The association between leukocidin M and F' expression by *Staphylococcus aureus* strains and the clinical severity of ruminant mastitis

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Abstract

Staphylococcus aureus is an important causative agent of ruminant mastitis. The bacterium has several ways of immune evasion, of which the production of bicomponent leukocidins is one. It is suggested that LukMF', a leukocidin almost exclusively produced by ruminant *S. aureus* strains, is the most important leukocidin *in vitro*. To elucidate the importance of this toxin in ruminant mastitis, milksamples were taken from sheep and cows to determine the prevalence of the LukMF' genes, study the *in vivo* and *in vitro* production of the toxin and relate the production of LukM to the somatic cell count (SCC) and clinical features of the concerning animals. A high prevalence of LukMF' was found in both the sheep- and the cow isolates, but presence of the genes was not associated with the severity of mastitis. LukM production *in vitro* couldn't be associated with the severity of mastitis but an association was found between the presence of LukM *in vivo* and the severity of mastitis.

Key words

Staphylococcus aureus, leukocidin MF', ruminant mastitis.

Introduction

Ruminant mastitis is a disease that affects sheep and cows worldwide, costing farmers a lot of money. These economic losses are caused by several factors, such as milk production losses, treatment costs, discarded milk, decrease in product quality, materials and investments needed for mastitis management, culling, and other diseases associated with mastitis.[1] The effect of mastitis on an individual animal depends on the virulence of the causative pathogens, the time of onset relative to the lactation state and the host's immunity.[2] Symptoms of mastitis can therefore range from visibly not present to general illness. However, an elevation of the SCC is a typical symptom that is almost always detectable in the milk of affected animals. In case of subclinical mastitis, this might be the only indication of inflammation. This makes the SCC a valuable tool for the screening on mastitis.[3]

Staphylococci are the main causative agents of mastitis in dairy and meat ewes, and hereof *S. aureus* is the most important pathogen causing clinical mastitis.[3] In dairy cows, *S. aureus* seems to be the pathogen most often isolated from animals afflicted by subclinical mastitis.[4] The repertoire of virulence factors differs among the diverse *S. aureus* strains, and the variation between strains is augmented by the fact that many virulence genes are encoded on mobile genetic elements that can be transferred between strains by horizontal gene transfer.[5] *S. aureus* has several mechanisms of immune evasion which complicate elimination in the host, for example inhibition of the complement cascade,[6-9] blocking of the activation of leukocytes[10] and production of leukocidins.[11] Leukocidins are bi-component exotoxins, that destroy leukocytes at a distance through the formation of pores in the cell membranes. These pores facilitate the efflux of vital molecules and metabolites, which cause the cells to die.[5,12] All *S. aureus* strains are capable of producing at least three of the six known bi-component leukocidins, which are HlgAB, HlgCB and LukAB/HG.[13] One additional leukocidin, known as LukMF', targets mainly CCR1 on leukocytes which is expressed by ruminants and other mammals such as mice.[12-14] CCR2 and CCR5 are minor targets of LukMF' toxicity. CCR1 is present on both bovine and human monocytes while CCR1 expression on neutrophils is restricted to cows. Since LukMF'-mediated killing of neutrophils is CCR1 dependent, human neutrophils are resistant toward LukMF' cytotoxicity.[12] Human *S. aureus* strains however contain a variant of the LukMF' toxin called Panton-Valentine Leukocidin (PVL), which is also a bi-component leukocidin and a very potent killer of human neutrophils.[5] Both the LukMF' genes and the genes encoding for PVL, LukS-PV and LukF-PV, are carried by temperate phages that became prophages after lysogenization into the *S. aureus* chromosome, and can be horizontally transmitted between *S. aureus* strains.[15,16] However, PVL targets human complement receptors C5aR and C5L2, of which C5aR is the major target.[17] Rabbit neutrophils are also susceptible for PVL leukotoxicity, but PVL is not active against cow neutrophils.[18]

S. aureus isolates equipped with LukMF' genes of ovine origin seem to produce supernatants more leukotoxic than those of bovine origin. This might be related to the fact that cases of *S. aureus* mastitis are more often clinical in ewes than in cows, even though ovine PMN proved to be five times more resistant than bovine PMN to the spreading effect of LukMF'. However, culture supernatants of LukM-positive *S. aureus* isolates from both species were considerable more leukotoxic than supernatants of LukM-negative isolates.[19]

All bi-component leukocidins consist of a slow and a fast component. In the case of LukMF', LukM is the slow component, which initially recognises and binds to the chemokine receptor CCR1 on the surface of target cells. LukM then recruits the fast subunit, called LukF. Together they form dimers and then oligomers, that eventually assemble into an octameric prepore structure containing alternating slow (S) and fast (F) subunits. Eventually a membrane insertion and subsequently a β-barrel pore that spans the host cell lipid bilayer arise through a major structural change in the stem domains of the S and F units. At this point the pore typical for bi-component leucocidins has formed through which vital molecules and metabolites flow out, causing the cell to die (**Figure 1**).[13,20]

LukMF' seems to be the most potent leukocidin *in vitro*,[12] and former research suggests that LukMF' could be the most active leukotoxin produced by mastitis isolates.[21] Since ruminant leukocytes are killed by LukMF', host elimination of *S. aureus* bacteria producing this toxin is probably impaired. It is therefore suspected that LukMF' might play an important role in the onset and the severity of mastitis caused by *S. aureus* bacteria. The aims of this study are to determine the prevalence of LukMF' in ovine and bovine *S. aureus* strains and establish the clinical relevance of LukMF'. Therefore the presence of the LukMF' genes as well as the LukM production *in vivo* and *in vitro* of ovine and bovine isolates will be determined, and the results will be linked to the clinical records of the animals from which the samples were taken. The severity of mastitis will be defined by the SCC and the form of mastitis (subclinical or clinical) that affects the animal. Aforementioned serves to answer the following question: is there an association between LukMF' expression by *Staphylococcus aureus* and the clinical severity of ruminant mastitis?

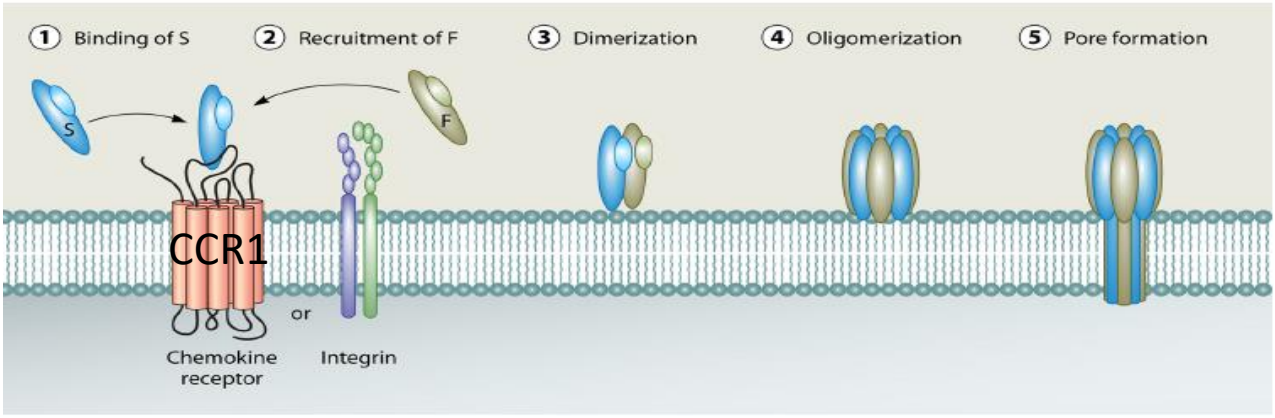

Figure 1: Modified from Alonzo and Torres 2014.[7] Current model of leukocidin pore formation. The slow (S) component, LukM, recognises and binds to the chemokine receptor CCR1 on the surface of target cells (1). LukM than recruits the fast (F) subunit called LukF (2). LukM and LukF subsequently form dimers (3) and then oligomers (4) that assemble into an octameric prepore containing alternating slow and fast subunits. A structural change in the stem domains of the S and F subunits eventually leads to a membrane insertion and the formation of a β-barrel pore that spans the host cell lipid bilayer (5), through which vital molecules and metabolites flow out.

Materials and methods

Sample collection. Milk samples from ewes were collected between February and April of 2015. Therefore 15 farms located in different provinces in The Netherlands were visited and a total of 225 ewes were sampled. 15 ewes per farm were selected by the farmer, if possible with a variety of parity. All ewes were sampled in the first stage of the lactation. Protective clothes were worn by the samplers at all visits, such as overalls, latex examination cloves and boots. Samples were collected aseptically from each ewe from both udder halves. Each teat was disinfected with cotton wool soaked in Ethanol (70%) and the first two streams of milk were discarded before a milk sample was collected in a sterile milk tube. During transportation to Utrecht University the samples were cooled in a cool box filled with cooling-elements.
For each sampled ewe, the condition of the animal and it's udder were assessed and the amount and consistency of the milk given by the ewe was examined. The ear tag number, parity, date of birth and parturition date were also noted. In case a farmer suspected an ewe of having a clinical mastitis, aseptic samples of both udder halves were taken by the farmer and collected in sterile milk tubes. These samples were ideally taken before the ewe received any treatment and were then frozen at -20°C, together with a questionnaire containing the ear tag number, parity, date of birth, parturition date and clinical parameters regarding the health status of the ewe. A total of 41 ovine samples containing *S. aureus* were included in the study.

Bovine samples were send to the ULP (Universitaire Landbouwhuisdieren Praktijk; university farm animal practice) by farmers when a cow showed a high somatic cell count or other signs of mastitis. Therefore cows were sampled throughout different stages of lactation. Clinical symptoms were noted in MPR (milk product registration). A total of 55 *S. aureus* containing bovine samples were collected.

Sample selection and diagnosis of mastitis. The goal of this study is to find out if there's an association between the severity of *S. aureus* mastitis and the production of LukM. Therefore samples not containing *S. aureus* bacteria were left out, since they were not relevant for this study.
An upper limit of 250.000 cells/ml was classified as a normal SCC for ewes.[22] Ewes with a cell count exceeding this number without showing clinical signs were classified as having a subclinical mastitis, while those with a cell count above the upper limit and clinical signs were classified as having a clinical mastitis. Ovine samples were used for the detection of bacteria present in the milk and the determination of the SCC. Sampling and establishment of the clinical symptoms of the animals was carried out on the same day. Milk was plated on blood agar plates and colonies were identified using MALDI/TOF MS. Blood agar plates containing three types of bacterial colonies or more were excluded from the study.
When a farmer sent in a bovine sample, the SCC and the clinical symptoms were read from the data in MPR. Cows were regularly sampled whereby the SCC was determined and noted in MPR. Therefore time elapsed between SCC determination and sampling of the cow could be up to 37 days. Cows were classified as having subclinical or clinical mastitis by the ULP based on the data given by the farmer. Upper cell count limits of 150.000 cells/ml for heifers and 250.000 cells/ml for second-calf and older cows were wielded, in accordance with the upper cell count limits stated by MPR. Samples were plated on blood agar plates and the ones containing *S. aureus* were used for this study. *S. aureus* colonies were identified using the motley row. For the bovine samples included in this study *S. aureus* was the only bacterial species present on the plate, except for one sample where *S. aureus* was predominantly present in a mixed bacterial culture.

DNA extraction. DNA from the bacteria was isolated by boiling. Therefore bacteria were plated on blood agar plates so that the next day isolated colonies could be resuspended in 1 ml of autoclaved water in microfuge tubes. Tubes were then centrifuged for 1 minute at 9000-13000 G, whereafter the supernatant was removed from the pellets. Subsequently 200 µl of autoclaved water was added

to the tubes, and after vortexing they were placed in a 100°C heat block for 10 minutes. Finally working dilutions could be made in autoclaved water. Working dilutions were stored at 4°C and the undiluted stock at -20°C.

Conventional PCR on LukM, LukF and FemA genes. LukM and LukF primers were designed based on the sequence of the genes using ThermoFisher scientific software. After a few experiments new LukM primers were designed to obtain clearer results. FemA primers were designed by Francois et al.[23] FemA was included as a control gene; because of its specificity for *S. aureus* it was used to ascertain that the DNA isolate contained *S. aureus* bacteria.[24]
Conventional PCR amplification was performed in 0,2 ml tubes (BIOplastics) for a 25 µl reaction volume consisting of mastermix (**table 1**), forward and reverse primers and nuclease free water. For screening on the LukM gene 5 vol% forward and reversed primer was used per sample. For the duo PCR on LukF and FemA the primer quantities were first also 5 vol% per primer, but this was later changed to 5,8 vol% for the LukF primers and 4,2 vol% for the FemA primers to obtain a better ratio between amplification of the LukF and FemA DNA. Template DNA was diluted 1:100 for the LukF/FemA PCR and 1:10 for the LukM PCR. Nuclease free water was added to the mastermix to complement till a volume of 25 µl per sample.
Subsequently DNA amplification took place in a Bio-rad T100^{tm} thermal cycler machine. Primer sequences and PCR protocols can be found in **table 2** and **table 3** respectively.
The amplified products were electrophoresed on 1.5% agarose gel stained with midori green. A 50- or 100bp ladder was run as a molecular marker. Presence of DNA products could subsequently be made visible using the Bio-rad gel imager (**figure 2**).

Table 1: Mastermix

Component	Vol%
5x GoTAQ green buffer	20
MgCl2 solution 25mM	6
PCR Nucleotide mix(4mM each)	5
GoTaq DNA polymerase (5µ/µl)	0,5
Template DNA	40

Table 2: Primers

Primer number	**Name primer**	**Primer forward or reversed**	**Primer sequence in 5'- 3' orientation**	**Product size (bp)**
1	Lukf'1	Forward	actcaggctatacccaaccca	425
2	Lukf'2	Reversed	cgagctactctgtctgccac	
3	FemA1	Forward	tgcctttacagatagcatgcca	142
4	FemA2	Reversed	agtaagtaagcaagctgcaatgacc	
5	LukM1	Forward	tgagtgggtatggcatgaaaga	572
6	LukM2	Reversed	tggacattttgtgttacacccc	
7	LukM3	Forward	aaacgcgcagttaataaaaag	975
8	LukM4	Reversed	agcattaggtcctcttgtcg	

Table 3: PCR protocols

PCR product	5' primer	3' primer	# cycles	Denaturing step	Annealing step	Elongation step
LukF	1	2	35	95°C 30 sec	59.5°C 30 sec	72°C 35 sec
FemA	3	4	35	95°C 30 sec	59.5°C 30 sec	72°C 35 sec
LukM	5 (old) 7 (new)	6 (old) 7 (new)	34	95°C 2 min	55°C 45 sec	72°C 60 sec

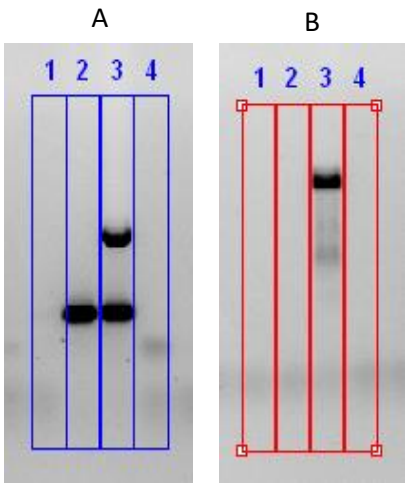

Figure 2. Picture of a PCR on LukF and FemA (a) and on LukM (b). Lane 1 shows DNA of a non-*S. aureus* control, lane 2 DNA of a LukMF' negative *S. aureus* control, lane 3 DNA of a LukMF' positive *S. aureus* control and lane 4 nuclease free water.

Duplo ELISA for LukM antigen measurement *in vivo*. EIA/RIA 96 well plates (Corning Costar) were coated overnight at 4°C or during one hour on a shaker at room temperature with 50 µl of 32 µg/mL Bovine IgG, isolated from colostrum of a cow with high antibody titers against LukM using a protein G column, in azide bicarbonate buffer 0,05% (pH 9,5). With exception of the overnight coating, all incubation steps were executed on a shaker. Plates were washed three times with PBS containing 0,05% Tween 20 (PBS-T) and blocked with 200 µl 4% ELK milk powder (Campina) diluted in PBS-T for at least 30 minutes at room temperature. Samples and standard curves were diluted two-fold in 1% ELK PBS-T and then boiled at 95°C for 10 minutes. Hereby antibody-antigen binding was disrupted in order for LukM to be detected. Subsequently a fourfold dilution in 1% ELK PBS-T followed, which brings the final dilution to 1:8. 100 ul of this dilution per sample was incubated after one washing of the plate. Calibration curves were made from recombinant LukM (KoMa43, Podiceps, The Netherlands). Subsequently plates were washed two times, after which 50 µl of 3 µg/ml mouse anti-LukM antibodies were added. After another 60 minutes of incubation and three more washes 100 µl 1:1000 diluted goat anti-mouse antibodies conjugated with peroxidase (biolegend, clone poly 4053) were incubated. An hour later the antibodies were removed and the plates were washed three times, after which 100 ul TMB was added to each well. After that plates were wrapped in aluminium foil to prevent the TMB from reacting with light, after which the plates were placed on the shaker for 15 to 20 minutes. Finally, 100 µl of sulfuric acid was added to terminate the reaction and the optical density was measured at 450 nm on a Multiskan™ FC Microplate Photometer (Thermo Scientific) within an hour. The quantity of LukM of every sample was measured for each plate by interpolating the unknowns from the calibration curve with GraphPad Prism version 6.05. LukM quantities lower than 0,4 ng/ml could not be detected.

Culturing and harvesting of supernatant. Bacteria were plated on blood agar plates and incubated at 37°C. The next day, single colonies were picked from the plate and inoculated in 3 ml Todd Hewitt Broth (THB) growth medium. Inoculation occurred by a pipet tip, in which case the pipet tip was used to pick a colony and then put into the THB, or with a sterilized inoculation loop. Tubes containing THB and a pipet tip without a bacterial colony were included as negative controls. The pipet tips were replaced by the inoculation loop after it was found that pipet tips often caused bacterial contaminations of the negative controls. Samples were discarded if controls were contaminated.

In order for bacterial growth to optimally occur, tubes were put in the shaking incubator (225 rpm) at 37°C. After 24 hours of growth supernatant could be harvested. Therefore, first the optical density of the solution was determined using the Pharmacia Biotech Ultrospec 2000® spectrophotometer at a wavelength of 660 nm (OD_{660}). THB from a control tube was set as reference. Then the tubes containing THB with bacteria were centrifuged for 10 minutes at 3500 G by 4°C. Thereafter supernatants were sterilized using a 0.2 micron filter (Corning®).

Another way of culturing supernatant was by inoculation of 3 ml THB with bacteria directly from the glycerol stock. Tubes were then put in the shaking stove and after 24 hours of bacterial growth the solution was diluted with THB until an optical density of 0,01 was reached. Thereon 3 ml of this

solution was allocated over several tubes, where after another 24 hour growth period in the shaking stove followed. Finally the OD was measured and the supernatant harvested, the same way as described before.

ELISA for LukM antigen measurement *in vitro*. For the determination of LukM quantities *in vitro* the procedure resembled the LukM ELISA on milk. The only difference was in the dilution and treatment of samples and standard curves; supernatant was a thousand fold diluted in 1% ELK PBS-T and samples were not boiled. Positive control supernatants of *S. aureus* strains producing high and intermediate amounts of LukM and negative ones of a Staphylococcal non-*S. aureus* strain not able to produce LukM were also included.

Statistical analysis. Data were analysed using GraphPad Prism version 6. Results were considered significant if the P-value was 0,05 or lower. For the Fisher's exact test used in figure 4 a cell count higher than 3.500.000 cells/ml was considered high for ewes, while a cell count exceeding 1.500.000 cells/ml was for cows. In figure 8, a LukM production beneath 900 ng/ml was considered low while a LukM production above this value was considered high.

Results

I Sample analysis

A high prevalence of LukMF' genes was found in *S. aureus* isolates of sheep and cows. Using PCR both the ovine and bovine *S. aureus* samples were screened for the presence of the LukMF' genes. Where bovine samples were only available from animals with mastitis, most of the ovine samples were collected randomly and thus also contained samples of healthy sheep. Therefore the prevalence of LukMF' could be calculated for all ovine samples but also for only the ewes with mastitis. The prevalence LukMF' found for the complete ovine *S. aureus* dataset was 0,927 with a 95% confidence interval of 0,806 < prevalence < 0,975. For the ewes with mastitis a prevalence of 0,941 with a 95% confidence interval of 0,73 < prevalence < 0,99 was found. A prevalence of 0,964 was found for the bovine samples, with a 95% confidence interval of 0,877 < prevalence < 0,99.

Table 4: prevalence of LukMF' found in the *S. aureus* samples of ewes and cowes

Data	Prevalence	95% confidence interval*
ovine samples (complete)	0,927	0,806 < P < 0,975
ovine samples (only sheep with mastitis)	0,941	0,73 < P < 0,99
bovine samples	0,964	0,877 < P < 0,99

* The Wilson interval was used for the calculation of the confidence intervals.

The presence of the LukMF' genes is not associated with the severity of mastitis. After the establishment of a high prevalence of LukMF' in the collected ovine and bovine samples, the presence of the genes was related to the form of mastitis seen in the animals (**figure 3**). Therefore a distinction was made between the presence and absence of mastitis in sheep and between subclinical and clinical mastitis in both the sheep- and cow isolates. The results suggest that solely the presence of the LukMF' genes has no effect on the severity of mastitis.

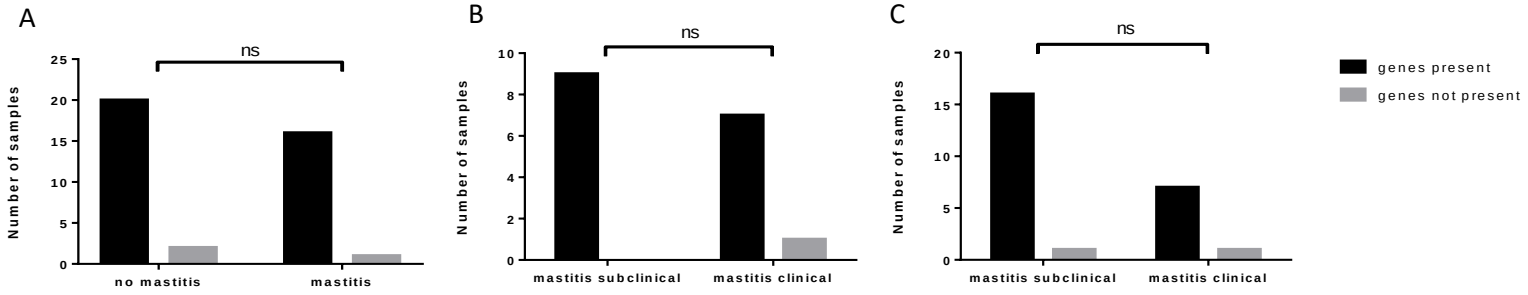

Figure 3. The presence of LukMF' in ovine and bovine *S. aureus* isolates. The Fisher's exact test was used to analyse the data. (a) The presence of LukMF' genes in sheep without and with mastitis. (b) The presence of LukMF' genes in sheep with subclinical and clinical mastitis. (c) The presence of LukMF' genes in cows with subclinical and clinical mastitis.

The presence of LukM *in vivo* might be associated with the severity of mastitis. Since presence or absence of the LukMF' genes could not explain the varying severity of mastitis in the animals, the presence of LukM toxin in the milksamples was studied. Therefore the presence of LukM *in vivo* was established by ELISA, to subsequently associate these outcomes to the cell counts of each animal. Within the ovine samples only a few showed presence of LukM *in vivo*, although samples showing no presence of LukM in the ELISA could have contained quantities below the detection limit of 0,4 ng/ml. Unfortunately some milk samples showing presence of LukM had to be left out because the corresponding SCC was unknown. However, in the ovine samples a significant association was found between a high cell count and the presence of LukM *in vivo* (**figure 4a**). This association was not significant in cows (P = 0,4605) (**figure 4b**).

After it was found that there might be a relation between the SCC and the presence of LukM *in vivo*, the existence of a possible link between the presence of LukM in the milk and the form of mastitis was studied. Within the sheep samples a distinction could be made between presence and absence of mastitis and between subclinical and clinical mastitis, while for the cow samples only discrimination between subclinical and clinical mastitis was possible. In ewes without mastitis LukM was significantly less frequently present *in vivo* than in sheep with mastitis (**figure 5a**). No significant difference regarding LukM presence was found between sheep with subclinical and clinical mastitis (**figure 5b**). For cows however a significant difference was found between the presence of LukM in milk of animals suffering from subclinical and clinical mastitis, meaning that LukM was significantly more present in the milk in case of clinical mastitis. (**figure 5c**).

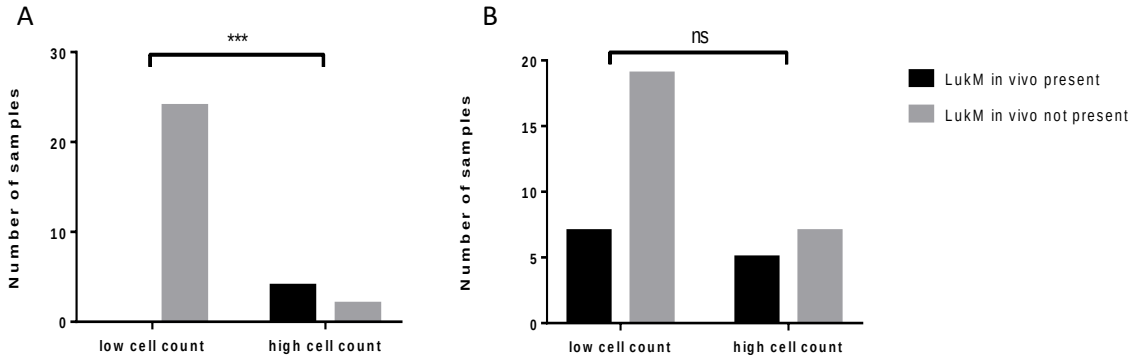

Figure 4. The height of the SCC (high or low) was associated with the presence or absence of LukM *in vivo* using the Fisher's exact test. For the sheep samples (a) a P-value of 0,0005 was found (***). No significance was found in the cow data (b).

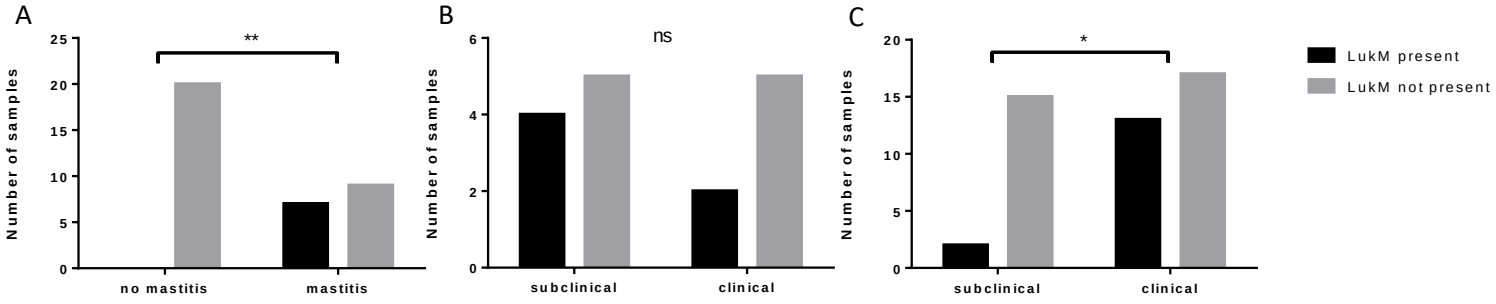

Figure 5. The severity of mastitis was associated with the presence of LukM in the milk of ewes (a and b) and cows (c) using the Fisher's exact test. P values of <0.05 (*) and <0.01 (**) were found.

The LukM production *in vitro* shows no association with the severity of mastitis of the ewes concerned. LukM production *in vitro* was measured to find out if the quantity of LukM produced by bacteria in optimal growth conditions could be linked to the severity of mastitis. *In vitro* LukM production was only determined for the ovine isolates. First it was examined if a correlation between the SCC and the quantity of LukM production *in vitro* was present (**figure 6**). No significant correlation was found. Subsequently the association between the severity of mastitis and the *in vitro* production of LukM was assessed (**figure 7**). A distinction was made between no mastitis, subclinical and clinical mastitis. The results show no association between LukM production *in vitro* and the severity of mastitis.

Lastly the quantity of LukM production *in vitro* was associated with the presence or absence of LukM *in vivo* (**figure 8**). No association was found, which indicates that LukM production *in vitro* can't be used to predict the quantity of LukM produced *in vivo.*

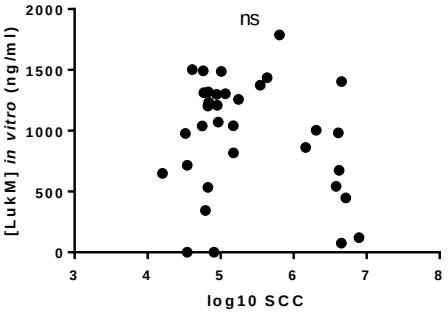

Figure 6. The correlation between the 10log of the SCC and the concentration of LukM produced *in vitro* by bacteria isolated from ewes' milk. For P and R^2 values of 0,2557 and 0,04017 were found respectively.

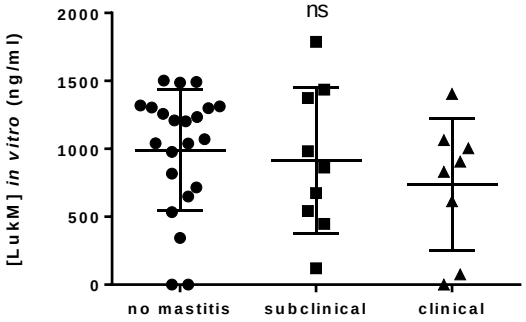

Figure 7. The *in vitro* production of LukM toxin in nanogram per millilitre by *S. aureus* strains isolated from ewes with no mastitis, subclinical or clinical mastitis. The ordinary one-way ANOVA was used to analyse the data. No significant differences were found between the groups.

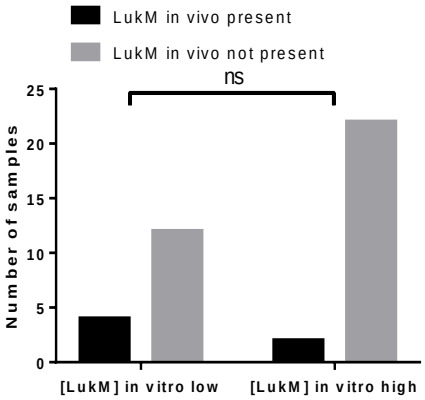

Figure 8. The quantity of the LukM production *in vitro* was associated with the presence or absence of LukM *in vivo* using the Fisher's exact test. No significant association was found.

II Analysis of the LukM production *in vitro*

Variation in LukM production might be caused by small colony variants. During the execution of the LukM ELISA's on in triplo harvested supernatant, large variation in LukM production between triplo's within some isolates was observed (**figure 9**). Considering that all colonies inoculated in THB within triplo's originated from the same *S. aureus* strain, it was expected that the LukM production would be in the same range. Therefore different hypotheses were formed; the prophage with the LukMF' genes on it could be activated and cut out of the genome of certain bacteria or regulation of LukMF' expression could result in different LukM levels between tubes.

To test if LukMF' was still present in bacteria producing little or no LukM *in vitro*, supernatants of two strains, one usually producing high and one intermediate quantities of LukM, were harvested in two different ways. For the first way THB containing bacteria was diluted to an optical density of 0,01. The second approach was the harvesting of supernatant produced by single colonies picked from blood agar plates. THB with bacteria was plated out on blood agar plates and DNA was isolated in threefold. Subsequently an ELISA was carried out on the supernatant in which LukM was detected in none of the samples. PCR showed that the genes for LukM were still present in all the samples. The experiment was repeated two more times, one time giving exactly the same results as described here, and one time LukM was detected in some samples but not all. Again PCR showed presence of the LukMF' genes in all samples.

Bacterial colonies of one LukM positive strain which now showed negative results in the ELISA were morphologically different from ordinary *S. aureus* colonies; they were smaller, white instead of yellowish and showed no bèta haemolysis (**figure 10**). This showed a striking resemblance with so-called small colony variants (SCV's). SCV's are an altered bacterial phenotype that form slow growing, small colonies on agar plates, show reduced or no pigmentation, and express a changed pattern of virulence factors.[25] Nothing is yet known about the production of LukM by small colony variants, but it is known that the production of other virulence factors can be increased or decreased in the small colony variant state. [26]

To find out what the effect of the SCV state on the production of LukM was supernatant from the suspected SCV and bacterial colonies that looked like normal *S. aureus* colonies were harvested, after which LukM quantities were determined by ELISA. It turned out that, within this bacterial strain, SCV's produced little to no LukM while the colonies with ordinary phenotypes showed a clear production. These results suggest that production of LukM could be downregulated in small colony variants of *S. aureus*.

When colonies of the SCV were plated onto another blood agar plate, they remained in the small colony variant state. Same goes for when a SCV colony was inoculated in THB; the supernatant showed no LukM production and plating of the THB showed presence of small colony variants only. This suggests that LukM production in SCV's is not regained when colonies are put in optimal growth conditions.

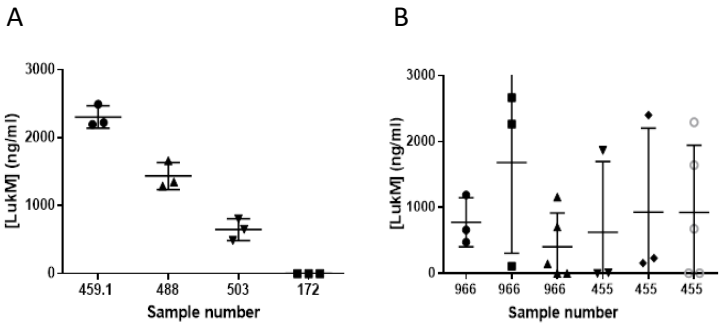

Figure 9. LukM production within multiple supernatants of different *S. aureus* isolates. (a) shows four isolates producing constant quantities of LukM in triplo. (b) shows two isolates displaying a major variation in LukM production. Supernatant was harvested multiple times in triplo or in fivefold.

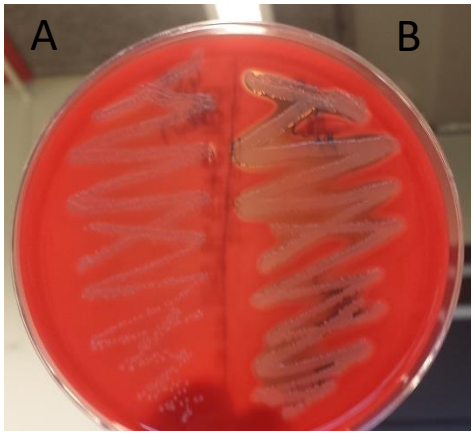

Figure 10. Blood-agar plate showing the small colony variant (a) and the normal (b) phenotype of one *S. aureus* isolate.

LukM quantities drop considerably after 24 hours of growth *in vitro*. THB inoculated from the glycerol stock was grown in triplo for for 6, 12 and 24 hours, after which a part of the solution was diluted to an OD of 0,01 followed by another 24 hours of growth. This was done for two LukM positive *S. aureus* strains to find out at which time point maximum LukM levels were reached. Supernatant was harvested of both the undiluted- and the diluted solutions. It appeared that LukM levels after 12 hours of growth in the undiluted solutions were much higher than after 24 hours (**figure 11**). A similar production of LukM for all the colonies within one strain was found in the diluted solutions after another 24 hours of growth (**figure 12**). Apparently the second 24 hour growth phase had more influence on the quantity of LukM in the supernatant than the time of growth before the solution was diluted.

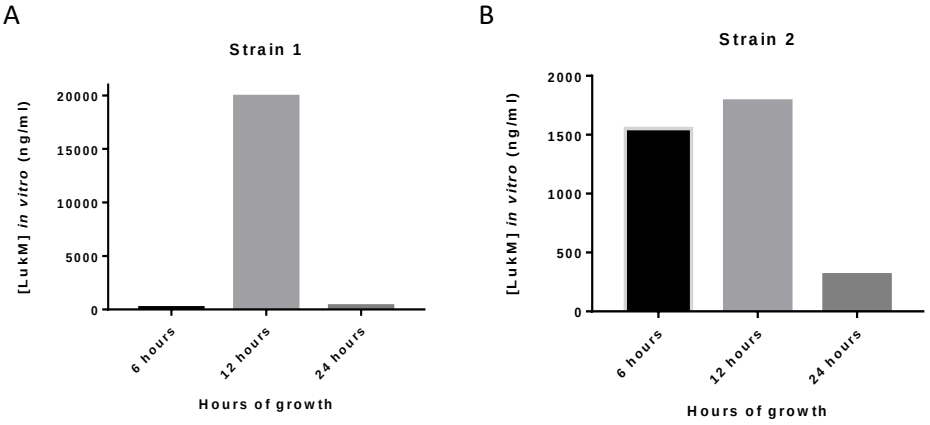

Figure 11. LukM production *in vitro* was measured for a high-producing (a) and a low-producing (b) *S. aureus* isolate after 6, 12 and 24 hours of growth. LukM levels were much higher after 12 hours of growth than after 24 hours.

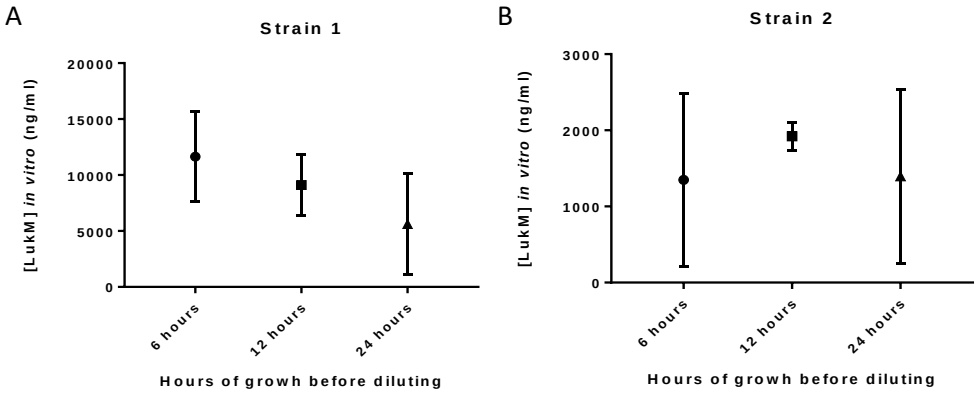

Figure 12. LukM production *in vitro* was measured in triplo for a high-producing (a) and a low-producing (b) *S. aureus* isolate. Therefore a growth fase of 6, 12 or 24 hours was followed by dilution of the solution to an optical density of 0,01 succeeded by a second growth fase of 24 hours. Similar LukM quantities were produced by all supernatants within one strain.

Discussion

Former studies seem to indicate that LukMF' is an important toxin among those produced by *S. aureus*.[5,12,19] However, if this is really the case or how LukMF' affects the severity of udder infection in ruminants is not yet known. Therefore the goal of this study was to find out if there is an association between LukMF' expression by *S. aureus* and the severity of ruminant mastitis.

A high prevalence of LukMF' genes in the *S. aureus* isolates was found for both the ovine- and bovine samples, respectively 0,941 and 0,964. For sheep isolates, the prevalence of LukMF' in literature ranges from 0,00 to 0,97. [19,30,31] A prevalence of 0,00 was found among meatsheep in Brasil, while prevalences of 0,851 and 0,97 were found in *S. aureus* strains from dairy sheep in Europe. Perhaps geographical differences and the production goals of the sheep included in the studies had to do with found differences. The prevalence of LukMF' found in our study suits with the numbers found in other studies within Europe. Prevalences for LukMF' in cow *S. aureus* isolates in literature range from 0,190 to 0,861.[16,19,31,32] Prevalences of 0,625 and 0,861 were found in Japan while values of 0,190, 0,354 and 0,799 were found in Europe. In the study from Vautor et al. (LukMF' prevalence of 0,190) only cows with subclinical mastitis were sampled. In the study from Schlotter et al. (LukMF' prevalence of 0,799) samples were only taken from clinically healthy cows and cows with clinical mastitis were excluded from sampling. The prevalence of LukMF' in cow isolates found in our study exceeds those found in former studies, both in Europe and Japan. This might be related to geographical differences and the fact that in our study only cows suffering from mastitis were sampled.

After finding a high prevalence of LukM and LukF in our isolates, the presence of these genes was related to the severity of mastitis found in the animals. No significant results were obtained for both the ovine- and the bovine samples, indicating there is no association between the presence of the LukMF' operon and the severity of the mastitis found in the animal.

The LukM production was studied *in vivo* and *in vitro*. When interpreting the results, it should be taken into account that the time expired between the determination of the clinical symptoms and SCC and the sampling of the cows could be up to 37 days, making the bovine cell counts very inaccurate.

First the presence of LukM *in vivo* was associated with the SCC. For the ovine samples a significant relation between these parameters was found. The small dataset makes it impossible to draw hard conclusions, but we can argue that there is a trend towards association between a high SCC and presence of LukM *in vivo*. No such trend was found in the bovine samples.

Subsequently the presence of LukM *in vivo* was related to the form of mastitis. No significant difference in presence of LukM toxin was found between sheep with subclinical and clinical mastitis, but there was between sheep with and without mastitis. In the cow milk samples a significant difference was found between cows with subclinical and clinical mastitis. There were much more milk samples from cows available containing LukM than from sheep. However, LukM quantities lower than 0,4 ng/ml might have been present, but this was below the detection limit of the ELISA. In further studies it would be better to have more LukM containing ovine samples at disposal or design a more sensitive ELISA.

LukM quantities detected *in vivo* are not only dependent on the LukM production of individual bacteria, but also on the number of bacteria present, the location of toxin production and the dilution of toxin by the milk. To determine the LukM production of single colonies, LukM production *in vitro* was studied. Supernatant was also harvested in another way; by inoculating THB directly from the glycerol stock and later dividing the diluted solution over different tubes, bacterial populations between the tubes were presumably more uniform than between tubes that were each inoculated with a different bacterial colony.

While harvesting and testing culture supernatant of the sheep isolates, a lot of variation was seen between in triplo harvested supernatant of some strains. Therefore *in vitro* production was only measured for the sheep isolates. Cow isolates will be analysed when this variation is ruled out.

For analyses of the *in vitro* production of LukM the average values of LukM produced per strain were used. No association was found between the LukM production *in vitro* and the severity of mastitis, nor a significant association between the quantities of LukM found *in vitro* and *in vivo*. However, no strong conclusions can be drawn due to the aforementioned variation in LukM production *in vitro*. For further experiments it is expedient to rule out this variation, in order to obtain unambiguous results.

Several possible causes were identified for the variation in LukM production *in vitro*. For one sheep isolate small colony variants were detected among phenotypically normal colonies in THB. In supernatant harvested from multiple SCV colonies no LukM was detected although PCR demonstrated that the genes encoding for the toxin were still present. However, small colony variants were only isolated from one *S. aureus* strain, which makes it unlikely that all variation was caused by SCV's. It is more plausible that variation in LukM quantities between tubes was caused by breakdown of LukM. We have demonstrated that more LukM is present after 12 hours than after 24 hours of growth. This indicates that at some time point between 12- and 24 hours of growth a plateau phase is reached in which bacteria start to fall short on nutrients, causing LukM to be broken down *in vitro*. When this deficiency starts probably depends on the number of bacteria present in the tube. Numbers of bacteria weren't precisely uniform between tubes.

Overall, a high prevalence of LukMF' was found in both the ovine- and the bovine isolates. For presence of the LukMF' genes is not associated with the severity of mastitis, finding out the association between LukM production *in vitro* and the severity of mastitis is essential to predict the virulence of individual *S. aureus* strains. No such association was found in this study, but results might be different when the variation of LukM production *in vitro* is ruled out and quantities can be determined unambiguously. However, we did find an association between LukM production *in vivo* and the severity of mastitis. For future research variation in *in vitro* LukM production should be

eliminated and the SCC should be determined at the day of sampling for all samples in order to obtain more accurate data. Also a bigger dataset would be recommended.

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References

1. Halasa T, Huijps K, Osteras O, Hogeveen H. Economic effects of bovine mastitis and mastitis management: A review. *Vet Q*. 2007;29(1):18-31. doi: 10.1080/01652176.2007.9695224 [doi].

2. De Vliegher S, Fox LK, Piepers S, McDougall S, Barkema HW. Invited review: Mastitis in dairy heifers: Nature of the disease, potential impact, prevention, and control. *J Dairy Sci*. 2012;95(3):1025-1040. doi: http://dx.doi.org/10.3168/jds.2010-4074.

3. Bergonier D, Berthelot X. New advances in epizootiology and control of ewe mastitis. *Livest Prod Sci*. 2003;79(1):1-16. doi: http://dx.doi.org.proxy.library.uu.nl/10.1016/S0301-6226(02)00145-8.

4. Keane OM, Budd KE, Flynn J, McCoy F. Pathogen profile of clinical mastitis in irish milk-recording herds reveals a complex aetiology. *Vet Rec*. 2013;173(1):17. doi: 10.1136/vr.101308 [doi].

5. Otto M. Staphylococcus aureus toxins. *Curr Opin Microbiol*. 2014;17:32-37. doi: http://dx.doi.org/10.1016/j.mib.2013.11.004.

6. Jusko M, Potempa J, Kantyka T, et al. Staphylococcal proteases aid in evasion of the human complement system. *J Innate Immun*. 2014;6(1):31-46. doi: 10.1159/000351458 [doi].

7. Rooijakkers SH, van Wamel WJ, Ruyken M, van Kessel KP, van Strijp JA. Anti-opsonic properties of staphylokinase. *Microbes Infect*. 2005;7(3):476-484. doi: S1286-4579(05)00036-5 [pii].

8. Hammel M, Sfyroera G, Ricklin D, Magotti P, Lambris JD, Geisbrecht BV. A structural basis for complement inhibition by staphylococcus aureus. *Nat Immunol*. 2007;8(4):430-437. doi: ni1450 [pii].

9. Jongerius I, Kohl J, Pandey MK, et al. Staphylococcal complement evasion by various convertase-blocking molecules. *J Exp Med*. 2007;204(10):2461-2471. doi: jem.20070818 [pii].

10. de Haas CJ, Veldkamp KE, Peschel A, et al. Chemotaxis inhibitory protein of staphylococcus aureus, a bacterial antiinflammatory agent. *J Exp Med*. 2004;199(5):687-695. doi: 10.1084/jem.20031636 [doi].

11. Menestrina G, Dalla Serra M, Comai M, et al. Ion channels and bacterial infection: The case of beta-barrel pore-forming protein toxins of staphylococcus aureus. *FEBS Lett*. 2003;552(1):54-60. doi: S0014579303008500 [pii].

12. Vrieling M, Koymans KJ, Heesterbeek DA, et al. Bovine staphylococcus aureus secretes the leukocidin LukMF' to kill migrating neutrophils through CCR1. *MBio*. 2015;6(3):e00335-15. doi: 10.1128/mBio.00335-15 [doi].

13. Alonzo F,3rd, Torres VJ. The bicomponent pore-forming leucocidins of staphylococcus aureus. *Microbiol Mol Biol Rev*. 2014;78(2):199-230. doi: 10.1128/MMBR.00055-13 [doi].

14. Fromageau A, Gilbert FB, Prevost G, Rainard P. Binding of the staphylococcus aureus leucotoxin LukM to its leucocyte targets. *Microb Pathog*. 2010;49(6):354-362. doi: 10.1016/j.micpath.2010.07.002 [doi].

15. Kaneko J, Kamio Y. Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: Structures, pore-forming mechanism, and organization of the genes. *Biosci Biotechnol Biochem*. 2004;68(5):981-1003. doi: 10.1271/bbb.68.981 [doi].

16. Yamada T, Tochimaru N, Nakasuji S, et al. Leukotoxin family genes in staphylococcus aureus isolated from domestic animals and prevalence of lukM-lukF-PV genes by bacteriophages in bovine isolates. *Vet Microbiol*. 2005;110(1-2):97-103. doi: S0378-1135(05)00253-1 [pii].

17. Spaan AN, Henry T, van Rooijen WJ, et al. The staphylococcal toxin panton-valentine leukocidin targets human C5a receptors. *Cell Host Microbe*. 2013;13(5):584-594. doi: 10.1016/j.chom.2013.04.006 [doi].

18. Spaan AN, Schiepers A, de Haas CJ, et al. Differential interaction of the staphylococcal toxins panton-valentine leukocidin and gamma-hemolysin CB with human C5a receptors. *J Immunol*. 2015;195(3):1034-1043. doi: 10.4049/jimmunol.1500604 [doi].

19. Rainard P, Corrales JC, Barrio MB, Cochard T, Poutrel B. Leucotoxic activities of staphylococcus aureus strains isolated from cows, ewes, and goats with mastitis: Importance of LukM/LukF'-PV leukotoxin. *Clin Diagn Lab Immunol*. 2003;10(2):272-277.

20. Lin YC, Peterson ML. New insights into the prevention of staphylococcal infections and toxic shock syndrome. *Expert Rev Clin Pharmacol*. 2010;3(6):753-767. doi: 10.1586/ecp.10.121 [doi].

21. Barrio MB, Rainard P, Prévost G. LukM/LukF'-PV is the most active staphylococcus aureus leukotoxin on bovine neutrophils. *Microb Infect*. 2006;8(8):2068-2074. doi: http://dx.doi.org/10.1016/j.micinf.2006.03.004.

22. Pengov A. The role of coagulase-negative staphylococcus spp. and associated somatic cell counts in the ovine mammary gland. *J Dairy Sci*. 2001;84(3):572-574. doi: S0022-0302(01)74509-2 [pii].

23. Francois P, Pittet D, Bento M, et al. Rapid detection of methicillin-resistant staphylococcus aureus directly from sterile or nonsterile clinical samples by a new molecular assay. *J Clin Microbiol*. 2003;41(1):254-260.

24. Berger-Bachi B, Barberis-Maino L, Strassle A, Kayser FH. FemA, a host-mediated factor essential for methicillin resistance in staphylococcus aureus: Molecular cloning and characterization. *Mol Gen Genet*. 1989;219(1-2):263-269.

25. Tuchscherr L, Heitmann V, Hussain M, et al. Staphylococcus aureus small-colony variants are adapted phenotypes for intracellular persistence. *J Infect Dis*. 2010;202(7):1031-1040. doi: 10.1086/656047 [doi].

26. Sifri CD, Baresch-Bernal A, Calderwood SB, von Eiff C. Virulence of staphylococcus aureus small colony variants in the caenorhabditis elegans infection model. *Infect Immun*. 2006;74(2):1091-1096. doi: 74/2/1091 [pii].

27. Sharma N, Singh NK, Bhadwal MS. Relationship of somatic cell count and mastitis: An overview. *Asian-Australas J Anim Sci*. 2011;24(3):429-438. Accessed 29 March 2016.

28. Godden SM, Jansen JT, Leslie KE, Smart NL, Kelton DF. The effect of sampling time and sample handling on the detection of staphylococcus aureus in milk from quarters with subclinical mastitis. *Can Vet J*. 2002;43(1):38-42.

29. Smith EM, Monaghan EM, Huntley SJ, Green LE. Short communication: Preliminary investigation into the effect of freezing and a cryopreservant on the recovery of mastitis pathogens from ewe milk. *J Dairy Sci*. 2011;94(10):4850-4855. doi: 10.3168/jds.2010-4076 [doi].

30. de Almeida LM, de Almeida MZ, de Mendonca CL, Mamizuka EM. Comparative analysis of agr groups and virulence genes among subclinical and clinical mastitis staphylococcus aureus isolates from sheep flocks of the northeast of brazil. *Braz J Microbiol*. 2013;44(2):493-498. doi: 10.1590/S1517-83822013000200026 [doi].

31. Vautor E, Magnone V, Rios G, et al. Genetic differences among staphylococcus aureus isolates from dairy ruminant species: A single-dye DNA microarray approach. *Vet Microbiol*. 2009;133(1–2):105-114. doi: http://dx.doi.org.proxy.library.uu.nl/10.1016/j.vetmic.2008.06.006.

32. Schlotter K, Ehricht R, Hotzel H, Monecke S, Pfeffer M, Donat K. Leukocidin genes lukF-P83 and lukM are associated with taphylococcus aureus clonal complexes 151, 479 and 133 isolated from bovine udder infections in thuringia, germany. *Vet Res*. 2012;43:42-9716-43-42. doi: 10.1186/1297-9716-43-42 [doi].