, the sensitivity was 37% for a SCC threshold value of 293,000 cells/mL. The positive predictive value of this test is 53% and the negative predictive value is 63%.
Staphylococcus aureus and *Mannheimia haemolytica* are major pathogens for intramammary infection, in this study 16 of the 388 samples were *S. aureus* or *M. haemolytica* positive. These samples were tested in the same way as the positive versus negative samples. In Figure 2 the ROC-curve of *S. aureus* and *M. haemolytica* can be seen, the AUC is 0.740. This test had a sensitivity of 88% with a specificity of 43%. But for a sensitivity

of 81% the specificity would be 53% for a SCC threshold value of 80,500 cells/mL.
In this study 8 samples were positive for *Escherichia coli* and *Enterococcus faecalis*. SCC test properties of these 8 samples in comparison with samples without *E. coli, E. faecalis, S. aureus* and *M. haemolytica* is shown in Figure 3. The AUC is 0.612. For a sensitivity of 88% the specificity of this test will be 25%.

	Bacteria		Somatic Cell Count			
	Count	Column N %	Mean	Std Dev.	Minimum	Maximum
Staphylococcus spp.	54	11%	376	802	14	3910
Staphylococcus equorum	28	6%	155	276	9	1271
Staphylococcus simulans	19	4%	2287	2682	24	9467
Staphylococcus chromogenes	18	4%	411	432	26	1688
Streptococcus spp.	13	3%	534	695	60	2089
Mannheimia haemolytica	10	2%	1932	2852	66	7715
Staphylococcus aureus	9	2%	1531	2056	33	5192
Brevibacillus parabrevis	8	2%	63	33	28	113
Bacillus spp.	5	1%	211	85	136	287
Enterococcus faecalis	4	1%	65	61	18	155
Escherichia coli	4	1%	2181	1902	161	3822
Staphylococcus epidermidis	4	1%	542	355	248	936
Aerococcus viridans	2	0%	113	109	36	190
Corynebacterium spp.	2	0%	96	77	41	150
Micrococcus luteus	2	0%	27	19	13	40
Rothia nasimurium	2	0%	2410		2410	2410
Staphylococcus warneri	2	0%	3912		3912	3912
Trueperella pyogenes	2	0%	10		10	10
Corynebacterium mastitidis	1	0%	41		41	41
Kocuria rhizophila	1	0%	64		64	64
Pantoea agglomerans	1	0%	34		34	34
Staphylococcus auricularis	1	0%	713		713	713
Staphylococcus capitis	1	0%	51		51	51
Staphylococcus haemolyticus	1	0%	2470		2470	2470
Staphylococcus pasteuri	1	0%	68		68	68
Staphylococcus vitulines	1	0%	161		161	161
Staphylococcus xylosus	1	0%	332		332	332
Unknown	14	3%	144	189	16	697
Mixed culture	44	9%	708	1181	12	5482
Negative	206	44%	215	683	5	5202
No milk	11	2%				
Total	472	100%	464	1.123	5	9467

Table 1. **Bacteriological and Somatic Cell Counts results (SCC x 1000 = cells/ml)**

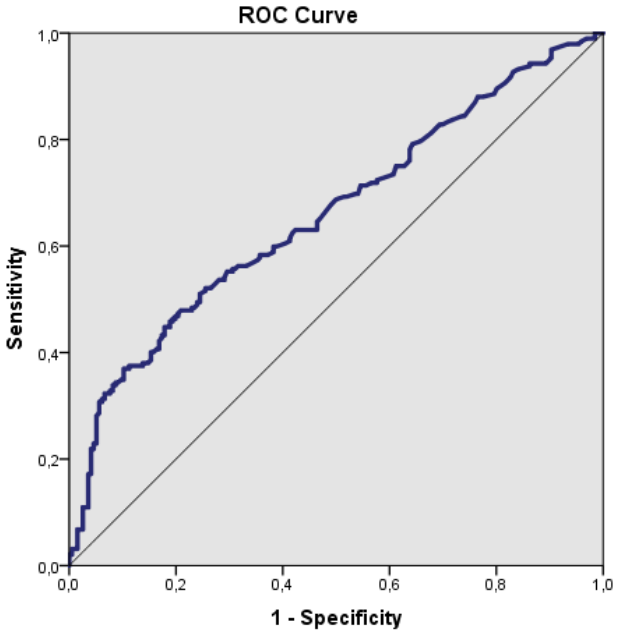

Figure 1. **ROC-curve positive and negative samples in comparison with SCC as test-group.**
AUC = 0.660
Std. Error = 0.028
95% Confidence Interval = 0.606-0.714

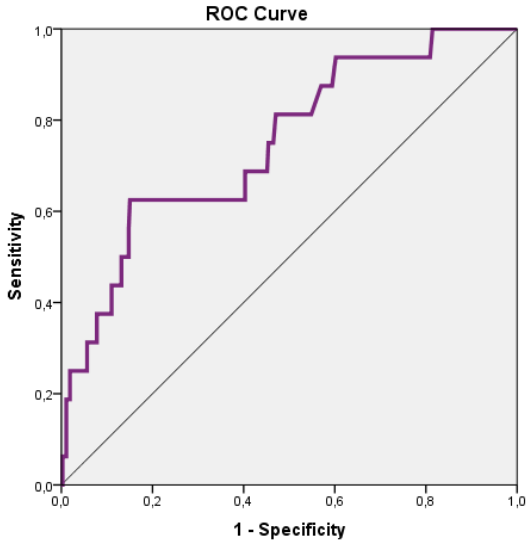

Figure 2 **ROC-curve samples with or without S. aureus and M. haemolytica in comparison with SCC as test-group.**
AUC = 0.740
Std. Error = 0.064
95% Confidence Interval = 0.625-0.874

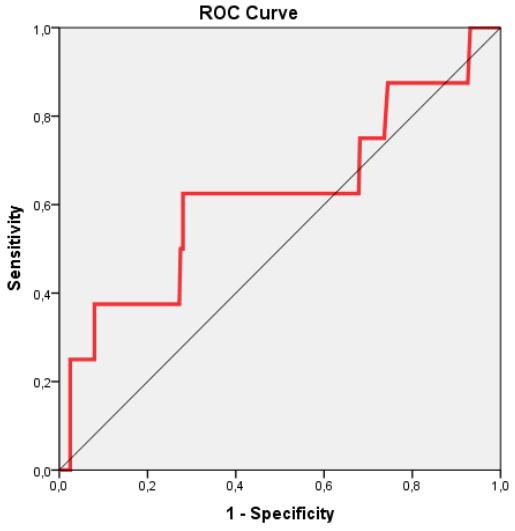

Figure 3 **ROC-curve samples with or without E. coli and Enterococcus faecalis in comparison with SCC as test-group. Samples without E.coli and E. faecalis were also without S. aureus and M. haemolytica too.**
AUC = 0.621
Std. Error = 0.118
95% Confidence Interval = 0.389 – 0.853

Discussion

In this study, the test properties of Somatic Cell Count were analyzed. The sensitivity and specificity of SCC as a diagnostic test for intramammary infections were calculated. Different SCC threshold values were tested, a threshold value with a high sensitivity has a very low specificity and a SCC threshold value with a high specificity has a low sensitivity. So SCC cannot be used as a diagnostic test for intramammary infection. Also the test properties for detection of *Staphylococcus aureus* and *Mannheimia haemolytica* were examined, this results in the same conclusion. The sensitivity and specificity were some better, but also not good enough for a useful diagnostic test.

In the literature it is not clearly described which percentage of the samples could be positive or negative. Berthelot et al. describes 95% of the (3758) milk samples were culture-negative.[24] But Contreras et al. describes 5-30% of the small ruminants has an intramammary infection.[25] And Rovai et. al describes a percentage of 42,9% and 50% ewes with subclinical intramammary infection.[26] In this study 192 of the 388 samples were positive and 196 were negative, so 49% of these milk samples has a bacteria in it, so the ewe has an intramammary infection. The ewes in this study were selected by the farmer, two hours before the visit. They were asked to select the animals randomly, while taking the parities into account. It could be that they had a preference for ewes with suspected udder inflammation, to get more information about them. In the data of this study most bacteria were *Staphylococcus spp.*, this is consistent with the literature.[25,27]

The gold standard for detection of intramammary infections is the bacteriological culture. This test has a sensitivity around 83% and a specificity around 90%, so in fact the bacteriological culture is not a true gold standard test.[28] The desired Somatic Cell count test requires a high sensitivity (>83%), like the bacteriological culture and a relative high specificity (>70%), so it can be used as a diagnostic test for intramammary infection with the same reliability as bacteriological culture, but this test could be easier to use, is cheaper and faster. If the sensitivity is high (83%) in this study, the specificity will be low (29%) and vice versa (Figure 1). The positive predictive value for the SCC-test is 53% and the negative predictive value is 63%. These values are also relatively low, so this test has a low predictable value for the presence of intramammary infections. "As the prevalence of the disease is raised, there are more animals with the disease in the population, and a greater confidence that a positive test result is correct; the positive predictive value of the test increased and the negative predictive value decreased. The reverse is true as the prevalence of the disease is lowed." (quoted from: *Statistics for Veterinary and animal science, A. Petrie and P. Watson, 1st ed., 14.2.7: Usefuless of the test: positive and negative predictive values, page 204*). Concluded; intramammary infections in meat sheep cannot be reliably diagnosed by Somatic Cell Count in the first three weeks after lambing.

Similar studies have been done in cattle, by Vissio et. al. The conclusion in this study was "there was no evidence of dependence between SCC and bacteriological culture."[28] This corresponds with our study in sheep. In some studies SCC will be used as a diagnostic test, but the wide range of the SCC values does not permit to suggest any threshold value.[5,10,12,14,29]

For detection of *S. aureus* or *M. haemolytica* this test is more useful, because when the SCC is 80,500 cells/mL the sensitivity is 81% with a specificity of 53%. However this test does not meet the requirements of this study (high sensitivity and specificity). The test for detection of *E. coli* or *E. faecalis* is not useful for the same reason as mentioned before, the specificity is too low for a high sensitivity and vice versa.

Bacteriological examination (gold standard) has no perfect sensitivity and specificity.[28,30] Especially the sensitivity is lower than 100%. So when bacteriological examination is negative, it is not sure that there are no bacteria in the milk.[5,12,14,26] For example *Staphylococcus aureus* is a highly pathogenic cause of mastitis, it can hide from the immune system. If it is in the udder, it is not always

secreted into the milk. So it can be missed by bacteriological examination.[12,31] This means that the sensitivity of SCC actually will be higher, because more samples which were positive in the SCC are positive in real, resulting in fewer false-positive samples.

The somatic cell count is normally variable during the lactation. At the beginning of the lactation the SCC is always higher than later in the lactation.[32,33] So maybe SCC is a better diagnostic test later in the lactation.[32] Furthermore, the analysis of various samples was delayed because of logistic reasons. In case, samples were delayed by a period of >24 hours, somatic cell counts could decrease slightly and influence the SCC values in this study.[34] However, only 79 out of 388 samples were delayed 2 days. This would change the final results hardly.

In this study not only Texelaar-ewes were used. Some of the sampled ewes were Blue-Texelaar sheep, Vlamingen, Swifters, Zwartbles Sheep and some other breed. The breed could have some influence on the results of intramammary infection and the milk yield, some breeds are more susceptible to intramammary infections than others.[35-37]

In this study each udder half sample was considered as an independent test result, but one ewe has two udder halves, so the two measurements are dependent. It is possible that one udder half affect the other udder half. The lambs can drink on both udder halves, so any infection of an udder half can be moved to the other udder half. This allows an infected udder half to infect the other half as well. In addition the udder halves are close together, so they never can be independent of each other. [1,2,10,12-14,38]

In short, this study shows that SCC cannot be used as a diagnostic test for intramammary infections in meat sheep. Because the sensitivity or specificity will be too low.

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