Mastitis in meat sheep

Etiology and clinical symptoms

P.H.M. van Dam, solis-ID 3754642

Supervisor: dr. G. Koop

Research, 2015

Mastitis in meat Sheep

P.H.M. van Dam*

** Solis-ID 3754642, Utrecht University, Faculty of Veterinary Medicine, Yalelaan 1, 3584 CL Utrecht, The Netherlands, Supervisor: dr. G. Koop*

Abstract:

A study was conducted in meat sheep in The Netherlands between February and April of 2015. A prevalence of positive milk samples of 51.7% in bacteriology was found overall. The prevalence on individual basis was 68.9%, in which an ewe was found to be positive if one or both udder halves were positive in bacteriology. Identification of the bacterial strains was performed by the Matrix Assisted Laser Desorption Time-of-Flight Analyser (MALDI/TOF MS) at University Medical Center Utrecht (UMC). Most commonly isolated from the milk were *Staphylococcus* spp. Besides *Staphylococci spp.*, major pathogens such as *Staphylococcus aureus* and *Mannheimia haemolytica* had been isolated. The presence of *S. aureus* or *M. haemolytica* was significantly correlated to the presence of clinical symptoms ($p < 0.005$). Another interesting finding was the identification of *Rothia nasimurium* on 2 farms, which was associated with high geometrical Somatic Cell Counts. Furthermore this study showed great variation in the number of positive samples and in the prevalence of major pathogens between the participating farms, possibly related to differences in farm management.

Keywords:

Mastitis, meat sheep, MALDI/TOF MS, *Rothia nasimurium,* clinical symptoms

Contents: **Page**

Abstract: 2
Introduction: 3
Methods and Materials: 4
Animals and clinical data: 4
Data and sample collection: 4
Additional samples: 5
Bacteriology: 5
Identification by the MALDI/TOF MS: 6
Somatic Cell Count (SCC): 7
Statistics: 7
Results: 7
Etiology: 7
Symptoms: 8
Additional samples: 9
Distribution amongst the farms: 9
Discussion: 10
Farms: 11
Selection of the ewes and sample collection: 11
Bacteriology and identification: 12
Treatment: 13
Conclusion: 13
Acknowledgements: 14
Bibliography: 15

Introduction:

At around 12.000 sheep farms are established in The Netherlands. A total of 958.602 sheep lived in The Netherlands in 2014, of which over half a million ewes[1]. The majority of sheep in The Netherlands are kept for the production of meat and to provide for future breeding stock.

Mastitis is an infection of the mammary gland and it can cause serious problems in ewes. Dutch farmers say it is one of the most common problems amongst meat sheep and they claim it is an important cause of culling of the ewes. Farmers said that as much as 80% of culled up ewes have been affected by mastitis, this however is based on personal experiences. Mastitis can cause ewes to become unfit for breeding, due to the fact that one or both udder halves do not longer produce any milk, wherefore they are no longer able to provide their lambs with enough milk. In addition to the culling of the ewes, there are more economical consequences of mastitis, which include increased veterinarian costs, reduced growth of the lambs, mortality of the lambs and ewes and increased labour expenses[2, 3].

Mastitis can present itself in different forms and usually a subdivision is made between clinical mastitis and subclinical mastitis. Clinical mastitis is an infection of the mammary gland in combination with general symptoms (weakness, anorexia, etc.) and/or clinical symptoms (swelling of the udder, possible necrosis, abnormal milk, etc.). Contrary to cases of clinical mastitis ewes with sub-clinical mastitis do not show any clinical symptoms. Sub-clinical mastitis can be detected by testing the ewes' milk, as an enumeration of inflammatory cells can be found in the milk by the Somatic Cell Count (SCC). Chronic mastitis is an inflammation of the udder that is lasting over an extended period of time and can be either clinical or sub-clinical [2-8].

Previous researches stated that clinical mastitis has a low incidence in sheep; as low as 5% of the sheep per year, which counts for both meat and dairy flocks[2-4]. Sub-clinical mastitis was found to have a prevalence of about 10-50% per lactation[2]. In order to detect sub-clinical mastitis various methods had been used, which included individual Somatic Cell Count (SCC), the Californian Mastitis Test (CMT) and bacteriological culturing of the milk[2, 9-11].

Staphylococcus aureus has been frequently isolated from ewes with clinical mastitis (in 17-53% of the cases), whereas Coagulase-negative *Staphylococci* (CNS) were commonly found in ewes with sub-clinical mastitis. CNS species that have commonly been isolated from the milk are *S. epididermidis, S. xylosus, S. simulans* and *S. chromogenes*[2-4, 12, 13].

Somatic Cell Counts (SCC) can be performed to detect mastitis, as an enumeration of cells can indicate ongoing cases of mastitis. Cell counts with values in between 200.000 and 400.000 cells per ml will identify the majority of ewes with sub-clinical mastitis[3, 14]. Samples with cell counts starting from 300.000 up to 1.000.0000 cells per ml can show

changes in the composition of the milk, which can indicate mastitis[3].
Most recent studies regarding mastitis in sheep have been conducted on dairy sheep and have been executed abroad. These studies have been based on different management systems, sheep breeds and hygiene protocols. Up to now, no study has been conducted describing the etiology of mastitis in The Netherlands in meat sheep flocks.
This study has been executed solely on meat sheep; breeds commonly kept for this purpose in The Netherlands (the Texel - and the Swifter sheep breed) were included in the study.
The aim of this study is to describe the bacterial species that are present in the milk of meat sheep and to find associations between clinical symptoms and the etiology. If an association can be found, farmers could select ewes based on the clinical findings to reduce the number of mastitis cases in their flocks.

Methods and Materials:

Animals and clinical data:

Milk samples and data regarding udder health were collected from 225 ewes in The Netherlands. A total of 15 flocks were included. The farms were spread over different provinces and their geographical spread is shown in **Figure 1**. The sample collection has been carried out in the period between February and April of 2015.

Figure 1 *The geographical spread of the 15 farms included in the study, marked by red and pink star(s). The pink star and the neighbouring red star represent two farms in the same village*

Data and sample collection:

All farms were visited twice in the period between February and April of 2015; the first visits took place within the first three weeks post-parturition and the second visits between three to six weeks post-parturition.
15 ewes per farm were selected and if possible a variety of parity was chosen. Ideally 5 ewes from the first parity, 5 ewes from the second parity and 5 ewes from the third parity were chosen by the farmer.
The ear tag number or neck number, date of birth (DOB), date of parturition and clinical condition of the udder were collected from all ewes individually. The condition of the udder was assessed by clinical parameters, which included an

overall assessment of the udder (e.g. the presence of nodules, the symmetry between the left and right udder half) and the milk (amount and consistency) given by the ewe. In order to obtain enough milk for bacteriological culturing and the SCC, the lambs were separated from the selected ewes for two hours prior to the sampling moment. After sampling the ewes and their lambs were immediately reunited.
Milk samples for bacteriological examination were collected aseptically from each ewe from both udder halves. Two streams of milk were discarded before a milk sample was collected and each teat was disinfected with cotton wool soaked in Ethanol (70%). Approximately 2 ml of milk per udder half was collected in a sterile milk tube. During transportation to Utrecht University the samples were cooled in a cool box filled with cooling-elements.
The samplers wore protective clothing at all visits, as in overalls, latex examination gloves and boots. The latex examination gloves were replaced if it was thought to be necessary to the opinion of the samplers (e.g. contact with dirt, pus, severe cases of Ecthyma and contact with ewes with clinical mastitis).

Additional samples:

In cases a farmer suspected a ewe of clinical mastitis in the period prior to the first visit or in between the first visit and the second visit, aseptic samples of both udder halves were taken by the farmer. The samples were collected in sterile milk tubes. These samples were taken before the ewe received any treatment and the samples were frozen at -20° Celsius. Samples were stored together with a questionnaire which contained the following information about the ewe; ear tag number, parity, DOB, parturition date and clinical parameters regarding udder health. The samples were collected by the researchers from the farmer at the first and/or second visit and transported in cooled state to Utrecht University for bacteriological culturing and identification.

Bacteriology:

All samples were examined and processed on the day of sampling at a laboratory of Clinical Infectiology (KLIF) at Utrecht University, Faculty of Veterinary Medicine. The samples were gently mixed before the milk was processed. Per milk sample 10 µl of milk was plated over on a Sheep Blood Agar plate by using a sterile inoculating loop. One Sheep Blood Agar plate was used per ewe and two milk samples were divided each over half a plate. The remaining milk sample was placed in the incubator overnight by 37° Celsius (5% CO_2).
After approximately 24 hours of incubation the Sheep Blood Agar plates were inspected for the presence of colonies.
In cases in which the bacteriological culture was negative, 10 µl of the incubated milk was plated out on a fresh Sheep Blood Agar plate. Both batches of agar plates were then placed in the incubator overnight at 37° Celsius (5% CO_2) and were checked the second day for colonies.
If a bacteriological culture was positive the colonies were scored on

their appearance (number of types, colony shape, colour, presence of haemolysis and the number of CFU's).
If one or two CFU types were present on the Blood Agar plate, the CFU('s) were plated over on a fresh Sheep Blood Agar plate. Fresh CFU's of approximately 17 hours of growth were then further analysed by the MALDI/TOF MS.
In case a bacteriological culture was positive out of the incubated milk, at least 5 CFU's had to be present, otherwise the sample was discarded.
Samples that were most likely to be contaminated (≥ 3 CFU types on the Blood Agar plate) were excluded from further bacteriological identification. Contaminated samples received the label "Mixed culture".
In case a contaminated sample contained a haemolytic CFU, this CFU was isolated and plated over on a fresh Blood agar plate. In case the haemolytic strain was successfully isolated it was further analysed by the MALDI/TOF MS.

Identification by the MALDI/TOF MS:

Identification of bacterial species by the Matrix Assisted Laser Desorption/Ionisation Time-of-Flight Analyser is fairly new and it had not yet been used for identification of strains in mastitis research in meat sheep. However the MALDI/TOF MS has been used for identification of strains in dairy cattle, in which it has been referred to as an alternative method to identify strains[15, 16]. Therefore it seemed useful to apply the MALDI/TOF MS in this study.

The MALDI/TOF MS of University Medical Center Utrecht (UMC) was used for identification. Fresh colonies (+/- 17 hours of growth, incubated at 37° Celcius, 5% CO_2) were streaked onto the MALDI/TOF target. Each target contains 96 spots. The first four spots were used for verification at all times with known bacterial species (*Escheria coli* 25922 and *Staphylococcus pseudintermedius* 2081218007*)*, both strains were smeared in duplicate. Streaking was performed with an autoclaved skewer. All samples were smeared onto the target in duplicate, in so doing this increased the chance of finding a useful score value. One MALDI/TOF target offered space to 47 samples per run. All samples were covered by 10 µl of Formic Acid (FA) and were allowed to dry by air for approximately 5-10 minutes. Directly after the spots were visibly dry 10 µl of matrix (Bruker Corporation, Germany) was added to the spots. Score values ≥ 2 indicated an identification on the level of species. In case score values were lower than 2, but greater than or equal to 1,7 only the bacterial genus was adopted. Score values < 1,7 were dismissed, as no reliable identification could be performed by the MALDI/TOF MS. Samples that had been dismissed were analyzed for a second time by the MALDI/TOF MS in order to obtain score values ≥ 2.
In cases the MALDI/TOF MS found *Streptococcus* spp., only the genus was adopted as the MALDI/TOF MS is not able to distinguish different *Streptococcal* spp. from each other.

In case score values were < 1,7 samples were analyzed for a second time; this procedure was repeated no more than three times. If the strain still had not been identified, the sample was labeled 'Unknown'. These samples have been frozen and stored (-80° Celsius) at Utrecht University.

Somatic Cell Count (SCC):

Somatic Cell Counts (SCC) were performed using a Delta instruments Combiscope 600. Approximately 10 ml of milk per udder half was in which the samples were collected were provided by Vereniging voor Veehouderijbelangen 'Veluwe-IJsselstreek' and contained a conservative for the milk. The conservative used in this study is Sodium azide (5%). Per liter one teaspoon of blue colouring agent was added. Each milktube contained 0,05 mL conservative and colouring agent.
The samples were brought to the laboratory for testing at the day of sampling or one or two days after sampling, in the intermediate time the samples were stored in a cool room (5° Celsius) at Utrecht University, Faculty of Veterinary Medicine.

Statistics:

A Chi-square test has been used to determine the significance of the presence of symptoms in combination with a positive bacteriology. Analyses were performed with IBM SPSS Statistics 22 (IBM Corporation, Armonk, New York 10504-1722, United States).

Results:

Etiology:

A total of 476 udder half samples were collected in the period between February and April of 2015. The prevalence of mastitis was 51.7% of all udder half samples included in the study (n= 476). A prevalence of 68.9% was found on ewe level, in which a ewe was considered to be positive if bacterial species were isolated from one or both udder halves.
Samples that grew one or more CFU's on the 48h Blood Agar plate were considered as mastitic as well as samples that grew ≥ 5 CFU's after 24h incubation out of the pre-enriched milk.
An overview of the isolated bacterial species and corresponding mean geometrical SCC are presented in **Table 1**. This table displays the species that have been identified by the MALDI/TOF MS. In case two types per sample were isolated only one type (major pathogen or most CFU's) has been included in **Table 1.**
Fourteen samples contained two species and these consisted of *Staphylococcus* spp. combined with another spp. (unknown spp. / *S. equorum* / *Streptococcus* spp. / *Pseudomonas* spp. or *Aerococcus* spp.), *Staphylococcus equorum* combined with another spp. (*Corynebacteriumm stationis*/ *Aerococcus viridans* or *unknown* spp.), *Staphylococcus chromogenes* combined with another spp. (*Mannheimia haemolytica* / *Streptococcus* spp.

	Count	%	Mean geometrical SCC (x 1000 cells/ml)
Staphylococcus spp.	132	27.7%	170
Bacillus spp.	21	4.4%	83
Streptococcus spp.	13	2.7%	308
Staphylococcus aureus	9	1.9%	384
Brevibacillus parabrevis	8	1.7%	56
Mannheimia haemolytica	7	1.5%	1603
Aerococcus viridans	4	0.8%	115
Enterococcus faecalis	4	0.8%	48
Escherichia coli	4	0.8%	1221
Mannheimia spp.	2	0.4%	75
Micrococcus luteus	2	0.4%	23
Rothia nasimurium	2	0.4%	2410
Trueperella pyogenes	2	0.4%	10
Corynebacterium mastitidis	1	0.2%	41
Corynebacterium spp.	1	0.2%	41
Kocuria rhizophila	1	0.2%	64
Pantoea agglomerans	1	0.2%	34
Unknown spp.	14	2.9%	68
Mixed culture	54	11.3%	175
Negative	177	37.2%	72
No milk	17	3.6%	-
Total	476	100%	6960

Table 1 An overview of the identified bacteria

or unknown spp.) and *Corynebacterium amycolatum* combined with *Corynebacterium* spp.. As mentioned before, only the species that was a major pathogen or had the most CFU's was displayed in **Table 1**.

Amongst *Staphylococcus* spp. were the following; *S. equorum*, *S. auricularis*, *S. capitis*, *S.chromogenes*, *S. epiderdimis*, *S. equorum*, *S. haemolyticus*, *S. pasteuri*, *S. simulans*, *S. vitulinus*, *S. warneri* and *S. xylosus*.

Symptoms:

A classification of the symptoms found during clinical examination is given in **Table 2**.

The amount of positive samples for *Mannheimia haemolytica* or *Staphylococcus aureus* and the distribution of symptoms amongst them is displayed in **Table 3**.

Classification	Symptoms
No symptoms	No symptoms found during clinical examination
Minor symptoms	Clinical findings that are less likely to be caused by mastitis. Findings such as: - Little milk - Irregularities in the udder - Abnormalities of the teats
Severe symptoms	Clinical findings that are most likely to be caused by mastitis. Findings such as: - Hardness of the udder - Painfulness of the udder - No milk - Changes in the milk

Table 2 An overview of the classification that has been made amongst the symptoms

		S. aureus or *M. haemolytica* Positive	%	Total
Symptoms	No	7	3%	280
	Minor	3	4%	80
	Severe	8	18%	45
Total		18	4%	405

Table 3 *This table shows the amount of samples from which Mannheimia haemolytica or Staphylococcus aureus was isolated. Furthermore the distribution of clinical symptoms has been displayed in this table*

Positive symptoms for mastitis were positively related to the presence of one of these major pathogens ($p < 0.05$).
Contrary to the findings with *M. haemolytica* and *S. aureus*, no significant relationship could be found between symptoms and positive samples in general (Chi-square test, $p > 0.05$). Nor did *E. coli* samples (n= 4) prove to have a significant relationship with symptoms in this study (Chi-square test, $p > 0.05$).
A total of 75 of the positive samples in bacteriology were cultured from incubated milk. The samples from incubated milk had a mean geometrical cell count of 243.000 cells/ml compared to a mean geometrical cell count of 808.000 cells/ml in 48 h samples. The distribution of symptoms found in the samples from incubated milk and 48 h samples is displayed in **Table 4.**
No significant relationship could be found between the presence of symptoms and a positive result in bacteriology from the incubated milk ($p > 0.05$).

		Fresh	Pre-incubated
Symptoms	No symptoms	101	47
	Minor symptoms	31	18
	Severe symptoms	21	10
Total		153	75

Table 4 *The distribution of symptoms over the positive samples. Samples cultured from the fresh milk samples and samples cultured from the pre- incubated milk are displayed*

Additional samples:

Additional samples were collected from ewes suspected of mastitis. These samples had been collected by the farmer. A total of 10 samples was collected. The following species had been identified from the samples; *Staphylococcus* spp. (n= 5), *Staphylococcus aureus* (n= 1), *Mannheimia haemolytica* (n= 1), Unknown spp. (n= 1) and Mixed cultures (n= 2). No SCC samples were collected, due to this no results of the mean geometrical Somatic Cell Count can be given. These samples have not been included in the Chi-square tests described earlier.

Distribution amongst the farms:

The outcome of the bacteriology per flock is displayed in **Figure 2**. A large variation exists between the different farms, which is visible in the number of animals affected by mastitis and the number of cases can be assigned to a major pathogen such as *Mannheimia haemolytica* or *Staphylococcus aureus*.

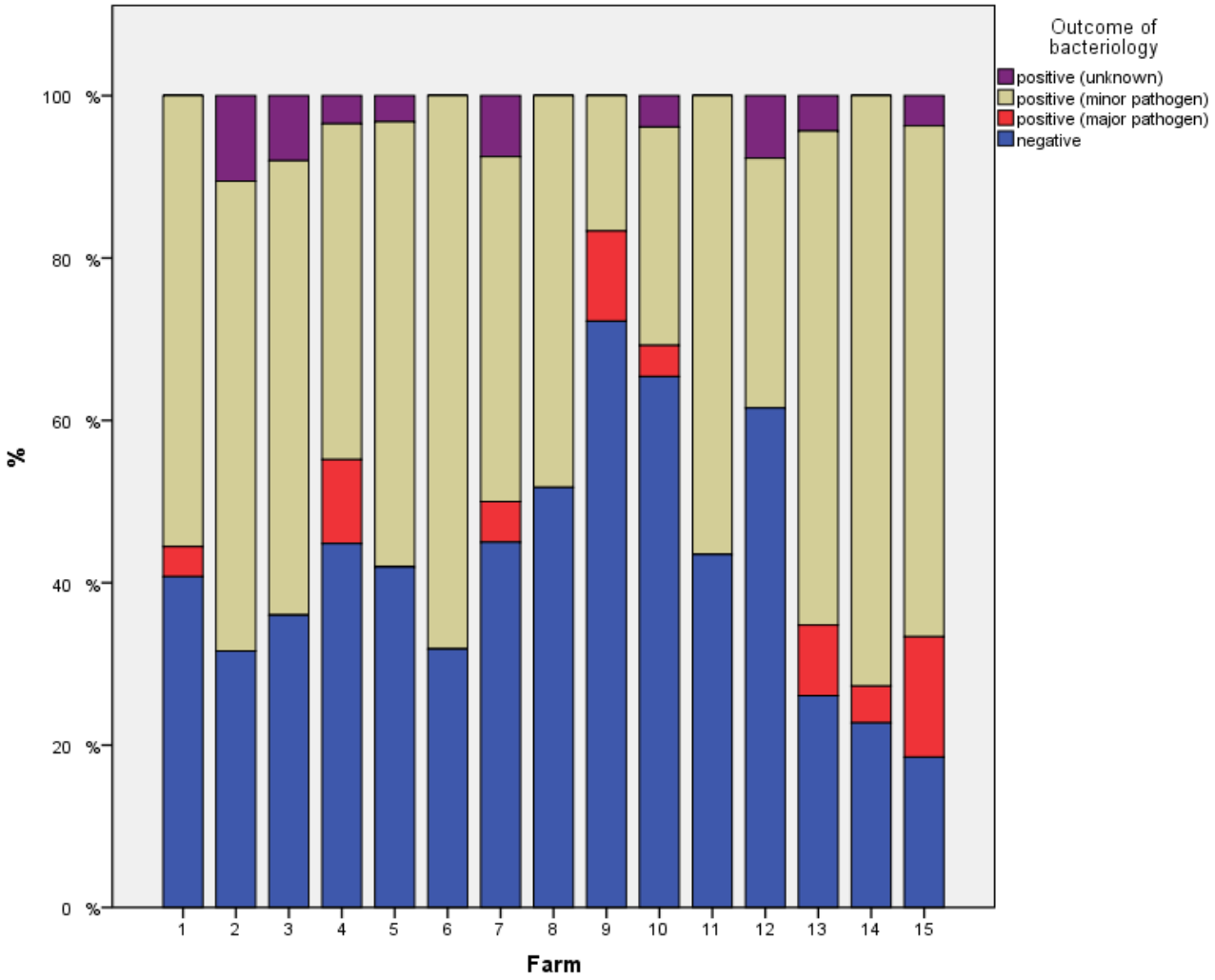

Figure 2 *This figure shows the distribution of positive - and negative samples over the 15 different farms. A sub division has been made amongst positive samples, namely unknown spp., minor pathogens and major pathogens. In this figure the major pathogens that are displayed are either M. haemolytica or S. aureus*

Discussion:

This study aimed to describe the bacterial species found in milk from Dutch meat sheep. Furthermore this study aimed to find an association between the etiology of mastitis in meat sheep and clinical symptoms.
The most commonly identified species in this study were *Staphylococcus* spp., which is in agreement with results of other studies in sheep[3, 5, 17]. This study found similar CNS as previous studies, these included *S. xylosus, S. simulans, S. chromogenes* and *S. epiderdimis*[2, 4].
In previous studies clinical mastitis is frequently attributed to *S. aureus* and *M. haemolytica*[2, 18]. This study showed symptoms were significantly related to the identification of *S. aureus* and *M. haemolytica* from milk samples. *M. haemolytica* and *S.* aureus had already been associated with severe clinical symptoms (e.g. hardness of the udder and enlargement of the udder)[5, 18], which corresponds with the results found in meat sheep in The Netherlands.

However this study did not find a significant relationship between the identification of *E. coli* and clinical symptoms. An explanation could be found in the number of *E. coli*'s identified in this study (n = 4). Due to the low number of *E. coli* samples in this study, no definite conclusion can be drawn if a relationship exists between symptoms and milk samples positive for *E. coli*. However, *E. coli* has previously been described to cause sub-clinical mastitis, in which no symptoms are to be seen[19].

Furthermore this study demonstrates pre-enrichment of milk has an added value in studies concerning mastitis in meat sheep, as a total of 75 of the positive samples were cultured from pre-enriched milk. 5 of these samples were *M. haemolytica* or *S. aureus*, which made up for 27.7% of the major pathogens that were isolated in this study. The mean geometrical SCC (243.000 cells/ml) found in the positive samples from pre-enriched milk was lower than the mean geometrical SCC (808.000 cells/ml) of 48 h samples.

As the samples from pre-enriched milk were not significantly related to clinical symptoms and had a lower mean geometrical SCC it is most likely the greater part of the positive samples from pre-enriched milk originated from sheep with sub-clinical mastitis.

A possibility exists that some samples were false-positive, as samples might have been contaminated with bacteria from the surroundings or from the skin of the udder. This however seems less likely, due to the fact the teats were carefully disinfected and the samples were collected accurately.

The number of positive samples which have been identified after incubating the milk demonstrates the usefulness of incubating the milk overnight and plating the milk out in case of negative bacteriological cultures were found. It is therefore recommended to apply this in mastitis researches in sheep.

Farms:

This study has been conducted under field circumstances. It is necessary to take into consideration that the participating ewes were housed under different circumstances. Furthermore the flocks had different breeding lines and goals and different sheep breeds (Texel-, Swifter -, Vlaming- and Zwartbles sheep) were present in this study. These factors might have had an influence on the outcome of this research. A study of Pereira at all. already showed that management and particular sheep breeds can be a risk factor for developing clinical mastitis[20]. Although that study was executed in Brazil, it is likely that differences between the farms also explain the variances seen between the farms in The Netherlands (**Figure 2**)[20].

Selection of the ewes and sample collection:

All farmers were asked to select 15 ewes from their flock, which was not further controlled by the researchers. For this reason it remains questionable if the selection has been carried out purely at random. The possibility exists that farmers selected ewes that were more prone to mastitis to their opinion or that animals have been

selected that had an excellent udder health status. This could mean the research would not be completely at random and the outcome of the research does not completely reflect the real situation. If further research is to be conducted, it is recommended that ewes are randomly selected by the researchers, based on stable lists, rather than to let the farmers select the animals. By so doing the farmers preferences are excluded, which makes the research more trustworthy.
Furthermore the farmers were given instructions to separate the ewes from their lambs 2 hours prior to the sampling moment. In cases the ewes had been confined in groups it was inevitable dust and debris from the surroundings were blown up in the air as a result of movements. A chance exists that as a consequence some samples were contaminated with dust and debris that fell into the samples, by which sterility was lost. As far as possible these samples were excluded from the study, as samples with ≥ 3 types of CFU's were marked as 'contaminated'.

Bacteriology and identification:

Bacterial identification was performed by the MALDI/TOF MS at UMC Utrecht. The purpose of the MALDI/TOF MS is to identify human strains on daily basis. As for this reason it has an extensive library of human strains, though less veterinary strains are included in the library. This could be one of the reasons why not all CFU's have been identified on genus and species level, which were labeled 'Unknown spp.'. Additional microbiologic testing will be needed for identification of these strains.
Furthermore a possibility exists that some samples have been incorrectly found to be negative in bacteriology. This could be declared by the fact that the secretion of bacteria is not identical if the milk sampling is repeated. Therefore it is possible samples were thought to be negative, which in fact have low number of bacteria in their milk. If this would be the case, the results shown in **Table 1** might be an underestimation of the real situation[21].
An interesting finding was the identification of *Rothia nasimurium* from two samples at two different farms. *Rothia* spp. are pleiomorphic and gram-positive bacteria that has been found in the oral flora of humans and tonsils of piglets[22, 23]. Up until now *R. nasimurium* has not been associated with mastitis, though in this research samples of *R. nasimurium* showed a high mean geometrical SCC of 2.410.000 cells/ml. Possibly *R. nasimurium* is a bacteria that is located in the oral flora of lambs, as it has already been isolated from tonsils in piglets[23].
Another explanation for the identification of *R. nasimurium* in this research is a flaw in the MALDI/TOF MS output. The MALDI/TOF MS might has mistaken another bacterial spp. for *R. nasimurium*, as the time of flight of both species could be extremely alike. This however seems less likely, as the MALDI/TOF score value of the two samples was > 2. A total of 27 *Rothia* spp. were present in the MALDI/TOF MS library of UMC Utrecht.
S. aureus showed a difference between the mean geometrical

SCC of the 48 h samples and the 24 h (incubated milk) samples. The 24 h samples had much a lower mean geometrical SCC of 243.000 cells/ml (n= 4) compared to a mean geometrical SCC of 606.000 cells/ml (n= 4).

Treatment:

During sampling collection it had been noticed that nearly all farmers treated ewes with clinical mastitis immediately with an antibiotic which they had in stock on the farm. In practically all cases a veterinarian was not consulted and ewes were treated based on the farmer's insight. According to the farmers that have collaborated in this research, treatment was unsuccessful in most cases. This poses the question if farmers are not amplifying the existing mastitis problems on their farms, by selecting for (multi-)resistant bacteria by using the same antibiotic treatment on all ewes with clinical mastitis. It is plausible to assume bacteria are able to develop resistance to certain antibiotics on farm-level. This makes it more difficult to treat cases of mastitis.
It is interesting to find out whether the *S.aureus* strains isolated in this study were Methicillin-resistant (MRSA), as MRSA poses a health risk to the farmer, their relatives and employees[24]. MRSA's have already been found amongst goats and dairy cows, which leaves the opportunity for MRSA's to be present as well in ovine mastitis samples[25]. Further testing would be necessary to rule out the presence of MRSA[25].

Conclusion:

This study showed that the same etiological agents can be found in meat sheep as in dairy sheep and most commonly *Staphylococcus spp*. are identified. A significant relationship was found between clinical symptoms and the identification of major pathogens (*M. haemolytica* or *S. aureus*). This means clinical symptoms can be used as a selection tool to reduce the number of animals affected by *S. aureus* and *M. haemolytica*.
The identification of *R. nasimurium* was interesting and it appears that this is a newly identified pathogen causing mastitis in ewes. Further investigation would be needed to specify the role of *R. nasimurium* in (sub)clinical mastitis in sheep.
The appliance of incubation of milk samples in mastitis research in sheep is useful and it enables identification of both major and minor pathogens. Variances between different farms are present, which can be declared by differences in management.

Acknowledgements:

This research has been a collaboration between Utrecht University and Nederlandse Schapen en Geiten Fokkerij Organisatie (NSFO).
I am grateful to Gerrit Koop for the opportunity to execute my research under his supervision. It has been an extremely educational research period, in which practical and theoretical skills were both present.
Furthermore I would like to thank the personnel of KLIF and the VMDC of Utrecht University, Faculty of Veterinary Medicine. A special word of thanks goes out to A.J.J.M. Daemen (Research and Education Assistant) and A.J. Timmerman (Department of Infectious Diseases and Immunology) for their excellent assistance and guidance.

Bibliography:

References

1. CBS StatLine - Landbouw; gewassen, dieren en grondgebruik naar regio, February 14, 2015, http://statline.cbs.nl/StatWeb/publication/?DM=SLNL&PA=80780NED&D1=440-450,482,486-487,490&D2=0&D3=0,5,%28l-2%29,%28l-1%29,l&HDR=G1,G2&STB=T&VW=T.

2. Bergonier, D. & Berthelot, X. New advances in epizootiology and control of ewe mastitis. *Livestock Production Science*. 79, 1-16 (2003).

3. Jan, O. & Jaskowski, J. M. Mastitis in small ruminants. *Medycyna weterynaryjna*. 70, 67-72 (2014).

4. Bergonier, D., de Crémoux, R., Rupp, R., Lagriffoul, G. & Berthelot, X. Mastitis of dairy small ruminants. *Vet. Res.* 34, 689-716 (2003).

5. Contreras, A., Sierra, D., Sánchez, A., Corrales, J.C., Marco, J.C., Paape, M.J., Gonzalo, C. Mastitis in small ruminants. *Small Ruminant Research* 68, 145-153 (2007).

6. Doğruer, G., Doğruer, G., Saribay, M.K., Ergün, Y., Aslantaş, O., Demir, C., Ateş, C.T. Treatment of subclinical mastitis in Damascus goats during lactation. *Small Ruminant Research* 90, 153-155 (2010).

7. Fotou, K., Tzora, A., Voidarou, C., Alexopoulos, A., Plessas, S., Avgeris, I., Bezirtzoglou, E., Akrida-Demertzi, K., Demertzis, P.G. Isolation of microbial pathogens of subclinical mastitis from raw sheep's milk of Epirus (Greece) and their role in its hygiene. *Anaerobe* 17, 315-319 (2011).

8. White, E. C. & Hinckley, L. S. Prevalence of mastitis pathogens in goat milk. *Small Ruminant Research* 33, 117-121 (1999).

9. Kirk, J. H. Mastitis in ewes. *Compendium : Continuing Education For Veterinarians* 18, 582-590 (1996).

10. Melero, M. J. C. Mastitis en la oveja Latxa: epidemiologia, diagnostico y control. , 398 (1994).

11. Watson, D. J. & Buswell, J. F. Modern aspects of sheep mastitis. *British Veterinary Journal*. 140, 529-534 (1984).

12. da Silva, E. R., Siqueira, A. P., Martins, J. C. D., Ferreira, W. P. B. & da Silva, N. Identification and in vitro antimicrobial susceptibility of Staphylococcus species isolated from goat mastitis in the Northeast of Brazil. *Small Ruminant Research* 55, 45-49 (2004).

13. Onni, T., Vidili, A., Bandino, E., Marogna, G., Schianchi, S., Tola, S. Identification of coagulase-negative staphylococci isolated from caprine milk samples by PCR-RFLP of groEL gene. *Small Ruminant Research* 104, 185-190 (2012).

14. Ruegg, P. L. *Mastitis in small ruminants* (44th Annual Conference of the American Association of Bovine Practitioners, Small Ruminant Session, 2011, September 22-25).

15. Tomazi, T., Gonçalves, J.L., Barreiro, J.R., De Campos Braga, P.A., Prada E Silva, L.F., Eberlin, M.N., Dos Santos, M.V. Identification of coagulase-negative staphylococci from bovine intramammary infection by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Journal of Clinical Microbiology*. 52, 1658-1663 (2014).

16. Gonçalves, J. L., Tomazi, T., Barreiro, J.R., Braga, P.A.D.C., Ferreira, C.R., Araújo Junior, J.P., Eberlin, M.N., dos Santos, M.V. Identification of Corynebacterium spp. isolated from bovine intramammary infections by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Veterinary Microbiology*. 173, 147-151 (2014).

17. Blagitz, M. G., Souza, F.N., Batista, C.F., Diniz, S.A., Haddad, J.P.A., Benites, N.R., Melville, P.A., Libera, A.M.M.P.D. Clinical findings related to intramammary infections in meat-producing ewes. *Tropical Animal Health and Production*. 46, 127-132 (2014).

18. Omaleki, L., Browning, G. F., Allen, J. L. & Barber, S. R. The role of Mannheimia species in ovine mastitis. *Veterinary Microbiology*. 153, 67-72 (2011).

19. Gebrewahid, T. T., Abera, B. H. & Menghistu, H. T. Prevalence and etiology of subclinical mastitis in small ruminants of tigray Regional state, North Ethiopia. *Veterinary World* 5, 103-109 (2012).

20. Pereira, P. F. V., Stotzer, E. S., Pretto-Giordano, L. G., Müller, E. E. & Lisbôa, J. A. N. Risk factors, etiology and clinical aspects of mastitis in meat ewes of Parana, Brazil. *Pesquisa Veterinaria Brasileira*. 34, 1-10 (2014).

21. Rovai, M., Caja, G., Salama, A.A.K., Jubert, A., Lázaro, B., Lázaro, M., Leitner, G. Identifying the major bacteria causing intramammary infections in individual milk samples of sheep and goats using traditional bacteria culturing and real-time polymerase chain reaction. *Journal of Dairy Science*. 97, 5393-5400 (2014).

22. Mahobia, N., Chaudhary, P. & Kamat, Y. Rothia prosthetic knee joint infection: report and mini-review. *New Microbes New Infect* 1, 2-5 (2013).

23. Baele, M., Chiers, K., Devriese, L.A., Smith, H.E., Wisselink, H.J., Vaneechoutte, M., Haesebrouck, F. The Gram-positive tonsillar and nasal flora of piglets before and after weaning. *Journal of Applied Microbiology*. 91, 997-1003 (2001).

24. 1. Pantosti, A. & Venditti, M. What is MRSA? *European Respiratory Journal*. 34, 1190-1196 (2009).

25. Alves, P. D. D., McCulloch, J.A., Even, S., Le Maréchal, C., Thierry, A., Grosset, N., Azevedo, V., Rosa, C.A., Vautor, E., Le Loir, Y. Molecular characterisation of Staphylococcus aureus strains isolated from small and large ruminants reveals a host rather than tissue specificity. *Veterinary Microbiology*. 137, 190-195 (2009).